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STUDIES ON THE MODE OF ACTION OF AUXIN IN PLANT DEVELOPMENT – ANALYSIS OF THE *PIN6* GENE IN MERISTEM FUNCTION AND CELL DIFFERENTIATION

VIOLANTE DE FARIA E MAIA PACHECO DE MEDEIROS

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Tese orientada pela	Professora Doutora Maria Salomé Soares Pais Professora Catedrática da Faculdade de Ciências da Universidade de Lisboa, Portugal
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"Philosophy (nature) is written in that great book which ever lies before our eyes. I mean the universe, but we cannot understand it if we do not first learn the language and grasp the symbols in which it is written. The book is written in the mathematical (scientifical) language... without whose help it is humanly impossible to comprehend a single word of it, and without which one wanders in vain through a dark labyrinth."

Galileo Galilei

Aos meus pais

e à avó Lú

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RESUMO

A proteína PIN6 é um dos membros menos caracterizados da família de proteínas PIN, associadas ao transporte de auxina em *Arabidopsis thaliana*. Nesta planta modelo, o transporte polar auxínico tem sido associado a diversos processos fisiológicos. Por exemplo, o movimento acropetal de IAA, do caule para a raíz, tem sido implicado no desenvolvimento de raízes laterais, enquanto que o movimento basipetal de IAA, do ápice radicular para a junção caule-raíz, tem sido associado à resposta à gravidade. As proteínas PIN desempenham um papel crucial no transporte polar auxínico e desta forma medeiam o estabelecimento e manutenção de meristemas, a iniciação e posicionamento de órgãos laterais, a formação do tecido vascular e as diversas formas de tropismo. Neste trabalho procedeu-se ao estudo da função do gene PIN6 recorrendo a diversas técnicas de engenharia genética e de biologia molecular.

O gene *PIN6* apresenta níveis de expressão génica relativamente baixos e específicos, sendo detectado apenas em determinadas células e em etapas específicas do desenvolvimento vegetal. O *PIN6* é expresso durante a formação de raízes laterais desde a fase de iniciação da raíz, quando as células do periciclo, nos vasos condutores, são activadas para entrarem em divisão celular, até fases mais tardias, como a da emergência da raíz lateral. Na fase de indução de raízes laterais o gene *PIN6* encontra-se a marcar as células fundadoras da futura raíz lateral no periciclo. Numa fase posterior, de emergência das raízes laterais, PIN6 é expresso nas margens da raíz lateral, na zona de contacto com a raíz principal, formando uma estrutura em forma de anel. O *PIN6* está, ainda, presente no meristema apical caulinar, em domínios restritos localizados sob os locais de formação de novos órgãos e nos vasos condutores dos mesmos. Este gene parece estar maioritariamente relaccionado com o desenvolvimento de novos órgãos laterais ao nível dos meristemas.

Os avanços recentes ao nível da Biologia disponibilizaram uma elevada diversidade de estratégias para caracterização funcional de um determinado gene ou família de genes. O recurso a técnicas de genética reversa permite a caracterização funcional de um gene pela determinação dos efeitos causados pela sua ausência. Para a caracterização funcional do PIN6 procedeu-se a uma pesquisa de alelos provenientes de colecções de mutantes com inserções de T-DNA. Foram ainda criadas linhas transgénicas com redução dos níveis de expressão do *PIN6*. Para o efeito, recorreu-se à estratégia de RNA de interferência (RNAi).

Os fenótipos das diferentes linhas foram analisados em diversas condições de crescimento. As mutações resultaram em taxas de crescimento da raíz primária superiores e num aumento da produção de raízes laterais em relação às plantas de fenótipo selvagem. Tendo em conta esse fenótipo, a proteína PIN6 é, na generalidade, repressora dos processos de crescimento.

O fenótipo dos mutantes *pin6* é, ainda, afectado pelo fotoperíodo, uma vez que os fenótipos observados nas plantas crescidas em condições de dia longo foram mais drásticos do que os patentes nas crescidas em presença de luz contínua. Para avaliar uma possível regulação ao nível da

percepção da luz, as plantas foram crescidas sob luz contínua com radiações de diferentes comprimento de onda: branco, azul, vermelho, vermelho-longínquo e UV-B. Os mutantes *pin6* apresentam maior de-estiolação do hipocótilo sob radiação vermelho-longínqua e UV-B, tendo sido observada uma indução da expressão de *PIN6*, o que sugere uma interacção, se bem que indirecta, com pigmentos receptores de luz, dos quais se destaca o fitocromo A.

Adicionalmente, os mutantes germinaram mais cedo e fazem a transição meristemática da fase vegetativa para a fase reprodutiva antes do fenótipo selvagem, produzindo menos folhas roseta e menos ramificações secundárias nos caules e nas inflorescências. Estes resultados apontam para uma relação estrita entre o *PIN6* e genes de identidade dos meristemas vegetativo e floral.

Os mutantes *pin6* são ainda hipergravitrópicos, sugerindo um papel para PIN6 na percepção da gravidade, provavelmente em conjunto com PIN2. A proteína PIN2 foi já caracterizada como envolvida na resposta à gravidade e o seu gene é expresso juntamente com o *PIN6* nos tecidos na raíz. Os níveis de expressão destes dois genes são afectados por alguns factores comuns.

Actualmente está disponível uma vasta gama de informação proveniente de experiências de análise de transcritos a larga escala e de pesquisa *in vitro* de parceiros de interacção, especialmente em *Arabidopsis thaliana*. Cada vez mais se torna necessário recorrer a estratégias de biologia de sistemas que permitam análises transversais dos resultados obtidos, cruzando dados de fontes diversas para obtenção de informações sobre determinados genes ou processos de interesse. Uma análise *in silico* dos dados existentes forneceu informação adicional necessária à caracterização do gene *PIN6* no que diz respeito à compreensão da sua função e dos mecanismos de regulação que o regem nos processos de desenvolvimento vegetal mencionados, nomeadamente no que diz respeito às respostas a outras hormonas vegetais.

Dessa análise concluiu-se que a expressão de *PIN6* é afectada a diversos níveis, nomeadamente factores de transcrição para os quais existem elementos reguladores na sequência do promotor de *PIN6* e que estão envolvidos na iniciação de fases do desenvolvimento. A nível da iniciação das germinação e desenvolvimento foliar, o *PIN6* parece ser induzido pela diminuição dos níveis de LEC1, um factor de transcrição envolvido na dormência e em fases embrionárias do desenvolvimento foliar. *MAX4*, membro de uma família de genes envolvida na ramificação do caule, parece ser também um repressor da expressão do *PIN6*, uma relação já descrita para outros genes *MAX* e *PIN*. FLC e VIP estão envolvidos no controlo temporal da floração e percepção da vernalização, respectivamente, e interagem um com o outro para promover a iniciação floral estando, possivelmente, envolvidos no controlo da expressão do *PIN6*. Ao nível da formação de raízes laterais, a proteína PIN6 pode interagir com MDR4, uma proteína envolvida no transporte basipetal auxínico. A sobre-expressão de E2Fa-DPa, um factor de transcrição envolvido na divisão celular e ligado à resposta auxínica, resulta na repressão do *PIN6*. Adicionalmente, estão presentes no promotor do *PIN6* elementos reguladores do ciclo celular, bem como da síntese de componentes da parede celular, sugerindo que a função do PIN6 nas células meristemáticas ocorre principalmente ao nível da divisão celular.

As auxinas aumentam os níveis de transcriptos do *PIN6* e induzem a sua expressão ectópica em tecidos da raíz. Há evidências desta regulação ocorrer através da via que envolve TIR1-Aux/IAA-ARF e que corresponde à degradação dos repressores Aux/IAAs dependente do proteassoma, libertando os factores de transcrição ARF que, por sua vez, induzem possivelmente a expressão génica do *PIN6*.

Constata-se a existência de redundância aonível dos membros da família de proteínas PIN: no mutante *pin1* o domínio de expressão do PIN6 expande-se para filas adicionais de células companheiras dos vasos condutores, de modo a equiparar a falta de proteína PIN1 nessas células.

Com este trabalho pretendeu-se sugerir hipóteses explicativas dos mecanismos pelos quais PIN6 faz a ligação entre o transporte auxínico e os processos de desenvolvimento em que esta proteína está envolvida, nomeadamente na transição de fase, no estabelecimento e manutenção do meristema apical caulinar, na formação de novos órgãos, folhas e raízes laterais, e na resposta gravirópica. Neste trabalho propõem-se, ainda, estratégias para abordagem dos diferentes pontos que ainda carecem de esclarecimento.

Palavras-chave: transporte auxínico; manutenção do meristem; fotoperíodo; transição de fase; gravitropismo.

ABSTRACT

PIN6, a member of the PIN protein family of auxin transporters, is expressed in relatively low levels and in particular cells at distinct time points. This gene seems to be mainly involved with new lateral organ development. It is expressed in lateral roots, since early stages when pericycle cells are activated for cell division, and in the shoot apical meristem, in restricted domains directly below sites of new organ formation. Screening for T-DNA insertional mutant alleles and generation of knock-down transgenic lines for *PIN6* provided the tools to characterize this gene's function. Phenotypes of those lines were analyzed under different growth conditions and included faster growth rates, longer roots and production of more lateral roots. PIN6 is therefore likely to be a negative regulator of overall growth processes. *pin6* phenotype is regulated by photoperiodism, as phenotypes were more drastic under specific photoperiodic conditions. Furthermore, *pin6* mutants germinate and make meristem transition from vegetative to reproductive phase earlier than WT, producing less rosette leaves, less secondary branches and inflorescence stems. These results imply a tight regulation between PIN6 and both vegetative and floral meristem identity genes. In addition, *pin6* mutants are hypergravitropic, proposing a role for PIN6 in gravity perception, probably in a concerted fashion with PIN2. Auxin upregulates *PIN6* expression levels and induces its ectopic expression in additional root tissues. A certain degree of redundancy exists among PIN protein family members. In fact, in the *pin1* mutant PIN6 protein localizes to additional cell files, thus compensating for the absence of PIN1. An additional analysis of *in silico* available data from microarray experiments provided extra information required to better understand PIN6 function and its regulation, namely by other hormones. Explanations regarding the mechanisms by which PIN6 links auxin transport to developmental processes as phase transition, new lateral organ emergence and gravitropism, are proposed.

Keywords: auxin transport; meristem maintenance; photoperiodism; phase transition; gravitropism.

ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
µg	Microgram
µl	Microliter
2,4-D	2,4-Dichlorophenoxy acetic acid
A_{***}	Absorbance at **** nm
aa	Amino acids
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylate
AGI	Arabidopsis Genome Initiative
Amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammoniumperoxodisulfate
A.tumefaciens	*Agrobacterium tumefaciens*
AVG	Aminoethoxyvinylglycine
BCIP	5-bromo-4-chloro-3'-Indolyphosphate p-toluidine salt
BFA	Brefeldin A
Bisacrylamide	N,N'-methylenebisacrylamide
bp	Basepair
BP	BP recombination (Gateway®)
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CSPD	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	Cetyl trimethyl ammonium bromide
DEPC	Diethylpyrocarbonate
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetate
g	Gram
GA_3	Giberellic acid
Gm	Gentamycin
GUS	β-Glucuronidase
h	Hour
HOAc	Acetic acid
IAA	3-Indole acetic acid
IgG	Immunoglobulin G
IPTG	Isopropyl-ß-D-thiogalactopyranoside
J	Joule
Kan	Kanamycin
Kin	Kinetin
KOAc	Potassiumacetate
kb	Kilobase
kDa	KiloDalton
l	Liter
LR	LR recombination (Gateway®)
m	Meter
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mol	Mole
mRNA	Messenger ribonucleic acid
1-NAA	[alpha]-Naphthalene acetic acid
NaOAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
NH_4OAc	Ammonium acetate
nm	Nanometer
NP40	Nonylphenyl-polyethylene glycol
NPA	N-(1-naphthyl)thalamic acid
Nr.	Number
o/n	Overnight
OD	Optical density
oligo(dT)	Oligodeoxythymidylic acid
PAGE	Polyacrylamide gel electrophoresis
PCIB	p-Chlorophenoxyisobutyric acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
QC	Quiescent centre
Rif	Rifampicin
R.T.	Reverse Transcriptase
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation or revolutions per minute
RT	Room temperature
SDS	Sodium dodecylsulfate
ssDNA	Salmon sperm DNA
TEMED	,N,N´,N´-tetramethylethylenediamine
TIBA	2,3,5-Triiodbenzoic acid
Tris	Tris(hydroxymethyl) aminoethane
Triton X-100	Polyoxyethylene-p-isooctylphenol
tRNA	transfer RNA
Tween 20	Polyoxyethylene (20) Sorbitan Monolaurate
U	Unit of enzyme activity
UTR	Untranslated region
UV	Ultraviolet
V	Volt
vol.	Volume
X-Gluc	5-bromo-4-chloro-3-indoxyl β–D-glucuronidase
w	Weight
WT	Wildtype
Ω	ohm

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	Tryptophane
Y	Tyr	Tyrosine

NUCLEOTIDES

A	Adenine	
C	Cytosine	
G	Guanine	
T	Thymine	
U	Uracil	
R	A or G	Purine
Y	C or T	Pyrimidine
W	A or T	Weak hydrogen bonding
S	C or G	Strong hydrogen bonding
M	A or C	Amino group at common position
K	G or T	Keto group at common position
B	C, G or T not A	
D	A, G or T not C	
H	A, C or T not G	
V	A, C or G not T	
N	A, C, G or T	Any nucleotide

dNTP	2′-deoxynucleotide-5´-triphosphate
dTTP	2′-deoxycitidine-5´-triphosphate
UTP	uracil-5′-triphosphate

1 INTRODUCTION

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

Sir William Bragg

1.1 AUXIN IN PLANT DEVELOPMENT

Plants are sessile organisms and their non-motility is reflected in the way they grow. Only through a tight developmental control can they adjust to changes in the surrounding environment. Plants grow toward resources such as light, nutrients or water, adjust to soil changes and resist pathogens, among others. It is the aim of developmental biology to understand how growth, cell differentiation, and pattern formation are regulated at the cellular, biochemical, genetic and molecular levels.

Arabidopsis thaliana is a model plant that provides many advantages for molecular research. It is a small plant, easy to handle, has a short generation time and produces a large number of offspring. At the moment, there are mutations available for theoretically every gene. Its genome is relatively small and since its complete sequencing (Arabidopsis Genome Initiative, 2000), analysis of specific processes and characterization of individual or families of genes/proteins became more accessible (reviewed by Somerville and Meyerowitz, 2001).

Plant hormones (phytohormones) are small organic molecules that affect diverse developmental processes specifically. In contrast to animal hormones, which are produced in specific organs, phytohormones are produced throughout the plant. Virtually every aspect of plant development from embryogenesis to senescence is under hormonal control. Generally, this developmental control is exerted by controlling cell division, expansion, differentiation and cell death. Many developmental processes can be controlled in this way, including formation of the apical-basal and radial pattern, seed germination, determination of plant architecture, flowering, fruit ripening and shedding (reviewed in Bishopp *et al.*, 2006). For instance, auxin is required for the formation of nodule structures upon wounding stress in hop (Santos, 2006), facilitating mycorrhization in chestnut (Barker and Tagu, 2000), among other processes. Cytokinin is required for calla lily regreening process of the spathe (Pais and Chaves das Neves, 1982/83), a natural process involving plant senescence. Plants have a wide array of hormones, including steroids and peptides, as well as the five classical classes of phytohormones: auxins, abscisic acid, cytokinins, ethylene and gibberellins. There is a certain degree of crosstalk and interaction between pathways downstream of these hormonal signals.

Development and organization of plant structures imply that cells are highly sensitive to positional information. The hormones auxin and cytokinin are primary signalling molecules (Barker and Tagu, 2000;

Gattolin *et al.*, 2006; Jasinski *et al.*, 2005; Kiba *et al.*, 2004; Okada *et al.*, 1991; Uggla *et al.*, 1996; Uggla *et al.*, 1998). Polar auxin transport provides the positional cues required for specifying organized plant structures, acting as a morphogen (Barker and Tagu, 2000; Benková *et al.*, 2003; Bennett *et al.*, 2006; Berleth and Sachs, 2001; Bhalerao *et al.*, 2002; Blilou *et al.*, 2005; Friml *et al.*, 2003; Noh *et al.*, 2003; Reinhardt *et al.*, 2003; Weijers *et al.*, 2005b). The fate of developing tissue can therefore be determined by the sensitivity of growing cells to auxin and the relative concentrations of other phytohormones. Auxin is readily conjugated to larger molecules that render it inactive. In fact, the majority of indole-3-acetic acid (IAA) in the plant is in the form of inactive conjugates. Auxin conjugation and catabolism can therefore decrease active auxin levels. *De novo* synthesis and hydrolysis of conjugates counterbalance the developmental regulation of auxin homeostasis by increasing active auxin levels (Ljung *et al.*, 2005). Young aerial tissues and roots, particularly in the RAM, are sites of auxin synthesis. Auxin is synthesized from indole through tryptophan-dependent and tryptophan-independent pathways (reviewed in Woodward and Bartel, 2005). The existence of multiple pathways for IAA biosynthesis and the absence of fully auxin-deficient mutants identified (suggesting that mutations eliminating auxin are lethal) is a reflection of how important this hormone is in plant development (reviewed in Teale *et al.*, 2006).

In higher plants, auxin is involved in embryogenesis, organogenesis, root meristem maintenance, vascular tissue differentiation, hypocotyl and root elongation, apical hook formation, apical dominance, fruit ripening, growth responses to environmental stimuli, among others.

Perturbations involving auxin may result from interference with auxin signalling at four levels (Berleth and Sachs, 2001): auxin synthesis, and of the relations between the age of the tissue or environmental conditions; auxin transport, which can be critical to the localisation of auxin in responding tissues; changes within cells might affect the activity of auxin on receptors; and auxin metabolism might be disrupted in some way.

The developmental patterning processes appear to be flexible and to emerge from complex intercellular crosstalk. It is plausible that auxins act as intercellular messengers in patterning processes in embryos, meristems and vascular development, and that auxin-mediated long-distance signalling could simultaneously integrate morphogenesis throughout the plant (Berleth and Sachs, 2001). Our understanding of the precise mechanisms by which auxin regulates morphogenic processes is, at best, fragmentary. The development of new technologies for classical biochemical approaches, and the widespread use of model plants such as *Arabidopsis thaliana* for genetic and molecular studies have led to great advances in the biology of plant hormones. Several genes involved in synthesis, conjugation, transport, perception and/or signal transduction of IAA have already been identified (Abel and Theologis, 1996; Benková *et al.*, 2003; Bennett *et al.*, 1996; Bennett *et al.*, 2006; Dharmasiri *et al.*, 2005a; Friml *et al.*, 2002a; Friml *et al.*, 2002b; Gälweiler *et al.*, 1998; Geisler *et al.*, 2005; Gray *et al.*, 1999; Guilfoyle, 1998; Jaillais *et al.*, 2006; Müller *et al.*, 1998; Noh *et al.*, 2001; Okada *et al.*, 1991; Steinmann *et al.*, 1999; Ulmasov *et al.*, 1997a; Ulmasov *et al.*, 1997b), providing us with both the conceptual structure and the experimental tools to investigate plant structure and morphogenesis at the molecular level. The use of these mutants led to the isolation of genes encoding auxin receptors, such as the TIR1 auxin receptor (Dharmasiri *et al.*, 2005a;

Kepinsky and Leyser, 2005; Tan *et al.*, 2007), transporters and proteins involved in subsequent signal transduction processes, thus linking auxin to several morphogenic processes.

Pleiotropic effects of the exogenous application of hormones complicate full understanding of the underlying mechanisms controlling specific hormone activities in the SAM. Crosstalk between different classes of hormones, such as auxin and cytokinin, or cytokinin and gibberellin, is common in plant development. Both auxin and ethylene contribute to several developmental processes. In root development, ethylene-regulated growth is dependent on auxin transport from the root apex via the lateral root cap and on auxin responses occurring in multiple elongation zone tissues (Swarup *et al.*, 2007). The ability of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to inhibit root cell elongation was significantly enhanced in the presence of auxin. By upregulating auxin biosynthesis, ethylene facilitates its ability to inhibit root cell expansion (Swarup *et al.*, 2007). Synergistic effects of auxin and ethylene have been reported for the regulation of hypocotyl elongation (Smalle *et al.*, 1997; Vandenbussche *et al.*, 2003), root hair growth and differentiation (Pitts *et al.*, 1998), apical hook formation (Li *et al.*, 2004), root gravitropism (Buer *et al.*, 2006), and root growth (Pickett *et al.*, 1990; Rahman *et al.*, 2001), suggesting that these two signalling pathways also interact at the molecular level. A component involved in this auxin-ethylene crosstalk is the POLARIS (PLS) peptide (Chilley *et al.*, 2006). Mutation of PLS results in an enhanced ethylene-response phenotype, defective auxin transport and homeostasis, and altered microtubule sensitivity to inhibitors (Chilley *et al.*, 2006). *PLS* expression is repressed by ethylene and induced by auxin (Chilley *et al.*, 2006). The authors suggest that PLS is a negative regulator of ethylene response, modulating cell division and expansion via downstream effects on microtubule cytoskeleton dynamics and auxin signalling, thereby influencing root growth and lateral root development (Chilley *et al.*, 2006).

1.2 AUXIN MODE OF ACTION: PERCEPTION AND SIGNALLING

Auxin-regulated gene expression triggers most processes controlled by this hormone. Many auxin-induced genes are regulated by the interplay of two classes of transcription factors: auxin-response factors (ARFs) and the Aux/IAA repressors (Hagen and Guilfoyle, 2002). Several Aux/IAA genes are transcribed within minutes of exposure to auxin or protein synthesis inhibitors. They form homo- and heterodimers with one another, as well as with members of the ARF family (reviewed in Ulmasov *et al.*, 1997b; Kim *et al.*, 1997). Aux/IAA repressors are composed of four conserved domains, of which domain II is essential for the instability of these proteins - it contains a degron sequence (GWPPV) which is the target of the SCF ubiquitin ligase complex (Gray *et al.*, 1999; Kepinsky and Leyser, 2004). ARFs bind to auxin-response promoter elements (AuxREs) of auxin-responsive genes (Kim *et al.*, 1997; Ulmasov *et al.*, 1997b). At basal concentrations of auxin, Aux/IAA repressors are relatively stable – through domains III and IV they can

homodimerize and heterodimerize with ARF. ARF-bound Aux/IAA proteins block transcription from auxin-responsive promoters by controlling the amount of free ARF transcription factors (Ulmasov *et al.*, 1997a). Conversely, when auxin concentrations rise above a certain threshold level, Aux/IAA repressors are destabilized (Tiwari *et al.*, 2001; Zenger *et al.*, 2001). An increase in auxin levels promotes the proteasome mediated degradation of Aux/IAAs, which results in an increasing number of active ARF proteins and transcriptional activation of auxin regulons (Tiwari *et al.*, 2003). In this situation, ARF-ARF dimer binding to AuxREs is facilitated and thereby enables the expression of certain auxin response genes. The large number of Aux/IAA and ARF allow for diverse combinatorial interactions between Aux/IAA and ARF, establishing a regulatory code that programmes auxin responses in a spatial-temporal defined window.

Some of the most related ARF and Aux/IAA proteins share similar expression patterns (Weijers *et al.*, 2005a). In early embryogenesis, MONOPTEROS (MP)/ARF5 and BODENLOS (BDL) /IAA12 physically interact and are coexpressed (Hamann *et al.*, 2002). SHORT HYPOCOTYL2 (SHY2)/IAA3 and NON-PHOTOTROPIC HYPOCOTYL4 (NPH4)/ARF7 or ARF19 pairs regulate auxin response in the root (Weijers *et al.*, 2005a), while MASSUGU2 (MSG2)/IAA19 and NPH4/ARF7 interact during hypocotyl growth and lateral root development (Tatematsu *et al.*, 2003).

The SCF (SKP-Cullin-F-box) complex is involved in a wide range of signal transduction processes by ubiquitylating target proteins that are selected by F-box proteins. SCF complexes select and covalently modify their target proteins through addition of several ubiquitin peptides, forming a multi-ubiquitin chain that targets them for degradation by the 26S proteasome (reviewed in Teale *et al.*, 2006). The F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) was identified in the past few years as a receptor for IAA (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005). There are over 700 F-box proteins in *Arabidopsis* (Gagne *et al.*, 2002) and TIR1 belongs to a small subfamily of seven related genes (Dharmasiri *et al.*, 2005b). TIR1 is a component of the SCF^{TIR1} ubiquitin-protein ligase protein complex (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005), which also includes the scaffold protein cullin (CUL1), SKP1-like proteins (ASK1/ASK2), and the ring-domain protein RBX1 (Gray *et al.*, 1999; reviewed in Moon *et al.*, 2004). The core cullin, SKP1-like and RBX1 proteins provide the catalytical activity necessary for the transfer of the activated ubiquitin to the target protein, whereas target specificity is conferred by the F-box protein (Gray *et al.*, 2001).

Loss-of-function *tir1* mutants show mild alterations in auxin response and development (Dharmasiri *et al.*, 2005b; Ruegger *et al.*, 1998). A quadruple mutant for *TIR1* and its three most closely related genes, *AUXIN SIGNALLING F-BOX PROTEINS 1*, *2* and *3* (*AFB1-AFB3*) shows a more severe phenotype and is auxin insensitive. Moreover, AFB proteins interact with Aux/IAAs in an auxin-dependent manner, indicating that auxin binding is collectively mediated by TIR1 and the AFB proteins (Dharmasiri *et al.*, 2005b). The phenotypic variability and presence of some auxin signalling in the quadruple mutant may be explained by residual AFB activity or by the presence of other genes coding for auxin receptors (reviewed in Bishopp *et al.*, 2006).

TIR1 recruits its substrates, Aux/IAA repressors, in an auxin concentration-dependent manner, targetting them for degradation. More recently, its crystal structure was resolved, revealing how auxin

molecules fit into a surface pocket of TIR1, filling a hydrophobic cavity at the protein interface, thus enhancing the interactions between Aux/IAA repressors and TIR1 (Tan *et al.*, 2007). Crystal structures revealed the mushroom shaped complex formed between TIR1 and ASK1, with the leucine-rich-repeat domain of TIR1 forming the cap, and the F-box of TIR1 along with ASK1 forming the stem. On top of the TIR1 leucine-rich-repeat domain lays a "pocket" which functions in both auxin binding and substrate recruitment (Tan *et al.*, 2007). The Aux/IAA peptide binds in close proximity to the auxin-binding site in the upper part of the pocket. The GWPPV motif is packed directly against auxin and covers the auxin binding site. This "traps" auxin in the binding pocket until the Aux/IAA peptide is released. Not only does TIR1 bind IAA, it also binds two synthetic auxins, 1-NAA and 2,4-D, with different affinities, IAA showing the highest (Tan *et al.*, 2007).

It is possible that the SCF^{TIR1} complexes do not account for all of the responses of *Arabidopsis* to auxin, such as plasma membrane-associated proton pump. AUXIN BINDING PROTEIN1 (ABP1) 1 is a candidate for such an extracellular receptor, as changes in ion transport associated with early stages of auxin-induced growth can be inhibited by extracellular treatment with anti-ABP1 antibodies (Leblanc *et al.*, 1999). ABP1 is a soluble, ER-located, dimeric glycoprotein, involved in cell expansion, stomatal closure, plasma-membrane hyperpolarization and cell division (Chen *et al.*, 2001; Rück *et al.*, 1993; Steffens *et al.*, 2001). However, there is no data available on downstream signalling events after the binding of auxin to ABP1 or whether there might be a link to the SCF^{TIR1} pathway. Considering the almost instantaneous auxin responses that ABP1 can mediate, it is possible that gene expression is not required to be involved in certain aspects of auxin signalling (reviewed in Teale *et al.*, 2006).

1.3 AUXIN TRANSPORT

Auxin transport and distribution are essential for polar development in plants, and their capacity to respond to environmental stimuli. Auxin is synthesized in young, apical tissues, but acts in virtually all parts of the plant.

Two main pathways describe the transport of auxin: a fast, non-directional transport in the phloem and a slower, directional, so-called polar auxin transport in various tissues. The phloem transport occurs in both basipetal (from the apex towards the base) and acropetal (from the base towards the apex) directions, proceeds relatively fast (5-20 cm/h) and correlates well with transport of assimilates. This transport often corresponds to the transport of inactive auxin conjugates. In contrast, polar auxin transport is specific for active free auxins, occurs in a cell-to-cell manner and has a strictly unidirectional character. Polar auxin transport requires energy, is saturable and sensitive to protein synthesis inhibitors, which taken together suggest the existence of specific auxin transport proteins. The Chemiosmotic Hypothesis (Rubery and Sheldrake, 1974; Raven, 1975) explains polar auxin transport: in the relatively acidic environment of the cell

wall (pH around 5.5) about 15% of IAA exists in its protonated form (IAAH). This non-charged, lipophilic molecule passes easily through the plasma membrane by diffusion. In the cytoplasm, where the pH is more basic (around 7) IAAH dissociates and the resulting IAA^- anion is imprisoned inside the cell due to its low membrane permeability (Fig. 1.1.). Therefore, the existence a specific efflux carrier was postulated and the polarity of the flux is explained by the protein's asymmetric distribution in cells.

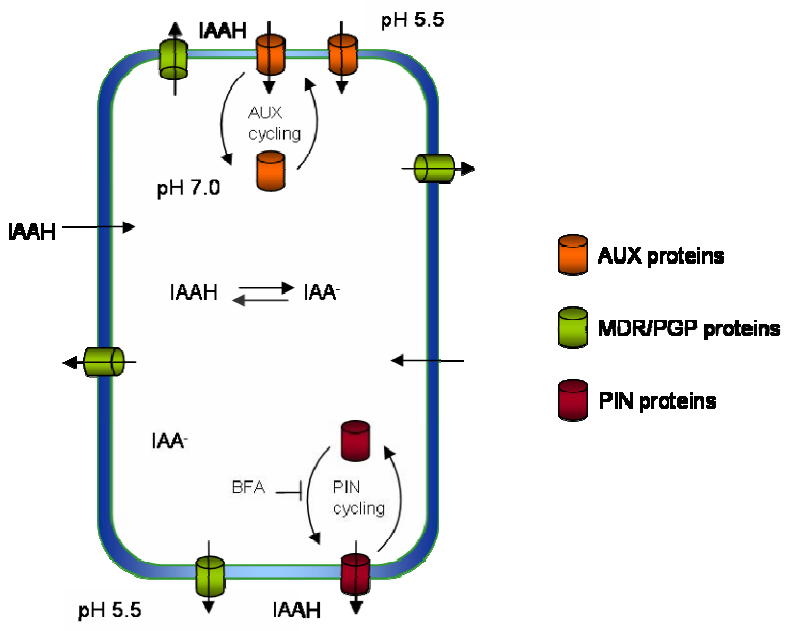

Fig. 1.1. Chemiosmotic hypothesis of polar, cell-to-cell, auxin transport. A pH gradient across the plasma membrane leads to the accumulation of IAA in the cell. A higher pH inside the cell promotes auxin molecules (IAAH) dissociation, rendering them unable to pass passively back through the membrane. Therefore, IAA^- becomes trapped inside the cell. Auxin efflux carriers (PINs, MDR/PGPs) are thus required to transport auxin out of the cell. In addition, auxin influx carriers (AUX) transport auxin anions (IAA^-) into the cell. The polar subcellular localization of PIN proteins is important for directional auxin transport, and is accompanied by constitutive endocytic cycling of the PIN proteins. Auxin itself inhibits endocytosis of PINs, increasing their levels at the cell surface. The inhibitor of vesicle trafficking BFA represses the exocytosis of PINs but not of AUX1. The subcellular dynamics of PGP proteins is as yet unclear.

Polar auxin transport is then required for local accumulation of auxin, necessary for developmental processes such as embryo axis formation (Friml *et al.*, 2003), organ development (Reinhardt *et al.*, 2000), root meristem maintenance (Sabatini *et al.*, 1999) or tropic growth responses (Friml *et al.*, 2002b; Ottenschläger *et al.*, 2003). Several approaches enable us to visualize such local accumulations of auxin and the gradients created thereby, which include the auxin responsive promoter DR5 (Ulmasov *et al.*, 1997b), immunolocalization of IAA (Avsian-Kretchmer *et al.*, 2002) or direct auxin measurements in tissue sections (Casimiro et al., 2001; Marchant *et al.*, 2002; Ljung *et al.*, 2005).

1.3.1 INFLUX AND EFFLUX TRANSPORTERS

Even though the rates of auxin synthesis and conjugation are important for the overall auxin status of the plant, it is the fine concentration gradients across only a few cells that have powerful effects on plant development, as they provide vectorial information to the tissues (reviewed in Teale *et al.*, 2006).

AUX1 is an auxin cellular influx carrier present in positions consistent with vascular loading at the sources of synthesis (in leaves) and unloading at the sink tissues (in roots; Marchant *et al.*, 2002). Establishment of apical-basal epidermal polarity in Arabidopsis roots is also dependent on AUX1 activity, since *aux1* mutant displays apical shifting of root hair initiation consequence of change on trichoblast polarity (Grebe *et al.*, 2002). *aux1* shows a strong agravitropic phenotype, suggested to be a result from disruption of basipetal auxin transport in root and/or loss of AUX1 expression in gravi-sensing columella cells (Marchant *et al.*, 1999; Swarup *et al.*, 2001). AUX1 transports auxin directly (Yang *et al.*, 2006) and its asymmetric subcellular localization is dependent on an ER protein AXR4 specifically involved in AUX1 trafficking to the plasma membrane (Fig. 1.1**.;** Dharmasiri *et al.*, 2006). The other members of the *LAX* (*AUX--like*) gene family remain to be characterized.

Polar auxin transport is also dependent on a group of ABC transporters belonging to the MULTIDRUG RESISTANCE (MDR)-like family, also known as the P-GLYCOPROTEINS (PGPs; Sanchez-Fernandez *et al.*, 2001; Martinoia *et al.*, 2002; Noh *et al.*, 2001). Knockout mutations in the *Arabidopsis thaliana MDR1* gene blocks 80% of basipetal transport in seedling hypocotyls and in the inflorescence stem (Noh *et al.*, 2001). PGP1, the family member most closely related to MDR1, is also involved in basipetal auxin transport in stems (Noh *et al.*, 2001). These MDRs may directly transport IAA, as shown in protoplasts, cell suspension cultures or by expression in mammalian cell lines (Geisler *et al.*, 2005; Bouchard et al., 2006; Petrášek *et al.*, 2006), or aid in polar localization of PIN efflux facilitators (Noh *et al.*, 2003), or both. MDR1's promoter is active throughout the root (Noh *et al.*, 2001), and *MDR1* is one of the highly expressed family members in most root tissues (Birnbaum *et al.*, 2003). *MDR4* may also contribute to auxin transport in the root, as it is expressed in the root cap and the epidermal cells of the root apex (Birnbaum *et al.*, 2003; Terasaka *et al.*, 2005). A 30% reduction in root basipetal auxin transport was measured in *pgp4-1* mutant, an allele of *mdr4* (Terasaka *et al.*, 2005).

1.4 AT*PIN* FAMILY OF AUXIN EFFLUX CARRIERS

The *PIN* gene family of *Arabidopsis* consists of eight transmembrane proteins involved in auxin efflux and whose sequences differ mainly in the central hydrophilic region. Homologous genes have already been found in other plant species (reviewed in Paponov *et al.*, 2005). The PIN family shows a relatively high

similarity in the two groups of membrane-spanning domains, located at the N- and C-termini of the proteins, and a high heterogeneity in the central hydrophilic region (Fig. 1.2.). PIN5 and PIN8 are different in that they lack a central loop domain. The identity between any two members of the family ranges from 32% to 85% (PIN3 and PIN7; Paponov *et al.*, 2005).

The predicted topology of the PIN proteins is similar to several membrane transporters in bacteria and prokaryotes. Indeed, immunolocalization assays located PIN1 to the plasma membrane, in a polar fashion, at the basal side of xylem and cambial cells in the inflorescence axis (Gälweiler *et al.*, 1998). This pattern supports further the hypothesis that PIN1 is regulating auxin transport throughout the plant, as proposed by the chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975). Recently, a set of experiments performed in HeLa cells, a non-plant system, resolved the question of whether PIN proteins play a catalytic role in auxin efflux or solely act as positive regulators of endogenous plant auxin efflux carriers (Petrášek *et al.*, 2006). Indeed, PINs are transporting auxin, given that cells transfected with PIN2 and PIN7 showed strong PIN expression and a substantial increase in the net flux of natural auxin IAA (Petrášek *et al.*, 2006). Whether or not other plant-specific co-factors are required for auxin efflux is not yet known. Furthermore, at least four of the other PINs (PIN1, PIN4, PIN6 and PIN7) are rate-limiting for auxin efflux from plant cells (Petrášek *et al.*, 2006).

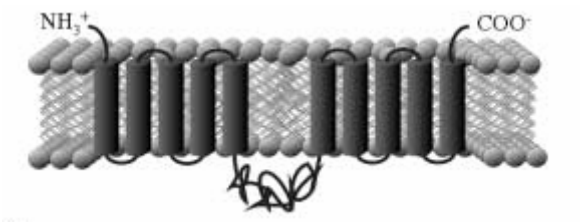

Fig. 1.2. Typical PIN protein topology: two membrane-spanning domains composed of five to six transmembrane sequences linked by a central hydrophilic loop.

The five PIN genes characterized so far have been linked to organogenic (PIN1 and PIN4), embryogenic (PIN1, PIN4 and PIN7), gravitropic (PIN2 and PIN3) and phototropic (PIN1 and PIN3) processes (Friml *et al.*, 2002a; Friml *et al.*, 2002b; Müller *et al.*, 1998; Paponov *et al.*, 2005; Sabatini *et al.*, 1999). PIN2 localizes to the upper membrane of the epidermal and cortical cells of root apices, regulating acropetal auxin transport essential for gravitropic responses (Müller *et al.*, 1998). PIN2 protein in roots is predominantly present at the basal side of epidermal cell files oriented towards the elongation zone and further on the apical side in root cortical cells. PIN3 is mainly localized in the lateral walls of the endodermal cells in the shoot and in root columella cells it has a uniform distribution (Friml *et al.*, 2002b). Upon gravistimulation, PIN3 becomes polarized to the lateral side of the columella cells and later to lateral root cap (Friml *et al.*, 2002b). The polar localization of PIN4 in the root quiescent centre directs the auxin flow towards the initial columella cells, the location of main auxin accumulation in the root apex (Friml *et al.*, 2002a; reviewed in Friml, 2003; Sabatini *et al.*, 1999). PIN7 is apically localized in the basal cell of the embryo and later in the suspensor cells (Friml *et al.*, 2003). PIN7 has been ascribed to the establishment of

the apical-basal axis by efflux-dependent auxin gradient during embryogenesis, as it is expressed immediately after the zygotic division, being the first marker for polarity (Friml *et al.*, 2003).

Absence of PINs results in aberrant local auxin accumulation patterns and most aspects of the respective mutant phenotypes can be phenocopied by chemically inhibiting polar auxin transport (Friml *et al.*, 2003; Friml *et al.*, 2002a; Friml *et al.*, 2002b; Luschnig *et al.*, 1998). Loss-of-function *pin1* mutants grow a single naked pin-shaped stem after floral transition, without any floral organs (Okada *et al.*, 1991), a phenotype due to lower rates of auxin transport (Gälweiler *et al.*, 1998). In fact, application of auxin to the *pin1* stem results in promoting lateral growth (Reinhardt *et al.*, 2003).

Members of the PIN protein family are considerably homologous and show significant functional overlap among them, as shown by the increasingly severe phenotypes of the multiple *pin* mutants (Friml *et al.*, 2003; Blilou *et al.*, 2005; Vieten *et al.*, 2005). Moreover, the most similar family members can complement each other in knockout mutants. For example, in the *pin1* background, PIN4 expression domain extends into the cells where PIN1 would have been present (Blilou *et al.*, 2005).

To this moment, all PIN proteins analyzed show subcellular asymmetric localization within auxin transport-competent cells, even though some may be found in specific cell types without pronounced polarity. Polarity of PIN localization correlates with the direction of auxin transport and/or with the local accumulation of auxin in adjacent cells, suggesting that PIN polar localization directs the intercellular auxin flow (Benková *et al.*, 2003; Blilou *et al.*, 2005; Friml *et al.*, 2003; Friml *et al.*, 2002a; Friml *et al.*, 2002b; Gälweiler *et al.*, 1998; Heisler *et al.*, 2005; Müller *et al.*, 1998). Recently, by manipulating PIN polarity and monitoring auxin transport it has been proposed that PIN localization is sufficient to direct auxin flow in plants (Wiśniewska *et al.*, 2006).

Auxin induces the expression of many PIN proteins in an Aux/IAA-dependent manner (Vieten *et al.*, 2005). However, correct expression of PIN proteins is dependent on pre-existing cell patterning rather than auxin concentration (Xu *et al.*, 2006). Moreover, auxin alone is not sufficient for root specification in the absence of PIN-mediated auxin transport (Weijers *et al.*, 2006). It is therefore unlikely that PINs alone determine cell specification or polarity. Taken together, the results suggest that PINs mediate distinct developmental signals as part of a wider developmental programme (Xu *et al.*, 2006).

It is essential to understand the molecular mechanism of targetting auxin transport components to opposite sides of the cell. The relationship between PINs and other auxin transporters is still unclear. It seems that there are at least two different polar targetting machineries, as AUX1 and PIN proteins are controlled by different subsets of vesicle trafficking pathways, showing different sensitivities to various inhibitors (Kleine-Vehn *et al.*, 2006). PIN1 is mislocalized in the *pgp1 mdr1* double mutant, suggesting a certain extent of control of the MDR/PGPs over PIN localization (Noh *et al.*, 2003). PGP1 and PGP19 co-localize with PIN1 in the shoot apex and with PIN1 and PIN2 in root tissues. In addition, PGPs and PINs interact in yeast 2-hybrid and co-immunoprecipitation essays, suggesting that PIN-PGP interactions are required to enhance auxin transport activity (Blakeslee *et al.*, 2007). Quite contradictory, it has also been shown that PIN1 function is not dependent on the presence of other factors, which includes MDR/PGPs (Petrášek *et al.*, 2006).

Active auxin transport mediates cellular auxin concentration and is therefore a determinant part in the creation of gradients crucial for coordination of plant development. There are additional factors contributing to the relationship between auxin signalling and transport. Many kinases are regulated by auxin (Beltran-Peña *et al.*, 2002; Kovtun *et al.*, 2000; Mockaitis *et al.*, 2000) and members of the MAP kinase cascade, including MAP KINASE KINASE-7 (MKK7), have been demonstrated to negatively regulate polar auxin transport (Dai *et al.*, 2006). RAC-LIKE (ROP) GTPases may also have a role in auxin action. Auxin activates ROP3 (Tao *et al.*, 2002), causing Aux/IAA proteins to aggregate. These nuclear protein bodies activate the 26S proteasome to mediate degradation of Aux/IAA proteins. Consequently, this could be the connection between auxin perception at the plasma membrane and control of gene regulation in the nucleus (Tao *et al.*, 2002; Tao *et al.*, 2005).

1.4.1 PINs IN PLANT DEVELOPMENT

1.4.1.1 PINs in leaf development and phyllotaxis

During embryogenesis the basic plant body plan is established, including the formation of meristems that will perpetuate cell division and originate new organs in the mature plant. The apical-basal axis is defined, with the shoot apical meristem being located at one end of the axis and the root apical meristem in the opposite. While during embryogenesis all cells undergo division, after germination further growth and development becomes restricted to special areas of the plant that maintain embryonic character - the meristems. Meristem cells divide to create the tissues and organs responsible for the general architecture, shape and size. Primary root and shoot meristems are formed during embryogenesis. Most plants also develop secondary meristems during postembryonic development, such as axillary meristems, floral meristems, intercalary meristems and lateral meristems (*e.g.* lateral roots). The vegetative shoot apical meristem is usually indeterminate in its development, *i.e.*, shows no predetermined limit to growth. In contrast, floral meristems are determinate: all meristematic activity stops when the last floral organs have been generated.

Meristematic undifferentiated cells that retain the ability for cell division indefinitely are stem cells. Similarly to animal stem cells, when plant stem cells divide, one of the cells retains the identity of the mother stem cell while the other undergoes a specific developmental program leading to its differentiation.

The shoot apical meristem (SAM) is located at the shoot apex and leaves, stems and axillary meristems are produced from its derivative cells. The SAM is composed of three concentric tissue layers: L1 (outermost), L2 and L3 (innermost). It can also be divided into different histological zones. The peripheral zone (PZ) flanks the central zone (CZ). Underneath lies the rib zone (RZ), which gives rise to the internal tissues of the stem. Lateral organs initiate from the PZ, at the meristem flanks. The CZ contains

self-maintaining, slowly dividing cells – the stem cells, which provide initials for both the PZ and the RZ (Bowman and Esched, 2000).

Coordinating the formation and differentiation of pluripotent stem cells in apical meristems seems to depend on transcriptional regulation and intercellular signalling. Several classes of transcription factors have been shown to take part in SAM maintenance. The indeterminate nature of the meristem and the formation of organ boundaries require class I *KNOTTED1*-like homeobox (*KNOX1*) genes, expressed in many plant species in specific patterns in the SAM (reviewed in Hake *et al.*, 2004). Members of the *NAC* group of transcription factors, including *NO APICAL MERISTEM* (*NAM*), *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *CUC3*, are also essential for meristem establishment and organ boundary formation. Their expression in narrow strips in the SAM will correspond to the future organ–organ and meristem-organ boundaries (Laufs *et al.*, 2004; Mallory *et al.*, 2004).

The patterning of the SAM and its stem cell niche is tightly controlled by a central regulatory mechanism in which the homeodomain protein WUSCHEL (WUS) specifies stem cell identity. In the active shoot meristem, WUS is expressed in a small cell group underneath the presumed position of the stem cells and positively regulates the synthesis of the small secreted peptide CLAVATA3 (CLV3) ligand at the stem cell niche (Laux *et al.*, 1996). CLV3, in turn, acts together with the receptors CLV1/CLV2, found mostly in an underlying domain of the L3 layer, to repress *WUS* expression, thereby creating a negative feedback loop that restricts the size of the stem cell population (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Mayer *et al.*, 1998; Schoof *et al.*, 2000; Trocochaud *et al.*, 2000). In addition, *CLV3* has been shown to be required for the dynamic regulation of meristem size, restricting the domain of its own expression in the CZ by preventing re–specification of PZ cells as CZ cells (Reddy and Meyerowitz, 2005). CLV3 has a long-range effect on cell division rate to restrict SAM size. Increasing CLV3 levels at its native expression domain results in repression of WUS expression. Balance signalling between CLV3/WUS seems to coordinate the cell-fate at the meristem: cells with higher CLV3 expression allow cells to adopt peripheral cell identity while WUS-expressing cells keep meristematic identity (Müller *et al.*, 2006). Additional signalling components may be necessary to stabilize WUS expression at the meristem organizing center.

The formation of new organs during plant development obeys predetermined genetic programmes, as well as possesses an inherent plasticity allowing the plant body to organize and adapt in response to environmental cues. Correct SAM function requires the maintenance of a delicate balance between the production of lateral organs from its flanks and indeterminate growth at its center. Leaf primordia arise from a small subset of cells in the L1 and L2 layers of the PZ that acquires leaf founder cell identity. Cells from the L1 layer originate epidermis, cells from the L2 layer differentiate as photosynthetic mesophyll cells and cells from L3 give rise to vascular elements and bundle sheath (reviewed in Sinha, 1999). These leaf primordia are separated from the main shoot through the establishment of a boundary between the organ-forming cells and the meristem cells. Once leaf primordia initiate, leaves grow rapidly through active cell division and expansion from the

initial primordia cells. Mature organs present three developmental axes of polarity: the proximodistal, the dorsoventral (adaxial-abaxial), and mediolateral axes.

A number of mutants displaying leaf formation defects have been isolated in *Arabidopsis thaliana*. Plants mutated in either *ASYMMETRIC LEAVES1* (*AS1*) or *AS2* form asymmetric, rumpled, lobed leaves with ectopic leaflet-like organs on the petioles (Byrne *et al.*, 2000; Semiarti *et al.*, 2001; Sun *et al.*, 2002). AS1 encodes a MYB domain–containing putative transcription factor (Byrne et al., 2000) and AS2 is a member of the plant-specific LATERAL ORGAN BOUNDARIES (LOB) domain (LBD) family of proteins, encoding a widely expressed protein with a Leu zipper motif (Iwakawa *et al.*, 2002). AS1 and AS2 function in overlapping developmental pathways (Serrano-Cartagena *et al.*, 1999; Ori *et al.*, 2000; Semiarti *et al.*, 2001) and physically interact with one another (Xu *et al.*, 2003). Both genes repress expression of three class I *KNOX* genes, *BREVIPEDICELLUS* (*BP*), *KNAT2*, and *KNAT6*, which promote the activity and maintenance of the SAM (Byrne et al., 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001). While *AS1* and *AS2* in turn repress *BP*, *KNAT2*, and *KNAT6* activity in the initiating primordia, class I *KNOX* gene *STM* negatively regulates *AS1* and *AS2* expression in the SAM maintaining a pool of meristematic cells (Byrne *et al.*, 2000, 2002). This results in a reciprocal negative molecular interaction that promotes leaf initiation at the periphery of the meristem.

Phyllotaxis, the regular way in which leaves are arranged around the stem, is therefore a consequence of the precise spatial regulation of growth within the apex, reflected in the number and order in which leaf primordial form. The relative stage of lateral primordium development is described in terms of plastochron (P), whereby the latest emerging primordium is termed P1, the next oldest primordium is P2 and so forth. The region within the meristem from which the next lateral organ primordium will be formed is then termed P0. Phyllotaxis is characterized by the divergences angles between two consecutively generated organs. The most common patterns in nature are spirals with divergence angles of 137.5^{o}. Mathematical modelling suggests that spiral leaf arrangements are superior for light capture or as a solution to a packing problem (reviewed in Kuhlemeier, 2007).

The current model - the Canalization Hypothesis - explaining the regulation of leaf primordium formation and phyllotaxis by auxin transport proposes that auxin is transported by PIN1 in epidermal cells towards the SAM, where it further induces PIN1 expression, which will, in turn, promote auxin accumulation at the site of incipient primordium formation (Reinhardt *et al.*, 2003). Leaf primordia then drain auxin through their central midveins, causing auxin depletion in the surrounding cells. Through a combination of positive feedback (*i.e.* auxin accumulation) and lateral inhibition (*i.e.* depletion of auxin from adjacent tissues), auxin accumulates at certain distances from the existing primordia, allowing for the phyllotactic patterning. Consistently with this model, direct auxin application or auxin transport inhibition enlarges primordia or reduces their lateral separation, eventually leading to primordium fusion (Reinhardt *et al.*, 2000). Additionally, inhibition of polar auxin transport through chemicals or by mutations in PIN1 specifically inhibits organogenesis. A naked meristem that grows normally but forms no lateral organs will arise. The defect can be rescued by the application of a microdroplet of auxin to the peripheral zone of such a pin-shaped meristem (Reinhardt *et al.*, 2000). Auxin accumulates at sites of incipient organ formation

(Heisler *et al.*, 2005) and localized application of auxin can induce ectopic organs (Reinhardt *et al.*, 2000). Therefore, auxin is required for the induction of lateral organs.

The cycle of auxin accumulation/depletion can be explained by the pattern of PIN1 expression. In the *pin1* mutant, *PIN1* mRNA is uniformly present throughout the PZ (Reinhardt *et al.*, 2003; Vernoux *et al.*, 2000), proving the absence of a prepattern. The position and concentration of the auxin applied to a *pin1* meristem determine the position and size of the induced primordium. All auxins can induce organogenesis, but correct positioning requires the endogenous hormone (reviewed in Kuhlemeier, 2007). PIN1 is primarily present in epidermal cells. As the leaf primordium develops, the pattern of PIN1 accumulation suggests that auxin flows from surrounding tissue towards the centre of the primordium. Later, PIN1 accumulates in abaxial cells of the elongating primordium, directing auxin flow towards the leaf tip. Gradually PIN1 becomes restricted to provascular elements within the leaf, where it contributes to vascular development itself (Reinhardt *et al.*, 2003).

It is plausible that an autoregulatory loop between auxin, *PIN1* expression and polar localization of PIN1 creates auxin maxima by transport against concentration gradients, which then activate downstream processes (reviewed in Kuhlemeier, 2007; Paciorek *et al.*, 2006). Curiously, during phyllotactic patterning, PIN1 in the L1 polarizes towards auxin concentration maxima, whereas during vein formation it orients oppositely (Heisler *et al.*, 2005; Scarpella *et al.*, 2006; Smith *et al.*, 2006). The molecular mechanism is unclear, but it might involve a protein kinase such as PINOID (PID), which causes reversal of PIN1 orientation (Friml *et al.*, 2004) and a feedback regulatory loop that affects PIN protein stability in response to auxin (Sieberer *et al.*, 2000) and by AUX/IAA-dependent PIN1 expression (Vieten *et al.*, 2005).

Recent studies have suggested that relative high auxin concentrations in the P0 region downregulate the expression of *CUC* and *KNOXI* genes, thus allowing for primordium initiation. Analysis of the dynamics of gene expression in the *Arabidopsis* floral meristem during the development of lateral primordia suggested that domains of auxin maxima are nearly complementary to the domains of *STM* and *CUC2* expression (Heisler *et al.*, 2005). Mutant combinations in *PIN1* and *PID* and the unknown gene *ENHANCER OF PINOID* (*ENP*) showed various degrees of defects in lateral organ formation (*e.g.* failure to produce cotyledons in *pin1 pid* and *pid enp* double mutants), including expansion of the expression domains of *CUC* and *STM* genes into the P0 region or the incipient cotyledon in the embryo (Furutani *et al.*, 2004; Trembl *et al.*, 2005; Vernoux *et al.*, 2000). Elimination of CUC1 function in triple mutants *cuc pin pid* restores cotyledon development. A partial restoration is also observed in *stm pin1 pid* mutants, thus indicating that the expansion of *CUC1* and *STM* expression domains mediates the failure of *pin1 pid* mutants to develop cotyledons (Furutani *et al.*, 2004). Moreover, lack of high auxin levels in the cotyledon primordia allows CUC accumulation throughout the apical domain, thus preventing cotyledon outgrowth.

Local auxin gradients are facilitated by auxin transport regulators, generating an auxin peak in the P0 and a smaller peak at the periphery of the meristem. The high auxin concentrations in the P0 further decrease cytokinin concentrations by downregulation of cytokinin biosynthetic genes. In the CZ of the SAM, a high cytokinin:auxin ratio, together with low gibberellin activity, promotes the maintenance of

indeterminate growth. High gibberellin and low cytokinin:auxin ratios promote lateral organ initiation through repression of KNOX1 and CUC expression and the resulting specification of P0.

Auxin might similarly downregulate *CUC* levels in initiating lateral primordia by inducing miR164, which targets CUC (Laufs *et al.*, 2004). Genes expressed in the boundary may regulate both meristem and organ development. Similar to LOB, CUC proteins are also expressed in boundaries (reviewed in Aida and Tasaka, 2006; Vroemen *et al.*, 2003). Furthermore, regulation of auxin transport and gene expression at boundaries are interdependent, since *PIN1* gene expression is downregulated at boundaries, and mutants that affect auxin distribution, such as *pin1*, misexpress the boundary gene *CUC1* (Furutani *et al.*, 2004; Vernoux *et al.*, 2000). Recently, a link between *KNOX* gene expression and auxin transport has been found. *JAGGED LATERAL ORGANS* (*JLO*), a member of the L*ATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) gene family, is initially required for progression of embryogenesis beyond the globular stage. At later stages, *JLO* is expressed in boundaries, and misexpression experiments reveal that JLO induces *KNOX* gene expression (of *KNAT1* and *STM*) but represses *PIN1*, drastically reducing bulk auxin transport. JLO could therefore act from the boundary to orchestrate the drastic changes in gene expression that entail the initiation of plant lateral organs. JLO function is required to maintain the integrity of boundaries between cell groups with indeterminate or determinate fates (Borghi *et al.*, 2007).

1.4.1.2 PINs in vascular patterning

Plant cells undergo a specific differentiation process to produce vascular tissues. Tracheary elements are transporting water and solutes through the plant. They are highly active and construct a secondary wall before reaching maturity. The cell wall material is deposited in discrete rings or spirals which are pulled apart as the cells grow and primary walls extend. At the end of the process, programmed cell death is required to finalize tracheary element differentiation. Vein progenitor cells (pre-procambial cells) become incorporated into veins by selection from equivalent subepidermal leaf cells. Pre-procambial cells then divide and elongate along a common axis, forming a continuous vein network. According to the canalization hypothesis, auxin is the main patterning agent. A positive feedback between auxin flow through a cell and the cell's capacity to transport auxin leads to preferred paths that will differentiate as veins (reviewed in Rolland-Lagan and Prusinkiewicz, 2005).

The PIN1 protein marks incipient vein cells earlier than any other pre-procambial marker described so far (Scarpella *et al.*, 2006). Specification of a PIN1 convergence domain and auxin accumulation at the convergence point result in higher PIN1 expression and polarization, leading to the gradual selection of a narrow strand of pre-procambial cells (Scarpella *et al.*, 2006). Pre-existing veins polarize surrounding cells, therefore recruiting preprocambial cells that will then connect to form a closed network. This step repeats itself to originate higher order veins in the whole network, and bipolar cells are the likely meeting points of

these growing vein ends (Scarpella *et al.*, 2006). From the last added veins the connections remain open, as the process is halted due to differentiation of mesophyll cells (Scarpella *et al.*, 2004). At the moment remains unclear the answer to how the epidermal PIN1 convergence points connect to the polar expression domains of the internal tissues.

1.4.1.3 PINs in root growth

Auxin effect in root development can be affected by many different factors. Normal levels of auxin biosynthesis, transport and/or response are required for the growth inhibitory effect of ethylene in roots but not in hypocotyls. Conversely, ethylene is not required for the auxin-mediated inhibition of hypocotyl and root growth in etiolated seedlings (Stepanova *et al.*, 2007). Ethylene and auxin can reciprocally regulate each other's biosyntheses, influence each other's response pathways, and/or act independently on the same target genes (Stepanova *et al.*, 2007). Ethylene triggers activation of *DR5-GUS*, probably due to an increase in auxin signalling and response in the cells of root transition zone upon exposure to ethylene (Stepanova *et al.*, 2007).

In the *Arabidopsis* root, files of all different cell types originate from the meristem that has a regular, rather stereotyped cellular organization. Roots constitute an adequate system for analysis of cell lineages, therefore facilitating molecular genetics studies on the role of patterns of cell division in root development. Four developmental zones can be distinguished in a root tip: the root cap (providing protection of the meristem from mechanical injury), the meristematic zone (where cells have maximum rates of division, promoting continuous growth of the RAM), the elongation zone (where cells attain maximum elongation rates) and the maturation zone (in which cells acquire their differentiated characteristics).

In the RAM there are two crucial groups of cells that control root development. The first comprises the slowly dividing cells of the organizing centre, which are known collectively as the quiescent centre (QC). The QC cells give rise to the other group of cells, the stem cells, which surround the QC and are more rapidly dividing pluripotent cells that originate the different cell files of the root (reviewed in Maughan *et al.*, 2006). The cortical-endodermal stem cells surrounding the QC undergo one anticlinal division followed by periclinal divisions to give rise to cortex and endodermis. Columella stem cells are situated apically to the QC. Root cap-epidermal stem cells form a ring surrounding the QC and the columella cells and originate epidermis and lateral root cap. Stele stem cells are localized basally to the QC and originate the pericycle and vascular tissues (for a scheme, see Fig. 1.3.).

In roots, in contrast to the SAM, no lateral organs are produced by the apical meristem. Instead, as the main root elongates, lateral roots arise internally and form only in mature, non-growing regions of the

root. Lateral roots arise from division of pericycle cells in mature regions of the root, thus resulting in secondary meristems that grow out through the cortex and epidermis, with an established new axis. The primary and secondary root meristems behave similarly in that divisions of the cells in the meristem give rise to progenitors of all the cells in the root. Mature lateral roots anatomically resemble the primary root, with an apical meristem, cortex, and stele, and are capable of producing new lateral roots (Malamy and Benfey, 1997).

Lateral root initiation and growth are regulated to suit the physical and chemical aspects of the surrounding soil environment, contributing to water-use efficiency and facilitating micro- and macronutrients extraction from the soil. Lateral roots form from a subset of pericycle cells (pericycle founder cells) at points adjacent to the protoxylem poles (Dolan *et al.*, 1993; Dubrovsky *et al.*, 2001; Malamy and Benfey, 1997; Beeckman *et al.*, 2001). Two pericycle founder cells undergo almost simultaneous polarized asymmetric transverse division, giving rise to two short cells flanked by two longer cells – stage I (Casimiro *et al.*, 2001). Daughter cells continue to divide both symmetrically and asymmetrically, upwards and downwards, creating groups of maximum ten short cells similar in length (Casimiro *et al.*, 2001; Malamy and Benfey, 1997). An identical series of mitotic divisions also occurs in both flanking pericycle cell files. At least three pericycle cells files are involved in lateral root formation, thus requiring a minimum of six founder cells in total (Dubrovsky *et al.*, 2000). After a period of radial expansion, the central short daughter cells divide periclinally, generating an organized primordium with inner and outer cell layers - stage II (Malamy and Benfey, 1997). In stage II, outer layer cells undergo periclinal divisions to create a three-layered primordium (Malamy and Benfey, 1997). Next, the inner cell layers divide periclinally, generating a four-layered primordium – stage IV (Malamy and Benfey, 1997). Stages V to VII promote further primordium growth through the parent cortex, finally emerging at stage VII (Malamy and Benfey, 1997). The lateral root primordium expands noticeably as it emerges from the parent root, at which point the number of cells near the lateral root apex increases (reviewed in Casimiro *et al.*, 2003).

Cell cycle control is essential for root development. The correct timing of root emergence during germination depends on the activation of cell division in the embryo root apex (Masubelele *et al.*, 2005). The D-type cyclin (CYCD)–RETINOBLASTOMA-related (RBR)–E2F pathway is a master external regulator of the cell cycle (de Jager *et al.*, 2005). The functionally different E2Fs are believed to recruit RBR and associated chromatin-modifying complexes to sets of genes, including genes necessary for progression through G_1/S and G_2/M transitions, thereby controlling cell cycle timing (Reis and Edgar, 2004). CYCD-cyclin-dependent kinase (CDK) complexes phosphorylation releases RBR repression of E2F-bound promoters, promoting cell division. Moreover, the abundance of the E2Fb protein is regulated by auxin (Magyar *et al.*, 2005). Even though *RBR* downregulation promotes stem cell proliferation, stem cell maintenance still requires QC cells, thus suggesting that *SCR* might act to downregulate *RBR* in QC cells, promoting its maintenance. *SCR* would then be upstream of the CYCD-RBR-E2F pathway (Wildwater *et al.*, 2005).

Three types of ethylene responses are predicted to exist: (1) auxin-mediated responses, such as part of the growth inhibition, in which ethylene effects are an indirect result of the increase in auxin, (2)

auxin-dependent responses, in which the activation by ethylene, although direct, is modulated by the status of the auxin pathway, and (3) auxin-independent responses, in which the ethylene responses are not affected by the levels of auxin (Stepanova *et al.*, 2007).

Recent findings suggest that a substantial part of the root growth regulation by ethylene is mediated through the basipetal delivery of auxin to cells of the elongation zone, where elongation is controlled (Růžička *et al.*, 2007). Loss of AUX1 and PIN2, both of which are required specifically for the basipetal transport of auxin through the outer root cell layers (Marchant *et al.*, 1999; Rashotte *et al.*, 2000), results in ethylene insensitivity (Růžička *et al.*, 2007). Furthermore, analysis of auxin reporters suggests that ethylene induces ectopic accumulation of auxin in the outer layers of the root meristem and in the elongation zone, where it activates a local auxin response (Růžička *et al.*, 2007). Ethylene regulates root growth through downstream local auxin signalling in the epidermis and elongation zone of the root (Růžička *et al.*, 2007). Even though the ethylene effect on root growth occurs mostly via the auxin pathway, there is also an ethylene-specific, auxin response–independent component to this regulation (Růžička *et al.*, 2007). Moreover, ethylene regulates auxin biosynthesis in different plant organs. However, exogenously applied ACC could not stimulate auxin biosynthesis additionally (Růžička *et al.*, 2007). Ethylene also requires a functional auxin transport system to accumulate auxin in responsive tissues of the outer root meristem and in the elongation zone, thus inhibiting cell elongation of cells leaving the root meristem (Růžička *et al.*, 2007). *PIN1*, *PIN2*, *PIN4* and the influx carrier *AUX1* are transcriptionally upregulated in response to ethylene. The major components of auxin transport in root tissues, AUX1 and PIN2, mediate auxin delivery into cells of the elongation zone, where auxin accumulates and induces local auxin responses that inhibit cell elongation and overall root growth (Růžička *et al.*, 2007).

1.4.1.4 PINs in transition from vegetative to reproductive development

To date, no PINs have been associated with phase change. The transition from vegetative to reproductive development is achieved by an increase in cell division within the central zone of the SAM. During the vegetative phase of growth, the *Arabidopsis* vegetative apical meristem produces phytomeres with very short internodes, resulting in a basal rosette of leaves. As plants initiate reproductive development, the vegetative meristem is transformed into an indeterminate primary floral meristem that produces floral meristems on its flanks. In parallel, lateral buds of cauline leaves (inflorescence leaves) develop into secondary inflorescence meristems, and their activity repeats the pattern of development of the primary inflorescence meristem.

Floral meristems initiate four different types of floral organs: sepals, petals, stamens, and carpels (reviewed in Coen and Carpenter, 1993). These sets of organs are organized in concentric whorls around the flanks of the meristem. In the wild type *Arabidopsis* flower, the first (outermost) whorl consists of four

sepals, which are green at maturity. The second whorl has four petals, white at maturity. The third whorl contains six stamens, two of which are shorter than the others. The fourth (innermost) whorl consists in a single complex organ, the gynoecium or pistil, composed of an ovary with two fused carpels, each containing numerous ovules, and a short style capped with a stigma.

To date, three classes of genes are ascribed to the regulation of floral development. Floral organ identity genes directly control floral identity. They usually are transcription factors that control expression of other genes involved in the formation and/or function of floral organs. Cadastral genes act as spatial regulators of the floral organ identity genes by setting boundaries for their expression. Meristem identity genes, the positive regulators of floral organ identity, are necessary for the initial induction of the organ identity genes. They must therefore be active so that the primordium formed at the flanks of the apical meristem becomes a floral meristem.

A long-distance signal, named florigen, triggering the transition to the reproductive phase, has been postulated to be transmitted through the phloem vascular system from the leaves to the apical meristem. Its identity was shown recently – a product of the gene *FLOWERING TIME* (*FT*). FT mRNA is expressed transiently in leaves, the transcribed protein is transported via phloem to the apex (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Matthieu *et al.*, 2007). Under long-day, CONSTANS (CO) activates transcription of FT in phloem companion cells of leaves (An *et al.*, 2004). FT protein interacts at the shoot apex with the bZIP transcription factor FLOWERING LOCUS D (FD) to induce downstream targets (Abe *et al.*, 2005).

1.4.1.5 PINs and lateral root formation

Lateral root initiation is triggered by auxin. Application of the auxin transport inhibitor naphthylphthalamic acid (NPA) to the root-shoot junction inhibits lateral root initiation (Reed *et al.*, 1998). The *pin1* mutant, presumably defective in root acropetal transport, initiates lateral root primordia but produces fewer mature lateral roots than the wild type (Benková *et al.*, 2003). Mutation of the auxin influx carrier AUX1 reduces production of lateral roots as a result of reduced auxin transport (Marchant *et al.*, 2002). The AUX1 protein localizes to the basal plasma membrane of root protophloem cells (Swarup *et al.*, 2001), where acropetal transport of IAA occurs. Also *tir3*, a mutant with reduced sensitivity to auxin transport inhibitors, has reduced basipetal auxin transport in stems and produces fewer lateral roots (Ruegger *et al.*, 1997). Root tip produced, basipetally transported IAA is therefore required for the initiation of lateral root primordia, while acropetally transported IAA from the shoot is required for its subsequent emergence and growth (Bhalerao *et al.*, 2002).

The importance of auxin-dependent TIR1-driven Aux/IAA degradation signalling pathway in lateral root initiation is highlighted in Aux/IAA mutants such as *solitary root*/*IAA14* (*slr*/*IAA14*), which

develops a primary root but fails to develop lateral roots (Vanneste *et al.*, 2005). Downstream of the TIR1-Aux/IAA pathway are NAC transcription factors (Xie *et al.*, 2002). NAC proteins belong to a large family including the CUC proteins that define boundaries in SAM development (Aida *et al.*, 2004). Ectopic expression of NAC1 and NAC2, like exogenous auxin treatment, induces lateral root production, indicating that the NAC transcription factors are probable targets of auxin-response pathways and necessary for auxin perception (Guo *et al.*, 2005; He *et al.*, 2005). They are also required for re–activating the cell cycle in the pericycle cells of the xylem pole. One of the earliest responses to auxin is the reduction of the KIP-related CDK-inhibitor protein (KRP2/ICK2) levels, thus suggesting that, although the auxin response results in transcriptional activity, there might be a crucial pre-existing block at the G1 checkpoint that must be overcome, by decreasing KRP2 levels, before cell cycle progression can occur (Himanen *et al.*, 2004). Auxin-regulated proteins such as SINAT5 (Xie et al., 2002) could attenuate the mitotic activity of auxin to just a few pericycle cells. *PICKLE* (*PKL*), a homologue to a mammalian chromatin-remodelling factor, has been recently shown to specifically restore lateral root formation in the *slr/IAA14* mutant, thus implying chromatin remodelling in auxin-mediated lateral root initiation in *Arabidopsis* (Fukaki *et al.*, 2006).

Lateral root formation is also regulated by two transcriptional activators of early response genes, ARF7 and ARF19. They directly activate the auxin-mediated transcription of the downstream targets *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16/ASL18*) and *LBD29/ASL16* in roots (Okushima *et al.*, 2007). Another putative downstream target of ARF7 and ARF19 is *PUCHI*, a gene encoding an AP2/ethylene-responsive element binding protein transcription factor involved in lateral root emergence (Hirota *et al.*, 2007). Loss of *PUCHI* results in swelling of the proximal region of lateral roots, leading to the formation of shorter lateral roots (Hirota *et al.*, 2007).

Genetic and physiological evidence suggests that auxin is required at several specific developmental stages to facilitate lateral root development. When excised from the primary root, young lateral root primordia are unable to continue to divide unless supplemented with exogenous IAA (Dubrovsky *et al.*, 2001). Between stages III and V, lateral primordia become independent of externally applied auxin, showing that they contain cell types that act as internal auxin sources. Several important questions remain unsolved: the nature of the main source of auxin that drives primordium formation before stages III–V, the role of auxin after stage V and the source of auxin after stage V. IAA accumulates at the root apex shortly after seedling germination (Bhalerao *et al.*, 2002). Roots lacking the auxin-influx-carrier component AUX1 fail to accumulate IAA at the root apex (Swarup *et al.*, 2001) and exhibit a 50% reduction in the number of lateral root primordia that initiate (Marchant *et al.*, 2002).

A pulse of auxin in the root system promotes the emergence of the lateral root primordia (Bhalerao *et al.*, 2002). Removal of shoot apical tissues abolishes the IAA pulse, blocking emergence of lateral roots (Bhalerao *et al.*, 2002). The root system might eventually become independent of auxin transported from the shoot. However, the observed initial dependence of lateral root development on a shoot apical source of auxin (Bhalerao *et al.*, 2002; Casimiro *et al.*, 2001; Marchant *et al.*, 2002) is a way to coordinate lateral root emergence with the development of primary leaves, allowing for a connection and balance in the carbon and nitrogen metabolism with their respective source organs (Casimiro *et al.*, 2003).

MDR transporters are also involved in lateral root formation. Knockout mutations in the *Arabidopsis MDR1* gene blocked 80% of basipetal transport in seedling hypocotyls and had a similar effect on transport down the inflorescence stem (Noh *et al.*, 2001). The existence of two phases in lateral root development, one dependent on MDR1 (postemergence elongation) and one independent (early primordium development), may coincide with the transition between the early stages of primordium formation and the later phase in which the emergent primordium formed its own apical meristem (Laskowski *et al.*, 1995). It is possible that the lower auxin levels in *mdr1* are sufficient for primordia production but suboptimal for postemergence elongation. However, MDR4-mediated root basipetal auxin transport does not affect root elongation or branching, although a 30% reduction in root basipetal auxin transport was measured in one allele of *mdr4* (*pgp4-1*; Terasaka *et al.*, 2005). Impaired root acropetal auxin transport due to mutation in *MDR1* causes 21% of nascent lateral roots to arrest their growth and the remainder elongate slower than the wild type (Wu *et al.*, 2007). Taken together, these results suggest that auxin levels established by MDR1-dependent acropetal transport in the main root control lateral root growth rates (Wu *et al.*, 2007).

It has been recently demonstrated how lateral root initiation is controlled in a spatiotemporal manner (De Smet *et al.*, 2007). Vertically grown *Arabidopsis* roots display a wavy pattern, accompanied by lateral root development at outer sides of bends. This wavy growth pattern is the consequence of an alternation between right-turn and left-turn root bending (Rutherford and Masson, 1996). Lateral roots form on top of the bends, so the wavy growth results in a left-right alternation of lateral roots with an equal distribution over both sides of the root (De Smet *et al.*, 2007). *aux1* agravitropic mutant (Bennett *et al.*, 1996) lacks the normal wavy growth pattern: *aux1* roots bent constitutively to the right and lateral roots appear predominantly on the outer (left) side of the coiling root (De Smet *et al.*, 2007). Targetted expression of *AUX1* to the lateral root cap and epidermis of *aux1* restores the number of emerging lateral roots, suggesting that AUX1 action in those tissues influences lateral root initiation and positioning (De Smet *et al.*, 2007). These findings suggest that the basal meristem represents a site of auxin accumulation distinct from the distal auxin maximum in the quiescent center and surrounding cells (De Smet *et al.*, 2007; Sabatini *et al.*, 1999). Consistently, *DR5-GUS* expression in the basal meristem was severely reduced in *aux1* seedlings (De Smet *et al.*, 2007). Therefore, the priming of pericycle cells for lateral root initiation takes place in the basal meristem, correlating with elevated auxin sensitivity in this part of the root. It is speculated that a radial gradient with an auxin maximum in the protoxylem cells might be required for lateral root initiation to take place (De Smet *et al.*, 2007). This local auxin responsiveness oscillates with peaks of expression at regular intervals of 15 hours, the time after which a consecutive lateral root will form. This recurrence of the auxin signal maximum may be a result of periodic gravitropism-induced fluctuations in auxin redistribution within the root apex (De Smet *et al.*, 2007), as the auxin pool in the root tip drives the initial stages of lateral root primordia formation (Bhalerao *et al.*, 2002). As lateral roots are almost never found in opposite positions, the appearance of the auxin signal simultaneously at both protoxylem poles requires regulation for determining in which side lateral roots emerge (De Smet *et al.*, 2007). SLR/IAA14 controls lateral root initiation by acting downstream of auxin signalling in the basal meristem and is not required for the

priming of the founder cells (De Smet *et al.*, 2007). The pericycle cells, in turn, might be primed through an SLR/IAA14-independent pathway. It is likely that other Aux/IAA proteins are involved in this process (De Smet *et al.*, 2007).

1.4.1.6 PINs in gravity perception

Gravity is a fundamental factor which affects all living organisms. In plants, it is essential for efficient exploration of the soil. Root gravitropism describes the orientation of root growth in relation to the gravity vector and is a consequence of differential cell elongation in the root meristem. This response requires the coordinated, asymmetric distribution of auxin within the root meristem, and therefore depends on the coordinated activities of PINs, MDR/PGPs and AUX1 auxin transporters.

Amyloplasts (heavy starch-filled plastids) function as intracellular gravity sensors, the statoliths. In shoots and coleoptiles, gravity is perceived in the starch sheath, a layer of cells surrounding the vascular tissues of the shoot. *Arabidopsis* mutants such as *scr*, lacking amyloplasts in the starch sheath, display agravitropic shoot growth but normal gravitropic root growth (Fujihira *et al.*, 2000), showing that the starch sheath is only required for gravitropism in shoots and not in the roots. In the roots there are large statoliths in the columella cells of the RAM. Removal of the root cap from otherwise intact roots abolishes root gravitropism without inhibiting growth (Shaw and Wilkins, 1973).

Little is known about the precise mechanism by which statocytes, amyloplasts-containing specialized root cap and columella cells, perceive gravity. Presumably, contact or pressure resulting from the amyloplast resting on the endoplasmic reticulum on the lower side of the cell triggers the response. The endoplasmic reticulum of columella cells is structurally unique, consisting of five to seven rough-ER sheets attached to a central nodal rod in a whorl, and might be involved in the gravity response (Zheng and Staehelin, 2001). Moreover, according to another model, the tensegrity model, sedimentation of the statoliths through the cytosol locally disrupts the actin skeleton, changing the distribution of tension transmitted to calcium channels on the plasma membrane (Yoder *et al.*, 2001). Nodal ER, which is also connected to channels via actin microfilaments, may then protect the cytoskeleton from being disrupted by the statoliths in specific regions, thus providing a signal for the directionality of the stimulus (Yoder *et al.*, 2001).

Because the cap is some distance away from the elongation zone where bending occurs, a chemical messenger is required to mediate communication between the cap and the elongation zone. Auxin from the root cap is taken up by the cortical parenchyma of the distant elongation zone and transported basipetally through the elongation zone of the root. When the root is oriented horizontally, however, the root cap redirects the bulk of auxin to the lower side, thus inhibiting the growth of that lower side. Another evidence

of auxin in this process is the fact that roots of mutants defective in auxin transport, such as *aux1* and *pin2*, are agravitropic (Bennett *et al.*, 1996; Luschnig *et al.*, 1998; Rashotte *et al.*, 2000).

After gravistimulation, there is an increase in the auxin levels at the root apex in columella cells, the site of gravity perception, and an asymmetric auxin flux from these cells to the lateral root cap and towards the elongation zone (Ottenschläger *et al.*, 2003). Lateral auxin transport from columella to lateral root cap cells is efflux-dependent and basipetal transport from the lateral root cap to the elongation zone is both efflux- and influx-dependent. Endogenous gravitropic auxin gradients develop even in the presence of an exogenous source of auxin (Fig. 1.3.; Ottenschläger *et al.*, 2003). Additional evidence suggests that a change in intracellular pH is the earliest detectable change in columella cells responding to gravity (Fasano *et al.*, 2001).

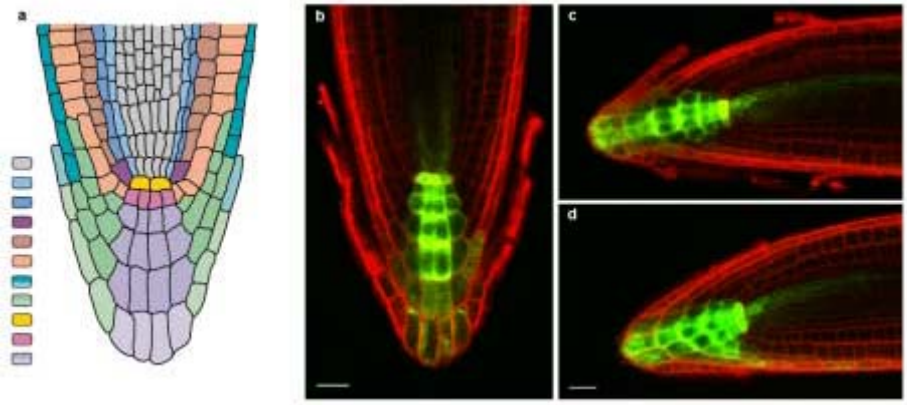

Fig. 1.3. Changes in gravity vector orientation induce asymmetric DR5-GFP expression in lateral root cap cells. (a) Schematic representation of the Arabidopsis root apex. The colour code represents different root tissues (from top to bottom: stele, pericycle, endodermis, cortex/endodermis initial, cortex, epidermis, proximal lateral root cap, distal lateral root cap, quiescent centre, columella initial, columella). (b) Confocal laser scanning microscopy images of GFP signals in quiescent center, columella initials and columella of vertically grown roots. (c) After 1.5 hours of gravistimulation GFP signals expand from columella to lower half of lateral root cap resulting in complete staining of lateral root cap after 3 hours (d, adapted from Ottenschläger *et al.*, 2003).

Auxin is transported apically in the stele of roots. Asymmetrically localized AUX1 and PIN1 transporters on the stele direct the acropetal transport of auxin from the phloem to a cluster of cells in the columella of the cap (Bennett et al., 1996; Blilou *et al.*, 2005; Marchant *et al.*, 1999). Upon reaching the root tip, auxin is transported laterally by a PIN3-mediated process before entering the basipetal stream that flows toward the base of the root through the epidermal cells (Friml *et al.*, 2002b). Upon changes in the gravity vector, PIN3 distribution becomes asymmetric, accumulating along the lower sidewall, which shifts the lateral auxin stream to the lower flank of the root. Auxin is presumed to enter the basipetal flow, which depends on the basally localized PIN2 protein (Abas *et al.*, 2006; Müller *et al.*, 1998) for its directionality. AUX1 localizes to a single layer of statolith-containing columella cell and is also strongly expressed in the lateral root cap cells and epidermal cell of the elongation zone thus putatively facilitating basipetal IAA transport in these tissues during the distributing of auxin following gravistimulation (Marchant *et al.*, 1999).

Auxin concentrations above the nanomolar range inhibit cell elongation in this region of the root, leading to faster cell expansion on the upper side and downward curvature. Consistently, mutations in PIN2 (Chen *et al.*, 1998; Müller *et al.*, 1998) or NPA treatment impair basipetal auxin transport and gravitropism.

Proteasome-dependent variations in PIN2 localization and degradation at the upper and lower sides of the root following gravistimulation result in asymmetric distribution of PIN2, thereby limiting the root response to gravistimulation (Abas *et al.*, 2006). Stabilization of PIN2 affects its abundance and distribution, and leads to defects in auxin distribution and gravitropic responses. Redistribution of auxin during the gravitropic response may thus be involved in the regulation of PIN2 (Abas *et al.*, 2006). Expression patterns of *MDR4* and *MDR1* in the root are more complementary than overlapping (Lewis *et al.*, 2007). MDR4 is expressed primarily in the outer cell layers of the root thus affecting acropetal auxin transport in roots (Birnbaum *et al.*, 2003; Lewis *et al.*, 2007; Terasaka *et al.*, 2005), whereas MDR1 is present primarily in cells of the central cylinder affecting basipetal transport (Wu *et al.*, 2007). Vertically grown *mdr1* roots produced positive and negative curvatures threefold greater than the wild type, probably due to the abnormal auxin distribution observed in the elongation zone, indicating that acropetal auxin transport maintains straight growth but contributes little to gravitropism. Conversely, *mdr4* roots grew vertically as straight as the wild type, but their gravitropism was enhanced. Upon reorientation, *mdr4* curvature developed faster, auxin was distributed more basally, and produced a greater total angle than the wild type. MDR1 and MDR4 co-localize with PIN1 and PIN2, respectively, once more suggesting their interaction with PIN function.

1.4.2 REGULATION OF PIN EXPRESSION

An auxin gradient between neighbouring cells is essential for organogenesis. In processes such as phyllotaxis (Heisler *et al.*, 2005) and vascular tissue formation (Scarpella *et al.*, 2006) or regeneration (Sauer *et al.*, 2006), local auxin accumulation leads to rearrangements in PIN polarity and therefore to the redirection of auxin flow. Moreover, PIN proteins polarize in response to an auxin gradient (Heisler *et al.*, 2005; Scarpella *et al.*, 2006). Exogenous application of auxin can elicit changes in PIN polar localization, showing that auxin is sufficient for PIN polar retargetting and that the direction of an auxin-induced PIN relocation response depends on the cell type where the response occurs instead of on the PIN protein (Sauer *et al.*, 2006). As mentioned, the molecular and cellular mechanism for the auxin effect on PIN polarity is unclear. Nevertheless, it should involve members of the Aux/IAA-ARF auxin signalling pathway, as in certain stabilized dominant *aux/iaa* mutants (*e.g. axr3/iaa17*) or in specific *arf* mutant combinations (*e.g.* in *arf 7,16,19*; Sorin *et al.*, 2005), auxin-induced retargetting of PINs is impaired (Sauer *et al.*, 2006). It is then plausible that different combinations of Aux/IAA and ARF proteins in different cell types might code for

distinct auxin-dependent rearrangements in PIN polar targetting required for the different developmental programs.

PIN proteins can be regulated at three levels: PIN protein levels (transcription, translation and silencing), PIN activity (post-translational modifications and degradation) and targetting (PIN abundance and presence at its correct polar subcellular location). PIN mRNA transcript levels are regulated by auxin, via auxin-dependent derepression of ARF transcription factors (Peer *et al.*, 2004; Vieten *et al.*, 2005). *PIN* transcription might be directly regulated by the ARFs or indirectly through other downstream signalling targets, such as AP2 transcription factors PLETHORA1 (PLT1) and PLT2 (Aida *et al.*, 2004). Ectopic *PLT* expression results in homeotic transformations that confer root identity to shoot cells, suggesting that these genes are key regulators of root stem cell identity (Aida *et al.*, 2004; Gallois *et al.*, 2004; Xu *et al.*, 2006). Loss of *PLT* function results in decreased root growth and fewer meristematic cells, implying *PLT* in the regulation of cell division. Not only is *PLT* expression expanded in some *pin* multiple mutant combinations, as well as *PIN* transcription is affected in multiple *plt* mutants. This suggests that auxin flow in the root meristem is established by the combined action of *PLT* and *PIN* genes (Blilou *et al.*, 2005). Characterized recently were two *Arabidopsis* loci, *MODULATOR OF PIN2* (*MOP2*) and *MOP3*, involved in post-transcriptional regulation of PIN proteins (Malenica *et al.*, 2007). *mop2* and *mop3* mutants show defects in auxin distribution and polar auxin transport, due to a pronounced reduction of PIN protein levels, without interfering neither with *PIN* transcript levels nor polar PIN protein localization (Malenica *et al.*, 2007). *MOP* genes must therefore be components of a regulatory network required for the fine-tuning of auxin distribution via post-transcriptional regulation of PIN protein levels (Malenica *et al.*, 2007). Additionally, mutant combinations between *PIN* and *MOP* genes suggests a functional crosstalk, further substantiated by the fact that ectopic PIN upregulation is compensated in the *mop* background (Malenica *et al.*, 2007).

Additionally, PIN activity can be controlled through protein degradation. Treatment with proteasome inhibitors affects root gravitropism and causes an increase in the ubiquitination status and level of PIN2, meaning that the degradation of PIN2 occurs in a proteasome-dependent manner (Abas *et al.*, 2006). Auxin itself seems to influence PIN2 levels in promoting PIN2 degradation (Sieberer *et al.*, 2000), but it is not yet determined whether this is the mechanism by which PIN2 asymmetry occurs during the root gravitropic response. Unpublished data also point to interaction between PIN1 and E3 ligases (Santos, 2006).

PIN expression is also regulated by phosphorylation. The *ROOTS CURL IN NPA-1* (*RCN1*) gene encodes a regulatory subunit of protein PHOSPHATASE-2A, a heterotrimeric serine/threonine protein phosphatase (Garbers *et al.*, 1996). *rcn1* mutants exhibit a higher level of root basipetal auxin transport (Rashotte *et al.*, 2001), mimicked by the treatment with inhibitors of protein phosphatases-1 and 2A (Rashotte et al., 1996; Shin *et al.*, 2005). In contrast, higher concentrations resulted in a significant reduction in root basipetal auxin transport, suggesting a biphasic response of the root to dephosphorylation (reviewed in Teale *et al.*, 2006).

1.4.3 PIN PROTEIN DYNAMICS

PIN localization is a very dynamic process. It can change rapidly in association with important developmental events, such as embryogenesis (Friml *et al.*, 2003) and gravity response (Friml *et al.*, 2002b). PIN proteins relocate rapidly through the recycling of PIN-containing endocytotic vesicles to and from the plasma membrane. Application of inhibitors of endosomal trafficking also inhibited polar auxin flux, thus revealing a close relationship between the two processes (Geldner *et al.*, 2001).

ADP ribosylation factors are monomeric GTPases involved in vesicular trafficking and their activity is regulated by ADP ribosylation factor guanine nucleotide exchange factors (ARF GEFs) and ADP ribosylation factor GTPase-activating proteins (ARF GAPs). Loss-of-function of these regulators, including ARF1 (ADP RIBOSYLATION FACTOR-1), results in defects in PIN function (Geldner *et al.*, 2003; Steinmann *et al.*, 1999). *GNOM* encodes an endosome-resident protein of the ARF-GEF family, which mediates recycling of a set of plasma membrane proteins from a subset of endosomes in a BFA-sensitive manner (Geldner *et al.*, 2003). GNOM regulates formation of exocytic vesicles at endosomes responsible for carrying PIN proteins and possibly other cargos to the plasma membrane, at the appropriate end of the cell (Geldner *et al.*, 2003). There might also be additional GNOM-independent routes for trafficking of other PINs proteins, *e.g.* PIN2 trafficking, involving Sorting Nexin 1 (SNX1)-containing endosomes (Jaillais *et al.*, 2006). Genetic suppression of the ARF-GEF function in *gnom* mutants, or chemical inhibition by the fungal toxin Brefeldin A (BFA) revealed that PIN1 and PIN3 proteins are constitutively cycling between endosomes and the plasma membrane (Geldner *et al.*, 2001). Additionally, the ARF-GAP VAN3 (also called SCARFACE) is required for normal intracellular transport of PIN1 from the plasma membrane to the endosome (Koizumi *et al.*, 2005, Sieburth *et al.*, 2006). Components of the actin cytoskeleton seem as well to be involved in PIN polarity regulation, whereas microtubules seem to be only indirectly involved in PIN trafficking (Geldner *et al.*, 2001; Kleine-Vehn *et al.*, 2006). It remains to be demonstrated how auxin accumulates in vesicles. Several authors have proposed that PIN cycling is integral to a subcellular system which translocates auxin trapped in endocytic vesicles along the actin cytoskeleton, analogously to neurotransmitters in animal cells (Baluska *et al.*, 2003; Friml and Palme, 2002c; Schlicht *et al.*, 2006). The fact that PIN1 might interact with a set of SNARE proteins (Santos, 2006), per excellence mediators of the intracellular destination of a transport vesicle, supports the view of a neurotransmitter-like auxin transport.

STEROL METHYLTRANSFERASE1, a protein required for synthesis of membrane sterols (Willemsen *et al.*, 2003), might also be involved in PIN regulation. Moreover, PIN2 seems to share the same recycling pathway as early sterol endocytosis (Grebe *et al.*, 2003). Unpublished data also point to a physical interaction or close proximity between PIN1 and an enzyme for sphingolipid synthesis (Santos, 2006). Taken together, these results link lipid membrane composition with PIN polarity control. In analogy to

mammalian systems, detergent-resistant microdomains (DRMs), structures similar to lipid rafts, could be important in plants for the trafficking of different cargos, including PINs. A recent proteomic analysis of DRM fractions in plants revealed association of many plant proteins with such domains including the PIN-interactors MDRs (Borner *et al*., 2005; Morel *et al*., 2006).

Significantly, PID, a Ser/Thr kinase, is the only factor unambiguously identified as mediating the polarity of PIN targetting. *pid* mutants show a similar mutant phenotype to *pin1* mutants: a naked inflorescence shoot with limited lateral organs and roots that are unable to respond to gravity, thus revealing auxin transport defects (Benjamins *et al*., 2001; Christensen *et al*., 2000; Furutani *et al*., 2004; Okada *et al*., 1991). In cotyledons and apices of *pid* mutants there is a shift, from apical to basal, of the polarity of several PINs, causing the meristem to collapse due to auxin depletion (Friml *et al*., 2004). The *35S::PID* root phenotype also shows changes in the polar localization of PIN1, PIN2 and PIN4 in primary roots and embryos (Friml et al., 2004). PID functions as a binary switch – if its level of expression rises above a certain threshold level it will lead to PIN apical localization. Moreover, this shift in PIN polarity was observed within a few hours of the increase in PID expression levels, implicating the direct regulation of polar PIN targetting by PID. It is possible that PID phosphorylates directly PIN proteins, marking them as apical cargo, so that dephosphorylated PIN would be sent to a basal targetting route (reviewed in Vieten *et al*., 2007). Nevertheless, as PID protein levels, and activity, are controlled by auxin, it is also likely to be a component of a feedback regulation between auxin signalling and transport. (Benjamins *et al*., 2001).

1.5 AIM OF THE THESIS

My research is focused on one of the less known genes of the PIN family, *AtPIN6*. Its function has been related to the initiation and emergence of lateral roots, its transcripts being present in lateral root meristems, specifically in pericycle founder cells (Benková *et al*., 2003).

The work hereby presented aimed at characterizing PIN6, its expression and function. *PIN6* expression throughout plant development was mapped resorting to reporter gene localization, immunolocalization and *in situ* hybridization approaches. In order to characterize the gene's function, I searched for mutants with disrupted PIN6 function and generated overexpressor and RNAi transformants. Their analysis provided insights as to understanding the contribution of *PIN6* gene to the processes in which it is involved - emergence of new organs at the SAM and the formation of lateral roots, as well as response to auxin, light and gravity. Moreover, transcript profiling data available from central compiling resources (GENEVESTIGATOR and eFP Browser) provided additional insights into PIN6 function and regulation.

2 MATERIALS AND METHODS

"Genius is one percent inspiration and
ninety-nine percent perspiration."
Thomas Eddison

2.1 MATERIALS

2.1.1 PLANTS

Plants used were *Arabidopsis thaliana* L. Cultivar Col0.

Wild-type
Atpin2/eir1-1 (Roman *et al.*, 1995)
pDR5::*GUS* (Ulmasov *et al.*, 1997b)
pPIN6::*GUS* (Schelhaas, 1999)
line SALK046393.41.20.x
line GABI-Kat 430 B01
line GABI-Kat 711 C09
line GABI-Kat 852 D10

2.1.2 BACTERIA

Agrobacterium tumefaciens GV3013 *pMP90 rk*	GV3013 C58C1, Rif^R, Gm^R (Koncz and Schell,1986) kan^R
Escherichia coli Bl 21 DE3	*B, F-, ompT, dcm, hsdS*
Escherichia coli DH10B	*F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80d lacZΔM15Δ lacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU galK nupG rpsL*
Escherichia coli DH5α	F- ø80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deo*R *recA*1 *endA*1 *hsdR*17(rk-, mk^+) *phoA supE*44 λ- *thi*-1 *gyrA*96 *relA*1

2.1.3 PLASMID VECTORS

pAM-PAT-GW	Gateway®-compatible vector containing 2x35S promoter for creating overexpression lines (kindly provided by B.Ulker, MPIZ, Cologne). Amp^R, TRP1
pCR®-Blunt II-TOPO®	Plasmid vector for direct insertion of blunt-end PCR products (INVITROGEN)
pDONR201	Entry vector for Gateway® cloning (INVITROGEN). Amp^R
pET-28a(+)	Protein expression vector (NOVAGEN). Kan^R
pJAWOHL8-RNAi	Gateway®-compatible vector containing antisense insertion sites for creating RNAi lines (kindly provided by B.Ulker, MPIZ, Cologne). Amp^R, TRP1

2.1.4 SYNTHETIC OLIGONUCLEOTIDES

The following synthetic oligonucleotides (primers) were derived from INVITROGEN, MWG BIOTECH or OPERON. Primer stocks were prepared diluting up to 100 µM in ddH_2O according to manufacturer's instructions. Aliquots of working concentration of 10 µM were used for both PCR amplification and sequencing.

OLIGONUCLEOTIDES	**SEQUENCE (5'→3')**
Screening/Genotyping for *pin6* mutants from the SALK collection	
SALK_046393 LP	gcgggttcggatttactcgac
SALK_046393 RP	cacgtgagcagatacagagtgatagcc
T-DNA Left Border a1 (Lba1)	tggttcacgtagtgggccatcg
T-DNA Left Border b1 (LBb1)	gcgtggaccgcttgctgcaact
Screening/Genotyping for *pin6* mutants from the GABI-Kat collection	
GABI_430B01_UP_1342_II	atgatgtgggagtctgggcaaagac
GABI_430B01_LP_2644_I	ctgcagcgccataaacaggc
GABI_711_852_UP_2653	gtggggcgaaaaagccaacaa
GABI_711_852_LP_3617	ttctccggtgtaatctttttcttcagtg
GABI T-DNA	cccatttggacgtgaatgtagacac
Gateway® BP entry primers	
attB1	ggggacaagtttgtacaaaaaagcaggcttcacc
attB2	ggggaccactttgtacaagaaagctgggtgtta
Cloning *PIN6* coding sequence into entry vector *pDONR201*	
PIN6_OX_GWfwd (with attB adapters)	aaaaagcaggctctatgataacgggaaacgaattctac
PIN6_OX_Gwrev (with attB adapters)	agaaagctggtctcataggcccaagagacgtag
Cloning a *PIN6*-specific 400 bp fragment for RNAi into entry vector *pDONR201*	
PIN6_35SRNAi_GWfwd (with attB adapters)	aaaaagcaggctctcctagagcctctaatctttcta
PIN6_35SRNAi_Gwrev (with attB adapters)	agaaagctgggtcttctctggcactgatccattg
Cloning a *PIN6*-specific 3'utr 202 bp fragment for RNAi into entry vector *pDONR201*	
PIN6_35SRNAi_GWfwd_utr (with attB adapters)	aaaaagcaggctctttcacacaacacactgaagaaa
PIN6_35SRNAi_Gwrev_utr (with attB adapters)	agaaagctgggtcacataaaattaaattattcac
Sequencing primers	
pDONR201_seq_fwd	tcgcgttaacgctagcatggatctc

pDONR201_seq_rev	gtaacatcagagattttgagacac
PIN6cdsSeq_1	atgataacgggaaacgaa
PIN6cdsSeq_401	tccaactcgttgttcttc
PIN6cdsSeq_801	gttttgtaacggagattt
PIN6cdsSeq_1201	caagaaatgccaagtgca
PIN6cdsSeq_1601	tgtttgcgagggagtaca
Confirming generated *pin6* transformants	
PIN_OX_fwd	atgataacgggaaacgaattctac
PIN_OX_rev	tcataggcccaagaggacgtag
PIN_35SRNAi_fwd	cctagagcctctaatctttcta
PIN_35SRNAi_rev	ctttctctggcactgatccattg
PIN_35SRNAi_fwd_utr	ttcacacaacacactgaagaaa
PIN_35SRNAi_rev_utr	acataaaattaaattattcac
35SFor	cgcacaatcccactatcctt
Amplifying the RNA probe for *In Situ* hybridization	
pin6ORFfor	caagtcgacgatgacgtcatttcc
pin6ORFrev	agccaccggagttgtgtcttttcc
T7 adapter	ccaagcttctaatacgactcactatagggaga
T3 adapter	aattaaccctcactaaagggaga
Generating a T-DNA probe for Southern Blot	
T-DNA_fwd	tcaattgccctttggtcttc
T-DNA_rev	ggtgctgctatcgatggttt
R.T.-PCR	
ACTF	atgatgcacctagagctg
ACTR	ttccagggaacattgttg
pin6-f	aggagccgccggaaaagacac
pin6-r	attcggcttcagtgtgttgtgtgaatc

2.1.5 ANTIBODIES

Guinea pig anti-PIN6 polyclonal antibody

Rabbit anti-PIN6 polyclonal antibody

Goat anti-rabbit IgG Alexa Fluor® 488 (MOLECULAR PROBES)

Goat anti-guinea pig IgG Alexa Fluor® 555 (MOLECULAR PROBES)

Goat Anti-Digoxigenin-AP Fab fragments (ROCHE)

Goat Anti-Digoxigenin-fluorescein Fab fragments (ROCHE)

2.1.6 ENZYMES

If not indicated otherwise, the enzymes used for experiments in this thesis were obtained from FERMENTAS.

2.1.7 CHEMICALS

If not indicated otherwise, chemicals used for experiments in this thesis were obtained from BAYER, BIORAD, CARL ROTH, DUCHEFA, FERMENTAS, GE HEALTHCARE, MERCK and SIGMA-ALDRICH.

2.1.8 OTHER MATERIALS

Autoradiofilm X-OMAT AR 5	KODAK
DIG Easy Hyb	ROCHE APPLIED SCIENCE
DIG Luminescent Detection Kit	ROCHE APPLIED SCIENCE
ECL Analysis System of Horseradish Peroxidase-coupled secondary antibodies	GE HEALTHCARE
Falcon Tubes	GREINER BIO-ONE
GFX PCR DNA and Gel Band Purification Kit	GE HEALTHCARE
Histoclear	NATIONAL DIAGNOSTICS
Hybond N+	GE HEALTHCARE
Microcentrifuge tubes (Eppendorfs)	ROTILABO
Parafilm® M	PECHINEY PLASTIC PACKAGING COMPANY
Paraplast®	MCCORMICK SCIENTIFIC
PCR DIG Labelling Mix	ROCHE
Petridishes	GREINER BIO-ONE
PVDF membrane	MILLIPORE
Revert-Aid M-MuLV Reverse Transcriptase & 1st Strand cDNA synthesis kit	FERMENTAS
Slowfade® Antifade Kit	MOLECULAR PROBES (INVITROGEN)
Slowfade® Gold antifade reagent with DAPI	MOLECULAR PROBES (INVITROGEN)
Sterile filtration units	MILLIPORE
Whatman 3MM paper	WHATMAN
Zero Blunt® TOPO® PCR Cloning Kit	INVITROGEN

2.1.9 PLANT MEDIA

Media were diluted in 1 l H_2O. For solid media agar-agar (16g/l) was added after pH adjustment. Media were sterilized by autoclaving for 20 min at 121°C. Whenever heat-labile supplements were necessary, sterile-filtered solutions were prepared and added afterwards.

AM (0.5x) 2.297 g MS basal salt medium, 10 g sucrose, pH=5.8-6.0.

When required, antibiotics/herbicides were supplemented to the following concentration:

BASTA®	40 mg/l
Cefotaxime	300 mg/l

2.1.10 BACTERIA MEDIA

Bacteria media were prepared according to Sambrook *et al.* (1989). Media were diluted in 1l H_2O. For solid media, 15g of agar-agar were added and autoclaved for 20 min at 121ºC. Whenever heat-labile supplements were necessary, sterile-filtered solutions were prepared and added after wards.

LB medium	10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, 15g agar-agar.
YEB medium	10 g yeast extract, 10g peptone, 5g NaCl.

When required and if not stated otherwise, antibiotics were supplemented to the following concentration:

Ampicillin	100 mg/l
Carbenicillin	50 mg/l
Gentamycin	25 mg/l
Kanamycin	50 mg/l
Rifampicin	50 mg/l

2.1.11 MICROSCOPES

Confocal Laser Scanning Microscopes	LSM-U-Zeiss LSM510vis upright microscope, with Axioplan imaging 2 processing with three emission channels. One transmission channel with a 530 ± 15 nm band pass filter for FITC/Alexa Fluor® 488 specific detection and a 580 nm band pass filter for autofluorescence detection. Coupled with an AxioCam MRc (ZEISS) camera. LSM-I-Live DUO inverted microscope, with Axioplan imaging 2 processing multiple emission channels, with a set of filters including a 530 ± 15 nm band pass filter for FITC/Alexa Fluor® 488 specific detection, a 530 nm long pass filter for Alexa Fluor® 555 detection and a 600 nm long pass filter for autofluorescence detection. Coupled with an AxioCam MRc (ZEISS) camera.
Fluorescence microscope	Axiovert 200M met (ZEISS) coupled with AxioCam MRc (ZEISS) and with SensiCam (PCO) cameras.
Binocular microscope	Zeiss STEMI SV11 Apo coupled with an AxioCam MRc (ZEISS) camera.

2.1.12 COMPUTER PROGRAMS

Adobe Photoshop 6.0	ADOBE
Axiovision LE 4.6	ZEISS
DNA star software package	LASERGENE
Image J 1.37v	RASBAND, W.S., NATIONAL INSTITUTES OF HEALTH, USA
GeneSNAP	SYNGENE
Zeiss LSM Image Browser	ZEISS

2.1.13 DATABASES

ClustalW	www.ebi.ac.uk/clustalw
eFP Browser	bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi
InterProScan	www.ebi.ac.uk/InterProScan
Genevestigator	www.genevestigator.ethz.ch/
Gene Ontology	www.geneontology.org/
MIRNA	sundarlab.ucdavis.edu/mirna/
National Center for Biotechnology Information (NCBI)	www.ncbi.nlm.nih.gov
NCBI Blast	www.ncbi.nlm.nih.gov/blast
NetOGlyc 3.1	www.cbs.dtu.dk/services/NetOGlyc/
NetPhos 2.0	www.cbs.dtu.dk/services/NetPhos/
PLACE	www.dna.affrc.go.jp/PLACE/
TAIR	www.arabidopsis.org
WolFPSORT	wolfpsort.org/

2.2 METHODS

Most of the methods used in this work are standard molecular biology methods, described in Sambrook *et al.* (1989) and Ausubel *et al*. (1996). Commercial kits were used according to manufacturer's protocols. Only in cases in which a standard method was modified or given its relevance for the reported work more details are given.

2.2.1 METHODS FOR THE CULTIVATION AND TRANSFORMATION OF ARABIDOPSIS

2.2.1.1 Arabidopsis seed sterilisation

Arabidopsis thaliana seeds (see section 2.1.1) were surface sterilized with a solution of 4% w/v calcium hypochloride and 0.1% Triton X-100 in 80% ethanol. After 2 washes in 80% ethanol and one rinse in absolute ethanol, seeds were left to dry under the sterile bench.

2.2.1.2 In vitro *germination of Arabidopsis seeds*

Sterile seeds were sown on AM agar medium. After vernalisation in the dark for 2 days at 4ºC, plates were transferred to climate chambers where seeds were germinated and grown under long-day (16 h light/8 h dark;

6.3 W/m² energy flow) conditions at 22ºC. When required, additional growth conditions were used, such as continuous light (24 hours; 7.2 W/m²), short-day (8 h light/16 h dark; 5.7 W/m²) or darkness at 22ºC.

For the light quality experiment, seeds sown on moist sterile filter paper were vernalised for 3 days at 4ºC and germinated under continuous white light at 22ºC and grown for 16. Then, they were exposed to different light sources (white, red, far-red and blue) for 3 days under continuous light at 25ºC. For UV-B light assay, plants grown on AM medium supplemented with 0.8% Bacto-agar, vernalised for 2 days at 4ºC, were germinated directly under UV-B light and grown for 4 days under continuous UV-B light. The set-up used is depicted in the table below.

Light quality	Energy and Photon Flow	Wavelength	Lamps and filters used
White light	5 W/m²	370 to 850 nm	fluorescent lamps 8x OSRAM L 16W/25
Blue light	10 W/m² 38.5 µmol/m²	436 nm (emission maximum)	fluorescent lamps PHILLIPS TL 40 W/18 combined with plexiglas PG 627/3 filter (RÖHM UND HAAS)
Red light	0.6 W/m² 3.4 µmol/m²	656 nm (emission maximum)	fluorescent lamps PHILLIPS TL 40 W/18 combined with plexiglas PG 501/3 filter (RÖHM UND HAAS)
Far-red light	3.5 W/m² 21.4 µmol/m²	730 nm (emission maximum)	fluorescent lamps OSRAM Linestra 120 W/235 V combined with heat absorption glas KG 3/2 (SCHOTT), plexiglas PG 501/3 and PG 627/3 filters (RÖHM UND HAAS)
UV-B	3.6 W/m² 1.5 µmol/m²	below 345 nm	fluorescent lamps OSRAM L18W/30 supplemented with PHILLIPS TL20W/01RS narrowband UV-B tubes, coupled with WG305 (for UV-B light only) and WG345 (a broader UV filter to use as a negative control) filters

2.2.1.3 Arabidopsis growth on soil

Moist soil mixed with vermiculite (4:1) and supplemented with 25 ml fertilizer was autoclaved at 121ºC for 20 min. When preparing the pots, insecticide tablets (Lizetan®, BAYER) were added (half per pot) to prevent proliferation of insects. *Arabidopsis* seeds were germinated as described above and 5-days old seedlings were transferred to soil.

2.2.1.4 Transformation of Arabidopsis WT plants

Agrobacterium tumefaciens clones carrying either *pAM-PAT-GW-PIN6*, *pJAWOHL-RNAi-PIN6-CDS* or *pJAWOHL-RNAi-PIN6-utr*, were cultured in 2 ml of YEB medium supplemented with carbenicillin (50 mg/l), gentamycin (25 mg/l), kanamycin (25 mg/l) and rifampicin (50 mg/l) o/n at 28ºC with continuous shaking. *A. tumefaciens* cultures were diluted 20x and further cultivated for 48 hours. Arabidopsis WT plants grown under greenhouse conditions at a density of 8 plants per pot (9 cm diameter) were transformed 5-10 days after clipping, according to the floral dip method (Clough and Bent, 1998).

2.2.1.5 In vitro *selection of transformed WT plants*

Following bacterial infiltration, Arabidopsis T1 plants were selected on AM agar plates containing 40 mg/l BASTA® and 300 mg/l cefotaxime to eliminate residual agrobacteria and later transferred to soil. Leaf samples were collected and the introduction of the T-DNA in the genome was confirmed by PCR reaction, using primers listed on 2.1.4. After propagation of T1 positives, the corresponding seeds (T2) were then used for further experiments.

2.2.1.6 Preparation of seedlings for microscopy

Prior to microscope inspection, Arabidopsis seedlings were mounted on microscope slides in 50% glycerol or in a solution of chloralhydrate/H_20/glycerol (2:1:1).

2.2.1.7 Measurements of root length, lateral root number and root curvature

Arabidopsis seedlings were scanned and several parameters such as root and hypocotyl length, number of lateral roots and root curvature were measured using ImageJ software.

2.2.2 METHODS FOR CULTIVATION AND TRANSFORMATION OF BACTERIA

2.2.2.1 Preparation of electrocompetent Escherichia coli *cells*

A single colony of a given *E. coli* strain (see section 2.1.2) was inoculated into 50 ml of LB medium and incubated o/n at 37ºC with continuous shaking. Subsequently, 10 ml of the culture were diluted in 500 ml of fresh LB medium and incubated at 18ºC (for highly competent cells) o/n or at 37ºC (for routine needs) until OD_{600}=0.4. Cells were harvested by centrifugation for 15 min at 4ºC and resuspended in 500 ml sterile ice-cold water. This washing step was repeated twice with 250 ml and 50 ml of ice-cold water, respectively. The supernatant was discarded and cells were gently resuspended in 800 µl of 7% DMSO. Samples were then divided into 40-60 µl aliquots, snap frozen in liquid nitrogen and stored at -70ºC.

2.2.2.2 Electroporation of Escherichia coli *cells*

DNA was added to an aliquot of thawed electrocompetent cells and transferred to an electroporation cuvette. A single electroporation pulse (25 µF, 2.5 kV and 200 Ω) was given and 1 ml of LB medium immediately added. After incubation for 1 hour at 37ºC with continuous shaking, cells were plated on selective LB medium and incubated o/n at 37ºC.

2.2.2.3 Preparation of electrocompetent Agrobacterium tumefaciens *cells*

A single colony of a given *A.tumefaciens* strain (see section 2.1.2) was inoculated into 50 ml of YEB medium and incubated o/n at 28ºC with continuous shaking. This culture was then diluted in 450 ml of fresh YEB medium

and incubated at 28ºC until OD_{600}=0.4-0.6. Cells were harvested by centrifugation for 15 min at 4ºC and resuspended in 500 ml sterile ice-cold water. This washing step was repeated twice with 250 ml and 50 ml of ice-cold water, respectively. Supernatant was discarded and cells were gently resuspended in 500 µl 50% glycerol. Samples were divided into 50 µl aliquots, snap frozen in liquid nitrogen and stored at -70ºC.

2.2.2.4 Electroporation of Agrobacterium tumefaciens *cells*

DNA was added to an aliquot of thawed electrocompetent cells and transferred to an electroporation cuvette. A single electroporation pulse (25 µF, 2.4 kV and 200 Ω) was given and 1 ml of YEB medium immediately added. After incubation for 2 hours at 28ºC with continuous shaking, cells were plated on selective YEB medium and incubated 48 hours at 28ºC.

2.2.3 CLONING STRATEGIES

Cloning strategies performed in the course of this thesis through Gateway® technology are summarized in the table below. Plasmids and synthetic oligonucleotides used for cloning procedures are listed in sections 2.1.3 and 2.1.4. The Gateway® Technology (INVITROGEN) is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move DNA sequences into multiple vector systems. Fragments of interest are inserted into the vector by creation of adapter attB sites at its ends. A first round of PCR with specific primers adds half of an adapter attB to each end of the sequence. A second round of PCR, with primers designed for the adapter sequence, results in a sequence of interest with two adapter attB sites at each end. The fragment is then combined with the entry vector through a BP clonase reaction, in which the region between P sites in the vector is swapped by the corresponding region, between B sites, in the sequence of interest, therefore resulting in the insertion of the fragment in the vector. With this insertion the BP sites are transformed into LR sites. The cloned sequence can then be exported to any type of destination vector, following a similar strategy. An LR clonase reaction swaps the region between L sites in the entry vector by the region between R sites in the destination vector.

Construct	Insert	Primers	Entry vector	Destination vector
pAM-PAT-GW-PIN6	complete PIN6 CDS (1713 bp)	*PIN6_OX_GWfwd* *PIN6_OX_GWrev*	pDONR201	*pAM-PAT-GW*
pJAWOHL8-RNAi-PIN6-CDS	402 bp of *PIN6* CDS (bps 678 to 1080)	*PIN6_35S_RNAi_GWfwd* *PIN6_35S_RNAi_GWrev*	pDONR201	*pJAWOHL8-RNAi*
pJAWOHL8-RNAi-PIN6-utr	197 bp sequence for the 3'-UTR *PIN6 (*starting from the TGA stop codon)	*PIN6_35S_RNAi_GWfwd_utr* *PIN6_35S_RNAi_GWrev_utr*	pDONR201	*pJAWOHL8-RNAi*

2.2.4 OLIGONUCLEOTIDE DESIGN

Oligonucleotides were designed with Primerselect DNAstar oligonucleotide design software with automatic estimation of the GC content, melting temperature, primer-dimers and hairpin formation. The usual length of the oligonucleotide chosen was 18-30 bases. The design was done in order for both primers (forward and reverse) to have similar T_m, within 2-4ºC and above 60ºC. Oligonucleotides were designed whenever necessary to genotype a mutant line of interest, to amplify by PCR a fragment of interest or for sequencing reactions. For DNA sequences that were inserted into Gateway®-compatible vectors, *attB1* and *attB2* adapters were added. For other DNA sequences that were subcloned, oligonucleotides contained at the 3'end a sequence overlap complementary to the sequence of interest, and whenever possible the region was long enough to achieve a T_m of 60ºC or more.

2.2.5 METHODS FOR NUCLEIC ACID ANALYSIS

2.2.5.1 Small scale plasmid isolation from Escherichia coli *and* Agrobacterium tumefaciens

Small scale plasmid isolation from either *E. coli* or *A. tumefaciens* was performed by alkaline lysis according to Sambrook *et al.* (1989). Briefly, 1.5 to 3 ml o/n grown bacterial culture was spinned down and the resulting pellet resuspended in 250 µl TE buffer (10 mM Tris-HCl pH 8.0–8.5; 1 mM EDTA pH 8.0; 100 µg/ml RNAse A). Cells were lysed by adding 250 µl NaOH/SDS buffer (200 mM NaOH; 1% SDS) and the reaction was allowed to proceed for 3 to 5 min at RT, followed by addition of 350 µl high salt buffer (5 M KOAc; 3 M HOAc) and 5 min incubation on ice. After 10 min centrifugation, the supernatant was transferred to a new reaction tube and DNA was precipitated by addition of an equal volume of isopropanol and then incubated at RT for 5 min. DNA was recovered by centrifugation for 10 min and the pellet washed with 70% ethanol and air-dried. Afterwards the pellet was resuspended in 20 to 30 µl of ddH_20 or 10 mM Tris-HCl pH 8.0–8.5.

2.2.5.2 Isolation of genomic DNA from Arabidopsis – crude protocol, for genotyping (adapted from Edwards et al.*, 1991)*

Genomic DNA from Arabidopsis was isolated according to Edwards *et al.* (1991). Plant tissue was extracted in 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS. After centrifugation, supernatant was precipitated by addition of isopropanol. Following several washes in absolute ethanol, sample was air-dried and DNA resuspended in 100 µl TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA).

2.2.5.3 Isolation of genomic DNA from Arabidopsis – CTAB method

When high-quality high-yield genomic DNA from Arabidopsis was necessary, we used the CTAB method. Extraction buffer (800 µl of 1% CTAB; 100 mM Tris-HCl pH 8.0; 20 mM EDTA; 1.4 M NaCl; 1% PVPP; 1% β-mercaptoethanol) were added to 100-200 µg frozen plant tissue grinded in liquid nitrogen and left to incubate at 65ºC for 30 min. Freshly prepared chloroform/isoamyl alcohol (24:1) was added, samples mixed and centrifuged to separate phases. To the aqueous phase were added 4 M NaCl and 2/3 vol of isopropanol and

0.1 vol of 3 M NaOAc and precipitation occurred at -20 or -80ºC. Following centrifugation, the resulting pellets were washed with 70% ethanol and 1 M KOAc-HOAc buffer pH 6.0, rinsed twice with 70% ethanol and left to air-dry prior to resuspension. DNA was treated with RNase for 1 hour at 37ºC to eliminate residual RNA contaminations from the samples and precipitated afterwards by ice-cold absolute ethanol and 100 µl NH_4OAc. Resulting pellets were rinsed with ethanol, air-dried and resuspended in 50-100 µl either ddH_2O or TE.

2.2.5.4 Separation of DNA fragments on agarose gel electrophoresis

DNA fragments were mixed with DNA loading buffer (50% glycerin; 50 mM EDTA; 0.25% bromophenol blue; 0.25% xylene cyanol) and separated on agarose gel electrophoresis. The agarose concentration used (0.8-1.5 %) was depending on the size of fragments to be resolved (Sambrook *et al.*, 1989). Electrophoresis was performed at 5 V/cm using TAE buffer (40 mM Tris; 20 mM acetic acid; 1 mM EDTA) and ethidium bromide (0.5 µg/ml) to visualize nucleic acids. 1 kb ladder size marker was used to estimate the size of DNA fragments. After electrophoresis, DNA was visualized on a transilluminator under UV light (254 nm).

2.2.5.5 Polymerase Chain Reaction (PCR)

PCR amplification was done according to Saiki *et al*. (1985). Typically, PCR reactions were prepared as represented in the table below.

	Mixture
DNA template	100 ng
Taq polymerase (1 U)	1 µl
5x Buffer	5 µl
2.5 mM dNTPs	10 µl
$MgCl_2$ or $MgSO_4$	according to manufacturer's requirements
primers [stock]=10 pmol/µl	5 µl each
Millipore H_2O	up to 100 µl

PCR reaction was performed by incubating the PCR reaction mix at three different temperatures corresponding to the denaturing, annealing and extension steps for each cycle of amplification. In a typical reaction, the DNA was denatured at 95°C, primers annealed at 40-60°C, and extension processed at 72°C. 30-40 amplification cycles were needed.

For cloning into Gateway®-compatible vectors, two rounds of PCR reaction were performed: the first round contained sequence-specific primers coupled with half of the *attB1* and *attB2* recombination sites, the second round primers for amplification of the full sequence of the *attB1* and *attB2* sites (for BP entry primers, see section 2.1.4).

For genotyping of the putative SALK_046392.41.20.x mutant line, PCR amplification was done using primers *SALK_046393 LP*, *SALK_046393 RP* and *Lba1* or *LBb1*. For GABI-Kat line 430 B01, primers *GABI_430B01_UP_1342_II*, *GABI_430B01_LP_2644_I* and *GABI T-DNA* were used. For GABI-Kat lines 711 C09

and 852 D10, the same set of primers were used, as both insertions are located to the same nucleotide. In this case, primers *GABI_711_852_UP_2653*, *GABI_711_852_LP_3617* and *GABI T-DNA* were used.

2.2.5.6 Reverse Transcriptase Polymerase Chain Reaction (R.T.-PCR)

Synthesis of cDNA was carried out using the Revert-Aid M-MuLV Reverse Transcriptase & 1st Strand cDNA Synthesis kit. Typically, 1-2 μg of denatured RNA were mixed with 1 μl of the Oligo (dT) and diluted in 10 μl RNAse-free water. The mixture was heated to 70ºC for 5 min and quickly chilled on ice. Following the addition of 4 μl of 5X RT buffer, 1 μl RiboLock RNAse inhibitor and 2 μl of 10 mM dNTP mix, reaction tubes were incubated at 37ºC for 5 min. M-MuLV RT (1 μl of 200 U/μl) was added and the overall incubated at 42ºC for 1 hour. Finally, the reaction was inactivated by heating at 70ºC for 10 min.

R.T.-PCR control reactions with Actin-1 gene (with primers ACTF and ACTR) were performed at an annealing temperature of 54ºC and ran for 35 cycles. PCR reactions for PIN6 (with primers pin6-f and pin6-r) were performed at an annealing temperature of 59ºC and during 35 cycles.

2.2.5.7 Purification of PCR products

2.2.5.7.1 PEG-Clean up

PCR products were purified using a PEG/NaOAc precipitation procedure. Equal amounts of DNA solution and PEG/NaOAc solution (26% v/v PEG 6 000; 6.5 mM $MgCl_2$; 0.6 M NaAc; pH 6.7) were mixed and incubated at RT for 10-15 min. DNA was precipitated by centrifugation for 5 min, washed twice with absolute ethanol and air-dried for 10-15 min. DNA was then resuspended in the desired volume of water.

2.2.5.7.2 Purification of plasmids or PCR products from an agarose gel

DNA fragments were purified from an agarose gel using the *GFX PCR DNA and Gel Band Purification Kit*, according to the manufacturer's instructions. Bands were cut from the gel and capture buffer was added. The mixture was incubated at 60ºC until the agarose fully dissolved (5-20 min). The mixture was then bound to a GFX column and afterwards eluted from it.

2.2.5.8 Purification of DNA by phenol/chloroform extraction

Equal volumes of DNA and phenol/chloroform/isoamyl alcohol (25:24:1) were mixed, vortexed and centrifuged. The upper, aqueous phase was carefully removed and transferred to a fresh reaction tube. This extraction procedure was repeated with chloroform/isoamyl alcohol (24:1). DNA in the aqueous phase was precipitated by addition of 10% (v/v) of 7.5 M NH_4OAc and 2.5% (v/v) of absolute ethanol at -20°C. After precipitation, DNA pellet was washed with 70% ethanol to remove salts.

2.2.5.9 Precipitation of DNA

DNA was added to 1/10 vol of 3M NaOAc pH 5.2 and 2.5 vol ice-cold absolute ethanol and placed at -80ºC for at least 1 hour. Following centrifugation, the pellet was washed twice with 70% ethanol and allowed to air-dry, prior to resuspending in TE.

2.2.5.10 Recombination of DNA fragments

For Gateway® BP ligation reactions, 5 µl in Tris 10 mM pH 8.0 containing 100-150 µg DNA template (purified PCR fragment to insert into the vector), 1 µl of 150 µg/µl entry vector, 2 µl BP Clonase Enzyme Mix (INVITROGEN) and 2 µl BP Buffer (INVITROGEN) were incubated for 1 hour to o/n at 25°C. Similarly, for LR reactions, a mixture of 100-150 µg DNA template (entry vector), 0.5 µl of 150 µg/µl destination vector, 1 µl LR Clonase Enzyme Mix (INVITROGEN), and 1 µl LR Buffer (INVITROGEN) to a total vol of 5 µl in Tris 10 mM pH 8.0, was incubated for 1 hour to o/n at 25°C.

2.2.5.11 Southern Blot

2.2.5.11.1 Preparation of probe and samples

A *ca.* 1 kb probe was designed against the T-DNA insertion (7 321-8 357 bp of the plasmid which originated the SALK mutant collection, *pBIN-pROK2*) and amplified by PCR using primers *T-DNA_fwd* and *T-DNA_rev*. Since LB of T-DNA in the vectors used by the SALK and GABI-Kat collections is conserved, we used the same probe for mutants of both seed collections. In fact, the probe used was designed for *pBIN-pROK2* (SALK) but also shared 98% identity with *pAC161* (GABI-Kat). DIG-labelled probe was prepared with *PCR DIG Labelling Mix*.

DNA was digested with *EcoRI* endonuclease. *EcoRI* generates two fragments by cutting once inside the *PIN6* gene. Since *EcoRI* does not cut inside the T-DNA probe, only one single labelled band will be observed on the membrane.

2.2.5.11.2 Separation of digested DNA fragments on agarose gel electrophoresis

Previously digested and purified DNA samples (1 µg /well) mixed with loading buffer were loaded on an 0.8 % agarose gel and electrophoresis was performed at low voltage (20-50 V) for better separation, over a period of 8 hours up to o/n. After electrophoresis, DNA was visualized on a transilluminator under UV light (254 nm).

2.2.5.11.3 DNA transfer on to a charged nylon membrane

Following electrophoresis, agarose gel was incubated in 0.25 M HCl solution for 10 min. The gel washed in ddH_2O was incubated in 0.5 M NaOH; 1.5 M NaCl solution for 30 min. Transfer was performed by upward capillary transfer with alkaline transfer buffer (0.4 M NaOH). Whatman filter paper was used as a bridge, on the bottom of the stack, having both ends inside the transfer buffer. Buffer transfer by capillary action from a region of high water potential to a region of low water potential is the driving force that moves DNA from the gel on to

the membrane. The gel was placed on top of Whatman paper in contact with the Hybond N+ dry membrane, positively charged, so that ion exchange interactions will bind the negatively charged DNA to it. Then consecutively, 3 sheets of Whatman paper, a stack (8-10 cm height) of Kimwipes, a plate of glass and a weight were placed on top to ensure good and even contact between the gel and the membrane.

2.2.5.11.4 Membrane hybridization

DNA was cross-linked to the membrane by exposing to UV (1200 J). The membrane was then pre-hybridized in hybridization solution (*DIG Easy Hyb*, ROCHE) for 4 hours at 42°C. DIG-labelled probe denatured at 95ºC for 5 min and cooled rapidly on ice was added to the hybridization solution and hybridized o/n at 42ºC. After hybridization, the membrane was washed in SSC buffer (150 mM NaCl; 15 mM sodium citrate) solutions as follows: twice with 2x SSC, 0.1% SDS for 15 min at 42ºC; once with 1x SSC, 0.1% SDS for 15 min at 42ºC, and finally once with 0.5x SSC, 0.1% SDS for 15 min at 42ºC.

2.2.5.11.5 Membrane development

The membrane was developed using *DIG Luminescent Detection Kit*. After rinsing in washing buffer (0.1 M maleic acid; 0.15 M NaCl; 0.3% Tween 20; pH 7.5), it was incubated in blocking buffer (1% Blocking Reagent, supplied with the kit; 0.1 M maleic acid; 0.15 M NaCl; pH 7.5), for 30 min at RT. Antibody incubation (1:10 000 Anti-DIG-AP in blocking buffer) occurred for 30 min at RT, followed by 2 washes in washing buffer. Afterwards the membrane was equilibrated in detection buffer (0.1 M Tris-HCl; 0.1 M NaCl; pH 9.5) for a few minutes and placed on a development folder, on top of which the Chemiluminescent Alkaline Phosphatase Substrate CSPD solution (1:100 CSPD, supplied with the kit, in detection buffer) was applied. The membrane was then covered with a second sheet of the folder to spread the substrate evenly and remove air bubbles. After 5 min incubation, the excess liquid was squeezed out and the edges of development folder sealed. Damp membrane was incubated for 15 min at 37ºC to enhance the luminescent reaction, followed by exposure to X-ray film XOMAT AR 5 (KODAK) for 2-25 min.

2.2.5.12 Isolation of total RNA from Arabidopsis – Trizol method

Trizol (380 ml/l phenol; 0.8 M guanidine thiocyanate; 0.4 M ammonium thiocyanate; 0.1 M NaOAc pH 5; 50 ml/l glycerol) reagent (1 ml) was added to 50-100 µg of frozen plant tissue grinded in liquid nitrogen, homogenized and centrifuged at 4ºC for 10 min. Supernatant was recovered and left at RT for 5 min. Samples were supplemented with chloroform (200 µl), mixed and centrifuged to separate phases. Isopropanol (500 µl) was added to the aqueous phase and the mixture was incubated at RT for 10 min. Resultant pellet was rinsed with 75% ethanol and resuspended in DEPC-treated water.

Water and solutions treatment with DEPC is done by addition of 1 ml DEPC (SIGMA) per liter of solution, stirring for 1 hour to o/n at 37ºC, followed by autoclaving to hydrolyze DEPC.

2.2.5.13 RNA quantification

RNA concentration was measured by its absorbance (A) at 260 nm in a spectrophotometer, according to Sambrook *et al*. (1989), considering that 1 of A_{260nm} = 40 µg RNA/ml. Purity of RNA was determined by A_{260nm}/A_{280nm} ratio, which ideally should be 1.8-2.0, and by fractioning RNA samples in a 1 % agarose gel. RNA samples were denatured at 65ºC for 5 min prior to being loaded on the gel.

2.2.5.14 In vitro *transcription of the RNA probe*

A 660 kb probe was designed for the 587-1246 bp region of PIN6 and amplified by PCR using primers *pin6ORFfor* and *pin6ORFrev*, which included the T7 and T3 transcription initiation sites, respectively. DNA template (0.3 µg) were added to 2.5 µl transcription buffer, 2.5 µl DIG-UTP mix, 1 µl RNAse inhibitor (20 U/µl) and 1-2 µl T7/T3 RNA polymerase (10 U/µl) and incubated for 2 hours at 37 ºC. yeast tRNA (4 µl of 10 mg/ml) and 2 µl DNAse I (1 U/µl) were added and the samples were incubated at 37ºC for 15 min more. Samples were placed on ice and 2 µl EDTA (0.2 M, pH 8.0), 2.5 µl LiCl (4 M) and 75 µl ethanol were added to precipitate RNA (at least 30 min at -70ºC). Afterwards, samples were centrifuged for 30 min at max speed at 4ºC, and the pellet washed twice with 80% ethanol. After drying, the pellet was resuspended in 80-100 µl RNase-free water.

2.2.6 METHODS FOR PROTEIN ANALYSIS

2.2.6.1 Determination of protein concentration

Protein samples were mixed with 200 µl of 5x Bradford solution to a final volume of 1 ml. After 5 min incubation at RT, the absorption of the colour complex was measured at 595 nm with a spectrophotometer. The protein concentration was estimated with a standard curve with different dilutions of BSA (Bradford, 1976).

2.2.6.2 Separation of proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of protein extracts was performed in a 10% polyacrylamide resolving gel (4 ml rotiphorese 30 containing 30% acrylamide and 0.8% bisacrylamide; 3 ml 1.5M Tris-HCl, pH 8.8; 120 µl 10% SDS; 600 µl glycerol, 40 µl APS). Separation gel mix was degassed and the polymerisation started by addition of 80 µl TEMED. The stacking gel (1.25 ml rotiphorese 30 containing 30% acrylamide and 0,8% bisacrylamide; 2.5 ml 0,5 M Tris-HCl, pH 6.8; 100 µl 10% SDS; 40 µl APS; 20 µl TEMED; 6.15 ml H_20) was prepared similarly and loaded on top of the separation gel. Before loading, samples were mixed with loading buffer (10% glycerol; 3% SDS; 3% β-mercaptoethanol; 0.3% bromophenol blue; 1 mM PMSF) and denatured (for 30 min at 37°C for membrane proteins or 5 min at 95°C for soluble proteins). Electrophoresis was performed at a steady current of 10 mA while samples were in the stacking gel and at 20 mA once they reached the running gel.

2.2.6.3 *Coomassie blue staining of SDS-PAGE gels*

The polyacrylamide gel was incubated at 65°C for 30 min with Coomassie blue (50% methanol; 10% acetic acid; 0.1% Brilliant Blue R250) for staining. Excess staining was removed by repeated washes in destaining solution (10% methanol; 10% HOAc).

2.2.6.4 *Western Blot*

After SDS-PAGE electrophoresis, proteins were transferred to a PVDF membrane. The PVDF membrane and gel were soaked in 100% methanol and twice equilibrated. The gel was placed adjacent to the PVDF membrane and pressed between sheets of 3MM paper and pads on the transfer cassette in the following order, starting from the side of the cathode: pad, 3MM paper, gel, membrane, 3MM paper and pad. The assembled transfer cassette was placed vertically into the electrophoresis tank and filled with transfer buffer. Transfer was with a 20 V current, o/n at 4°C, with agitation.

2.2.6.5 *Ponceau staining*

After transfer, PVDF membrane was washed in water and stained for 2 min in Ponceau staining solution (0.2% v/v Ponceau; 3% v/v trichloroacetic acid). The staining was removed with several washes in water with a few drops of NaOH, to facilitate removal of the strongly acidic dye from the membrane.

2.2.6.6 *Immunostaining of Western Blots*

Membrane with the bound proteins was soaked in ethanol and washed in PBST buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4; 0.1% Tween20) twice for 10 min. Blocking of the membrane was done by incubating it in 1% BSA in PBST for 1 hour. Primary antibody binding was performed with a dilution of 1:3000 in 1% BSA in PBST for 90-120 min at RT. Unbound antibodies were removed by washing with PBST buffer: 2x shortly, 1x 15min and 2x 5min at RT. After addition of the secondary antibody diluted 1:5000 in 1% BSA in PBST buffer, the membrane was incubated for 60-90 min at RT. Unbound antibodies were subsequently removed with PBST buffer washes: 2x shortly, 1x 15min and 2x 5min at RT. Finally, the membrane was incubated in a 1:1 mixture of detection solutions 1 and 2 from the *ECL Analysis System of Horseradish Peroxidase-coupled secondary antibodies* and exposed to X-ray film XOMAT AR 5.

2.2.6.7 *Epitope expression for antibody generation and purification*

A region of the PIN6 protein corresponding to aminoacids 175 to 395 was inserted in *pET-28a(+)* (NOVAGEN). *E. coli* strain BL21 DE3 was transformed with this construct and several clones containing the plasmid were selected for small-scale expression. The expression was verified by SDS-PAGE followed by Coomassie staining. Single colony transformants were inoculated as a pre-culture in 1.5 ml of culture media containing kanamycin.

From the pre-culture grown o/n at 37°C 2 ml were used to inoculate 50 ml LB liquid medium (pre-warmed) containing kanamycin. Cultures were incubated for 2 hours at 37°C until OD_{600} reached 0.7-0.9, split in 2x 4 ml and then induced with 1 mM IPTG. Induced cultures were then incubated 3h at 37°C for protein synthesis. Cells were afterwards harvested by centrifugation at 4 000 rpm for 20 min and pellets lysed with 200 µl buffer B (100 mM NaH_2PO_4; 10 mM Tris-HCl; 8 M urea; pH 8.0) at RT by gently vortexing. The pellet was obtained by centrifugation for 10 min at 14 000 rpm, and kept at 4°C. Of the 50 % Ni-NTA slurry (QIAGEN), 50 µl were added to each tube containing the pellet and centrifuged for 30 min at RT. The resin columns (QIAGEN), pre-equilibrated with buffer B, were then loaded with the cleared lysate supernatant and centrifuged for 2 min at 660 rpm. The columns were washed twice with 600 µl buffer C (100 mM NaH_2PO_4; 10 mM Tris-HCl; 8 M urea; pH 6.3). Protein was then eluted twice with 200 µl buffer E (100 mM NaH_2PO_4; 10 mM Tris-HCl; 8 M urea; pH 4.5). Purified protein fragment was used for immunisation of rabbits, mice and guinea pigs at EUROGENTEC anD NANOTOOLS and later on for antibody purification.

2.2.6.8 Affinity purification of antibodies

Purified PIN6 epitope (100 µg) was separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Western Blot, see section 2.2.6.4). After Ponceau staining, membrane area containing the antigenic epitope was isolated. The membrane strip was destained with TBS (35 mM Tris-HCl pH 7.4; 140 mM NaCl) and incubated for 2 hours in buffer 1 (1% BSA; 0.5% Tween 20 in TBS) to block non-specific binding sites. An aliquot of the immune serum was diluted with 4 vol of TBS and incubated with membrane strip for 2 hours at 4 °C to promote specific binding between the antibodies and the epitopes. The unbound antibodies were washed with TBS for 4x 5 min, at 4°C. Bound specific antibodies were eluted with 2x 0.75 ml buffer 2 (0.1 M Glycine; 0.5 M NaCl; 0.05% Tween 20; pH 2.6) for 1.5 min at 4 °C. Immediately after elution, the pH of both fractions was adjusted with 100 µl Tris-HCl pH 8.0. For stabilization, antibodies solution was supplemented with 0.5% BSA and 0.05% sodium azide.

2.2.7 METHODS FOR HISTOCHEMISTRY ASSAYS

2.2.7.1 Lugol staining

Prior to microscope inspection, Arabidopsis seedlings were stained with Lugol reagent (ROTH) for 10-15 min, twice rinsed in water and then mounted on microscope slides in chloralhydrate/glycerol (1:1).

2.2.7.2 Histochemical detection of reporter gene activity (GUS staining)

Activity of reporter enzyme β–glucuronidase (GUS) was analyzed in transgenic plants carrying a fusion of *PIN6* promoter with *uidA* gene, *pPIN6*::*GUS* (Schelhaas, 1999), and *DR5*::*GUS* (Ulmasov *et al.*, 1997b), X-Gluc was used as a substrate for enzyme activity localization. Seedlings were fixed for 1 hour in 90% ice-cold acetone on ice, then vacuum infiltrated with the staining solution (0.05% X-Gluc, 5 mM potassium ferrocyanide, 5 mM potassium

ferricyanide and 0.1% Triton X-100 in 0.1 M phosphate buffer) and incubated o/n at 37° C. After staining green tissues were bleached by o/n treatment with absolute ethanol.

2.2.7.3 Embedding plant tissue for sectioning

Plant material (inflorescences) was fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 10 mM phosphate buffer, 2.7 mM KCl, pH 7.0) with vacuum infiltration. Following two washes with PBS on ice, tissues were dehydrated in an increasing series of ethanol (30%; 50%; 70%; twice 100%), each step 30 min on ice. Tissues were stained with 0.1% Saffranin-O in absolute ethanol, for 1 hour on ice. The samples were then transferred to Falcon tubes and left in absolute ethanol o/n. After two washes with 100% ethanol (30 min on ice), the tissues were treated with a series of ethanol:Histoclear solutions (2:1; 1:1; 1:2; twice 100% Histoclear) at RT, 15-30 min each. Histoclear was removed until there was enough volume left to cover the samples. Paraplast tablets were added on top of them and left o/n at 60ºC, for the paraffin to melt. Paraffin was changed twice a day for at least 2 days, until samples were solidified into paraffin blocks. 7 µm sections were made using a Microtome MICROM HM 355.

2.2.7.4 In situ hybridization on sections

Slides were dewaxed in a series of Histoclear: ethanol solutions (3x Histoclear; 2:1; 1:2; 3x ethanol) at RT, 3 min each. Tissues were then rehydrated in 95%, 90%, 70%, 50% and 30% ethanol, for 2 min each. Slides were incubated in 0.2 M HCl for 20 min at RT, followed by rinsing in 0.85% NaCl in PBS and permeabilization with Proteinase K (2-5 µg/ml) in Buffer 1 (20 mM Tris-HCl pH 7.5; 2 mM $CaCl_2$) for 20 min at 37ºC. The enzymatic reaction was stopped by adding 0.2% glycine in PBS. After two washes in PBS, samples were post-fixed with 4% paraformaldehyde in PBS. After three rinses in PBS, tissues were carefully overlaid with prehybridization buffer (50% deionised formamide, 2x SSC, 50x Denhardt mix, 10 mg/ml yeast tRNA, 2% Dextran sulphate, 10 mg/ml Poly A, 10 mg/ml ssDNA, 100 mM DTT) and incubated in a humid sealed chamber at 37ºC for 2 hours. After 5 min rinse in 2x SSC, slides were overlaid with prehybridization buffer supplemented with 15 µl hydrolysed DIG-labelled RNA probe (see section 2.2.5.14), denatured at 65ºC for 5 min before applying it to the slides, and 1 µl RNAse inhibitor. Samples incubated 16 hours at 50ºC. Post-hybridization washes were done as follows: a quick wash in 1x SSC (10 mM DTT) at RT, 2x 15 min in 1x SSC (10 mM DTT) at 55ºC, 2x 15 min in 0.5x SSC (10 mM DTT) at 55ºC and 1x in 0.5x SSC (10 mM DTT) at RT. Hybridized probes were detected with anti-DIG antibody coupled to either AP or FITC. Alternatively, the samples were loaded in the slide unit of the INSITU PRO VS robot (INTAVIS), in which a program equivalent to this protocol was followed.

2.2.7.5 Whole-mount in situ hybridization

Slides were dewaxed in a series of Histoclear: ethanol solutions (3x Histoclear; 2:1; 1:2; 3x ethanol) at RT, 3 min each. Tissues were then rehydrated in 95%, 90%, 70%, 50% and 30% ethanol, for 2 min each. Slides were incubated in 0.2 M HCl for 20 min at RT, followed by rinsing in 0.85% NaCl in PBS and permeabilization with

Proteinase K (2-5 µg/ml) in Buffer 1 (20 mM Tris-HCl pH 7.5; 2 mM $CaCl_2$) for 20 min at 37ºC. The samples were loaded in the INSITU PRO VS robot (INTAVIS), in which a program equivalent to the protocol described above (see section 2.2.7.4) was followed.

2.2.7.6 Immunolocalization on sections

Slides were immersed in PBS and treated for immunofluorescence. After permeabilization with 1% cellulase (ONOZUKA) in PBS for 30 min at 37ºC, sections were rinsed twice in PBS with 0.002% Tween 20. In order to saturate the tissue and ensure specific antibody binding, sections were blocked for 1 hour with 3% BSA and 0.002% Tween 20 in PBS. Incubation with the primary antibody anti-PIN6 rabbit was carried out diluted 1:300 in 3% BSA in PBS o/n at 4ºC in a wet chamber. Following 3x 5 min rinsing steps in PBS with 0.002% Tween 20, sections were incubated for 2 hours with the secondary antibody (anti- rabbit IgG Alexa Fluor® 488) 1:300 in 3% BSA in PBS. For multiple labelling with anti-PIN1 and anti-PIN6 antibodies, anti-rabbit IgG Alexa Fluor® 488 and anti-guinea pig IgG Alexa Fluor® 555 were used. Finally, sections were washed twice in distilled water and mounted in anti-fade mounting medium and inspected by confocal microscopy. Alternatively, samples were loaded in the slide unit of the INSITU PRO VS robot (INTAVIS), in which a program equivalent to this protocol was followed.

3 RESULTS

"Science is nothing but developed perception, interpreted intent, common sense rounded out and minutely articulated."

George Santayana

3.1 *IN SILICO* ANALYSIS OF PIN6

PIN6 (At1g77110) has been annotated in TAIR as a rate-limiting factor in saturable efflux of auxins, by analogy with other described PINs. The *PIN6* genomic sequence is 3747 bp long and the corresponding coding sequence (CDS) comprises 1451 bp (Fig. 3.1. and Fig. 3.4.).

taacttaacctacatagtacataccaagaagaagaagaggcaaagaaaaag**ATG**ATAACGGGAAACGAATTCTACACGGTGAT
GTGTGCCATGGCACCACTCTACTTCGCCATGTTCGTGGCTTATGGATCGGTAAAGTGGTGCAAGATCTTCACGCCGGCGCAAT
GTTCCGGAATCAACCGTTTTGTCTCCGTTTTCGCCGTCCCAGTCCTCTCTTTCCACTTCATCTCACAAAACAACCCTTACAAA
ATGGACACAATGTTCATCCTCGCCGACACTTTGTCCAAAATCTTCGTCTTCGTTTTGCTCTCTCTATGGGCCGTTTTCTTCAA
AGCCGGTGGTCTCGATTGGCTCATCACTCTATTCTCAATCGCTACTCTCCCTAACACTCTCGTTATGGGCATACCTTTGCTTC
AGGCCATGTATGGAGATTACACTCAAACCCTCATGGTCCAACTCGTTGTTCTTCAATGCATCATTTGgtacgttatcaaatta
ctatccgttctttattcatttaccctgtgattgattagaaaacctcataataatggaattattttgtgatttcacatattttt
accacacatttttcatggattttgtaaccaaaacttaaatgaatttcgttactctaggatataattcaaccaaatcaaatgt
ttttactaacccaagcttcttattagttgaattatttaattataacgtatgataatataagctaagtgtatatacatgtatag
GTATACTCTGCTACTATTCCTCTTTGAACTCCGAGCAGCGAGGTTGCTTATCCGAGCAGAGTTTCCGGGTCAAGCAGCCGGAT
CAATCGCCAAGATCCAAGTCGACGATGACGTCATTTCCTTAGACGGCATGGATCCGCTCCGTACAGAAACTGAAACCGATGTT
AACGGTCGGATCAGGCTCAGGATCCGACGGTCCGTATCATCCGTACCTGATTCTGTTATGTCCTCCTCCTTGTGCTTAACCCC
TAGAGCCTCTAATCTTTCTAACGCCGAGATTTTCTCCGTCAACACTCCTAATAACCGTTTCTTCCACGGCGGAGGAGGAAGCG
GTACCCTTCAGTTTTATAACGGTAGTAACGAGATCATGTTTTGTAACGGAGATTTAGGCGGGTTCGGATTTACTCGACCCGGA
TTAGGTGCAAGTCCTAGAAGGCTCTCGGGTTATGCTTCCTCCGATGCTTACTCGTTGCAGCCAACACCACGTGCCTCGAACTT
TAACGAGCTTGACGTTAACGGAAACGGTACGCCGGTTTGGATGAAATCTCCTGCCGCCGGGAGAATATACCGGCAATCTTCGC
CGAAGATGATGTGGGAGTCTGGGCAAAGACATGCAGCCAAAGATATCAATGgtacgttttctttgaaagatgtgtcgttttcg
tttttcgttagcactgacacgacggcgtttagtcaatttcttctcttttcgtgtttttctttatcattggttctttttaaaag
atttaatgaatagaaaaacattgatagactacataaaatgcagcttttcagaaataaaaaaacttagcattgtctcttttact
ttccaaatccctaggaattaactaattagaaattcaatctaattttataaaatttataagtaatagtcactaaatcccactta
ttttatttttttgtgtggaaacttttgaattttgatacttcatgtttttgagcttaagattctataggacaacatgatgaagc
atgtaaacaaagcggacccctcataccacccacacacttactatcatcaataaaatcaccaaaatggtcaacatgatgttgac
ttttttgtttctcatgatgtttttggactcatcaaattaatgtccattctaaagaatttatttgggagattctatcattttcc
tctagctcacgtgtgtaatcatcagcgaaaccgacgcatggttttctatttttttcttccattactaaatccacaacctttat
taacaaaaagagttataactaattagttgctttcaaaatatcaagttttgtcctaactatcactctgtatctgctcacgtgta
ttttatataactaagGATCAGTGCCAGAGAAGGAGATTAGCTTCAGAGACGCACTTAAGGCTGCACCACAGGCTACGGCAGCC
GGTGGTGGTGCTTCCATGGAGGAAGGAGCCGCCGGAAAAGACACAACTCCGGTGGCTGCGATTGGCAAGCAAGAAATGCCAAG
TGCAATTGTAATGATGCGCCTCATACTAACAGTCGTGGGCCGCAAGCTTTCTCGAAACCCTAACACTTATTCCAGCCTCTTAG
GTCTTGTCTGGTCACTTATATCCTTCAAgtgagtcaaaaaataactcaaatatcaaaatttcaactataatcattgaatttgt
ttgttatataactttaactcatattttattttgctaaataatgagcagATGGAATATACCGATGCCAAATATAGTGGATTTCT
CGATAAAGATCATTTCAGATGCAGGTCTTGGGATGGCTATGTTTAGTTTAGgtatgaacatgttcttataaacctgcataata
aataaaattatgtataacgtattctaacaaaattaaactatagGCCTGTTTATGGCGCTGCAGCCAAAGATGATCCCTTGTGG
GGCGAAAAAGGCAACAATGGGGATGTTGATCAGGTTCATCTCAGGTCCTCTTTTCATGGCTGGTGCTTCCCTTCTTGTCGGAT
TAAGAGGTTCTAGGTTACATGCTGCTATCGTACAGgtaaactattcagagtttaccttgatttggcccatatatattattgtt
ggaaaattaaagaaaacatacttggtgatggagaaagtgtttaagattttgggagtgaattaggaagtttaacatatagaagg
gactaattagttaaaaaaaaatgaaggatagatatgttcatagctttcaaaagtacaaatatctatctcatataaacctaata
tagtctaccattatatctagacttatatgttacaaagtatctagatattatgaaaatatctagatattttacatgatgcaatt
ccctgtattagtctaaggaggtattaatattttaagatggttctaaagagactctttaacttgtttcaagttttgcgtttggt
ccaagaaattaatgatacctggatcattgacacaaattataactaatccaaaatcaagttaaatatttcaaaatataaattac
ccgtggctataatttaaacttcgtttcttataaaagttttcgattaacttatagtagagatatgtacatctaaatttgatgaa
gccgatatgttcttttagGCTGCTCTACCGCAAGGGATAGTACCATTTGTGTTTGCGAGGGAGTACAATTTGCATCCAGACCT
ACTCAGTACATTgtgagtctcttgctcttctttttgttgttcttcatttacatttacggttgaacgtttcaaaatgcggtatt
atggtttatgtgaaatctctgttcttggtgatttttttgttttgttttcagGGTTATCTTTGGGATGATAGTATCTTTACCAGT
AACTATTTTGTACTACGTCCTCTTGGGCCTA**TGA**ttcacacaacacactgaagaaaaagattacaccggagaaattaaatgat
ctatgaataaagaggtctggaaatagagaaatgtgagctcttaagagttgttcttgaggagcttgtgagaaagattaattaac
ggttaattacattgttgaatcaaattactggtaaatggttaggtgaataatttaattttatgt

Fig. 3.1. Full-length genomic DNA sequence of *PIN6* (At1g77110). Coding sequence (CDS) nucleotides are depicted in caps. Exons are located in bp 52-482, 748-1379, 2456-2541, 2617-2774, 3341-3417 and 3539-3605. Start codon ATG and termination codon TGA are signalled in bold letters.

Of the *PIN* gene family, *PIN3* and *PIN7* share the highest sequence similarity (Fig. 3.2.) and *PIN4* is their next closest relative. The gene most similar to *PIN6* is *PIN2* - they group together in the following cluster (Fig. 3.2.). DNA sequence from *PIN1*, *PIN5* and *PIN8* differ the most from the other *PIN*s. For *PIN5* and *PIN8* this is mainly due to the absence of a central hydrophilic loop.

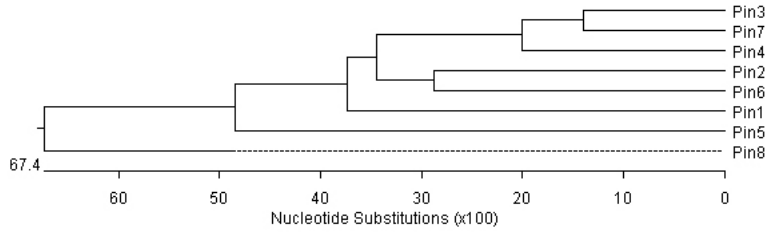

Fig. 3.2. Tree view of *PIN* genes sequence alignment. Length of the lines corresponds to the number of nucleotides substituted.

3.1.1 PREDICTION OF PROTEIN PROPERTIES

PIN6 protein (Fig. 3.3.) is 570 aa long with a predicted molecular weight of 62 KDa and an isoelectric point (pI) of 9.48 (TAIR).

```
  1 MITGNEFYTV MCAMAPLYFA MFVAYGSVKW CKIFTPAQCS GINRFVSVFA VPVLSFHFIS
 61 QNNPYKMDTM FILADTLSKI FVFVLLSLWA VFFKAGGLDW LITLFSIATL PNTLVMGIPL
121 LQAMYGDYTQ TLMVQLVVLQ CIIWYTLLLF LFELRAARLL IRAEFPGQAA GSIAKIQVDD
181 DVISLDGMDP LRTETETDVN GRIRLRIRRS VSSVPDSVMS PSLCLTPRAS NLSNAEIFSV
241 NTPNNRFFHG GGGSGTLQFY NGSNEIMFCN GDLGGFGFTR PGLGASPRRL SGYASSDAYS
301 LQPTPRASNF NELDVNGNGT PVWMKSPAAG RIYRQSSPKM MWESGQRHAA KDINGSVPEK
361 EISFRDALKA APQATAAGGG ASMEEGAAGK DTTPVAAIGK QEMPSAIVMM RLILTVVGRK
421 LSRNPNTYSS LLGLVWSLIS FKWNIPMPNI VDFSIKIISD AGLGMAMFSL GLFMALQPKM
481 IPCGAKKATM GMLIRFISGP LFMAGASLLV GLRGSRLHAA IVQAALPQGI VPFVFAREYN
541 LHPDLLSTLV IFGMIVSLPV TILYYVLLGL
```

Fig. 3.3. PIN6 amino acid sequence.

Analysis of PIN6 amino acid sequence using InterProScan identified an ER signal peptide in aa 1-20. This signal sequence often also referred to as leader peptide and is common to proteins sorted through the vesicular pathway. Moreover, transmembrane domains are predicted to be located in aa 10-28, 42-60,

75-95, 101-121, 131-151, 457-477, 492-512, 548-568 (Fig. 3.4.). As *PIN1* and *PIN2* are the PIN genes most co-expressed and co-regulated with *PIN6* (see sections 3.1.3 and 3.1.4), PIN1 and PIN2 protein transmembrane structures are also depicted in Fig. 3.4. The loop is predicted to be located at aa 152-456. According to WolFPSORT's peptide signal prediction, PIN6 is likely to be a plasma membrane protein. Furthermore, a putative vacuolar targetting motif (TLPN) is present at aa 109. A search for glycosylation sites (using NetOGlyc3.1) predicted one putative site at aa - 304. Additionally, 27 possible phosphorylation sites were retrieved from the sequence (using NetPhos2.0): 16 Ser (aa 27, 47, 210, 212, 213, 217, 233, 286, 291, 300, 308, 326, 337, 356, 363 and 382), 8 Thr (aa 131, 193, 195, 226, 242, 304, 320 and 393) and 3 Tyr (aa 8, 293 and 299).

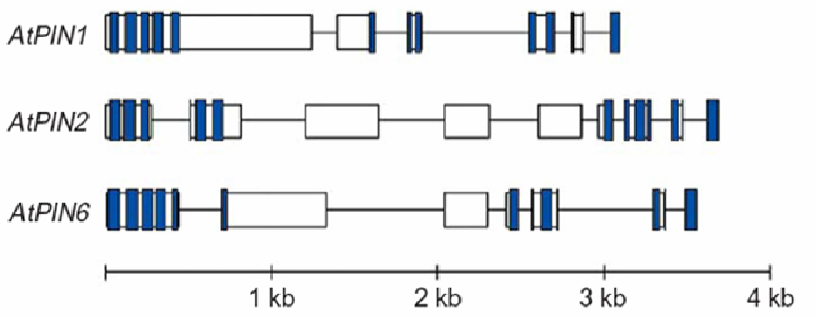

Fig. 3.4. Exon-intron structure of *PIN1*, *PIN2* and *PIN6* gene sequences. Exons are represented in boxes and in *PIN6* sequence they are located in bp 52-482, 748-1379, 2456-2541, 2617-2774, 3341-3417 and 3539-3605. Introns are depicted as black lines. Blue boxes represent protein transmembrane domains. Adapted from Paponov *et al.*, 2005.

PIN proteins share similar topology - five to six transmembrane domains linked by a small hydrophilic loop (see section 1.5.). The similarity between members of the PIN protein family ranges between 32% and 85% (Paponov *et al.*, 2005), the most similar being PIN3 and PIN7. PIN3, PIN4 and PIN7 group once more in the cluster for the most similar proteins. On protein level, the closest relative to PIN6 is PIN8 (Fig. 3.5.), followed by PIN5 and the previously mentioned cluster.

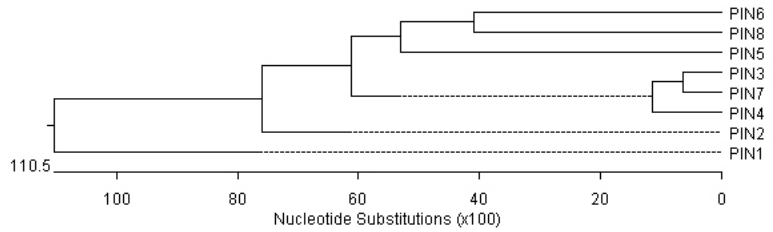

Fig. 3.5. Sequence alignment of PIN proteins. Length of the lines corresponds to the number of number of amino acids substituted (x100).

3.1.2 PROMOTER SEQUENCE ANALYSIS

The presence of regulatory elements in the promoter sequence provides hints on how genes are regulated by different factors, *e.g.* light, hormones and DNA binding proteins. Such promoter sequences are recognized as binding sites for transcription factors or transcription regulators, which after binding activate or repress gene expression. PLACE, a database of PLAnt Cis-acting regulatory DNA Elements covering vascular plants motifs with regular description updating, was screened with a 1794 bp sequence upstream of the ATG initiating codon sequence region for the presence of regulatory elements in *PIN6* gene promoter region (see Table 3.1.). The sequence corresponded to that previously used for generating the *pPIN6*::GUS construct (see Fig. 3.6.; Schelhaas, 1999).

```
-1794 ACAAACTTGCTTATCCTGAATTGTTTTAGTCAGATTCATGTGTG
-1750 TACTAATATATACAGACAATATAGGTTACATGAGGCTTTGATGCCATTAC
-1700 CCATCGGGGAAAATAATCATAACTATAAGTGATTATATATATTATCCTAC
-1650 ATAACTCTTGTCTGCACATTTTGGTTAGAAAGTAGGGTTACTTAATATTT
-1600 ATCTTAGGTTAGATATATAGGACGGAATCAATAATCCCAAACTTTTTTGT
-1550 AGCCTTTCGTTGACAAAGTAAATTATTTATTTTATAAGAAAAAGGTTACG
-1500 TATGACTTAGTTCTTGCAATTGCCAAAAGGCTAAAGGCTAAAGGCTTTTA
-1450 AGCTTGAGAGTAAAAGGCTAACACTGTAAGTCTCCACCCATTTCGCCGAT
-1400 ATCATTGATCTTAGATTCTATGAGGATCTCCTTTCACTTTCTTTATGTAT
-1350 ATATGTACAATTTCTGTATATCTATGTGAATAAATTAAGAACAAGGTAAT
-1300 CATGTCTCTGTGGACCCTTTGCTTAATTGTGACATATGTATTGTTAGTAC
-1250 AAGAAGCTCATTACATTCCTTTTATATGGTCAGAAGTTCCAAACATTTTC
-1200 TAGTTTCAGATATGTTAGCTTTCCATATTTTAAGAAAATTAGTAAACCTG
-1150 ACTCTACTTCATTATTGATTGTTTTAATTTGTTTTTTGAATAATTGATAA
-1100 CTGGTTCTTTTATATGTTAAAAGACGAATTTCATGATATTGTTCTTGAGC
-1050 ATAGCGAGACGAGGTATATAATGGTGGGAAGATACATCAAAAGAGTTGCA
-1000 TGAGACAAAAACAAAATGGAAATTGTCATGTCATTAATGACTGACCAATT
 -950 TCCGTAGCTTTAACTAGAAAGCTTCAAATTTGAAGTATATACTTTATCTT
 -900 CATTTTTCGTGCATGTATTTCTAGTAGATTTTGAATTGAATAGTCATATA
 -850 GCTTTTCGTCATAAAACACACCAGAAATAAGAAGTTTAATAATCAATAAA
 -800 AGAAAACAAAAGAGTTAGGGGGATAATTTGGGTGAAAGCCTATTTCGACA
 -750 AGACACCAAGTGTGATAGTTTTGGATAAACAGATATTTTTTATCCGAGTT
 -700 TGCTGCAACTGTATGCAAAAAGTGTAAAATACTGCAAAAGTAGATATACA
 -650 CTTGCTAAAAGTGAAAATACTGTAAAAGTAAAAATAAGAGAAAAGAAAAC
 -600 AAAAGAGTTAGGTCAATAATAAGGGTGCAAAGCTCACTTAAGCAAAACAC
 -550 AGAATTTTCAATTTTGGGTAAACAAAGTGTACTTTTTTCTTGAATTACGA
 -500 AATTTTGTTTATTATATAGATTTCTGAAATAAGAGACTTATGCGTGTACA
 -450 TATTGAGAATGATTATACGGGTTACAAAGCAAATCTATTGCATGAAGTTA
 -400 TTCTAAATATTCTATTAAAATCTTTTTCCCGGTTTGGATTAGATTTTCAA
 -350 TAATTTAATTATGAAGTTGAATTAAAAAAAAAAAAAGAGTAACAAATCAAC
 -300 TTATTAATTAATTTTAAAATTGTGATGAGTCTATTTAATGAGAATTTTAG
 -250 AGATTAAAATAATGATGAGGTCCAGAAAAAGAAAGTGCTTTATATATAGA
 -200 TTTCCAACTATATATGTATAGCAGCAGTATTTTACCAATCTATATAATAA
 -150 TCACTAATCCCTCCCTAATGCAATTTCATAACGAAGCTAACTAAGGGGTA
 -100 ATCTCGTCAACAAGTCTCTATATAACCCAACACACAAACATTTGGCACAT
  -50 AACTTAACCTACATAGTACATACCAAGAAGAAGAAGAGGCAAAGAAAAAG
```

Fig. 3.6. Promoter sequence of *PIN6* used previously for the *pPIN6::GUS* construct (Schelhaas, 1999), from -1794 bp to 0. The full 5'-UTR region of *PIN6* (from the STOP codon of the previous gene) comprises 3737 bp.

From the regulatory elements present in *PIN6* promoter sequence, the most interesting are listed in Table 3.1. These elements relate to different developmental stages such as embryogenesis and root development, also including seed-specific elements (*i.e.* endosperm-specific), and for expression in pollen

and fruits. Binding sequences for Dof transcription factors, involved in many different aspects of plant development, are also present in the promoter region.

Given that other *PIN* genes are regulated by auxin (Heisler *et al.*, 2005; Paciorek *et al.*, 2005; Sauer *et al.*, 2006; Scarpella *et al.*, 2006), I expected to find auxin responsive elements in the promoter sequence of *PIN6*. Consistently, such elements are present eight times in the promoter, with five being relatively close to the start codon (in the first -600 bp), including two elements involved in the rapid response to auxin (SAUR: small auxin up RNA).

In addition to auxin, there are also elements responsive to other hormones in the sequence, such as abscisic acid, cytokinin, ethylene and gibberellin.

It is also interesting to note the presence of elements involved in light-associated processes, such as a circadian expression regulatory sequence: the I-box, the GT-1 box and its closely related SBF-1 binding site. Additionally, elements associated with the meristem transition to reproductive phase, such as AG-, MYB- and PHY-related elements, can be found adding to the role of PINs in meristem maintenance.

Cell cycle regulatory elements (including E2F) are also represented in *PIN6* promoter. Besides common regulatory elements, there are two miRNA binding sequences in the *PIN6* promoter sequence, and MIRNA analysis revealed 6 potential miRNA targeted transcripts in the complete *PIN6* CDS sequence, located at 201, 206, 729, 784, 887 and 1749 bp (Adai *et al.*, 2005).

Table 3.1. Partial list of responsive elements present in *PIN6* promoter sequence. Common housekeeping regulatory elements such as A-boxes, C-boxes, T-boxes and TATA-boxes are not presented here.

Responsive Element	Sequence	Nr. times present in *PIN6* promoter	
		- 1794 to ATG	- 600 to ATG
AGAMOUS-binding sequence	ATTACCCATCGGGGAAAA	15	1
Auxin responsive element	TGTCTC	2	1
	ACTTA	1	0
	YTGTCWC	1	0
Auxin responsive module	CATATG	2	2
Cell cycle	CACGAAAA	1	0
Circadian expression regulating sequence	CAANNNNATC	3	2
	AAAATATCT	1	0
Cytokinin enhanced binding	TATTAG	1	1
Dof transcription factors binding sequence	AAAG	44	16
	CCTTTT	4	4
	TAAAG	6	3
E-box (ABA responsive)	CANNTG	14	6
Endosperm expression	TGHAAARK	3	0
	TGCAAAG	1	0
Ethylene responsive element	AWTTCAAA	1	0
Fruit specific expression	TGTCA	5	3
GA-response and sugar response	TATCCA	1	0
G-Box binding proteins sequence	GGTTACGTATGA	2	2
Light regulated genes GT-1 consensus sequence	GRWAAW	18	7
	GAAAAA	6	1
	GGTTAA	1	0
Light regulated I-box	GATAA	9	4
MYB transcriptional activators binding sequence	CAACACAC	5	3
	WAACCA	1	1
	TAACTG	1	0
	YAACKG	2	0
	CTAACCA	1	1
	CNGTTR	2	0
	TAACAAA	1	0
	GGATA	5	2
Phytochrome repression sequence	ATAGAA	2	1
	AACCAA	1	1
	CGGATA	1	0
Pollen-specific expression	GTGA	10	4
	AGAAA	17	6
	AGGTCA	1	0
RAV1-A (miRNA binding sequence)	CAACA	2	0
Root-specific expression	ATATT	11	5
RY element (seed-specific)	CATGCA	4	0
Small auxin up RNA (SAUR)	CATATG	2	2
SBF-1 binding site sequence	TTTTGGTTAGAAAG	14	14
Seed-specific	AACCCA	1	0
	RTTTTTR	2	0
SORLREP3 (miRNA binding sequence)	TGTATATAT	2	2

3.1.3 EXPRESSION PATTERN ANALYSIS

Microarray data from various sets of experiments have been recently made available through eFP Browser (which uses data sets from AtGenExpress, Schmid *et al.*, 2005) and GENEVESTIGATOR (Zimmermann *et al.*, 2004; Zimmermann *et al.*, 2005). Such toolboxes allow, for a particular gene of interest, to obtain the location of its transcripts in organs, tissues or even cells, differential expression under certain conditions, and modifications in the gene's expression in a still growing collection of mutants. A combined analysis of the above mentioned resources will be presented next.

PIN6 transcription levels are generally low, as much as 3-fold less than average *PIN1* expression levels. *PIN6* is mostly expressed in the shoot apex, from the vegetative phase (including cauline leaves) onwards, through transition phase, increasing up to the formation of the inflorescence, where the expression levels are the highest. *PIN6* is also expressed in young flowers (including carpels and ovary), in siliques and in seeds, including the early embryogenic development, from the heart-shaped to the torpedo phase. In young seedlings, *PIN6* is mainly expressed in the hypocotyl and in roots (including lateral roots, epidermis, root hair zone, elongation zone, root tip). *PIN6* mRNA is present in cell suspension cultures as well.

Of all *PIN* genes, *PIN1* and *PIN2* are the family members that share the most similar expression domains with *PIN6* during the life cycle. *PIN6* and *PIN1* are mainly co-expressed in shoot tissues, more specifically in flowers, stems, shoot apex, cotyledons, cauline leaves, hypocotyl, root hair zone and root elongation zone. *PIN2* co-expresses with *PIN6* specifically in root tissues (lateral roots, epidermis, root hair zone, root elongation zone, root rip).

3.1.4 PIN6 REGULATION

As reported above, PIN6 promoter is enriched with hormone regulatory elements. Microarray data shows that IAA treatment (1 μM for 1 h) induces *PIN6* expression (1.39-fold; refers to treatment/control). Auxin transport inhibitors NPA and TIBA upregulate *PIN6* expression levels (1.23-, and 1.74-fold, respectively). Impairment of auxin signalling by application of anti-auxin PCIB (Oono *et al.*, 2003) also up-regulates *PIN6* (1.43-fold) further proposing a role for Aux/IAA-ARF-dependent *PIN6* transcription. Besides hormones *PIN6* can be regulated by other factors. Cycloheximide (CHX), a protein biosynthesis inhibitor, induces *PIN6* 1.84-fold. This CHX-mediated increase of mRNA expression is explained by decreasing Aux/IAA short-lived transcriptional repressors as a consequence of CHX-directed inhibition of protein synthesis.

Moreover, mutants for primary auxin-response genes *axr1-12*, *arf7*, *arf19* and *max4* have higher levels of *PIN6* mRNA.

Abscisic acid (ABA) affects *PIN6* at the level of seed imbibition, inducing it 1.4-fold. Cytokinin treatment (zeatin 1 μM) downregulates slightly *PIN6* levels (0.87-fold). In 21-days old *ARR22*OX plants that displayed attenuated cytokinin responses treated with zeatin (20 μM), *PIN6* was induced 1.24-fold. *PIN6* is repressed by 30 min treatment with gibberellin (GA_3 1 μM; 0.8-fold). Conversely, reduction of GA levels in mutants (*ga1-5*) or by chemical inhibition results in induction of *PIN6* transcription. *PIN6* transcription seems to be variably responsive to ethylene: ACC (10 μM), a precursor of ethylene synthesis, has variable effect on *PIN6* levels, from downregulation (0.78-fold) after 30 min to upregulation (1.33-fold) after 1 h, again followed by downregulation (0.71-fold after 3 h), in accordance with the general view that ethylene can modulate polar auxin transport. On the other hand, inhibition of ethylene biosynthesis by AVG or of ethylene signalling by $AgNO_3$ (10 μM each) induce *PIN6* expression (1.22-and 1.29-fold, respectively). Moreover, *PIN6* expression was also induced in mutants for genes involved in responses to other hormones, such as *rdo2* (ABA, auxin, cytokinin, ethylene), *abi1-1* (abscisic acid), *ein2* (ethylene) and *gpa1* (ABA, ethylene, gibberellin). *PIN6* expression was further repressed in mutants for genes involved in responses to other hormones, such as *cesA3/zorro (*ethylene, jasmonate) and *ARR22OX* (cytokinin).

Additionally, light produces different effects on *PIN6* regulation. White and blue light have no effect on *PIN6* expression levels (1.03- and 1.07-fold, respectively), whereas red light, far-red light and UV-B slightly induce *PIN6* (1.15-, 1.17- and 1.28-fold, respectively).

The highest induction of *PIN6* transcription was observed in the *lec1-1.3* mutant (5-fold), with LEC1 protein involved in leaf development and embryogenesis. The strongest repression of *PIN6* transcription was observed in *cov1*, a mutant involved in apical dominance, leaf development and vasculature patterning. Mutants required for root hair development *lrx1* and *rhd2-1* also show reduced *PIN6* expression.

Similarly, other mutants related to leaf development, such as *add3*, *cls8*, *tcp13* and *tcp14* and the double mutant *tcp13 tcp14* have higher expression levels of *PIN6* transcripts. Mutants involved in branching also show increased levels of *PIN6* (*max4*, *tcp13*, *tcp14*, *tcp13 tcp14*). Regarding flower development, the male sterility gene *ms1* (in *ms1ttg*), the anther dehiscense-related mutant *myb61*, the pollen mutant *camta1-1* and the self-incompatibility mutant *sph1* show an increase in *PIN6* transcription levels. An increase in *PIN6* levels (1.83-fold) is seen in *det-1*, a mutant for a negative regulator of seedling photomorphogenesis.

In addition, mutants related with flower development as the flowering time mutant *flc-3*, the vernalization independent mutant *vip5* and the floral identity overexpressor *35S:LFY*, have downregulated levels of *PIN6*.

PIN6 is additionally downregulated in a cell cycle mutant, *E2Fa-DPa* a transcription factor that controls proliferation, endoreduplication and differentiation in Arabidopsis.

Noteworthy due to the presence of putative miRNA targets in the *PIN6* sequence, *PIN6* is overexpressed in the miRNA processing enzyme mutant *dcl1*. On the other hand, impairment of siRNA processing lead to *PIN6* downregulation in *rdr2* and *dcl3-1* mutants.

Some PIN genes are co-regulated by the same molecules and/or conditions. *PIN1* is the PIN most co-expressed with *PIN6* throughout plant development. *PIN1* and *PIN6* respond similarly to IAA, NPA, GA_3 and zeatin. *PIN2*, which co-expresses with *PIN6* specifically in root tissues, is regulated in the same way as *PIN6* by NPA, ACC, GA_3 and zeatin. It is interesting to note that *PIN6* is the only *PIN* gene whose transcript levels are upregulated by AVG treatment.

Table 3.2. List of mutants in which *PIN6* expression is up- or downregulated by at least ± 15%. At the top of the list are the mutants showing the highest variation in *PIN6* expression, followed by the second highest and hence forth. Relevant publications for each mutant are indicated.

Mutants with upregulation of *PIN6*		Mutants with downregulation of *PIN6*	
lec1-1.3	Lotan *et al.*, 1998	*cov1*	Parker *et al.*, 2003
det-1	Pepper and Chory, 1997	*cesA3 (zorro)*	Burn *et al.*, 2002; Wang *et al.*, 2006
add3	Picket *et al.*, 1996	*E2Fa-DPa*	Boudolf *et al.*, 2004
lerttg, ms1ttg	Thorlby *et al.*, 1997	*rdr2-1*	Gasciolli *et al.*, 2005; Lu *et al.*, 2006
gun1:gun5	Koussevitzky *et al.*, 2007	*cdb1*	Bezprozvanny *et al.*, 1997
rdo2	Léon-Kloosterziel *et al.*, 1996	*dcl3-1*	Gasciolli *et al.*, 2005
axr1-12	del Pozo *et al.*, 2002	*ARR22OX*	Kiba *et al.*, 2004
myb61	Liang *et al.*, 2005	*flc-3*	Martin-Trillo *et al.*, 2006
abi1-1	Allen *et al.*, 1999	*ape2*	Walters *et al.*, 2003; Walters *et al.*, 2004
max4	Bainbridge *et al.*, 2005	*dcl2-1*	Gasciolli *et al.*, 2005
arf7	Wilmoth *et al.*, 2005	*lrx1*	Diet *et al.*, 2006
ein2	Alonso *et al.*, 1999	*sfr3*	McKown *et al.*, 1996
vip5	Oh *et al.*, 2004	*rhd2-1*	Grierson *et al.*, 1997
gi11	Oliverio *et al.*, 2007	*35S::LFY*	Page *et al.*, 1999
dcl1	Gasciolli *et al.*, 2005		
arf19	Wilmoth *et al.*, 2005		
camta1-1	Mitsuda *et al.*, 2003		
sph1	Ride *et al.*, 1999		
gpa1	Chen *et al.*, 2006; Ullah *et al.*, 2002		
cls8	Garton *et al.*, 2007		
tcp13, tcp14, tcp13tcp14	Koyama *et al.*, 2007		

3.2 PIN6 LOCALIZATION

3.2.1 GENE EXPRESSION

Fusions between the promoter sequence of a gene of interest and reporter gene provide insights as to in which tissues or cells this gene is expressed and can be used to test conditions affecting this expression.

Promoter::GUS fusions are widely used. The presence of the β-glucuronidase gene (GUS) can be identified by a simple histochemical reaction in which a blue-coloured precipitate forms, thus allowing easy visualisation of promoter activity (Jefferson *et al.*, 1987). A *pPIN6::GUS* line previously developed in our group (Schelhaas, 1999) was used to characterize *PIN6* gene expression domains and *pDR5::GUS* (Ulmasov *et al.*, 1997b) as a measure of auxin distribution. *DR5* is a synthetic promoter containing 9 inverted repeats of auxin-responsive element TGTCTC. Expression of *DR5* transcriptional fusions have been shown to be proportionally responsive to a range of auxin and brassinolide concentrations (Sabatini *et al.*, 1999). However, it has been shown to be induced by brassinolide as well (Nakamura *et al.*, 2003). Nevertheless, it is the best available auxin reporter, as it has been shown to overlap spatially with auxin accumulation patterns detected by immunolocalizations with antibodies directed against IAA (Friml *et al.*, 2003). Therefore, *pDR5::GUS* reflects relative auxin levels exceeding a certain threshold and allows monitoring of changes in auxin distribution at cellular resolution.

At the hypocotyl shoot-root junction, *PIN6* is expressed in epidermis and cortex layers of the emerging adventitious root (Fig. 3.7.a). Auxin is present in the vasculature and at the root tip (Fig. 3.7.f,j). During lateral root formation, *PIN6* is localized in the lateral root founder cells in the pericycle (Fig. 3.7.b). As the lateral root emerges, it remains at the base of the lateral root, forming a ring-shaped expression domain (Fig. 3.7.c,d). *PIN6* and *DR5* localization in lateral roots are distinct – *PIN6* expression is limited to epidermal cells (Fig. 3.7.d) where auxin levels are lower (Fig. 3.7.h), while the auxin maximum is found at the tip of the root, in columella and root cap cells (Fig. 3.7. i). Interestingly, *PIN6* was absent from mature root tips suggesting *PIN6* not to be involved in the establishment and the maintenance of auxin gradient at the root tip (Fig. 3.7.e).

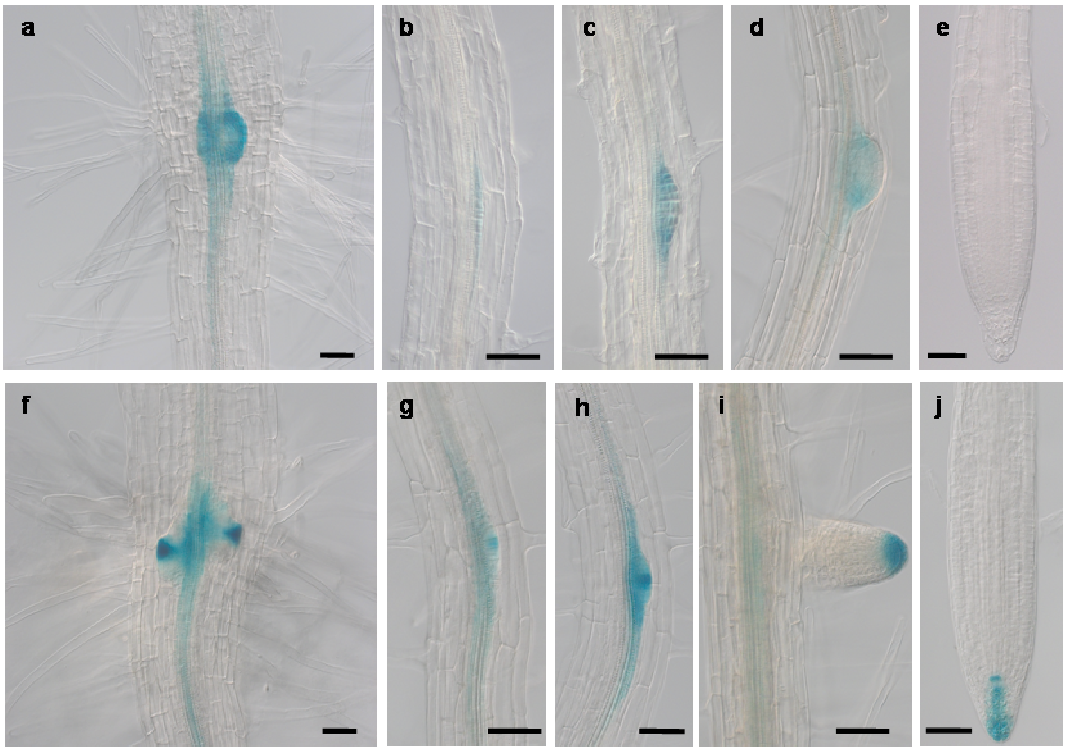

Fig. 3.7. *PIN6* and *DR5* expression in Arabidopsis roots. (a)-(e) *pPIN6::GUS* and (f)-(j) *pDR5::GUS* seedlings were grown on AM medium under long-days condition (16h light/8h dark). (a) and (f), adventitious root formation at the shoot-root junction. (b)-(c) and (g)-(i), lateral root formation. (e) and (f), main root. At the shoot-root junction, *PIN6* is expressed in epidermis and cortex layers of the emerging adventitious roots (a). *PIN6* is expressed initially in the lateral root founder cells at the pericycle (b). It remains at the base of the lateral root and the vascular tissue of the main root (c) during root emergence (d). *PIN6* is not present in the main root tip (e). Scale bar = 50 μm.

In shoot apex, *PIN6* expression coincides with auxin levels in the stipules (arrowed in Fig. 3.8.a). *PIN6* is expressed in hypocotyl and in shoot apex, in meristem region (closed arrow in Fig. 3.8.a). Following its expression in the hypocotyl vascular tissue (Fig. 3.8.a), *PIN6* is present without interruption in the vasculature of the new leaves, including the developing second order loops (open arrow in Fig. 3.8.a). As reported for PIN1 (Scarpella *et al.*, 2006), the presence of *PIN6* along vascular tissue suggests this gene is a potential regulator of vascular tissue development. Auxin, like *PIN6* expression, is detected along the vasculature and in leaves, but also accumulates at the tip of the leaf (Fig. 3.8.b).

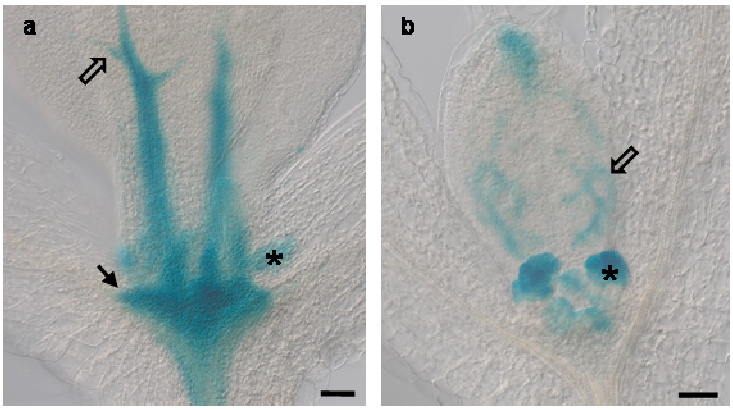

Fig. 3.8. *PIN6* and *DR5* expression in the Arabidopsis shoot apical meristem. (a) *pPIN6::GUS* and (b) *pDR5::GUS* seedlings were grown on AM medium under long-days condition (16h light/8h dark). *PIN6* is expressed at the shoot apex (a, closed arrow). Following expression in the hypocotyl vascular tissues, it shows a strong signal in the vasculature of the new leaves, including second order loops (a, open arrow). *PIN6* is also expressed in stipules (a, star). Auxin accumulates at the tip apex and in higher order vascular bundles of new leaves and in stipules (b, star). Scale bar = 50 μm.

In the floral meristem *PIN6* localizes to lateral meristematic cells at the sites of emergence of new organs (sepals, petals, stamens and carpels; closed arrow in Fig. 3.9.a). Moreover, it is strongly expressed in the underlying vascular tissue (Fig. 3.9.b). In mature pistils, *PIN6* is present at the stigma (open arrow in Fig. 3.9.b) and in the transmitting tissue surrounding the ovules (closed arrow in Fig. 3.9.b). In the male flower organs, *PIN6* is expressed specifically in the stamen vascular system directly below the anther (Fig. 3.9.c,d).

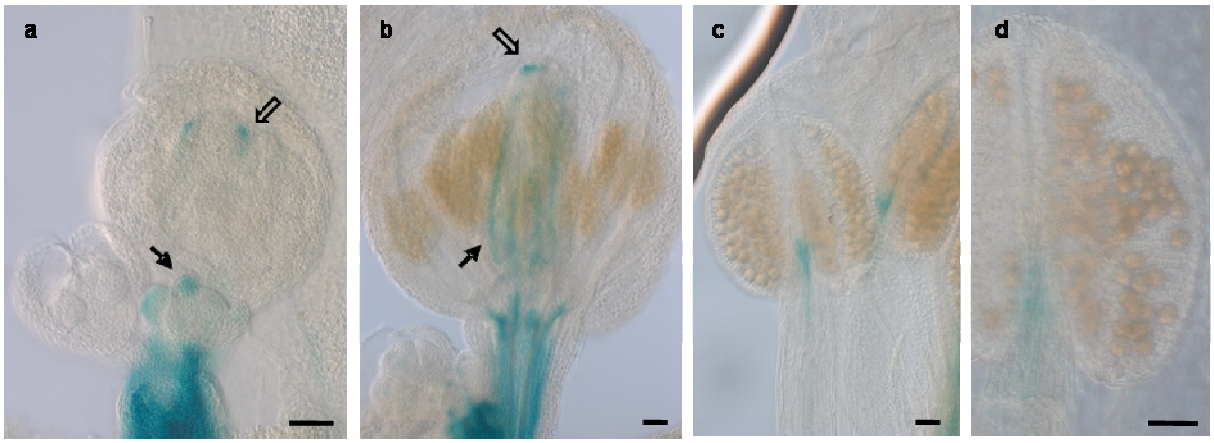

Fig. 3.9. *PIN6* expression in the Arabidopsis floral meristem. *pPIN6::GUS* seedlings were grown on AM medium under long-days condition (16h light/8h dark). *PIN6* expresses in lateral meristematic cells at the young meristem (a, closed arrow). *PIN6* is expressed in the transmitting tissue of the mature pistil (b, closed arrow) and at the tip of the stigma (a,b, open arrow). *PIN6* is also expressed in the vascular tissue supporting the anther (c) and (d). Scale bar = 50 μm.

In situ hybridization of *PIN6* mRNA transcripts in the floral meristem correlate with the *pPIN6::GUS* analysis and confirms its localization in lateral meristematic cells at the site of emergence of

new floral organs (arrowed in Fig. 3.10.b). *PIN6* is additionally localized to the provascular tissue of already developing lateral organs (arrowed in Fig. 3.10.a).

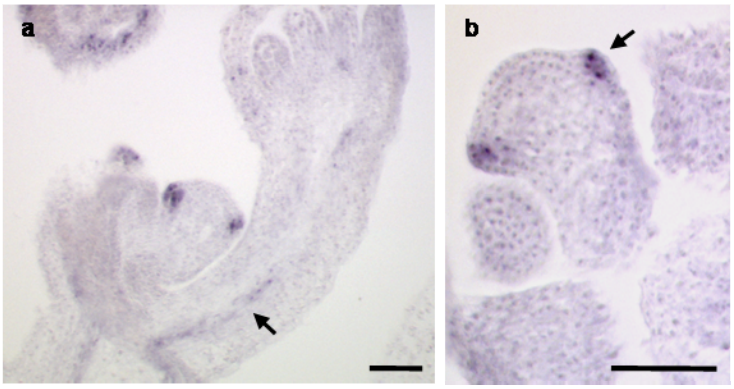

Fig. 3.10. *In situ* hybridization of *PIN6* mRNA transcripts in the floral meristem. *PIN6* expression is restricted to lateral meristematic cells and provascular tissue (arrowed). Scale bar = 50 μm.

Since *PIN6* is regulated by other hormones and hormone-related factors (see section 3.1.4), *pPIN6::GUS* seedlings were subjected to short-term treatments (120 min) of the following hormones: 2,4-D 1 μM, ABA 1 μM, GA_3 100 μM, IAA 1 μM and Kin 10 μM. Treatments with auxins 2,4-D and IAA upregulate *PIN6* expression levels (Fig. 3.11.d,e,n) and induce its ectopic expression in lateral and main root tips (Fig. 3.11.f,o,p). ABA, too, increases *PIN6* expression levels (Fig. 3.11.g). Cytokinin upregulates *PIN6* levels (Fig. 3.11.h,i), an induction most striking in adventitious roots formed at the shoot-root junction (Fig. 3.11.h). Gibberellic acid slightly induces *PIN6* levels throughout lateral root formation and emergence (Fig. 3.11.j,k,l,m). In summary, PIN6 expression is upregulated mainly by auxin and cytokinin, but in lesser extent also by ABA and gibberellin.

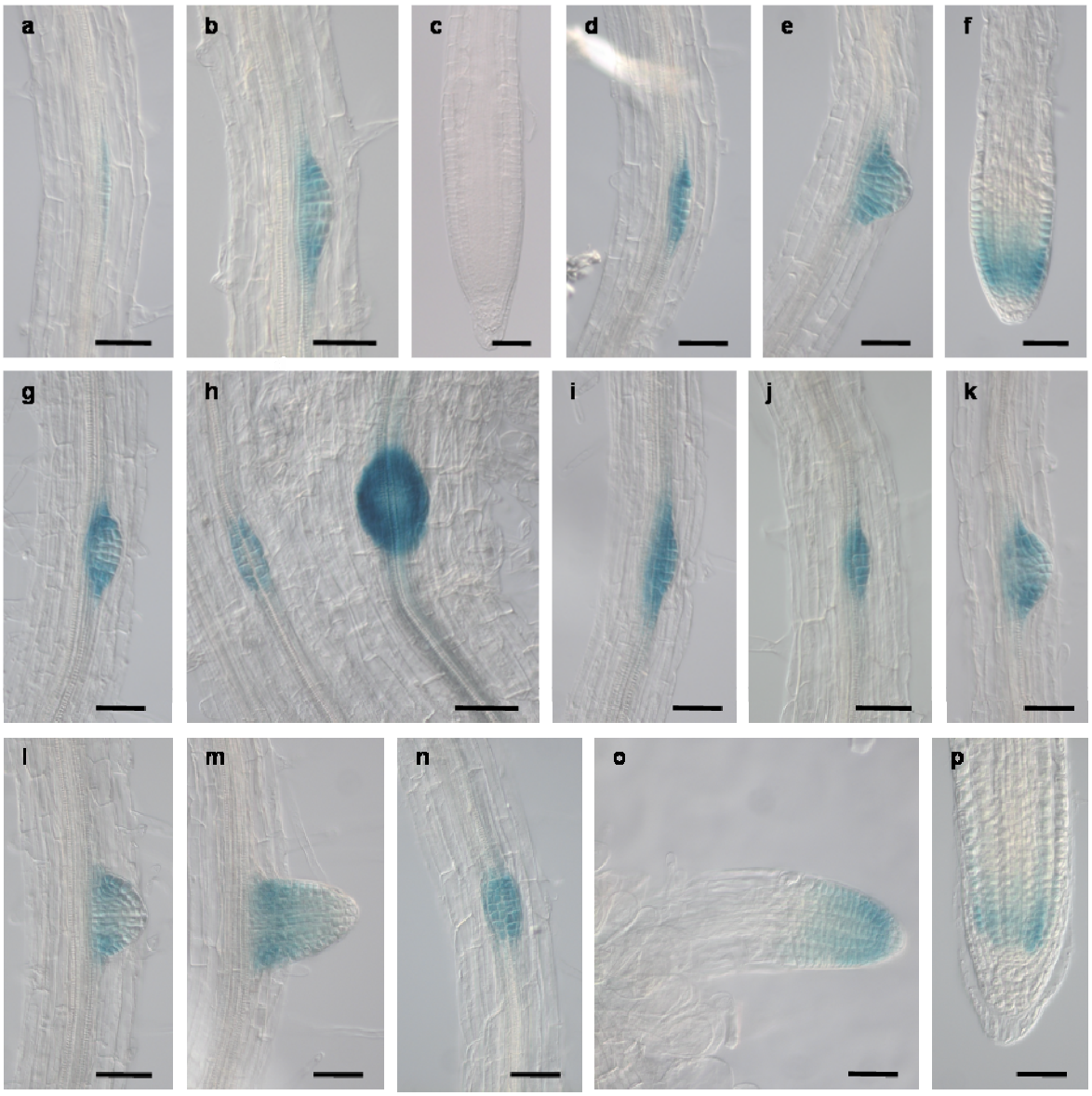

Fig. 3.11. *pPIN6::GUS* expression in 7-days old seedlings grown in AM medium under long-days condition (16h light/8h dark) and transferred for 120 min to liquid AM medium containing hormones. In control seedlings, *PIN6* is expressed in the first dividing pericycle cells (a), in the basal and lateral cells of the emerging root and not in the lateral root apex (b) nor in the main root tip (c). Treatment with 2,4-D 1 µM upregulates *PIN6* levels in lateral roots (d,e), inducing its ectopic expression at the main root tip (f). ABA 1 µM also increases *PIN6* expression levels (g). Cytokinin (kinetin 10 µM) upregulates PIN6 levels (h,i), especially in roots formed at the shoot-root junction (h). GA_3 100 µM slightly induces PIN6 expression throughout lateral root formation and emergence (j,k,l,m). Auxin treatment (IAA 1µM) upregulates PIN6 expression levels (n) and induces its expression in lateral and main root tips (o,p). Scale bar = 50 µm.

Even though GUS detection did not show the presence of *PIN6* in the main root tip, additional whole-mount *in situ* hybridization on roots has shown that PIN6 mRNAs locate to the epidermis and cortex layers (Fig. 3.12.a). Upon auxin treatment, IAA 1 µM for 120 min, PIN6 expression is upregulated and induced in the stele (Fig. 3.12.b).

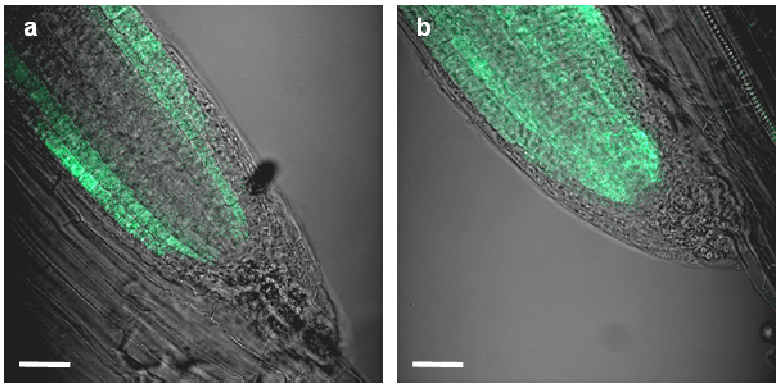

Fig. 3.12. *PIN6 mRNA* expression in 7-days old seedlings grown in AM medium under long-days condition (16h light/8h dark). (a) control. (b) seedlings transferred for 120 min to liquid AM medium containing 1 µM of IAA. Scale bar = 50 µm.

3.2.2 PROTEIN LOCALIZATION

So far, we have only succeeded in localizing PIN6 protein on sectioned plant material, where PIN6 shows polar localization (arrowed in Fig. 3.13.b). Whole-mount immunolocalization in Arabidopsis roots with the available anti-PIN6 antibodies, raised in mouse, guinea pig and rabbit, resulted in a scattered dotty pattern throughout the tissue, due to unspecific recognition of cellular epitopes.

In the floral meristem, PIN1 is localized in the L1 cell layer covering the whole the SAM (closed arrow in Fig. 3.13.a). PIN1 is also present in the vasculature underlying new emerging lateral organs (Fig. 3.13.a). Similarly, in SAM PIN6 is present in the L1 layer, but only at sites of new organ emergence, a domain localized below and outwards of PIN1's, and also in the vasculature underlying new organs(open arrow in Fig. 3.13.a). In vasculature of inflorescences, PIN1 localizes to the 6 most inner cell files in the central veins and PIN6 is present only in the two most inner cell files (Fig. 3.13.b). In the absence of PIN1, PIN6 expression domain spreads to all the cell files where PIN1 is normally expressed (open arrow in Fig. 3.13.c). In outgrowing lateral organs, PIN6 appears non-polarly and co-localizes partially with PIN1 (closed arrow in Fig. 3.13.d,e). In contrast, PIN6 and PIN1 are both located polarly in companion cells along vascular bundles (closed arrow in Fig. 3.13.f).

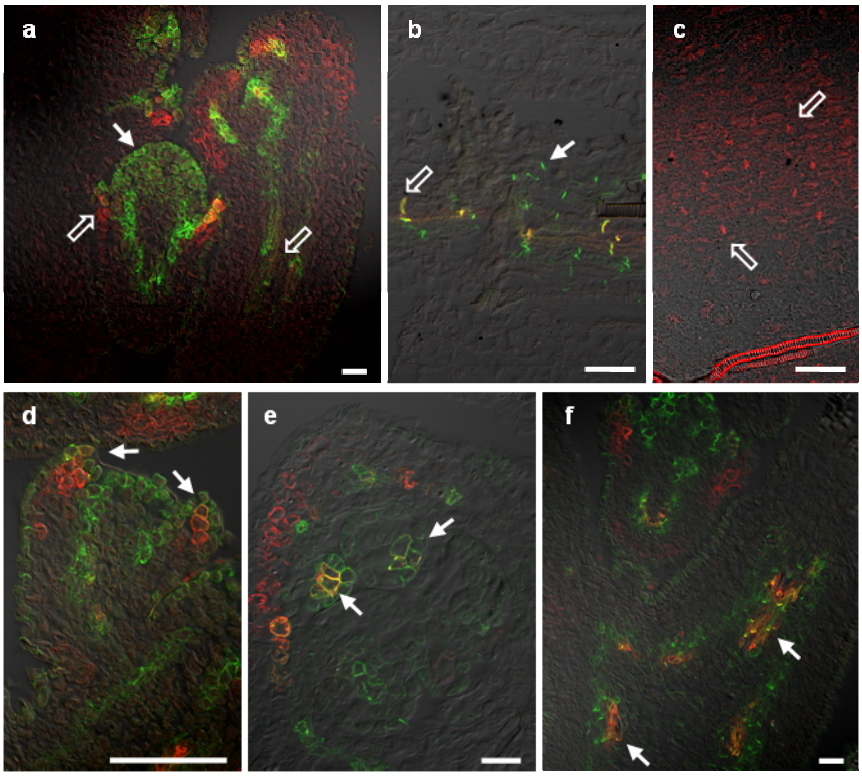

Fig. 3.13. Visualization of PIN expression in Arabidopsis. Localization of PIN6 (red) and PIN1 (green) proteins in longitudinal (a,b,c,d,f) and in transverse (e) sections of floral meristems. PIN1 localizes to the L1 cell layer at the shoot apex and to vascular tissue underlying the new emerging lateral organs (a, closed arrow). PIN6 is also present in the L1 layer, but at the sites of new organ emergence (a, closed arrow). In the vascular tissue (b), PIN1 localizes to the 6 cell files in the central vein (closed arrow) and PIN6 is present only in the two most inner cell files (open arrow). In the pin1 mutant vasculature (c), PIN6 expression domain spreads to cover the other cell files where PIN1 is normally expressed (open arrow). PIN6 appears non-polarly and co-localizes partially with PIN1 in outgrowing lateral organs (d,e, closed arrow). PIN1 and PIN6 are located polarly in companion cells along vascular bundles (f, closed arrow). Scale bar = 20 µm.

3.3 SCREENING FOR *PIN6* NULL MUTANTS

Nowadays reverse genetics is acquiring a determinant relevance in biological studies, to understand the biological function of a particular gene. Gene knockout is considered a major component of the functional genomics toolbox, as it aims to reveal the function of genes discovered through large-scale sequencing programs (Bouché and Bouchez, 2001). Gene-disruption methods include the use of large populations of plants randomly mutagenized by EMS (Ethylmethane Sulphonate) or, transposons or the T-DNA of *Agrobacterium tumefaciens* (Krysan *et al.*, 1999; Parinov and Sundaresan, 2000) - and targeted mutagenesis – *e.g.* RNAi (Lloyd *et al.*, 2005; Shaked *et al.*, 2005). The high gene density in the Arabidopsis genome is particularly favourable for random mutagenesis, as every second insertion will disrupt a gene sequence (Parinov and Sundaresan, 2000). These reverse genetic approaches are based on the use of polymerase chain

reaction (PCR) to screen DNA pools obtained from a population for the desired insertions (Parinov and Sundaresan, 2000). PCR primers specific for both the target gene and the insertion element are used (Parinov and Sundaresan, 2000) to discriminate between putative mutations.

Mutants analyzed in this work came from the SALK (Alonso *et al.*, 2003) and the GABI-Kat (Li *et al.*, 2003; Li *et al.*, 2007; Rosso *et al.*, 2003; Strizhov *et al.*, 2003) collections for T-DNA insertion mutants (Table 3.3.). In addition, other collections were also screened for putative *pin6* mutants –GARLIC and WISCOSIN T-DNA pool libraries and TILLING collection of EMS mutants with point mutations (data not shown). However, no mutant lines were found in the GARLIC and WISCOSIN collections. TILLING lines have a high frequency of point mutations, often resulting in more than one point mutation per line, making it impractical to use these mutants.

Table 3.3. T-DNA insertion lines used for screening *pin6* mutants. Exons are located in bp 52-483, 760-1392, 2105-2367, 2472-2558, 2631-2789, 3357-3434 and 3554-3621.

T-DNA insertion line	Approximate location (bp)	Location (sequence)	*pin6* allele
SALK_046392.41.20.x	1731	II. Intron	*pin6-1*
430 B01	2321	III. Exon	*pin6-2*
711 C09	3412	VI. Exon	*pin6-3*
852 D10	3412	VI. Exon	*pin6-4*

A combination of both, gene of interest's specific primer and a primer designed for the T-DNA insertion border (LB) reveals which plants carry the mutant allele. Another search with only gene's specific primers that reveals WT allele, will allow distinguish between homozygous and heterozygous plants: the homozygous for the mutation will be those lines for which amplification was obtained with the gene and T-DNA primers combination, and not with the gene specific primers only.

3.3.1 GENOTYPING

Seeds received from the stock collections were termed F0 generation. They were grown on soil and produced F1 seed progeny. During growth, leaves were collected for DNA extraction and subsequent PCR analysis and heterozygous or homozygous lines for *pin6* were selected. Next, F1 seedlings were analyzed by PCR and the homozygous status of F2 seeds was confirmed thereafter. This analysis was repeated for, at least, the next two generations, to ensure that the homozygous mutant lines obtained were stable and that no false positives existed in the seed population. In case of the SALK collection, the lines were followed by PCR inspection through 4 generations (down to F5). For the GABI-Kat lines, putative stable homozygous mutants were obtained on F3 generation. Hence, seedlings from F3 to F5 generations (or its progeny) were used for further phenotypic analysis.

Each putative mutant line was screened by PCR reaction, as described on section 3.3 and section 2.2.5.5. *pin6* mutants characterized by PCR were further analyzed by Southern Blot to verify the number of T-DNA insertions. DNA samples were prepared from the *pin6* mutant candidates. Samples from two lines from the original SALK046393 line (SALK046393_2_2_1_2 and SALK046393_2_2_1_2_3), two from the original 430 B01 line (430 B01_1_1 and 430 B01_1_3), two from the original 711 C09 line (711 C09_5_1 and 711 C09_7_3) and three from the original 852 D10 line (852 D10_5_1, 852 D10_6_1 and 852 D10_6_2) were digested with *EcoRI* endonuclease. As this enzyme does not cut inside the sequence used for the T-DNA probe, only a single labelled band is expected on the membrane. Accordingly, one single T-DNA insertion was observed in the genome of the following analyzed lines: SALK046393_2_2_1_2_3, 430 B01_1_1, 711 C09_5_1 and 852 D10_5_1 (Fig. 3.14.).

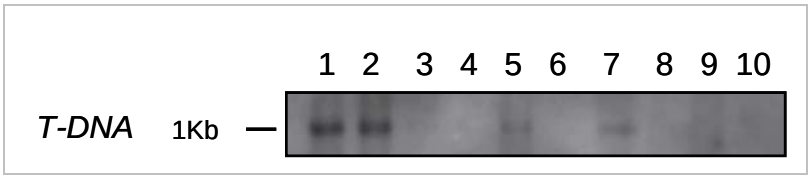

Fig. 3.14. Southern Blot analysis of the T-DNA insertion lines. DNA was extracted with the CTAB method for higher yield and purity from 3 weeks old *in vitro* grown seedlings under continuous light condition. Samples (1) SALK046393_2_2_1_2, (2) SALK046393_2_2_1_2_3, (3) 430 B01_1_1, (4) 430 B01_1_2, (5) 711 C09_5_1, (6) 711 C09_7_3, (7) 852 D10_5_1, (8) 852 D10_6_1, (9) 852 D10_6_2, (10) WT DNA, were digested with *EcoRI* and blotted with a DIG-labelled probe against the T-DNA insertion.

Finally, allele names were assigned to the four T-DNA insertion lines obtained according to the position of the mutation in the gene, from the furthest upstream to the furthest downstream (see Table 3.3.).

3.3.2 *PIN6* EXPRESSION LEVELS IN *pin6* SEEDLINGS

Reverse-Transcriptase Polymerase Chain Reaction (R.T.-PCR) was used for a qualitative evaluation of *PIN6* mRNA levels in each of the alleles. Primers pin6-f and pin6-r were designed for CDS, located at the middle of the sequence and the far most 3'-end, respectively, and actin-1 was used as control (using primers ACTF and ACTR). *pin6-3* exhibits strongly decreased transcript levels: only a faint band can be seen on the gel (Fig. 3.15. lane 4). The other mutant lines show reductions of *PIN6* expression levels (Fig. 3.15.), yet less striking as *pin6-3*.

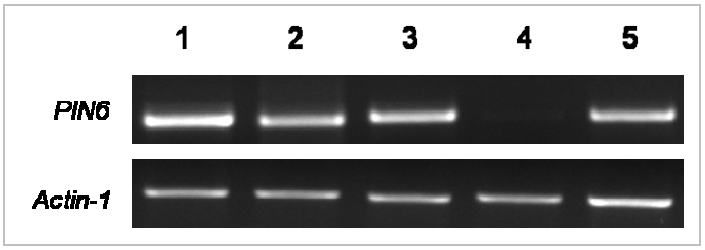

Fig. 3.15. R.T.-PCR analysis of the T-DNA insertion lines. RNA from 11 days old *in vitro* grown seedlings under long-days condition was extracted using the Trizol method. Samples are (1) WT, (2) *pin6-1*, (3) *pin6-2*, (4) *pin6-3* and (5) *pin6-4*.

3.4 GENERATING PIN6 OVEREXPRESSING PLANTS AND RNAI LOSS-OF-FUNCTION TRANSGENIC LINES

Besides insertion knock-outs, it is of interest to analyse transgenic plants with higher or lower levels of expression of the gene of interest. Overexpressor lines induce ectopic expression of the gene of interest, thereby enhancing the effects caused by its function in the plant. I therefore aimed at creating one overexpressor line for *PIN6*, by placing the *PIN6* CDS under the control of the strong CaMV 35S promoter. On the other hand, RNA interference (RNAi) strategies can be used to knockdown a gene of interest. dsRNA is formed from the annealing of sense and antisense strands present in the *in vitro* RNA. In this work two different RNAi constructs were designed for *PIN6* silencing, one targetted against a specific 402 bp sequence in the *PIN6* CDS, and the other one designed against 197 bp in the 3'-UTR region of the gene (see section 2.2.3.) .

For each of the constructs produced (see Table 3.4. and section 2.2.3.). WT plants (considered T0) were infiltrated with the *Agrobacterium tumefaciens* strain harbouring the construct. Subsequently, T1 seed progeny was grown on selective medium. In order to differentiate between transformed plants carrying construct *pAM-PAT-GW-PIN6* and those carrying empty vectors, primers *PIN_OX_fwd*, *PIN_OX_rev* and *35SFor* were used. Similarly, for the four plants transformed with *pJAWOHL-RNAi-PIN6-CDS* construct primers PIN_35SRNAi_fwd, PIN_35SRNAi_rev and 35SFor were used for PCR analysis. For plants transformed with *pJAWOHL-RNAi-PIN6-utr* construct primers *PIN_35SRNAi_fwd_utr*, *PIN_35SRNAi_rev_utr* and *35SFor* were used. T2 progeny of plant lines in which the presence of the construct was confirmed by PCR were used for further analysis as indicated in Table 3.4.

Table 3.4. Overexpressor and RNAi lines generated for *pin6*.

Line	Construct
3.11	*pAM-PAT-GW-PIN6*
8.1 (5)	*pJAWOHL-RNAi-PIN6-CDS*
8.1 (5a)	*pJAWOHL-RNAi-PIN6-CDS*
10.14 (4)	*pJAWOHL-RNAi-PIN6-utr*
10.14 (5)	*pJAWOHL-RNAi-PIN6-utr*

3.4.1 *PIN6* EXPRESSION LEVELS IN *pin6* TRANSGENIC SEEDLINGS

Reverse-Transcriptase Polymerase Chain Reaction (R.T.-PCR) was used as qualitative evaluation of *PIN6* mRNA levels. Primers pin6-f and pin6-r were designed for CDS and actin-1 was used as control (using primers ACTF and ACTR). Line 3.11, a putative overexpressor line, does not show an overexpression of *PIN6*, but instead, a strong reduction in the transcript levels, therefore it was considered for further experiments as a knockdown line in parallel with the RNAi lines. As expected, the RNAi lines show a reduction in *PIN6* transcript levels (Fig. 3.16.) and were thus used as knock-down of PIN6.

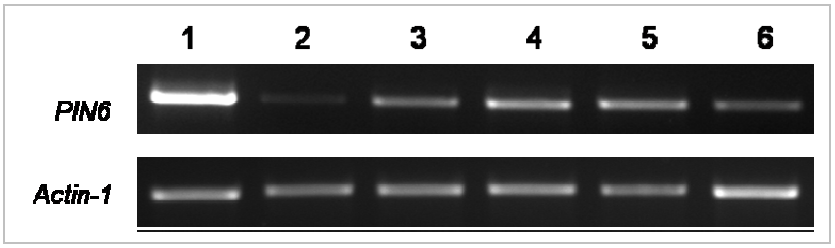

Fig. 3.16. R.T.-PCR analysis of the generated overexpressor and RNAi lines. RNA from 11 days old *in vitro* grown seedlings under long-days condition was extracted using the Trizol method. Samples are (1) WT, (2) 3.11, (3) 8.1 (5), (4) 8.1 (5a), (5) 10.14 (4) and (6) 10.14 (5).

3.5 PHENOTYPIC ANALYSIS OF *pin6* MUTANT ALLELES

Nowadays it is possible to isolate of knockout lines for virtually every Arabidopsis gene (Parinov and Sundaresan, 2000). However, when compared to wild type most knockout lines do not show visible

changes in standard culture conditions (Bouché and Bouchez, 2001). To circumvent this problem, *pin6* mutants were tested under a wide range of environmental conditions.

3.5.1 PHOTOPERIOD EFFECT ON *pin6* GROWTH

Because light responsive elements are well represented in *PIN6* promoter sequence (see section 3.1.2), it was interesting to know whether light quality or the circadian rhythm could regulate *PIN6* expression. *Pin6* seedlings growth was therefore followed under different photoperiodic conditions – long-day (16h light/8h dark) and continuous light.

Under continuous light, all mutant lines grew faster than the WT. Compared to 10-days old WT seedlings' main roots, *pin6-1, pin6-2, pin6-3* and *pin6-4* roots were, respectively, 3% 18%, 47% and 53% longer (Fig. 3.17.). Moreover, *pin6* alleles showed increased growth rates. While WT seedlings grow at a rate of 0.66 mm/day (measured between 2 and 4 days), *pin6-1* grows at 0.91 mm/day, *pin6-2* at 1.32 mm/day, *pin6-3* at 0.93 mm/day and *pin6-4* at 0.96 mm/day.

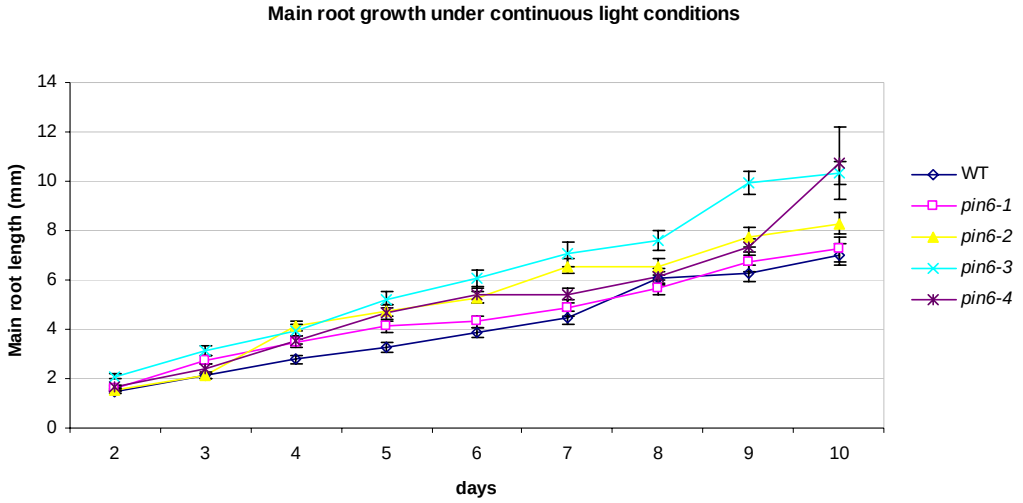

Fig. 3.17. Main root length (mm) of 2- to 10-days old WT and *pin6* seedlings grown under continuous light. Values represent 2 technical and at least 60 biological replicates for each time point. Data are mean ± SE.

In a similar way, under continuous light all knockdown lines analyzed showed longer roots than the WT. Compared to 10-days old WT roots, 3.11, 8.1 (5), 8.1 (5a), 10.14 (4) and 10.14 (5) roots were, respectively, 6% 8%, 35%, 9% and 10% longer (Fig. 3.18.). Moreover, all these lines showed increased growth rates. While WT seedlings grow at a rate of 1.61 mm/day (measured between 2 and 4 days), 3.11 grows at 1.68 mm/day, 8.1 (5) at 1.67 mm/day, 8.1 (5a) at 1.93 mm/day, 10.14 (4) at 1.89 mm/day and 10.14 (5) at 1.73 mm/day.

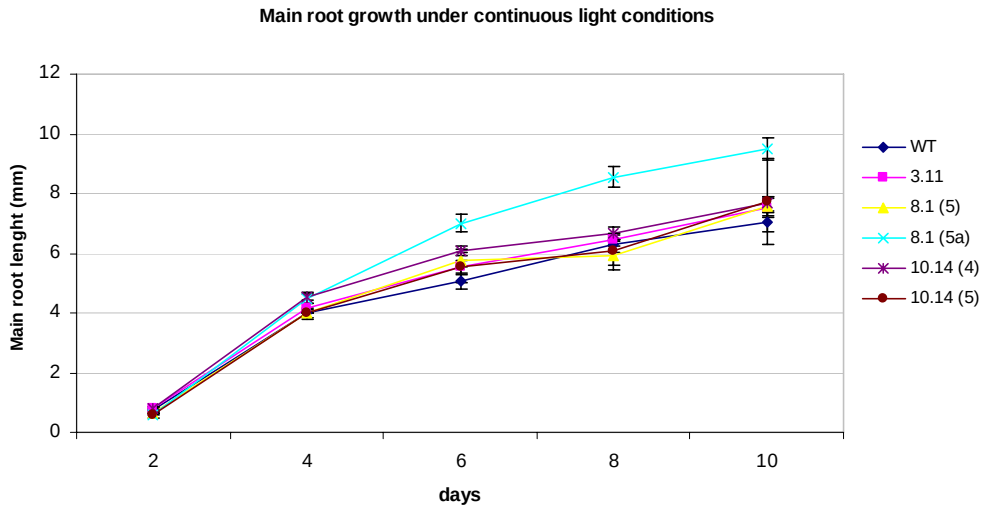

Fig. 3.18. Main root length (mm) of 2- to 10-days old WT and *pin6* knockdown lines grown under continuous light. Values represent at least 40 biological replicates for each time point. Data are mean ± SE.

Under long-days condition, results were similar to those observed with plants grown under continuous light. Root length of all mutant lines analyzed was longer than WT's. Compared to WT, *pin6-1, pin6-2, pin6-3* and *pin6-4* roots were, respectively, 29%, 1%, 76% and 30% longer (Fig. 3.19.). Mutant lines also showed increased growth rates. WT seedlings grow at a rate of 0.54 mm/day (measured between 2 and 4 days), while *pin6-1* grow at 0.88 mm/day, *pin6-2* at 0.70 mm/day, *pin6-3* at 1.40 mm/day and *pin6-4* at 0.95 mm/day.

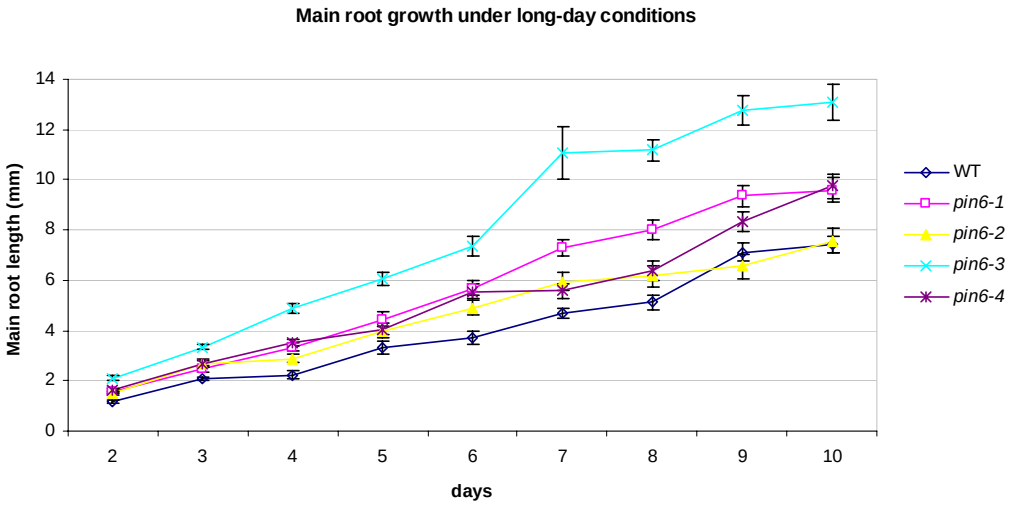

Fig. 3.19. Main root length (mm) of 2- to 10-days old WT and *pin6* seedlings grown under long-days condition (16h light/8h dark). Values represent 2 technical and at least 60 biological replicates for each time point. Data are mean ± SE.

Likewise, under long-days condition all knockdown lines analyzed showed longer roots than the WT. Root length of transgenic lines was longer than the WT's. 3.11, 8.1 (5), 8.1 (5a), 10.14 (4) and 10.14 (5)

roots were, respectively, 98% 94%, 16%, 23% and 17% longer (Fig. 3.20.). As described above for continuous light, these lines also showed increased growth rates. While WT seedlings grow at a rate of 1.37 mm/day (measured between 2 and 4 days), 3.11 grows at 1.60 mm/day, 8.1 (5) at 1.40 mm/day and 8.1 (5a) at 1.69 mm/day. Lines 10.14 (4) and 10.14 (5) showed slower growth rates: 1.32 mm/day and 1.26 mm/day, respectively.

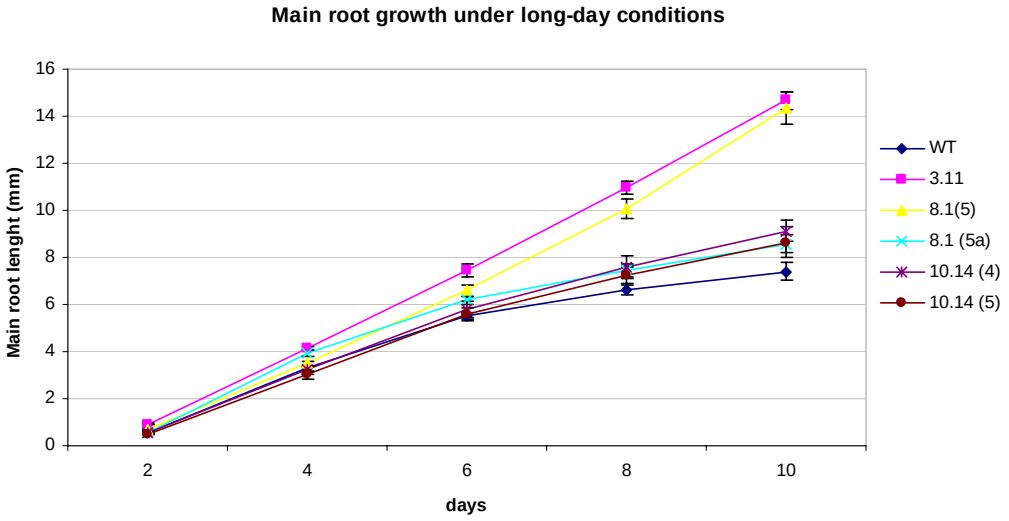

Fig. 3.20. Main root length (mm) of 2- to 10-days old WT and *pin6* knockdown lines grown under long-days condition (16h light/8h dark). Values represent at least 40 biological replicates for each time point. Data are mean ± SE.

In absence of *PIN6*, roots are longer, suggesting a role for PIN6 as a negative regulator of growth processes. The longer root phenotype is more striking under long-days condition (Fig. 3.19.; Fig. 3.20.) when compared to continuous light condition (Fig. 3.17.; Fig. 3.18.). Taken together, and considering the presence of circadian expression regulatory sequences in *PIN6* promoter (see section 3.1.2), these results suggest that *PIN6* might be regulated by photoperiodism.

Additionally, it was observed that most *pin6* mutants germinated faster than the WT (Table 3.5.). Two days after vernalisation, more mutant seedlings had germinated than WT, suggesting that *pin6* plants are affected in break of dormancy.

Table 3.5. Percentage of germinated WT and *pin6* seedlings 2 days after vernalisation. 10-20 seedlings were analyzed per line.

Line	Germinated seedlings after 2 days (%)
WT	79 %
pin6-1	67 %
pin6-2	92 %
pin6-3	95 %
pin6-4	82 %

To further clarify the regulation of *PIN6* by different photoperiodic regimes, hypocotyl length of 6-days old seedlings grown under the following conditions were measured: continuous light, long-day (16h light/8h dark), short-day (8h light/16h dark) and darkness.

Under continuous light, hypocotyl length of *pin6-1* and WT were similar, *pin6-2* showed a 10% reduction in hypocotyl length, whereas *pin6-3* and *pin6-4* displayed longer hypocotyls than the WT (7% and 8%, respectively; Fig. 3.21.). In long-days condition there were no statistically significant differences between hypocotyl growth of *pin6-1*, *pin6-3*, *pin6-4* and WT. *pin6-2* showed a 5% reduction in hypocotyl length when compared to WT (Fig. 3.21.). As for short-days condition, it was also not possible to differentiate between the groups analyzed (Fig. 3.21.). Grown in darkness, all mutant alleles analyzed showed different hypocotyls lengths than the WT. Hypocotyls of *pin6-1* were 8% longer, unlike those of *pin6-2*, *pin6-3* and *pin6-4*, which were 15%, 11% and 5% shorter than the WT, respectively (Fig. 3.21.). All in all, these data suggest that PIN6 is also a repressor of hypocotyl growth under different light regimes and that in the darkness it represses etiolated growth.

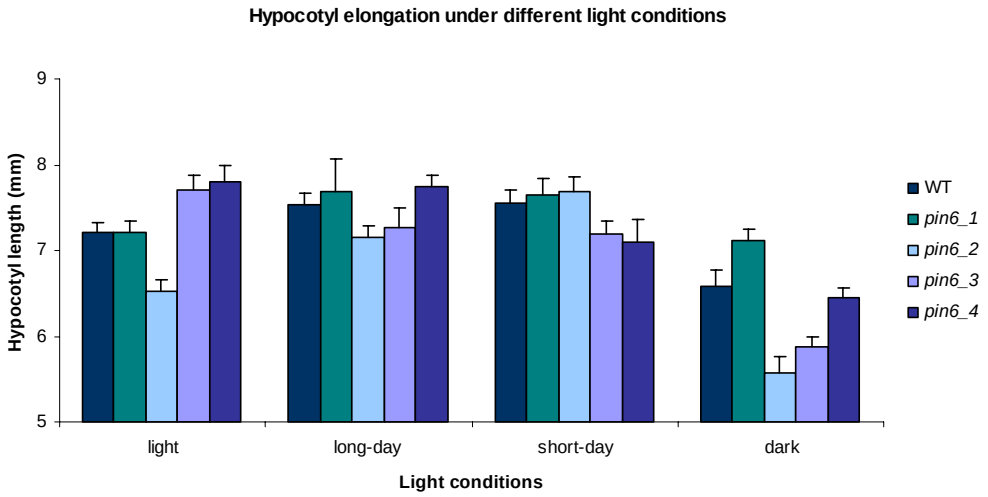

Fig. 3.21. Hypocotyl length (mm) of 6-days old WT and *pin6* seedlings grown under continuous light, long-days (16h light/8h dark), short-days (8h light/16h dark) and darkness. 30 seedlings were analyzed per treatment. Data are mean ± SE.

To evaluate possible changes in *PIN6* expression, *pPIN6::GUS* seedlings were grown under the same light and photoperiodic conditions (continuous light, long-day, short-day and darkness) as the T-DNA mutant lines. *PIN6* expression domains did not change between the different conditions (Fig. 3.22.). However, *PIN6* expression levels were highest under continuous light (Fig. 3.22.a,b,c), followed by long-day and short-day, an effect that could be seen both at the SAM and in lateral roots (Fig. 3.22.a-f). *PIN6* expression levels in short-day and darkness were similar (Fig. 3.22.f,g).

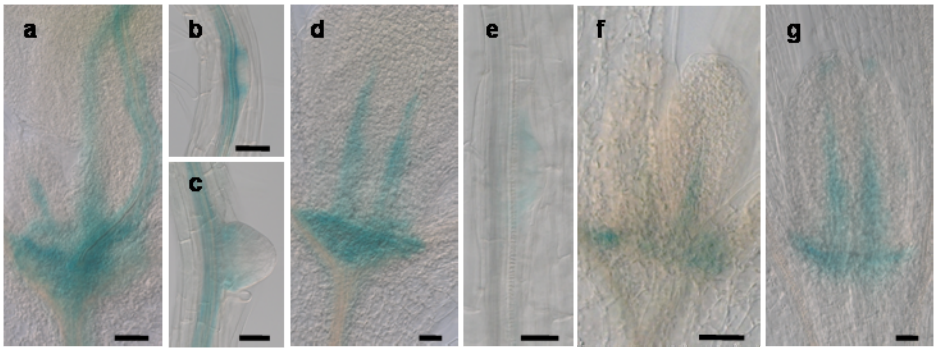

Fig. 3.22. *PIN6* expression in 6-days old *pPIN6::GUS* seedlings grown under different photoperiodic regimes. (a)-(c) continuous light, (d)-(e) long-day (16h light/8h dark), (f) short-day (8h light/16h dark) and (g) darkness. (a), (d), (e) and (g) depict shoot apices, whereas (b), (c) and (e) show lateral roots. Seedlings grown under short-day and darkness do not have lateral roots at this stage of development. Scale bar = 50 μm.

Since *pin6* mutants show longer roots than WT plants, and that this effect is enhanced by a short darkness period, it seems that light and the existence of an alternating light/darkness photoperiod induce *PIN6* and repress growth.

3.5.2 TRANSITION FROM VEGETATIVE TO REPRODUCTIVE PHASE IN *pin6* PLANTS

During its development, a shoot progresses from a juvenile to an adult phase of vegetative growth and from a reproductively incompetent to a reproductively competent state. After perception and processing of several environmental and internal signals, the shoot apical meristem undergoes a change in fate, and an inflorescence meristem is produced. During flowering, auxin promotes organ outgrowth and development of the gynoecium, the female reproductive organ of angiosperms (Okada *et al.*, 1991; Vernoux *et al.*, 2000).

pin6 plants make the transition to the reproductive phase earlier than WT. After 4 weeks *in vitro* culture under long-days condition, all *pin6* mutants had developed inflorescence stems, whereas in the WT 30% of the plants did not have them yet (Fig. 3.23.a, compare with b,c,d,e). Moreover, many of the *pin6* stems already possessed floral meristems, an event more pronounced in *pin6-3* (Fig. 3.23.d), in contrast to the WT, in which no inflorescences had developed yet.

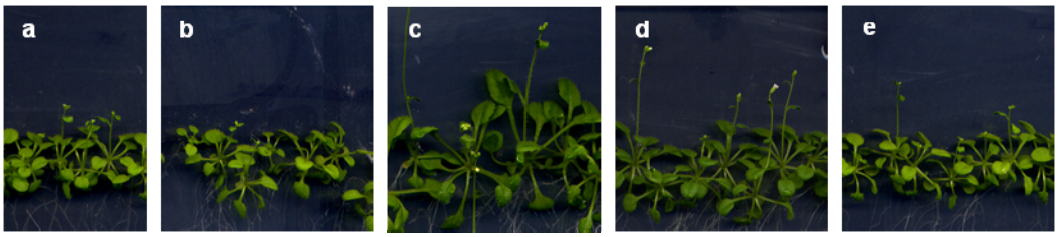

Fig. 3.23. Flowering status of 4-weeks old seedlings grown under long-days condition. (a) WT, (b) *pin6-1*, (c) *pin6-2*, (d) *pin6-3* and (e) *pin6-4*. All *pin6* mutants have developed stems, whereas 30% of the WT plants have not. Many of the *pin6* stems already possess floral meristems (c,d).

To further analyze the involvement of PIN6 in the transition from the vegetative to the reproductive phase, plants were grown on soil under different photoperiodic regimes: continuous light, long-day (16h light/8h dark) and short-day (8h light/16h dark) conditions. In all conditions tested, the mutants made an earlier transition to the reproductive phase than the WT (Table 3.6.). Under continuous light, *pin6-3*, the allele with more reduced *PIN6* expression levels, was the first to develop inflorescence stems after 14 days, followed by *pin6-1*, *pin6-2*, *pin6-4* and later WT. In long-days condition, *pin6-1* were the first to develop stems after 21 days, followed by *pin6-2*, *pin6-2*, *pin6-4* and then WT. As for short-days condition, plants started producing stems after 32 days in the following order: *pin6-3*, *pin6-1*, *pin6-2*, *pin6-4* and WT.

Additionally, in all knockdown lines analyzed inflorescence stems were generated faster than in WT (Table 3.6.). Under continuous light, 10.14 (4) and 10.14 (5) developed stems first, as *pin6-3* allele after 14 days, followed by 3.11, 8.1 (5), 8.1 (5a) and then WT. In long-days condition, 3.11 were the first to develop inflorescence stems after 22 days, followed by 8.1 (5a), 10.14 (5), 10.14 (4), 8.1 (5) and later WT. As for short-days condition, plants produced stems after 32 days in the following order: 8.1 (5), 8.1 (5a), 10.14 (5), 10.14 (4), 3.11 and WT.

Table 3.6. Age of the plant (days) at the transition to reproductive phase (stem emergence). 30-40 plants were analyzed per line. Seedlings were grown in AM medium under continuous light, long-day (16h light/8h dark) and short-day (8h light/16h dark) conditions.

Line	Vegetative-to-reproductive phase transition		
	Light	Long-day	Short-day
WT	17	26	36
pin6-1	15	21	33
pin6-2	16	24	34
pin6-3	14	24	32
pin6-4	16	26	34
3.11	15	22	35
8.1(5)	15	26	32
8.1(5a)	16	23	33
10.14(4)	14	24	34
10.14(5)	14	23	33

In order to assess whether *pin6* earlier transition to the reproductive phase could be due to the fact that this mutant is growing faster, therefore flowering earlier, I counted the number of rosette leaves produced until the first flower emerged. Two alleles, *pin6-1* and *pin6-2*, showed no statistically significant difference in the number of leaves produced when compared to the WT plants (13.3 ± 2.9 and 12.5 ± 2.3 compared to 13.4 ± 3.2 leaves). On the other hand, *pin6-3* and pin6-*4* developed, in average, *ca.* 3 and 2 rosette leaves fewer than WT plants (10.7 ± 1.3 and 11.1 ± 2.0, respectively; Fig. 3.24.).

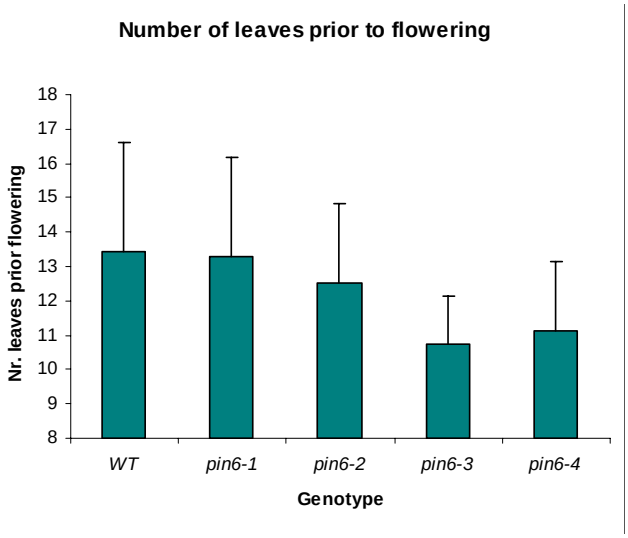

Fig. 3.24. Numbers of leaves developed prior to the transition to flowering. Seedlings grown in AM medium for 20 days were transferred to soil for another 20 days, under long-days (16h light/8h dark). 7-16 plants were analyzed per line. Data are mean ± SD.

Besides resulting from a faster growth rate and an economy in the number of leaves, *pin6* earlier flowering may additionally result from reduction in the number of stem lateral organs associated with loss of apical dominance. Next was measured the number of second order stem branches that arise from rosette nodes, as well as the number of cauline node inflorescence branches. In fact, all *pin6* plants produced less second order stem branches, the most significant reduction (44%) being observed for the *pin6-3* mutant allele (Table 3.7.). Moreover, *pin6* plants also generated less second order inflorescence branches, with a drastic 55% reduction in *pin6-1* mutant line (Table 3.7.).

Table 3.7. Numbers of second order stem branches and second order inflorescence branches arising from rosette and cauline nodes in WT and *pin6* plants. Seedlings were grown in AM medium for 20 days and then transferred to soil for another 20 days, under long-days (16h light/8h dark). Data are mean ± SD. 7-16 plants were analyzed per line.

Line	Nr 2nd Order Stem Branches	Nr 2nd Order Inflorescence Branches
WT	1.15 ± 0.55	3.46 ± 1.45
pin6-1	0.71 ± 0.49	1.57 ± 1.62
pin6-2	0.83 ± 0.58	2.08 ± 1.38
pin6-3	0.64 ± 0.63	2.07± 1.9
pin6-4	0.75 ± 0.45	2.94 ± 2.05

On the one hand, PIN6-driven auxin may be required to keep the vegetative identity of SAM; in parallel, PIN6 may regulate the ability of axillary branches to establish auxin transport themselves, which in turn results in break of apical dominance and induces their outgrowth.

3.5.3 LIGHT QUALITY EFFECT ON *pin6* GROWTH

It is likely that *PIN6* is regulated by the photoperiod, as *pin6* roots are longer under long-days condition (Fig. 3.19.; Fig. 3.20.) when compared to continuous light (Fig. 3.17.; Fig. 3.18.). Therefore, I evaluated whether light perception regulates PIN6 activity. Mutants affected in cryptochrome- and phototropin-mediated perception (cry1-, cry2-, phot1- and phot2-) de-etiolate when grown under blue light (reviewed in Fankhauser and Casai, 2004). Similarly, mutants affected in phytochromeB (phyB)-mediated perception de-etiolate under red light, but additional signalling components are involved in red light signalling (reviewed in Fankhauser and Casai, 2004). Under far-red light, mutants with disruption in phyA–mediated perception de-etiolate (reviewed in Fankhauser and Casai, 2004). Moreover, *PIN6* could be regulated by UV-B light, so growth under these conditions was also tested, under which *cop1* mutants, the only putative receptors identified for UV-B light so far, de-etiolate (Oravecz *et al.*, 2006).

WT and *pin6* seedlings were grown under different continuous light quality conditions: white, blue, red, far-red and UV-B light, and hypocotyl growth was then assessed. A simple way to measure light

response is to analyze de-etiolation upon light stimulus, in terms of reduction in hypocotyl length and opening of the cotyledons.

I measured hypocotyl growth under different continuous light quality conditions. WT etiolated hypocotyls were 9.32 mm long, whereas *pin6-1*, *pin6-2*, *pin6-3* and *pin6-4* measured 11.55 mm, 11.68 mm, 10.90 mm and 9.88 mm, respectively. Under white light and relatively to its etiolated growth, WT seedlings showed 86% de-etiolation, whereas hypocotyl growth was reduced in 89%, 88%, 87% and 87% in *pin6-1*, *pin6-2*, *pin6-3* and *pin6-4*, respectively (Fig. 3.25.a). Growth under blue light resulted in 82%, 85%, 85%, 84% and 83% de-etiolation in WT, *pin6-1*, *pin6-2*, *pin6-3* and *pin6-4*, respectively (Fig. 3.25.a). Under red light, WT seedlings showed 89% de-etiolation, whereas hypocotyl growth was reduced in 90%, 88%, 90% and 88% in *pin6-1*, *pin6-2*, *pin6-3* and *pin6-4*, respectively (Fig. 3.25.a). Under far-red light, the hypocotyl length reductions observed for WT, *pin6-1*, *pin6-2*, *pin6-3* and *pin6-4* were: 46%, 60%, 58%, 54% and 53% (Fig. 3.25.a). All mutants showed a slightly stronger de-etiolation upon the light treatments tested, which might suggest that *PIN6* is only indirectly affected by light and does not participate in the early perception machinery. The most striking difference between WT and *pin6* responses was observed under far-red light, which might be required indirectly for *PIN6* regulation.

Likewise, plants were grown under UV-B light, below 305 nm, and as control plants were grown below 345 nm, which includes other visible light type sources. Here, too, hypocotyl length suggested the level of de-etiolation. WT hypocotyls increased 36% in length, *pin6-1* increased 21%, *pin6-2* were 80% longer, *pin6-3* measured 49% more and *pin6-4* showed a 42% increase (Fig. 3.25.b). All mutants showed a slightly stronger de-etiolation upon UV-B light treatment, which once more suggests that *PIN6* is indirectly affected by UV-B light.

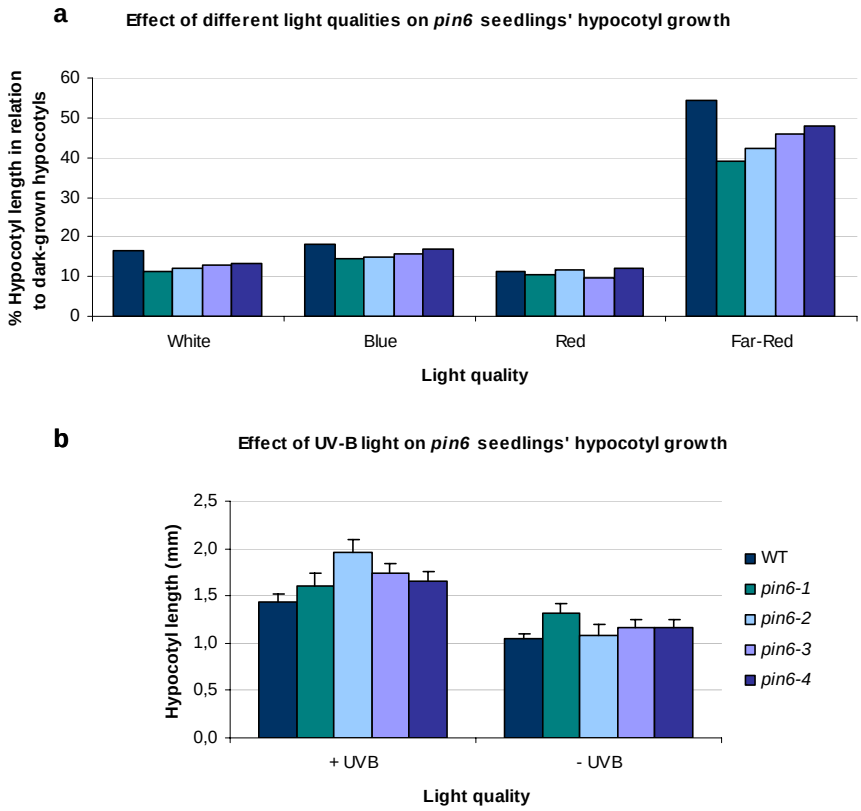

Fig. 3.25. Effect of different light qualities on hypocotyl growth of WT and *pin6* seedlings. 3-days old seedlings were grown under continuous white, blue, red, far-red light or in the dark on filter paper (a) and 4-days old seedlings were grown under UV-B light on AM medium prepared with Bacto-agar (b). (a) Hypocotyl length in relation to maximum hypocotyl length measured in etiolated seedlings grown in the dark. (b) Hypocotyl length (mm). Values represent 2 technical and at least 40 biological replicates for each time point. Data are mean ± SE.

pPIN6::GUS seedlings were grown under the same light quality sources (white, blue, red, far-red, dark and UV-B) to look for changes in *PIN6* expression pattern. *PIN6* expression domains were not affected by the different conditions (Fig. 3.26.). However, blue (Fig. 3.26.d), red (Fig. 3.26.f) and far-red (Fig. 3.26.h) light sources slightly induce *PIN6* expression in SAM. *PIN6* expression in adventitious roots at the shoot-root junction is instead mildly downregulated under blue (Fig. 3.26.e) and far-red light (Fig. 3.26.i, compare with b,k). UV-B treatment seems to have no effect on *PIN6* expression (Fig. 3.26.l, compare with m).

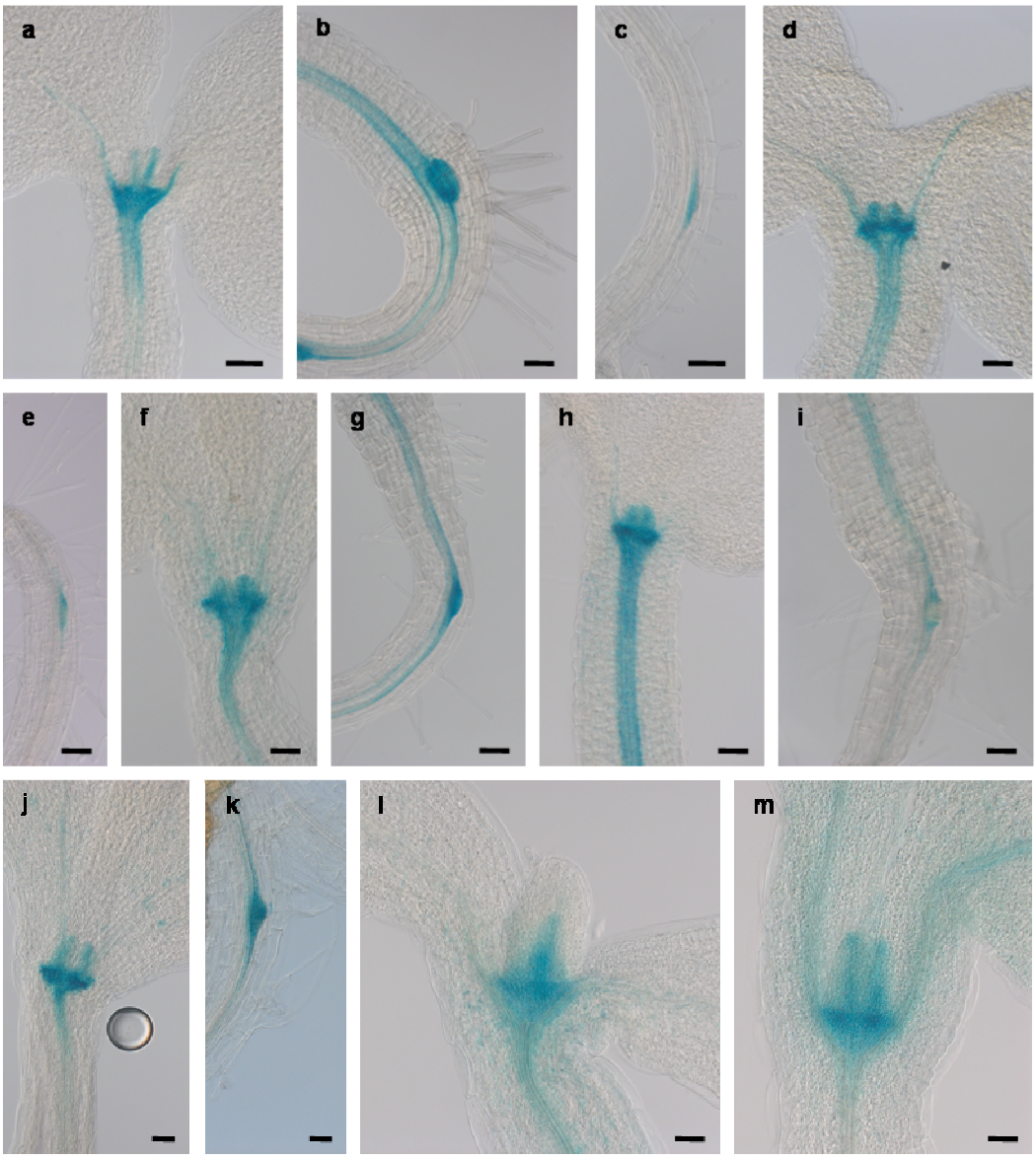

Fig. 3.26. Effect of different light qualities on *PIN6* expression. 3-days old *pPIN6::GUS* seedlings were grown under continuous white (a)-(c), blue (d,e), red (f,g) , far-red (h,i) light or in the dark (j,k) on filter paper and 4-days old seedlings were grown under UV-B light on AM medium prepared with Bacto-agar (l; m is the negative control). *PIN6* expression in SAM is slightly induced in blue (d), red (f) and far-red (h) grown seedlings. *PIN6* expression in adventitious roots at the shoot-root junction is downregulated under blue (e) and far-red light (i, compare with b,k). UV-B treatment has slight or no effect on *PIN6* expression (l, compare with m). Scale bar = 50 µm.

Therefore it seems likely that PIN6 activity is somehow induced by far-red and UV-B light, suggesting that PIN6 regulation is mediated by light perception machineries.

3.5.4 LATERAL ROOT DEVELOPMENT IN *pin6* SEEDLINGS

PIN6 is expressed during all stages of lateral root development (see section 3.2.1), but it is unknown to what extent it contributes to this process. Therefore I inspected the number of lateral roots formed in *pin6* mutants. Under our experimental conditions, 13-days old WT seedlings possess 9.36 ± 0.6 lateral roots (Fig. 3.27.). *pin6-1* and *pin6-2* seedlings had slightly less lateral roots than the WT, but this result is not statistically significant (Fig. 3.27.). Significant differences were observed for *pin6-3* and *pin6-4* lines, in which more lateral roots were formed (11.53 ± 1.25 and 11.06 ± 1.15, respectively; Fig. 3.27.).

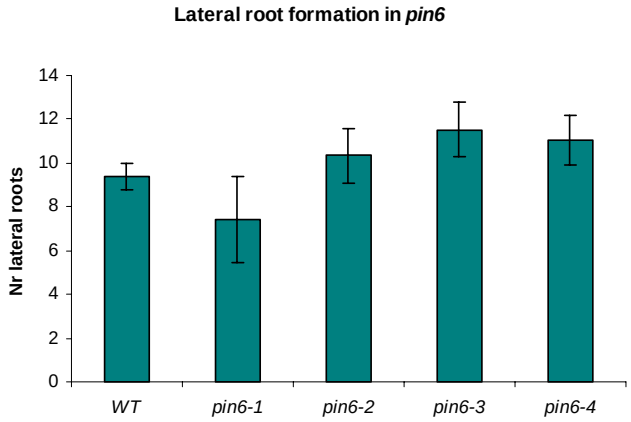

Fig. 3.27. Number of lateral roots in WT and *pin6* seedlings. 13-days old seedlings were grown in AM medium under long-days condition (16h light/8h dark). 30 seedlings were analyzed per line. Data are mean ± SE.

Regarding the knockdown lines, 10.14 (4) and 10.14 (5) do not show a significantly different number of lateral roots (9.00 ± 0.72 and 10.86 ± 1.36, respectively) when compared to the WT (9.36 ± 0.6; Fig. 3.28.). Significant differences (Student's T-test $p<0.05$) were observed for 3.11, a line which formed less lateral roots (7.41 ± 2.77) and for 8.1 (5) and 8.1 (5a), which formed more lateral roots (11.92 ± 3.33 and 13.67 ± 3.77, respectively; Fig. 3.28.).

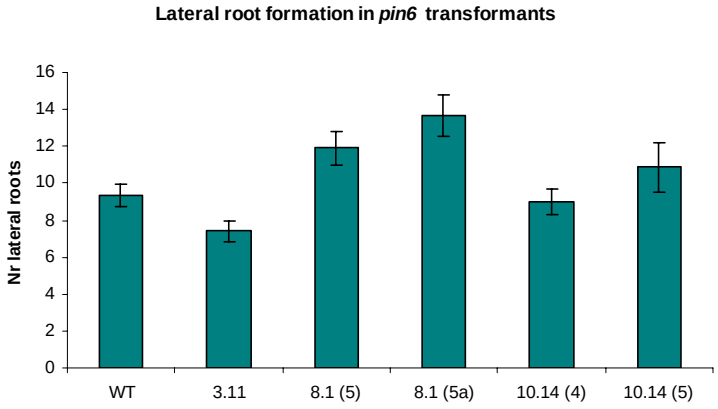

Fig. 3.28. Number of lateral roots in WT and *pin6* knockdown lines. 13-days old seedlings were grown in AM medium under long-days condition (16h light/8h dark). 30 seedlings were analyzed per line. Data are mean ± SE.

Taken in consideration that most mutant alleles for PNI6 result in an increased number of lateral roots, it is reasonable to assume that PIN6 acts by repressing the emergence of lateral roots.

3.5.5 GRAVITY PERCEPTION OF *pin6* SEEDLINGS

Root gravitropism is the orientation of root growth in relation to the gravity vector and is a consequence of gravity sensing in the root cap and differential cell elongation in the root elongation zone. Amyloplasts in the columella cells function as intracellular gravity sensors: they shift their position inside the cell, towards the lower side, where they deposit on top of the endoplasmic reticulum, triggering the differential growth response required for subsequent root bending. Upon gravistimulation, there is an asymmetric re-distribution of auxin from the columella into the lateral root cap cells (Ottenschläger *et al.*, 2003). Furthermore, auxin influx (*aux1*) and efflux (*eir1/pin2* and *pin3*) mutants are agravitropic (Bennett *et al.*, 1996; Friml *et al.*, 2002b; Luschnig *et al.*, 1998; Rashotte *et al.*, 2000). Moreover, *PIN6* and *PIN2* are co-expressed in root tissues (see section 3.1.3) and co-regulated by several factors (see section 3.1.4). It was therefore of interest to evaluate whether *pin6* mutants are also impaired in the gravitropic response.

After 4 hours of gravistimulation by turning the seedlings to 135°, WT seedlings are recovering the perception of the gravity vector and grow in several different directions, from 0° to 149°, a larger part of the plants growing at angles of 60 to 89° (Fig. 3.29.). *eir1*_1 agravitropic seedlings grow in all directions, the majority of them showing curvatures between 120° and 179° (Fig. 3.29.), indicating the lack of correct gravitropism. *pin6* mutants were distributed more evenly, growing more towards the gravity vector (0°) (Fig. 3.29.). *pin6-3* seedlings showed the most homogeneous and striking response, as curvatures restrict to angles between 0° and 89°, the majority lying between 30° and 59° (Fig. 3.29.). After 24h, both WT and

mutant seedlings have fully re-oriented their growth towards the new gravity vector (data not shown). *Pin6* mutants are therefore hypergravitropic – they respond faster to a change in gravity vector.

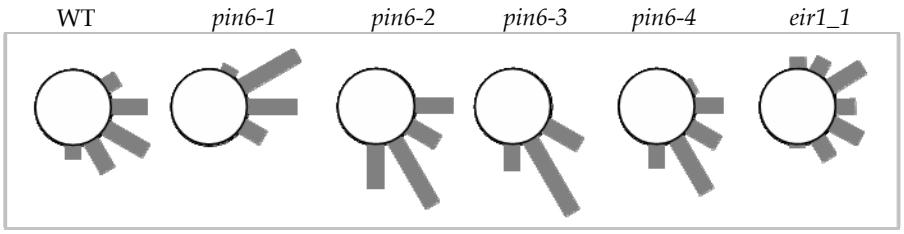

Fig. 3.29. Root curvature (°) induced by 4h of gravistimulation by turning seedlings to 135°. 6-days old seedlings were grown in AM medium for 6 days under long-days condition (16h light/8h dark) and then gravistimulated. Schematic representation of the angle formed between the root tip and the gravity axis (0°). Data are grouped in discrete classes of 30° (0-29°, 30-59°, 60-89°, 90-119°, 120-179°, 180-209°, 210-239°, 240-269°, 270-299°, 300-329° and 330-359°) and bar length is directly proportional to the percentage of plants in that angle class. At least 30 plants were analyzed per line.

In order to investigate reasons for altered gravitropism, amyloplasts that perceive gravity were stained in *pin6* mutant lines with lugol. Amyloplasts typically deposit in the lower side of the first three columella cell layers (from the meristem outwards: C1, C2, C3; Fig. 3.30.c). They also localize to the C4 layer, in scattered circles instead of organized agglomerates (Fig. 3.30.c). After 4 hours of gravistimulation, amyloplasts in all columella cell layers are disorganized, not forming the deposits, but tend to accumulate to the right side, consistently with the direction of the gravity vector applied (Fig. 3.30.j). Consistently with the results presented above (Fig. 3.29), amyloplasts in *pin6* respond to gravity stimulation earlier than the WT. All *pin6* mutants showed reorganization of the amyloplasts in C1, C2 and C3 layers after 4 hours of gravistimulation. Amyloplasts formed again deposits (as before the gravistimulation), oriented at angles to the right (Fig. 3.30.k,l,m,n). Amyloplasts in the C4 cell layer accumulate in the correct direction but did not form the agglomerates (Fig. 3.30.k,l,m,n). Also consistent with the previous data (Fig. 3.29), *pin6-3* seems to be the first mutant to recover gravity perception, shown by the re-organization of the amyloplast deposits (Fig. 3.30.m).

For this experiment I additionally used the *pPIN6::GUS* and *DR5::GUS* lines as additional controls. *pPIN6::GUS* showed no signal in the root tip (Fig. 3.30.a), as was already shown under normal conditions (Fig. 3.7.). *DR5::GUS* confirmed the presence of the auxin in the meristem, particularly forming a gradient in the columella cells layers (Fig. 3.30.b). After 4 hours of gravistimulation, an asymmetry of *GUS* distribution was observed and therefore of an auxin maximum, changing in the direction of the lower side of the curvature (Fig. 3.30.i; the lower side of the root corresponds to the right side in the figure), as previously reported by Ottenschläger and colleagues (2003). As before, upon stimulation, *pPIN6::GUS* showed no signal in the root tip (Fig. 3.30.h).

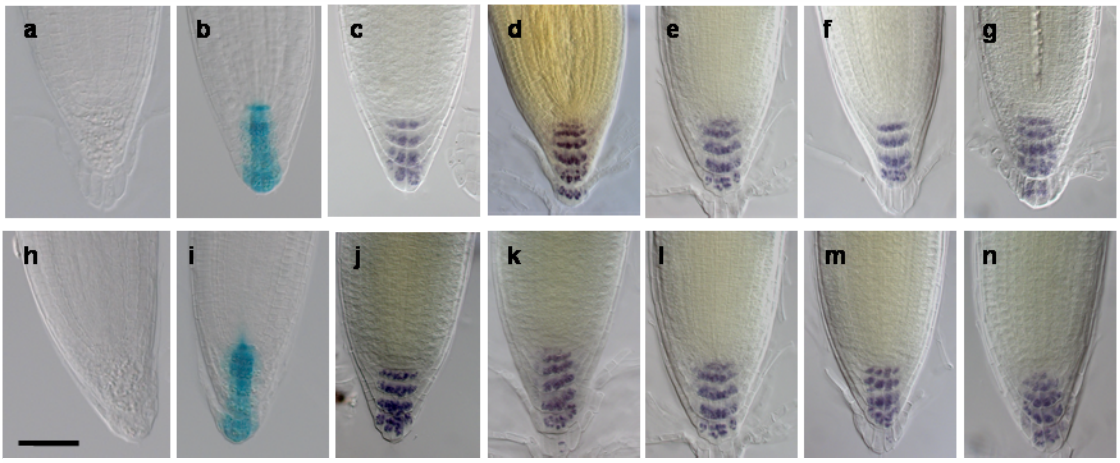

Fig. 3.30. Effect of 4h gravistimulation induced by turning seedlings to 135º. 6-days old seedlings were grown in AM medium for 6 days under long-days condition (16h light/8h dark) and then stained for GUS (a,b,h,i) or for starch grain analysis with lugol. (a)-(g) are the control roots and (h)-(n) the gravistimulated roots. (a) and (h), *pPIN6::GUS*, (b) and (i) *DR5::GUS*, (c) and (j) WT, (d) and (k) *pin6-1*, (e) and (l) *pin6-2*, (f) and (m) *pin6-3*, (g) and (n) *pin6-4*. Note that the lower half of gravistimulated roots is at the right-hand side. In (h) and for all samples scale bar = 50 μm.

3.5.6 AUXIN SENSITIVITY OF *pin6* SEEDLINGS

Auxin regulation of PIN expression has been reported (Heisler *et al.*, 2005; Paciorek *et al.*, 2005; Sauer *et al.*, 2006; Scarpella *et al.*, 2006). Moreover, auxin responsive elements are well represented in the *PIN6* promoter sequence (see section 3.1.2) and *PIN6* transcript levels are upregulated by IAA, NPA, PCIB and TIBA (see section 3.1.4).

Consequently, I analyzed the effect of auxin on *pin6* mutants' root growth. Under our experimental conditions, roots of 6-days old WT seedlings measure 6.58 ± 0.18 mm (Fig. 3.31.). When grown in presence of 1-NAA (1, 5 or 10 μM), an auxin that enters the cell by diffusion but requires a transporter for its efflux, WT root growth is inhibited in 10%, 15% and 25%, respectively. For *pin6-1* the observed inhibition was similar to the WT: 10%, 11% and 16.5%, respectively (Fig. 3.31.). Under the same conditions, *pin6-2* root length was reduced by 10.5%, 33.5% and 25% (Fig. 3.31.), still in the same range as WT. *pin6-4* is the allele most sensitive to 1-NAA: the inhibition observed is 20.4%, 23% and 31.6%, respectively (Fig. 3.31.). Of all genotypes analyzed, *pin6-3* severe allele is the most resistant to 1-NAA, its inhibition levels being of only 7.1%, 12.3% and 21.1% for growing in presence of 1-NAA 1, 5 or 10 μM, respectively (Fig. 3.31.).

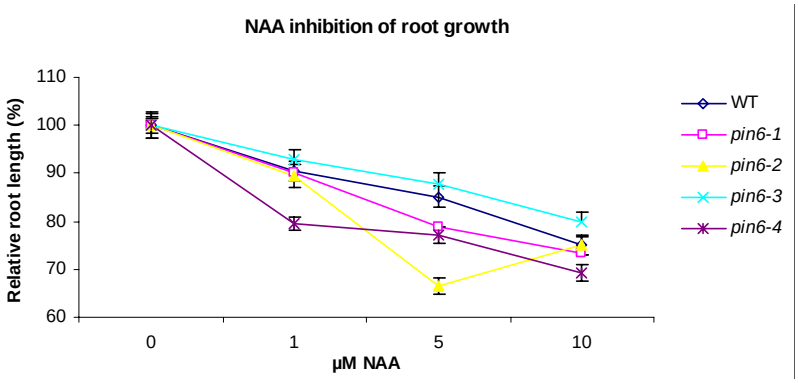

Fig. 3.31. 1-NAA inhibition of root growth. Relative root length (%) of 6-days old WT and *pin6* seedlings grown in AM medium containing 1-NAA 1, 5 or 10 µM under long-days condition (16h light/8h dark). At least 40 seedlings were analyzed per treatment. Data are mean ± SE.

In addition, it was observed that *PIN6* expression is upregulated by 1-NAA. 1-NAA induces the formation of lateral roots all along the main root, from the shoot-root junction to the distal elongation zone behind the root tip (Fig. 3.32.a,b,c). This was accompanied by an increase in *PIN6* levels in the vasculature below and at the sites of lateral root emergence (Fig. 3.32.a,b,c). At higher concentration of 1-NAA (10 µM), *DR5::GUS* auxin reporter accumulated in high levels in the root tissues, specially at the root tip (Fig. 3.32.f,h). Moreover, the root phenotype was more drastic – a shorter root and more ectopic lateral roots were formed, even closer to the root tip, in which *PIN6* expression was observed (Fig. 3.32.g).

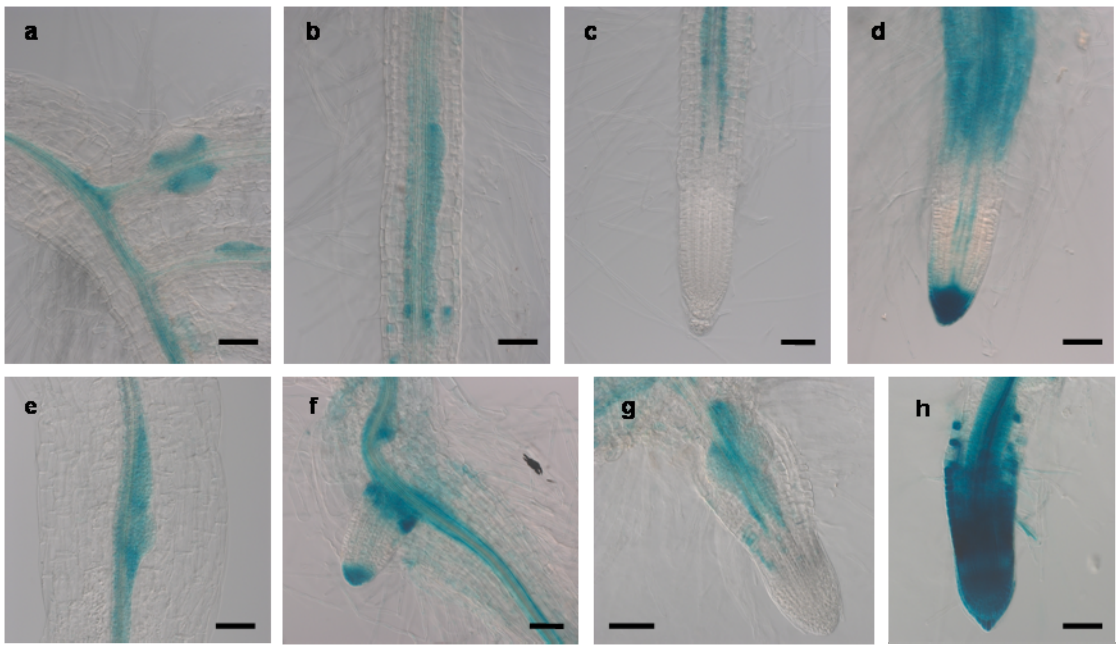

Fig. 3.32. Induction of *PIN6* expression by 1-NAA. 6-days old *pPIN6::GUS* and *pDR5::GUS* seedlings were grown on AM medium under long-day condition (16h light/8h dark). Pictures (a), (b), (c), (e) and (g) are staining for *pPIN6::GUS*, whereas the others are for *DR5::GUS*. (a)-(d) 1-NAA 1 µM induces the formation of ectopic roots with co-expression of *PIN6* at the shoot-root junction (a) and along the main root (b), stopping just above the root tip (c), where auxin levels are induced (d). (e)-(h) 1-NAA 10 µM induces the formation of ectopic roots with co-expression of *PIN6* at the shoot-root junction (e) where higher levels of auxin accumulate (f). Root phenotype is severed and lateral root formation is induced closer to the root tip (g), where auxin levels reach their maxima (h). Scale bar = 50 µm.

PIN6 transcript levels are upregulated by NPA and by other auxin transport inhibitors (see section 3.1.4). NPA inhibits WT root growth and lateral root formation (Fig. 3.33.). Six days old WT seedlings measure 6.58 ± 0.18 mm in length (Fig. 3.33.). When grown in presence of 1 or 10 µM of NPA, root growth is slightly inhibited in 2.6% and 4.8%, respectively. For *pin6-1*, root growth inhibition observed was higher than in the WT: 5% and 16%, respectively (Fig. 3.33.). Under the same conditions, *pin6-3* root length was reduced by 12% and 17% (Fig. 3.33.). *pin6-4* is the allele most sensitive to NPA: the inhibition observed was 14.6% and 18.5%, respectively (Fig. 3.33.). Of all genotypes analyzed, *pin6-2* is the most resistant to NPA, its inhibition levels being 1% and 1.5% for growing in presence of 1-NAA 1 or 10 µM, respectively (Fig. 3.33.).

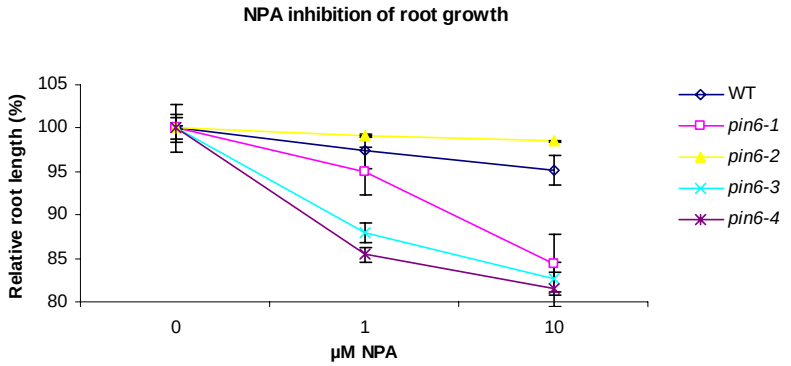

Fig. 3.33. NPA inhibition of WT and *pin6* seedlings root growth. Plants were grown in AM medium containing NPA 1 or 10 µM under long-days condition (16h light/8h dark). Relative growth length (%) of 6-days old WT and *pin6* seedlings grown in presence of NPA 1or 10 µM. At least 40 seedlings were analyzed per treatment. Data are mean ± SE.

Moreover, NPA also inhibited lateral root emergence. Under our experimental conditions, 13-days old WT seedlings grown in NPA (1 µM) produced 83% less lateral roots than control seedlings grown in absence of NPA (Fig. 3.34). Less severely affected were lines *pin6-1*, *pin6-2* and *pin6-4*, in which NPA caused a decrease in the number of emerging lateral roots of 82%, 82.5% and 81%, respectively (Fig. 3.34). On the contrary, *pin6-3* is more affected than the WT. In fact, it is the allele most sensitive – lateral root emergence was reduced by 98.5% (Fig. 3.34).

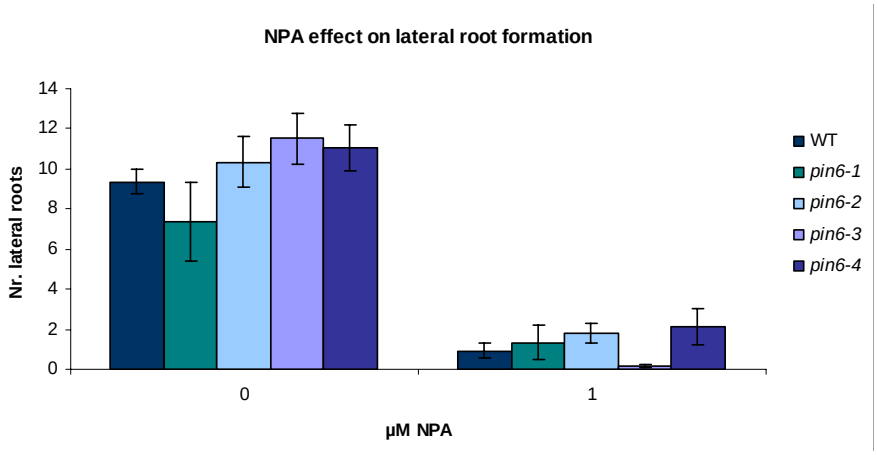

Fig. 3.34. NPA inhibition of WT and *pin6* seedlings lateral root development. Number of lateral roots of 13-days old WT and *pin6* seedlings grown in AM medium containing NPA 1 µM under long-days condition (16h light/8h dark). At least 40 seedlings were analyzed per treatment. Data are mean ± SE.

NPA blocks auxin efflux, thus inducing the accumulation of auxin throughout the vasculature and therefore inhibiting lateral root emergence (Fig. 3.35.c) Auxin transport inhibition can also be observed at the root tip, where higher levels of auxin accumulate (Fig. 3.35.d). Nevertheless, NPA induced *PIN6* expression in the vasculature above and below the shoot-root junction (Fig. 3.35.a) and at the fewer sites of lateral root formation (Fig. 3.35.b) where auxin accumulates.

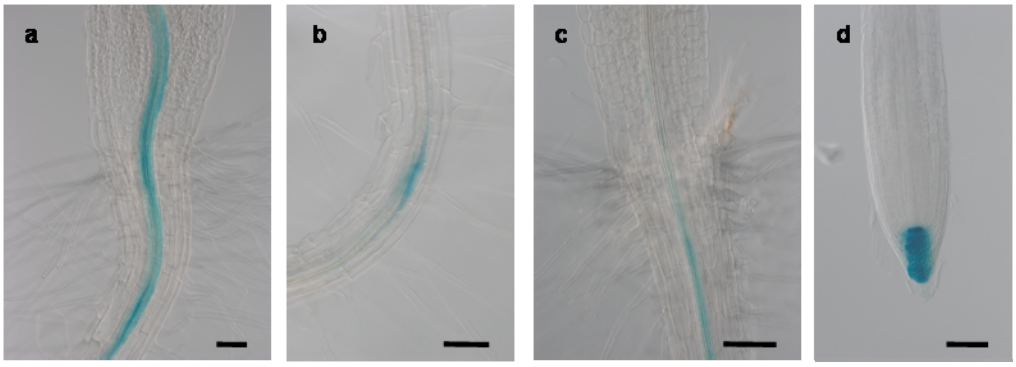

Fig. 3.35. Inhibition of lateral root emergence by NPA. 6-days old *pPIN6::GUS* (a,b) and *pDR5::GUS* (c,d) seedlings were grown on AM medium under long-days condition (16h light/8h dark). NPA 1 µM induces the expression of *PIN6* in the vasculature at the shoot-root junction and neighbouring tissues (a) and at the sites of lateral root formation (b). NPA µM inhibits lateral root emergence (c) by reducing auxin levels (c,d). Scale bar = 50 µm.

Afterwards was analyzed the effect of an auxin influx inhibitor, NOA, in lateral root development and in the regulation of *PIN6* expression. Similarly to the effect caused by the auxin efflux inhibitor NPA, NOA inhibited drastically lateral root emergence. Under our experimental conditions, 13-days old WT seedlings grown in NOA (50 µM) had 95% less lateral roots than control seedlings grown in absence of NOA (Fig. 3.36.). *pin6-1* was the most affected genotype, in which lateral root formation was completely abolished. *pin6-2* and *pin6-4* were affected in the same way as the WT: the number of emerging lateral roots

was decreased in 94% and 96%, respectively (Fig. 3.36.). On the other hand, *pin6-3* severe allele was the most resistant to NOA, in which lateral root emergence was reduced by only 69% (Fig. 3.36.).

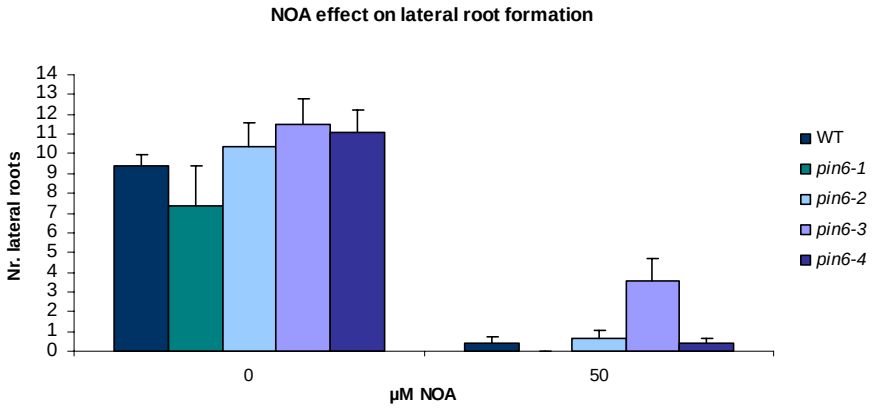

Fig. 3.36. NOA inhibition of lateral root development. 13-days old WT and *pin6* seedlings were grown in AM medium containing NOA 50 µM under long-days condition (16h light/8h dark). At least 30 seedlings were analyzed per treatment. Data are mean ± SE.

3.5.7 ETHYLENE EFFECT ON *pin6* SEEDLINGS

PIN6 is the only member of the *PIN* family upregulated by AVG (see section 3.1.4), an inhibitor of ethylene synthesis that blocks the conversion of S-adenosyl Methionine to ACC, the immediate ethylene precursor (Yang and Hoffman, 1984). Consequently, I checked the effect of altering ethylene levels in *pin6* mutants. To see whether *PIN6*-related lateral root formation was affected by ethylene, *pin6* seedlings were grown in presence of ACC, to increase endogenous ethylene levels, or AVG, to block ethylene synthesis and thus decreasing its levels.

Under our experimental conditions, ACC (0.5 and 5 µM) inhibited lateral root formation. Thirteen-days old WT seedlings grown in ACC (0.5 µM) produced 7% less lateral roots than control seedlings (Fig. 3.37.). Similarly, in *pin6-2* the inhibition was 10% (Fig. 3.37.). *pin6-1* and *pin6-3* showed stronger inhibition levels, the number of emerging lateral roots was decreased in 65% and 44%, respectively (Fig. 3.37.). The most affected genotype was *pin6-4* since lateral root emergence was reduced by 75% (Fig. 3.37.). Growth in presence of ACC (5 µM) results in 34% less lateral root production in the WT (Fig. 3.37.). Similarly, in *pin6-2* the inhibition was 24% (Fig. 3.37.). *pin6-1* and *pin6-3* showed stronger inhibition levels, the number of emerging lateral roots was decreased in 48% and 67%, respectively (Fig. 3.37.). The most affected genotype was *pin6-4* in which lateral root emergence was reduced by 83% (Fig. 3.37.). The consistent results obtained with a concentration of ACC ranging from 0.5 to 5 µM imply a

dose-response-dependent effect of ethylene on lateral root development and show that *pin6* mutants are more sensitive to increasing levels of ethylene.

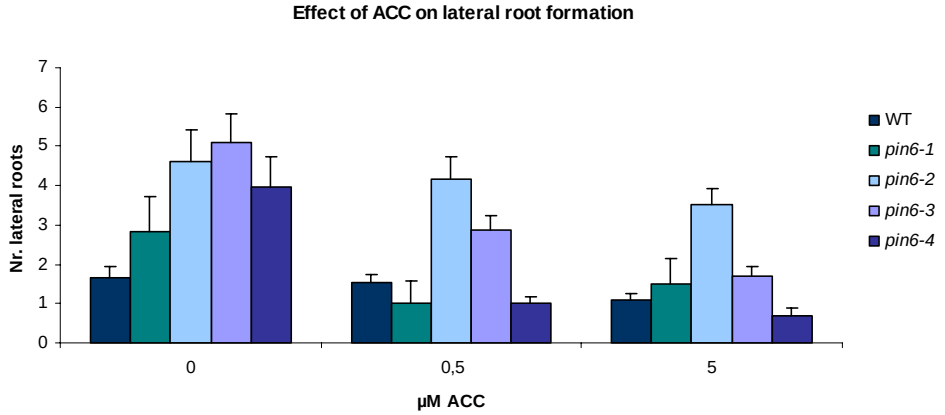

Fig. 3.37. Ethylene effect on lateral root formation of WT and *pin6* seedlings. Plants were grown in AM medium containing ACC 0.5 or 5 µM under long-days condition (16h light/8h dark). Number of lateral roots of 13-days old seedlings. 30 seedlings were analyzed per treatment. Data are mean ± SD.

As for AVG (5 µM), WT seedlings are the most resistant to it, producing 12% less lateral roots (Fig. 3.38.). Lateral root formation was reduced in only 32% in *pin6-2* line, which was the allele less sensitive to AVG (Fig. 3.38.). The other *pin6* alleles showed similar levels of lateral root formation inhibition by AVG, namely 69%, 73% and 68% for *pin6-1*, *pin6-3* and *pin6-4*, respectively (Fig. 3.38.).

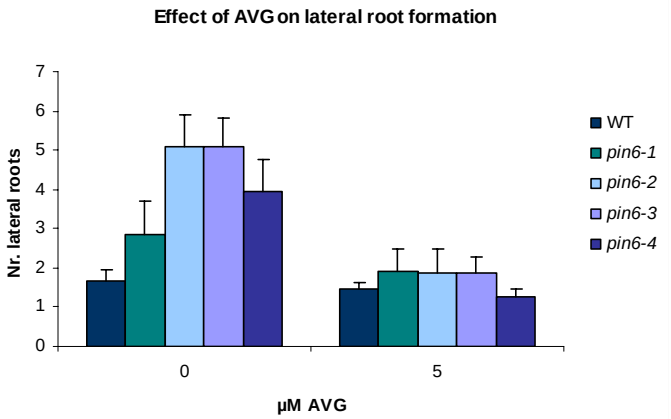

Fig. 3.38. Effect of blocking ethylene synthesis on WT and *pin6* seedlings lateral root formation. Plants were grown in AM medium containing AVG 5 µM under long-days condition (16h light/8h dark). Number of lateral roots of 13-days old seedlings. 30 seedlings were analyzed per treatment. Data are mean ± SD.

It appears contradictory that both increasing levels of ethylene with ACC or blocking of its synthesis reduce the number of lateral roots produced. AVG 5 µM induces the expression of *PIN6* where lateral roots emerge, namely at the shoot-root junction (Fig. 3.39.a). In turn, low ACC levels (0.5 µM) induce accumulation of low levels of auxin in these tissues (Fig. 3.39.c), whereas higher ACC levels (5 µM) upregulate *PIN6* along the vasculature in pericycle cells (Fig. 3.39.b). However, it seems that both

treatments induce *PIN6* expression, even if possibly at slightly different expression domains – ACC induces *PIN6* in ectopic points of lateral root initiation, as more pericycle cells have initiated cell division

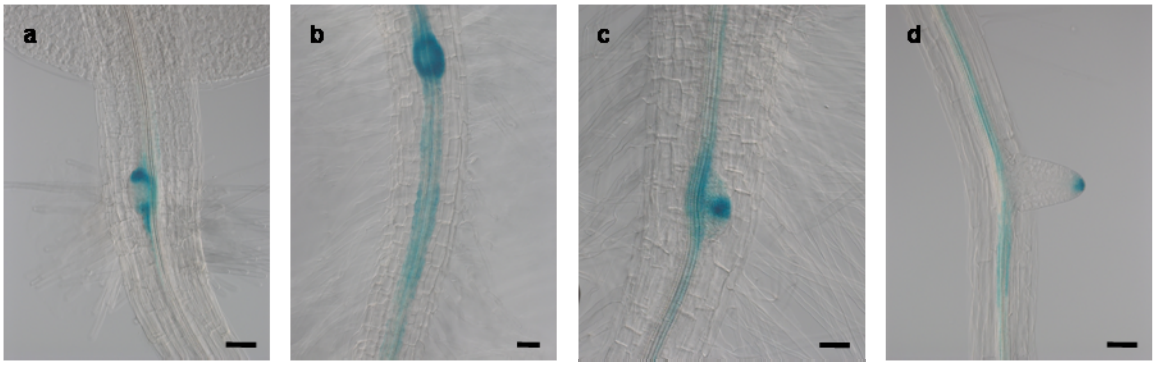

Fig. 3.39. Ethylene effect on lateral root emergence. 6-days old *pPIN6::GUS* (a,b) and *pDR5::GUS* (c,d) seedlings were grown on AM medium under long-days condition (16h light/8h dark). AVG 5 µM induces the expression of *PIN6* at the shoot-root junction where a lateral root emerges (a), ACC 5 µM upregulates *PIN6* along the vasculature in pericycle cells (b) ACC 0.5 µM induces accumulation of low levels of auxin *pDR5::GUS* at the shoot-root junction (c) and in the vasculature and lateral root tip (d). Scale bar = 50 µm.

To sum up, PIN6 appears to act as a repressor of growth involved in several distinct developmental programs: germination, lateral root formation and transition from vegetative to reproductive phase. It is additionally involved in gravity perception and auxin and ethylene cross-talk.

4 DISCUSSION

"Research is to see what everybody else has seen,
and to think what nobody else has thought."
Albert Szent-Györgi

4.1 CHARACTERIZATION OF A NOVEL PIN PROTEIN: PIN6

Polar auxin transport is required for many determinant processes in plant development. Coordination of auxin flow by influx (AUX) and efflux (MDR/PGP and PIN) transporters leads to local auxin accumulation patterns necessary for processes as embryogenesis (Friml *et al.*, 2003), organ development (Reinhardt *et al.*, 2000), root meristem maintenance (Sabatini *et al.*, 1999) and tropic growth responses (Friml *et al.*, 2002b; Ottenschläger *et al.*, 2003).

Among auxin transporters, PINs are the best studied, but it cannot be excluded that PIN proteins are part of larger signalling complexes. In fact, recent unpublished data showed that PIN1 interacts with several other transporters, namely MATE, hexose (STP13), purine (PUP13) and metal ion (NRAMP) transporters (Santos, 2006). These proteins seem to be involved in transporting nutrients, ions, hormones and its derivatives or inhibitors, respectively. Although speculative at present, the results point to a coordination of auxin transport with perception of and response to internal and external cues of nutrients, ions, hormone and biotic stress levels. Other PIN1 putative interactors include E3 ligases, further indicating that PIN1 regulation is proteasome-dependent (Santos, 2006; Yin *et al.*, 2007).

One of the most striking features of PIN proteins is their polar localization. AUXIN RESPONSE FACTOR (ARF)-GEF GNOM regulates PIN protein cycling between plasma membrane and endosomes (Geldner *et al.*, 2003). The molecular mechanisms by which PIN polarity is established are far from being understood. Recent data indicate that PIN1 might interact with proteins of the endosome and vesicle-docking machinery, further supporting a crucial role for this recycling for PIN function (Santos, 2006). Furthermore, the lipid environment seems to be important for PIN correct localization and perhaps function. Sterol-deficient mutants show delocalization of PIN1 (Willemsen *et al.*, 2003) and a putative PIN1 interaction partner plays a role in sphingolipid biosynthesis (ECR1; Santos, 2006).

The PIN protein family has been suggested to be involved in the regulation of polar auxin transport by directly transporting auxin molecules across cell membranes (Petrášek *et al.*, 2006). Characterized PIN proteins all show subcellular asymmetric localization within certain types of cells, a polarity required for auxin transport direction. They also have distinct expression domains, suggesting its specialization in transporting auxin in particular tissues or time points in plant development. PIN1 is present in the central stele of vasculature throughout the whole plant; PIN2 is localized in epidermis and cortex cells in root tissues; PIN3 localizes to columella cells, assuming its polar localization upon

gravistimulation; and PIN4 and PIN7 are present in the quiescent centre and adjacent cells since early embryo developmental stages (Friml *et al.*, 2002a; Friml *et al.*, 2002b; Gälweiler *et al.*, 1998; Müller *et al.*, 1998). In recent years, effort in our group has been put into characterizing the remaining three family members: PIN5, PIN6 and PIN8.

So far, the first insights in PIN6 subcellular localization come from immunolocalization of PIN6 protein in embedded plant material, where PIN6 is localized in a polar fashion (see section 3.2.2). PIN6 co-localizes with PIN1 in the companion cells – PIN1 domain is a few cell files broader and surrounds PIN6 expressing cells. Whole-mount immunolocalization in Arabidopsis roots resulted in a scattered dotty pattern throughout the whole tissue. This pattern might reflect unspecific binding of the antibody or cross-reaction with another closely related protein, such as PIN2. Microarray data have shown that *PIN2* and *PIN6* co-express and are co-regulated in root tissues. Furthermore, auxin induces *PIN6* mRNA ectopic expression in additional cell files in the main root, suggesting that PIN6 protein might only assume an organized polar localization upon certain stimuli as, for instance, auxin or gravity. In fact, PIN3 protein is distributed in the plasma membrane all around the cell and only assumes its lateral localization upon gravistimulation (Friml *et al.*, 2002b).

The aim of this work was to contribute to elucidation of PIN6 function in plant development and identify factors which may regulate PIN6 expression.

4.1.1 USING GENE KNOCKOUT AND -DOWN STRATEGIES AS TOOLS FOR ANALYZING PIN6 FUNCTION

Several strategies are available to study the biological function of a particular gene. Gene interaction experiments combined with a theoretical analysis allow identification of frequent or fixed developmental programs (Alvarez-Buylla *et al.*, 2007). Gene knockout is nowadays considered an important component of the functional genomics toolbox. Gene-disruption methods include randomly mutagenizing plants with insertion elements (Krysan *et al.*, 1999; Parinov and Sundaresan, 2000) and targetted mutagenesis (Lloyd *et al.*, 2005; Shaked *et al.*, 2005). Reverse genetic approaches are based on PCR screening of DNA pools obtained from a population with the desired insertions and allow for isolation of knock-out mutant lines for a gene of interest (Parinov and Sundaresan, 2000).

Agrobacterium-mediated transformation is a unique phenomenon of horizontal gene transfer between prokaryotes and eukaryotes that involves the processing of transferred (T)-DNA from the resident *A. tumefaciens* Ti (tumor-inducing) plasmid and its transfer to plants with the aid of virulence proteins (reviewed in Gelvin, 2003). T-DNA inserts on the order of 5 to 25 kb in length generally are well established tools to disrupt gene functions (Krysan *et al.*, 1999).

In this work four independently transformed T-DNA insertion lines were analyzed, each containing a single copy of the T-DNA sequence (see section 3.3.1). None of the mutant lines has complete disruption of *PIN6* gene expression, even though in *pin6-3* allele transcript levels are drastically reduced. Quite striking is the fact that two close (or coinciding) T-DNA insertions, in *pin6-3* and *pin6-4* alleles, result in very different levels of downregulation of *PIN6* (see section 3.3.2). Nevertheless, it is possible that the T-DNA insertion is partially or totally excised in *pin6-4*, leading to higher production of the correct mRNA. To find out whether PIN6 protein exists at all in the drastic *pin6-3* allele, and that it is correctly synthesized in all other mutants, one should undertake further analysis such as Western Blot or immunolocalization.

In addition to knock-out mutations, insights into gene function can come from generation of transgenic lines causing gain or loss of function mutants by expressing the gene constitutively or containing knockdown mutations which result in partial or complete gene silencing. In this work, transgenic constructs were prepared for both *PIN6* overexpression and *PIN6* silencing through RNA interference (RNAi). All transgenic lines generated have reduced *PIN6* expression levels (see section 3.4.1). Therefore, the diverse transgenic lines were considered as knockdown allelic series of *PIN6* and were inspected for phenotypes to elucidate PIN6 function.

Generally, *pin6* mutant analysis revealed a phenotype of longer main roots that grow faster than the WT (see section 3.5.1) and produce more lateral roots. *pin6-3* allele, the allele with almost no *PIN6* expression, shows the highest growth rates. Absence of *PIN6* resulted in additional growth, proposing PIN6 action as a negative regulator of root development. We could observe that *pin6* mutants germinate and make the transition from vegetative to reproductive phase earlier than the WT, accompanied by a decrease in the number of rosette leaves, as well as a reduced number of shoot and inflorescence branches generated (see sections 3.5.1 and 3.5.2). Additionally, *pin6* mutants are more resistant to hypocotyl etiolated growth under far-red and UV-B light, conditions that induce *PIN6* gene expression (see section 3.5.3). *pin6* mutants are also hypergravitropic (see section 3.5.5). It appears that *pin6_3*, the severe allele, is more resistant to 1-NAA root growth inhibition and the most sensitive to NPA inhibition of lateral root formation (see section 3.5.6). All mutants are sensitive to NOA inhibition of lateral root formation, an auxin influx inhibitor, thus implying PIN6 interaction with auxin influx regulators at some level (see section 3.5.6). Regarding ethylene response, *pin6* mutants are more sensitive to ACC and AVG inhibition of lateral root formation (see section 3.5.7).

Quite interestingly, line 3.11, originally designed to overexpress *PIN6*, is the transgenic line with the lowest levels of *PIN6* expression. Indeed, 3.11 and *pin6-3* are the most severe alleles presenting similar *PIN6* expression levels. The overexpression of PIN6 was driven by the Cauliflower mosaic virus (CaMV) *35S* promoter. This strong promoter is broadly used for ectopic and constitutive expression of a gene of interest. Nevertheless, cosuppression may occur, such as in line 3.11, leading to gene (*PIN6*) silencing. Posttranscriptional gene silencing (or cosuppression) occurs when mRNA turnover is increased because the product of gene expression reaches a threshold concentration as a consequence of a strong promoter (reviewed in Moissiard and Voinnet, 2006). Other events of CaMV-induced gene silencing have been reported (Al-Kaff *et al.*, 1998; Al-Kaff *et al.*, 2000), and only recently this mechanism has been explained

(Moissiard and Voinnet, 2006). CaMV carries an unusually extensive secondary structure known as translational leader. Biogenesis of leader-derived siRNA requires the coordinated and hierarchical action of the four Arabidopsis Dicer-like (DCL) proteins (Moissiard and Voinnet, 2006). DCL1 facilitates accumulation of DCL2-, DCL3-, and DCL4-dependent siRNAs derived from the *35S* leader (Moissiard and Voinnet, 2006).

Two different RNAi constructs were designed for *PIN6* silencing: one targetted to *PIN6* CDS and the other to 3'-UTR region of the gene. RNAi lines typically show a wide range of effects on gene expression, from complete inhibition to no alteration of target mRNA levels. The analysis of several independent *PIN6*-RNAi lines was therefore necessary, since the variability of the silencing effects in different plants and tissues can complicate interpretation of results. RNAi can be generally defined as the silencing of gene expression by double-stranded RNA (dsRNA), which has a complementary sequence to the target gene to be silenced. RNAi strategy involves dsRNA formation from annealing of sense and antisense strands present in the *in vitro* RNA. dsRNA is cleaved into *ca.* 23 bp short interfering RNAs (siRNAs) by the enzyme Dicer. The siRNA-Dicer complex recruits additional components to form an RNA-induced Silencing Complex (RISC) in which the unwound siRNA base pairs with complementary mRNA, thus guiding the RNAi machinery to the target mRNA, resulting in the effective cleavage and subsequent degradation of the mRNA (Pham *et al.*, 2004; Zamore *et al.*, 2000). The activated RISC can then target multiple mRNAs.

In overview, the generated knockdown transgenic lines present phenotypes similar to the T-DNA insertion mutants: longer main roots and they grow at the same speed or faster than the WT (see section 3.5.1). Interestingly, the four *pin6* alleles have slightly differently responses under the various conditions tested (see section 3.5). 3.11 and the severe *pin6-3* allele, the lines with lower levels of *PIN6* expression, show the highest growth rates and root lengths. The fact that silenced allelic series of *PIN6* result in more growth suggests that PIN6 is a negative regulator of root growth.

4.2 PIN6 AND AUXIN TRANSPORT MODULATION

The PIN proteins PIN1 and PIN2 have been demonstrated to be regulated by auxin (Heisler *et al.*, 2005; Paciorek *et al.*, 2005; Sauer *et al.*, 2006; Scarpella *et al.*, 2006). The presence of several auxin responsive elements in PIN6 promoter sequence suggests that this gene is also regulated by auxin, possibly through a TIR1-Aux/IAA-ARF-mediated mechanism.

In fact, upon auxin (IAA) treatment *PIN6* mRNA is ectopically induced in the stele of main roots (see section 3.2.1). *pin6-3* allele is more resistant to 1-NAA inhibition of root elongation while *pin6-4* is the most sensitive to 1-NAA growth inhibition, suggesting that PIN6 is required for correct auxin accumulation pattern and subsequent growth inhibition. PIN6 might, therefore, direct auxin transport, lowering auxin

accumulation levels at cell or tissue levels. This would result in a negative feed-back loop, with higher auxin levels inducing *PIN6* expression, in turn leading to less accumulation of auxin and so forth.

On the other hand, seedlings grown in presence of 1-NAA produce many ectopic lateral roots always accompanied by *PIN6* expression in the vasculature below and at the sites of lateral root emergence (see section 3.5.6). In contrast to IAA, 1-NAA is lipophilic and can thus enter cells bypassing the requirement of an auxin influx carrier (Delbarre *et al.*, 1996), whereas its efflux is strictly dependent on the presence of auxin transporters. Additionally, *pin6* mutants grown in presence of NOA, a specific auxin influx inhibitor, show a reduced number of lateral roots formed – in *pin6-1* allele lateral root emergence was completely abolished, suggesting that auxin influx is required for PIN6-mediated lateral root initiation. Opposite results were obtained with *pin6-3*, which proved to be more resistant to NOA. The results propose the existence of a certain crosstalk between both types of auxin transport that may occur at the level of PIN6. Consequently, auxin coming via influx might modulate PIN6 activity at distinct levels in the different alleles. All *pin6* mutants are resistant to inhibition of lateral root formation by auxin efflux inhibitor NPA and, except of *pin6-2*, sensitive to auxin transport inhibition effect on root growth. These results suggest that *PIN6* is required for root growth occurring in the root elongation zone. However, one cannot exclude the possibility of NPA blocking of auxin transport occurring in different tissues (*e.g.* root tip meristem) or cells that do not normally express *PIN6* and where it cannot be ectopically induced. Another possibility is that NPA binds PIN6 protein impairing auxin transport. The different alleles might have different affinities for NPA molecule, thus explaining the relative extent to which the alleles are affected by NPA treatment.

It is possible that auxin induction of PIN6 not only involves its local accumulation at the cellular level due to transport gradients, but also involves a TIR1-Aux/IAA-ARF-mediated mechanism. PCIB, an anti-auxin compound affecting Aux/IAA protein stability (Oono *et al.*, 2003), upregulates *PIN6*, hence indicating that some sort of transcriptional repressors are involved in *PIN6* regulation.

4.3 PIN6 AND OTHER HORMONES

Hormone crosstalk is an important component of plant development. Signalling pathways of auxin, abscisic acid (ABA), brassinosteroids, cytokinin (CK), ethylene, gibberellin (GA), jasmonate (MJ) and salicylic acid (SA) have been shown to converge in specific developmental programs and molecular components have been identified. In the study presented here, *PIN6* expression was shown to be altered upon hormone treatment.

CK enhanced binding sequences are present in *PIN6* promoter and kinetin treatment induced *pPIN6::GUS* expression (see sections 3.1.2 and 3.2.1). In contrast, zeatin downregulates *PIN6* expression, and *PIN6* is repressed in an *ARABIDOPSIS RESPONSE REGULATOR22* overexpressor line, in which cytokinin responses are globally attenuated (*ARR22OX*; see section 3.1.4; Kiba *et al.*, 2004). Such results suggest that

PIN6 might be involved in CK response. However, it is not clear how response to different cytokinins affects *PIN6*. The CK to auxin ratio has long been proposed to be determinant for organogenesis. Therefore, it is not surprising that a joint control of auxin transporters and regulators of CK response may be necessary for diverse stages of plant development.

Regarding GA, several responsive elements exist in *PIN6* promoter sequence. GA induces slightly *pPIN6::GUS* (see section 3.2.1). Contradictory results from microarray experiments state that a higher concentration of GA downregulates *PIN6* levels and use of GA inhibitors induces *PIN6* expression. These results cannot be directly compared due to different conditions tested, so one can only assume that it is likely that PIN6 is somehow involved in GA-mediated processes.

cesA3 (*zorro*), a cellulose synthase mutant involved in cell wall formation (Burn *et al.*, 2002; Wang *et al.*, 2006) and ethylene-jasmonate crosstalk, has *PIN6* mRNA levels downregulated (see section 3.1.4). This implies a connection between cell wall formation and proper *PIN6* expression. Considering PIN6 activity in meristems, at sites of new organs development, it is possible that cell wall synthesis, as a part of the cell division process, is required for PIN6-dependent organ initiation.

All evidence points to PIN6 involvement in crosstalk between auxin and several other classes of plant hormones. PIN6 might be an important player in the particular crosstalk between auxin and ethylene. In fact, it is the only *PIN* gene to be upregulated upon ethylene synthesis inhibition by AVG treatment (see section 3.1.4), as also shown in our results (see section 3.5.7). Several ethylene responsive elements are present in *PIN6* promoter, and *PIN6* expression is upregulated in the *ein2* mutant impaired in ethylene perception (Alonso *et al.*, 1999), as well as it is affected in other ethylene-related mutants (upregulated in *rdo2* and *gpa1* and downregulated in *cesA3*). *pPIN6::GUS* seedlings grown in presence of ACC, an ethylene precursor, have *PIN6* induction in root vasculature (see section 3.5.7). Analysis of *pin6* mutants showed that they are more sensitive to ACC inhibition of lateral root initiation than WT. Similarly, they are more sensitive to inhibition of lateral root initiation by AVG. It seems contradictory that both increasing endogenous ethylene levels by supplying the plant with its precursor and reducing its endogenous levels by blocking its synthesis both result in arresting lateral root initiation. Nevertheless, it is possible that *PIN6* expression is not affected and that instead, only lateral root emergence stages are impaired. Indeed, I observed that *PIN6* expression remains along the vasculature in the initial lateral root founder cells. One can propose that ACC upregulates *PIN6* along the vasculature in pericycle cells, where auxin is available, triggering those cells for cell division and that ethylene subsequently causes arrest of lateral root emergence.

To date, there have been no reports on ACC effect on lateral root development. The first insights in the role of ethylene in this particular developmental stage come from a recently described component involved in auxin-ethylene crosstalk, the POLARIS (PLS) peptide (Chilley *et al.*, 2006). *Pls* mutants have enhanced ethylene-response phenotypes, defective auxin transport and homeostasis, and altered microtubule sensitivity to inhibitors (Chilley *et al.*, 2006). In contrast to *pin6* mutants, *pls* mutants show a drastic reduction in the number of developed lateral roots. *PLS* expression is repressed by ethylene and induced by auxin (Chilley *et al.*, 2006). Similarly to PLS, *PIN6* is upregulated by ethylene inhibitors and

auxin. PLS is proposed to be a negative regulator of ethylene response, modulating cell division and expansion via downstream effects on microtubule cytoskeleton dynamics and auxin signalling, thereby influencing root growth and lateral root development (Chilley *et al.*, 2006). Similarly, PIN6 could be involved in auxin- and ethylene-effect modulation at the root level, being an additional component to the regulatory loop controlled by PLS.

4.4 PIN6 IN MERISTEM DEVELOPMENT

4.4.1 GERMINATION

Pin6 mutants germinate earlier than WT, observed most strikingly in *pin6-3* severe allele (see section 3.5.1). During seed maturation, the embryo enters dormancy, a quiescent phase in response to desiccation. Break of dormancy leads to germination, and the embryo resumes growth from the mature seed. Among other factors, such as light or cold triggers, germination is induced by a sharp drop in the ABA to GA ratio (reviewed in Taiz and Zeiger, 2002).

ABA induces slightly *PIN6* expression in lateral roots (see sections 3.1.4 and 3.2.1). Consistently with these results, many E-box ABA-responsive elements are present in *PIN6* promoter sequence (see section 3.1.2). The transcription factor ABSCISIC ACID INSENSITIVE3 (*ABI3*) and *FUSCA3* (*FUS3*) genes are necessary for seed maturation and the initiation of dormancy and are sensitive to the hormone abscisic acid, the signalling molecule initiating seed and embryo dormancy (Nambara *et al.*, 2000). Hence, ABA-responsive transcription factors could bind to *PIN6* and control its expression. Concurrently, in a*bscisic acid insensitive1* (*abi1-1*) mutant (Allen *et al.*, 1999), with enhanced ABA sensitivity, and in *gpa1* mutant involved in ABA, GA and ethylene perception in control of germination and cell division events (Chen *et al.*, 2006; Ullah *et al.*, 2002), *PIN6* is upregulated (see section 3.1.4).

Considering that *PIN6* is upregulated in *gpa1* mutants involved in both ABA and GA perception at the regulation level of germination and cell division events (Ullah *et al.*, 2002), it is possible that auxin, conveyed by PIN6, interacts with other hormones in the control of germination. *PIN2* transcript levels increase during early stages of germination and the gene is downregulated in *ga1-3* GA biosynthesis mutant, proposing a role for polar auxin transport in germination events (Ogawa *et al.*, 2003). Microarray analysis of GA-treated *ga1-5* seedlings showed an intense increase in *PIN6* transcript levels (see section 3.1.4). Taken together, these results suggest that PIN6 might be involved together with ABA- and GA-signalling pathways in early germination events.

The *LEAFY COTYLEDON1* (*LEC1*) transcription factor is another gene active in late embryogenesis. *Lec1* mutants cannot survive desiccation and do not enter dormancy. *LEC1* is expressed throughout embryogenesis, suggesting its role as a repressor of vegetative development (Lotan *et al.*, 1998).

Interestingly, *lec1-1.3* allele is, from the microarray data available, the mutant showing highest induction of *PIN6* expression (5-fold; see section 3.1.4). The close spatio-temporal expression of both LEC1 and PIN6 at later stages of embryogenesis – entering and breaking dormancy, respectively, suggests that LEC1 might act first, upstream of PIN6, and changes in ABA, GA and possibly auxin trigger its activation prior to germination. Considering *PIN6* strong induction in absence of *LEC1*, it is proposed that a decrease in *LEC1* expression levels after entering dormancy triggers *PIN6* expression.

Moreover, *rdo2* is an example of a mutant with reduced seed dormancy. This mutant shows upregulated levels of *PIN6* expression (see section 3.1.4). In general, *pin6* mutants germinated earlier than WT. The sharp drop in the ABA to GA ratio (reviewed in Taiz and Zeiger, 2002) may lead to increase of *PIN6* transcription. Therefore it is possible that PIN6 has a role in breaking seed dormancy by increasing polar auxin transport into target cells in coordination with other hormones.

4.4.2 LEAF PRIMORDIA EMERGENCE

Auxin seems to have an instructive rather then permissive role in the regular arrangement of organs. A role for polar auxin transport in phyllotaxis regulation has been proposed (Reinhardt *et al.*, 2003). PIN1 localization in Arabidopsis SAM and analysis of mutants with auxin-related defects propose a role for auxin in determining the regular arrangement of leaf primordia. Manipulation of polar auxin transport, genetically or pharmacologically, leads to altered phyllotactic patterning. Furthermore, in polar auxin transport-related mutants expression patterns of phyllotaxis regulatory genes are impaired. Interestingly, the authors have shown that not only PIN1 responds to auxin as a phyllotactic signal, but *per se* reinforces the signal by generation of local auxin elevations and depletions in the surrounding tissues creating a phyllotactic pattern. Thus, the expression of PIN1 not only responds to phyllotactic information but also functional PIN1 protein is necessary to create such patterns in a similar manner to organogenesis.

In aerial organs formation, auxin follows a "reverse fountain" flow - PIN-dependent accumulation of auxin at the tip of the primordium is drained into the interior of the primordium and transported through its middle. This flow establishes a route for neovascularization, connecting new organs to the existent vascular strands in the main body of the plant. PIN6 is localized to lateral meristematic cells below sites of new organ emergence, during a short period of time prior to organ initiation. It is possible that PIN6 is required to transport auxin away from those cells and tissues and into the new organ emergence site, leading to promotion of cell division and further organ outgrowth. Similarly to PIN1, PIN6 might determine which cells de-differentiate and specialize into vascular cells, thus making the connection between the main apex vasculature and the veins in the developing organs.

Early vasculature development requires *CONTINUOUS VASCULAR RING1* (*COV1*; Parker *et al.*, 2003). *Cov1* mutants have decreased levels of *PIN6* transcripts. PIN1 protein is the earliest known marker

for vascular development (Scarpella *et al.*, 2006). Definition of a PIN1 convergence domain and resulting auxin flow induce PIN1 expression levels and polarization, resulting in gradual selection of a narrow strand of pre-procambial cells (Scarpella *et al.*, 2006). PIN6 is localized to the vasculature underlying sites of new organ formation (see section 3.2.1). It is restricted to a much smaller domain than PIN1 and its expression appears to be shorter in time. It is possible that PIN6 is involved in vasculature formation of new organs, and that its expression is triggered by PIN1-mediated auxin flow in already established veins.

PIN6 is mainly associated with meristem development (see sections 3.2.1 and 3.2.2). The protein and its transcripts are present in lateral meristems directly below new organ initiation sites, at the sides of the apical meristem and in the vasculature of those emerging organs.

Expression of a chimeric repressor from TCP3, a member of TCP transcription factors in *Arabidopsis thaliana*, resulted in the formation of ectopic shoots on cotyledons and various defects in organ development (Koyama *et al.*, 2007). Additionally, induced ectopic expression of boundary-specific genes was observed, namely the *CUP-SHAPED COTYLEDON* (*CUC*) genes, and expression of miR164, whose product cleaves the transcripts of *CUC* genes, was suppressed (Koyama *et al.*, 2007). The pattern of expression of TCP3 did not overlap with that of the *CUC* genes. Thus, TCP transcription factors are required in morphogenesis control of shoot organs by negatively regulating the expression of boundary specific genes (Koyama *et al.*, 2007). Interestingly, other functionally-related TCP transcription factors, including TCP13, have functions similar to that of TCP3 (Koyama *et al.*, 2007). In fact, *tcp13*, *tcp14* and *tcp13tcp14* mutants all show increased levels of *PIN6* transcripts (see section 3.1.4). It seems that TCP transcription factors negatively regulate *PIN6* expression, besides boundary specific genes. It would be interesting to assess the relationship between *PIN6* and *CUC* genes, to see if *PIN6* gene is activated first for new organ formation and then followed by CUC definition of organ boundaries, or if these are already defined before *PIN6* is expressed.

Temperature-sensitive *arrested development3* (*add3*) mutants with upregulated *PIN6* levels show a phenotype defective in leaf blade expansion at high temperatures (Picket *et al.*, 1996). Another mutant impaired in leaf development is *crinkled leaves8* (*cls8*), which is also necessary for chloroplast division (Garton *et al.*, 2007) and its absence results in upregulated *PIN6* levels. ADD3 and CLS8 possibly act as *PIN6* repressors at the level of leaf emergence.

4.4.3 MERISTEM TRANSITION TO THE REPRODUCTIVE PHASE

SAM maintenance and pattern definition of the stem cell niche is tightly controlled by a central regulatory mechanism involving *WUSCHEL* (*WUS*), *CLAVATA3* (*CLV3*) and receptors CLV1/CLV2 (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Laux *et al.*, 1996; Mayer *et al.*, 1998; Schoof *et al.*, 2000; Trocochaud *et al.*, 2000). Keeping the nature of the meristem indeterminate and defining new organ boundaries requires class I

KNOTTED1-like homeobox (*KNOX1*) genes (reviewed in Hake *et al.*, 2004). The transition to the reproductive phase involves many molecular processes. Contrary to SAM, floral meristems are determinate: meristematic activity stops with the generation of the last floral organs. Also auxin is implicated in the floral transition, as has been recently observed in the late flowering mutant *arf*2 mutant (Ellis *et al.*, 2005).

PIN6 transcripts are present in inflorescence stems at sites of new organ emergence, as well as in the vasculature that lies underneath. *PIN6* is additionally expressed in the transmitting tissue of the mature pistil, at the tip of the stigma and in the vascular tissue supporting the anther. All *pin6* transgenic lines make the transition to the reproductive phase earlier than WT (see section 3.5.2). The basal rosette of leaves in Arabidopsis is a consequence of the vegetative apical meristem producing phytomeres with very short internodes. Two of the mutant alleles analyzed (*pin6-3* and *pin6-4*) produced less rosette leaves prior to emergence of the inflorescence stem. It seems that these mutants achieve higher growth rates and earlier flowering by decreasing the number of leaves produced in the rosette, thus saving the time and energy required to build those organs. In earlier stages of reproductive development, the vegetative meristem is transformed into an indeterminate primary floral meristem that produces floral meristems on its flanks, from which lateral buds develop into secondary inflorescence meristems. Concurrently, *pin6* mutant alleles produce less second order stem branches and inflorescence branches, thereby reducing the time and energy invested in this phase transition.

More axillary growth (*max*) mutants have increased capacity for auxin transport (Bainbridge *et al.*, 2005; Bennett *et al.*, 2006) and show phenotypes with loss of apical dominance, producing more axillary branches. *max1* mutant shows upregulation of *PIN1*, *PIN2*, *PIN3*, *PIN4*, *PIN6* and *PIN7*. It has been proposed that *MAX1*, *MAX3* and *MAX4* genes act to produce a long-distance signal which is transported up the plant perceived via *MAX2*-dependent signalling (Bennett *et al.*, 2006). *MAX2* codes for an F-box leucine-rich repeat protein (Stirnberg *et al.*, 2002). This long-distance signal leads to reduction in *PIN* gene transcription, reducing auxin transport capacity, and blocking export of auxin from the bud (Bennett *et al.*, 2006). The MAX pathway controls shoot branching by control of PIN activity and, therefore, of auxin transport that might indirectly control axillary bud dormancy. MAX proteins might be necessary to regulate *PIN6* expression. Consistently, *PIN6* levels are upregulated in *max4* mutant (see section 3.1.4). It seems that in *pin6* mutants apical dominance is stronger and buds are kept dormant. It would be interesting to analyse if PIN6 is important to establish a polar auxin transport route from the axillary buds to the main stem and hence allow breaking of bud's dormancy and lateral branches formation.

Recently, effort has been put into identifying florigen, the long-distance signal triggering the transition to the reproductive phase. *FLOWERING TIME* (*FT*) mRNA is expressed transiently in leaves, and the transcribed protein is transported via phloem to the apex (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Matthieu *et al.*, 2007), thus triggering phase transition. Additionally, Arabidopsis genes *AGAMOUS-LIKE 201* (*AGL20*), *APETALA1* (*AP1*), *AP2*, *AP3*, *LEAFY* (*LFY*) and *PISTILLATA* (*PI1*) are key regulatory genes in the genetic pathway that establishes floral meristem identity (Bowman *et al.*, 1989). AGL20 serves as a

master switch initiating floral development, by integrating signals from several different pathways involving both environmental and internal cues (Borner *et al.*, 2000). It triggers the expression of *LFY*, which turns on the expression of *AP1* (Simon *et al.*, 1996), which then stimulates expression of *LFY*, as they are both involved in a positive feedback loop pivotal in specifying floral meristems during floral transition. Many of the genes determining floral organ identity are MADS box genes, such as *AGAMOUS* (*AG*), *AP1, AP3, CAULIFLOWER* (*CAL*), *FRUITFULL* (*FUL*) and *PI1* (reviewed in Blásquez *et al.*, 2006). These floral identity genes act as transcription factors and share a conserved nucleotide sequence coding for the MADS DNA binding-domain (Borner *et al.*, 2000). Floral homeotic genes *APETALA2* (*AP2*) and *AG* specify the identities of perianth and reproductive organs, respectively. They act antagonistically to restrict each other domains within the floral meristem. MicroRNA *miR172* serves as an additional negative regulator of *AP2* in floral stem cells and in the delineation of the expression domain of another class of floral homeotic genes (Zhao *et al.*, 2007). It has been recently demonstrated that *AG* and *miR172* have distinct functions in flower development and act independently in the negative regulation of *AP2* (Zhao *et al.*, 2007).

Several lines of evidence point to a role for PIN6 in SAM phase transition. In addition to *pin6* mutants flowering earlier, *PIN6* is expressed in floral tissues, namely at the base of the anther. Pollen- and fruit-specific elements are present in *PIN6* promoter sequence and *PIN6* transcript levels are upregulated in pollen-specific mutant *camta1-1* (Mitsuda *et al.*, 2003), suggesting that *PIN6* levels are usually kept downregulated in pollen. Moreover, mutation in another pollination and reproduction-related gene, *SELF-INCOMPATIBILITY-PROTEIN HOMOLOGUE1* (*SPH1*; Ride *et al.*, 1999), leads to induction of *PIN6* mRNA levels, again suggesting its role at the pollen development. Furthermore, several AG-binding sequences are also present in its promoter, suggesting that AG transcription factor binds to *PIN6* in early stages of flower induction. In a line overexpressing *LFY* (Page *et al.*, 1999), *PIN6* expression levels are reduced. *LFY* is another gene involved in this phase transition and it seems to act as a negative regulator of *PIN6* activity. Considering the tight regulatory mechanisms controlling shoot apex organization, it is likely that different flower identity genes as *AG* and *LFY* have opposite effects in auxin transporters regulation, to ensure a correct auxin distribution.

Flowering time is determined by four different pathways. The photoperiodic (light-dependent) pathway involves phytochromes and cryptochromes. The interaction of these photoreceptors with the circadian clock initiates a pathway that activates the floral activator CONSTANS (CO), which encodes a zinc-finger transcription factor that promotes flowering. *CO* acts through other genes to increase the expression of the floral meristem identity gene *LFY*. The vernalisation and autonomous pathway acts through the floral repressor *FLOWERING LOCUS C* (*FLC*), an inhibitor of *LFY*. The carbohydrate pathway reflects the metabolic state of the plant. Sucrose stimulates flowering by increasing *LFY* expression. GA pathway promotes flowering via hormonal inputs. All these pathways regulate a common set of integrator gene targets, such as *SUPRESSOR OF OVEREXPRESSION OF CONSTANTS1* (*SCO1*), *FT* and *LFY* (reviewed in Roux *et al.*, 2006).

Disruption of any functional domain of floral repressive genes results in early flowering. Such genes include *FLOWERING LOCUS C* (*FLC*), its activators and homologues and genes from the GA

pathway. Increasing *FLC* expression results in delayed flowering (Martin-Trillo *et al.*, 2006; Michaels and Amasino, 1999). In absence of *FLC*, *PIN6* mRNA levels are downregulated, which would lead to earlier flowering (see section 3.1.4).

Other molecular components regulating flowering time might be related to PIN6. Mutations in *EMBRYONIC FLOWER1* (*EMF1*) and *EMF2* floral repressors confer an early-flowering phenotype (Boss *et al.*, 2004). In contrast, early flowering can also arise from mutations in floral promotive genes such as cry1, cry2 and phyA, which enhance their own protein stability, thus activating *CO* (reviewed in Roux *et al.*, 2006). Mutations in cry2 caused a delay in flowering and an inability to perceive inductive photoperiods (El-Din El-Assal *et al.*, 2001; Guo *et al.*, 1998).

Vernalization is another regulator of FLC. *Vernalization independence* (*vip*) mutants show cold-independent flowering and earlier flowering through suppression of *FLC* (Zhang *et al.*, 2003). Consistently, *vip5* (Oh *et al.*, 2004) mutant shows upregulation of *PIN6* expression (see section 3.1.4). Considering that PIN6 is downstream of FLC, which is in turn downstream of VIP, one would need additional assays to evaluate whether PIN6 and VIP proteins are directly related, or how PIN6 is involved in a possible regulatory VIP-FLC feedback mechanism.

4.4.4 RESPONSE TO LIGHT AND PHOTOPERIOD

Roots of *pin6* knockdown lines show differential growth under different light regimes. The most severe lines *pin6-3* and 3.11 develop much longer roots in long-day when compared to continuous light conditions. While light may increase PIN6 activity repressing growth, the dark period might repress PIN6 activity and allow for root growth. These results suggested that somehow dark stimulus is required for *PIN6* regulation, and that circadian rhythm must be a part of it. In fact, several circadian expression regulating sequences were found in *PIN6* promoter. Circadian rhythms are a consequence of earth's rotation that generates daily changes in the light and temperature environment (reviewed in Ding *et al.*, 2007). Many organisms have evolved endogenous oscillators, which are influenced by environmental cues, as are changes in light and temperature conditions.

In higher plants, circadian rhythms control many biological processes, including floral transition, leaf movement, stomata opening, seed germination, and hypocotyls elongation. Interestingly, *ca.* 10% of *Arabidopsis thaliana* transcripts are clock regulated at steady-state level (Harmer *et al.*, 2000; reviewed in Ding *et al.*, 2007). A self-sustaining endogenous oscillator is coupled to many physiological processes, such as leaf movement or photosynthesis, and cyclic phenomena such as circadian rhythms. Photoreceptors seem to influence the circadian clock by connecting light perception to downstream molecular and physiological responses.

Recently, phyB mutant analysis has shown it to have higher levels of *PIN3* and *PIN7* mRNA levels. Moreover, phyB mutants have altered auxin levels, as observed by alterations in *DR5::GUS* expression (Salisbury *et al.*, 2007). The authors propose that phytochrome controls *IAA1/IAA3* transcription, at least in part, via regulation of *PIN3/PIN7* levels. Interestingly, *PIN1* mRNA levels are not changed in phyB, indicating that PIN1 is not controlled by phyB at the transcriptional level (Salisbury *et al.*, 2007). Furthermore, it has been suggested that phytochromes may control auxin transport by altering the levels and/or the cellular localization of PIN proteins (Salisbury *et al.*, 2007).

There are three families of photoreceptors in Arabidopsis: phototropins, cryptochromes and phytochromes. Except of phytochrome C, all phytochromes are implicated in clock entrainment. Each phytochrome acts as a specific photoreceptor for red, far-red, or blue light. cry1 and cry2 participate in blue-light entrainment of the clock (Devlin and Kay, 2000), whereas phot1 and phot2 play a minor role for blue-light regulated transcription (Ohgishi *et al.*, 2004). phyA is involved in far-red light perception (Tepperman *et al.*, 2001), and both phyA and phyB are involved in red light mediated processes (Tepperman *et al.*, 2004). cry proteins also seem to be required for normal entrainment by red light. As they do not absorb red light, this requirement suggests that blue-light receptors cry1 and cry2 may act as intermediates in phytochrome signalling during entrainment of the clock (reviewed in Yanovsky and Kay, 2001). Additionally, light input can be carried out by the ZEITLUPE (ZLP) protein family, which includes phyC-phyE (Imaizumi *et al.*, 2003). ZLP has an F-box domain, suggesting its involvement in the proteasome-mediated degradation of light input regulators (Nelson *et al.*, 2000; Somers *et al.*, 2000; Schultz *et al.*, 2001). In fact, ZLT promotes dark-dependent degradation of TIMING OF CHLOROPHYLL A/B BINDING PROTEIN/PSEUDO RESPONSE REGULATOR1 (TOC1/PRR1) through the proteasome (Más *et al.*, 2003; Han *et al.*, 2004). No photoreceptor specialized in UV-B irradiation has been found so far. Recently, CONSTITUTIVELY MORPHOGENIC1 (COP1) has been shown to mediate UV-B response, by negatively regulating photomorphogenesis (Oravecz *et al.*, 2006).

In the current model of the Arabidopsis oscillator, TOC1/PRR1 is part of the central negative feedback loop with CIRCADIAN CLOCK ASSOCIATED (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Alabadí *et al.*, 2001). TOC1 positively regulates expression of *CCA1* and *LHY*, which in turn negatively regulate *TOC1* expression. GIGANTEA (GI) positively regulates *CO* and *FT* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Suarez-Lopez *et al.*, 2001) and its expression is only indirectly affected by TOC1, suggesting that GI acts both in series with and in parallel to TOC1 within the central circadian oscillator. Thus, GI assumes multiple roles in clock input, the central oscillator and clock output (Martin-Tryon *et al.*, 2007).

The photoperiodic (light-dependent) pathway involving phytochromes and cryptochromes is also involved in flowering time regulation. The interaction of these photoreceptors with a circadian clock initiates a pathway that activates the floral activator CONSTANS (CO), acting through other genes to increase expression of *LFY*.

Photoperiod also affects early flowering. Cry1, cry2 and phyA enhance their own protein stability activating CO (reviewed in Roux *et al.*, 2006). cry2 mutations lead to delayed flowering and an inability to

perceive inductive photoperiods (El-Din El-Assal *et al.*, 2001; Guo *et al.*, 1998). Additionally, mutations in phyB alter seed germination (Tonsor *et al.*, 2005), providing a link between photoperiod perception and germination. It is possible that PIN6 is one of the players involved in both mechanisms.

PIN6 promoter sequence also has light responsive elements, such as GT-1 box, I-box and SBF-1 binding sequence, as well as many MYB transcriptional activators binding sequences. Misexpression of *MYB61*, which encodes an R2R3-MYB transcription factor, was both necessary and sufficient to explain aspects of the phenotype of the *Arabidopsis thaliana* mutant *de-etiolated3* (*det3*; Newman *et al.*, 2004). Additionally, *myb61* mutants have upregulation of PIN6 levels (see section 3.1.4), suggesting they could bind to *PIN6* promoter, repressing its transcription. *det3* does not undergo etiolation processes, resembling light-grown plants when grown in complete darkness (Pepper and Chory, 1997). Mutation in another member of the de-etiolated gene family, *det-1*, leads to upregulation of *PIN6* transcript levels (see section 3.1.4), thus providing the link for the role of MYB transcription factors in *PIN6* regulation.

Photoperiod is likely to regulate *PIN6*, as *pin6* root growth phenotype is even more pronounced under long-day conditions when compared to continuous light conditions. As phytochrome repression sequences are present in the *PIN6* promoter, light perception in *pin6* mutants was analyzed. A simple way to evaluate light perception is to measure de-etiolation upon light stimulus, in terms of reduction of hypocotyl length. As guidelines, mutants affected in cry- and phot-mediated perception de-etiolate under blue light, mutants affected in phyB-mediated perception de-etiolate under red light and mutants with disruption in phyA-mediated perception de-etiolate under far-red light (reviewed in Fankhauser and Casai, 2004).

PIN6 expression domains were not affected by the different light sources investigated (white, blue, red, far-red, dark and UV-B; see section 3.5.3). However, blue, red and far-red light sources slightly induced *PIN6* expression in SAM, without affecting its expression domain. Mutant and WT de-etiolation under white, blue and red light was similar. *pin6* mutants seem to be more resistant to far-red light-induced etiolation than the WT, consistent with the fact that *PIN6* transcript levels are induced by far-red light treatment (see section 3.1.4). All mutants showed slightly stronger de-etiolation upon UV-B light treatment, more evident in *pin6-2* allele, suggesting that *PIN6* might also be indirectly affected by this type of light. In fact, UV-B light upregulates *PIN6* expression. Taken together, the results suggest that it is likely that PIN6 is regulated, even though only indirectly, by both far-red and UV-B light. PhyA, involved in far-red light perception, is also required at later stages of plant development for the detection of day length extension, therefore being involved in triggering flowering transition (Yanovsky and Kay, 2002). One can propose that phyA perceives light changes and induces release of the repressor-bound *PIN6*, which is then available for early flowering induction. In addition to its role in circadian oscillator, at the level of light perception GI regulates phyA-mediated photomorphogenesis (Oliverio *et al.*, 2007). Moreover, *gi* mutants show reduced seed germination and *gi11* has upregulated *PIN6* levels (see section 3.1.4), which suggests another level of crosstalk between processes in which PIN6 is involved, namely germination and circadian rhythm perception.

4.4.5 LATERAL ROOT DEVELOPMENT

PIN6 localization at the lateral root suggests its involvement in lateral root development. In fact, *PIN6* is expressed since early stages of lateral root formation, the first expression being detected in pericycle founder cells. These cells divide to give birth to a lateral root primordium and as it emerges, *PIN6* is expressed at its margins, in a ring-like shaped structure at the base of the emerging primordium, where it maintains contact with the main root. *PIN6* is strongly expressed in adventitious roots emerging at the shoot-root junction, in the most external cell layers of this structure. *PIN6* transcripts were additionally detected in the main root in the epidermis and cortex layers. *PIN6*'s expression in pericycle initial cells presumes a basipetal auxin flow away from the vascular tissue of the main root and into the site of lateral root emergence at later stages of its development. The effect of polar auxin transport in lateral root development has been characterised (Casimiro *et al.*, 2001; Laskowski *et al.*, 1995; Reed *et al.*, 1998) and basipetal and acropetal auxin transports are required for distinct phases of lateral root development. Root tip produced, basipetally transported IAA is required for the initiation of a lateral root (Casimiro *et al.*, 2001), while acropetally transported IAA from the shoot is required for its subsequent emergence and growth (Bhalerao *et al.*, 2002; Casimiro *et al.*, 2001; Reed *et al.*, 1998). Even though its presence is more determinant in the earlier stages of lateral root formation, *PIN6* expression domain remains during lateral root emergence at the margins, in the contact area between the lateral root and the main root originating it. In lateral root formation, auxin follows a "fountain" flow where auxin provided from the primary root vasculature is directed to the tip of the primordia. There is a further distribution of auxin laterally from the tip through the outer layer of the primordia. One can therefore propose that PIN6 is involved in directing both basipetal and acropetal auxin transport, required for both stages of lateral root development – initiation and emergence, respectively (Casimiro *et al.*, 2001). Recently, it has been shown that lateral root initiation is controlled in a spatiotemporal manner (De Smet *et al.*, 2007). Pericycle cells are primed for lateral root initiation in the basal meristem and a radial gradient with auxin maxima in protoxylem cells might be required for lateral root initiation (De Smet *et al.*, 2007).

In *pin1*, presumably defective in root acropetal transport, lateral root primordia initiate but fewer mature lateral roots are produced than the wild type (Benková *et al.*, 2003). Moreover, mutation of *AUX1* reduces production of lateral roots as a result of the disruption of AUX1-mediated transport between IAA sources (young leaves) and sink (root apex) tissues (Marchant *et al.*, 2002). In addition, the MDR/PGP auxin transport proteins are involved in the regulation of auxin transport and lateral root emergence. MDR4 regulates basipetal auxin transport required to trigger early stages of lateral root emergence, whereas MDR1 regulates acropetal auxin transport in the main root, thereby controlling lateral root growth rates in the postemergence elongation phase (Wu *et al.*, 2007). Consistently, mutants that overproduce auxin, such as *superroot1* (*sur1*; Boerjan *et al.*, 1995), *alf1 (*Celenza *et al.*, 1995), *rooty1* (*rty1*; King *et al.*, 1995), *sur2* (Delarue

et al., 1998), and *yucca* (Zhao *et al.*, 2001), produce more lateral roots, while reduced-sensitivity mutants, such as *axr4*, produce fewer lateral roots (Hobbie and Estelle, 1995). *pin6* mutants produce, generally, more lateral roots than the WT (see section 3.5.4). These data are also evidence for PIN6 acting as a repressor of growth and development, as in its absence more lateral roots initiate. PIN6, alone or together with MDR4, should restrain the supply of auxin from the vasculature into the pericycle initial cells. In its absence, more auxin accumulates in these cells, giving rise to more mature lateral root primordia.

Auxin-dependent TIR1-driven Aux/IAA degradation signalling pathway is important for lateral root initiation. Aux/IAA mutant *solitary root/IAA14* (*slr/IAA14*) develops a primary root but no lateral roots (Vanneste *et al.*, 2005). SLR/IAA14 controls lateral root initiation by acting downstream of auxin signalling in the basal meristem and is not required for the priming of the founder cells (De Smet *et al.*, 2007). It is likely that other Aux/IAA proteins are involved in this process (De Smet *et al.*, 2007). It has been recently demonstrated that *PICKLE* (*PKL*), a homologue to a mammalian chromatin-remodelling factor, specifically restores lateral root formation in the *slr/IAA14* mutant, thus providing a link between chromatin remodelling and auxin-mediated lateral root initiation in *Arabidopsis* (Fukaki *et al.*, 2006).

Lateral root formation is also regulated by NPH4/ARF7 and ARF19 transcriptional activators of early auxin response genes. They activate transcription of the downstream targets *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16/ASL18*) and *LBD29/ASL16*, in roots (Okushima *et al.*, 2007), and *PUCHI*, involved in lateral root emergence (Hirota *et al.*, 2007). *puchi* mutants lateral roots are swollen in the proximal region, resulting in shorter lateral roots (Hirota *et al.*, 2007).

Furthermore, *arf7* and *arf19* mutants have higher levels of *PIN6* mRNA (see section 3.1.4), suggesting they could be overall negative regulators of *PIN6* expression. On higher concentrations of auxin, Aux/IAA repressors are destabilized and targetted for destruction by the proteasome, leaving the previously bound ARF available to regulate target gene expression (Tiwari *et al.*, 2001; Tiwari *et al.*, 2003; Zenger *et al.*, 2001). Different combinations of Aux/IAA and ARF proteins in different cell types might regulate distinct PIN proteins. It is then plausible that *PIN6* expression during lateral root development is controlled by ARF7 and ARF19, working as repressors of PIN6 transcription.

Because lateral roots originate from a zone distal to the Arabidopsis primary root elongation zone, cell proliferation during lateral root development and cell proliferation of the apical meristem do not overlap (Dubrovsky *et al.*, 2000). Pericycle cells must therefore de-differentiate and then re-enter the cell cycle prior to lateral root formation. Expression of *CYCD4;1*, a D-type-cyclin gene, during lateral root initiation (De Veylder *et al.*, 1999), suggests a prominent role of the G1–S-phase transition as the trigger for lateral root initiation (reviewed in Casimiro *et al.*, 2003). On the other hand, CYCLIN B1 (CYCB1;1), involved in the G2-M checkpoint in cell cycle, is not required for lateral root initiation (Beeckman *et al.*, 2001; Doerner *et al.*, 1996). Downstream of the TIR1–Aux/IAA pathway are NAC transcription factors (Xie *et al.*, 2002), which are required for re–activating the cell cycle in the pericycle cells of the xylem pole. An early auxin response includes reduction of the KIP-related CDK-inhibitor protein (KRP2/ICK2) levels, suggesting KRP2 levels must be decreased to overcome a pre-existing block at the G1 checkpoint before cell cycle progression can occur (Himanen *et al.*, 2004). This process might involve auxin-regulated protein

SINAT5 in attenuating the mitotic activity of auxin to just a few pericycle cells (Xie et al., 2002). Upon application of auxin both, SINAT5 (an E3-ubiquitin ligase), and NAC1 (a lateral root activator of the NAM/CUC-like protein family) are induced and co-localize to lateral root initials. SINAT5 has been reported to attenuate auxin signal by targetting NAC1 for degradation, thus regulating lateral root formation (Xie *et al.*, 2002).

PIN6 acts as a repressor of growth. Further assays are required to establish whether the mutant's longer roots are a result of increased cell division or cell elongation rates. It is more likely that PIN6 involvement in lateral root development occurs too at the level of cell cycle control. Cell cycle regulatory elements are present in *PIN6* promoter sequence. In addition, overexpression of *E2Fa-DPa*, a transcription factor involved in the entry in S phase and mitosis and also a target for auxin signals (Magyar *et al.*, 2005), results in downregulation of *PIN6* levels. E2Fa-DPa might therefore bind to *PIN6* in order to repress its expression. PIN6 activity, in a limited number of pericycle cells, might attenuate the auxin-induced mitotic activity, restricting the number of cells primed for cell division and, consequently, the number of lateral roots initiated. Use of cell cycle marker genes and inhibitors will shed light into this particular aspect of PIN6activity in growth processes.

4.5 PIN6 AND GRAVITROPIC RESPONSE

Root gravitropism reflects the (re)orientation of root growth in relation to the gravity vector and is a consequence of differential cell elongation in the root elongation zone. Auxin mediates this differential growth response at the root tip. Elevated auxin levels are present in columella cells (Ottenschläger *et al.*, 2003). Upon gravistimulation an asymmetric efflux of auxin from the columella into the lateral root cap cells is created (Ottenschläger *et al.*, 2003). At later stages auxin moves through an influx- and efflux-dependent transport towards the root elongation zone, where it inhibits cell elongation thus resulting in the overall root bending (Ottenschläger *et al.*, 2003). Basipetal auxin transport is therefore essential for gravitropic response, whereas acropetal transport contributes very little to it (Lewis *et al.*, 2007).

Auxin influx (*aux1*) and efflux (*eir1/pin2)* mutants are agravitropic (Bennett *et al.*, 1996; Luschnig *et al.*, 1998; Rashotte *et al.*, 2000). As *PIN6* and *PIN2* are co-expressed in root tissues and co-regulated by several factors, it is possible that putative interaction might occur at the gravity response level. However, they respond in opposite ways: *eir1/pin2* mutants are agravitropic, whereas *pin6* mutants are hypergravitropic (see section 3.5.5). Proteasome-dependent degradation of *PIN2* is responsible for its protein localization at the correct side of the cell (Abas *et al.*, 2005). Similarly to PIN2, *PIN6* degradation via the proteasome in an ARF-Aux/IAA-dependent manner might be determinant for the PIN6 correct localization during gravity response. At the root tip, *PIN6* mRNA is expressed in epidermis and cortex cells, co-localizing with PIN2. By diverting auxin, it is possible that PIN6 modulates auxin flow in an antagonist

way to PIN2. One possibility is that PIN6 and PIN2 are differentially expressed on opposite sides of the root, resulting in the overall root bending. Upon gravity stimulation, PIN2 is preferentially localized to the upper side of bending roots, where auxin levels are lower and cells elongate (Abas *et al.*, 2005), whereas PIN6 would localize to the lower side of the root, contributing to the increase in auxin levels required for repression of cell elongation.

Alternatively, *PIN6* could be ectopically induced upon gravistimulation. In main root tips, auxin induces *PIN6* expression in additional cell files in the central stele (see section 3.2.1), also suggesting that PIN6 organized protein polar localization might only be assumed upon stimuli as auxin or gravity. A similar process has been described for PIN3, which is originally distributed evenly in the plasma membrane and only localizes to columella cells lateral walls after gravistimulation (Friml *et al.*, 2002b).

4.6 OTHER LEVELS OF PIN6 REGULATION

PIN6 might be regulated by additional factors and conditions. *PIN6* expression is affected in mutants related to RNA processing and gene silencing. There are 6 potential miRNA target transcripts in the *PIN6* cDNA sequence (located at 201, 206, 729, 784, 887 and 1749 bp; Adai *et al.*, 2005) and several promoter binding elements, thus suggesting that *PIN6* transcript levels might be regulated via miRNA degradation. Moreover, *PIN6* is overexpressed in *dicer-like1* (*dcl1*) and downregulated in *RNA-dependent RNA polymerase 2* (*rdr2*) and *dcl3-1* mutants (Gasciolli *et al.*, 2005; Lu *et al.*, 2006). Differences in *PIN6* expression observed between the *dcl1* and *dcl3* mutants might therefore be explained by generation of miRNAs or siRNAs with different sizes and probably from different target parts of PIN6 gene sequence, which subsequently activate specific Dicer-dependent downstream events.

4.7 PIN6 AND REDUNDANCY AMONG THE PIN FAMILY

PIN proteins have diverged long enough for the similarity in their expression patterns to be due to high conservation between their respective promoter regions (Paponov *et al.*, 2005). It is nevertheless unclear how the respective functions of these proteins have become interdependent over time. It has been proposed that an evolutionary convergence resulting in the co-expression of PIN1 and PIN6 under differing growth conditions and developmental stages results in complementary functions at least at an organ level (Paponov *et al.*, 2005). Indeed, *PIN1* and *PIN6* co-localize in vascular tissue and have close expression domains at the SAM. They also respond similarly to IAA, NPA, GA_3 and zeatin. *PIN2* co-expresses with

PIN6 specifically in root tissues and both proteins might be involved in distinct processes of root bending upon gravity stimulation. The two genes are regulated in the same way by NPA, ACC, GA_3 and zeatin. It would be interesting to screen for interaction partners for PIN6 and evaluate the extent to which there is an overlap between PIN6's interactors and those of PIN1 or PIN2. The close relationship between PIN1 and PIN6 in shoot tissues, and PIN2 and PIN6 in roots raises the intriguing question of whether PIN6 is responsible for the communication between the major auxin transporters PIN1, in aerial tissues, and PIN2, in roots, through balancing auxin levels, thus providing developmental cues to more distal tissues.

An interesting fact is that *PIN6* is the only *PIN* upregulated by AVG treatment. Blocking ethylene synthesis, thus lowering ethylene levels, leads to an increase in *PIN6* transcripts, suggesting a role for PIN6 in ethylene response. PIN6 might be responsible for the crosstalk between ethylene and auxin. As a consequence of a decrease in ethylene levels, PIN6 increased levels might increase auxin transport, diverting auxin and thus affecting its concentration in order to keep it low so that it does not affect the overall auxin to ethylene ratio in the tissues in question.

Promoter analysis raises other questions regarding the less characterized PIN family members. *PIN6* is expressed in anther vasculature and its promoter contains several pollen-responsive elements. PIN8 is reported to be highly expressed in pollen, so that both genes could be co-expressed in this tissue. Nevertheless, it is likely that PIN6 expression domain finishes just below PIN8's, and that PIN6 is coordinating the auxin flow required in anthers for correct pollen development, and that PIN8 is involved in the fine-tuning of those concentrations at the pollen grain level.

4.8 CONCLUDING REMARKS

The results discussed in this thesis suggest that PIN6 is a negative regulator of plant growth processes which is involved in germination, main root elongation, lateral root initiation, leaf emergence and specification of flowering time. Its involvement in new organ development is quite clear in lateral root emergence, and in leaves and floral organ emergence at the SAM. PIN6 is further involved in gravity and is regulated by auxin and ethylene. Mutant analysis combined with analysis of *in silico* data mining allowed to describe aspects of PIN6 regulation and its mode of action in the different developmental stages studied.

4.9 FUTURE PERSPECTIVES

Data presented in this work imply a role for PIN6 in particular aspects of meristem development. Further studies will provide answers to the several remaining open questions, particularly regarding PIN6 contribution to plant development and its placement in the development programs in which it is involved.

Mutant complementation is essential for proving that the observed phenotypes are uniquely due to the knocked-down gene. Even though it was ascertained that there are no extra T-DNA insertions in the mutant lines used in this work, one cannot exclude the possibility of existing deletions in the genome sequences that would result in the phenotypes observed. Considering the consistent phenotypic responses among different independently generated mutant lines, and its consistency with the additional RNAi lines generated, it is very likely that the phenotypes observed reflect *PIN6* gene disruption and not effects on any other genes. Nevertheless, *pin6* mutants were complemented with *PIN6* endogenous sequence (*pPIN6-PIN6*) to ensure that the phenotype is a consequence of *PIN6* gene disruption only. F2 progeny will be soon available for analysis.

Analyzing *PIN6* residual expression patterns in the mutant and transgenic lines available will be necessary to show how this gene is still required for plant development, possibly explaining why no complete null alleles have been found to date. For that purpose, crossings between *pPIN6::GUS* and the T-DNA and silencing line backgrounds have been prepared. Additionally, fluorescent (*pPIN6-PIN6-GFP*) and/or tagged versions of PIN6 (*pPIN6-PIN6-HA*) lines will provide insights into PIN6 expression and dynamics.

Another question that needs resolving is how auxin pattern and distribution are affected by PIN6, which could be solved by crossing the *DR5*::reporter constructs into *pin6* background. Crossings between *pin6* mutants and *pDR5::GUS* or *pDR5::GFP* have been prepared and the plants will be available for analysis soon.

Evidence points to regulation of *PIN6* expression via TIR1-Aux/IAA-ARF-mediated proteasome-dependent auxin induction. Since *nph4/arf7* and *arf19*, which have upregulated levels of *PIN6*, a more thorough analysis of these mutants together with *mp/arf5*, *bdl/iaa12*, *nph4/arf7* and *arf19* mutants should provide insights as to *PIN6* gene expression.

PIN protein cycling is one important feature required for its correct polar localization in plasma membranes. Genetic studies with *gnom* mutants, or chemical inhibition by the fungal toxin Brefeldin A (BFA) proposed that PIN1 and PIN3 proteins constitutively cycle between endosomes and the plasma membrane (Geldner *et al.*, 2001). Further analysis of PIN6 protein dynamics in the *gnom* mutant background are required to explain whether PIN6 polar membrane polar localization, putatively regulated by auxin, requires involvement of GNOM-mediated exocytic vesicle formation. Moreover, *PIN6* sequence contains a peptide and a vacuolar signal, further substantiating a possible compartmentalization of PIN6 within the cell.

To enlighten its role, particularly at SAM level, *PIN6* expression should be driven under the control of promoters for other genes required for SAM maintenance and which are strongly expressed in that region, such as *PIN1*, *CLV3*, *LFY* and *WUS*. Constructs for *pPIN1-PIN6*, *pCLV3-PIN6*, *pLFY-PIN6* and *pWUS-PIN6* are being prepared. Furthermore, it would be interesting to investigate closer PIN6 regulation of flowering timing, through analysis of the *flc* mutant.

PIN6 rescues PIN1 expression domains in the *pin1* background. Therefore, attempting to rescue *pin1* mutants with an effective PIN6 overexpressor sequence (*35S-PIN6*) would be theoretically possible, at least at SAM level. As for root development and gravitropic response, the relationship and putative crosstalk between PIN6 and PIN2 should be explained resorting to promoter-swap experiments, by generation of *pPIN2-PIN6* and *pPIN6-PIN2* lines. Nevertheless, using split-sensor systems to confirm *in vitro* protein-protein interactions between PIN6 and PIN1, and PIN6 and PIN2, shall complement these experiments. Additionally, screening for interaction partners for PIN6 shall provide a map of the regulatory mechanisms underlying PIN6 action, and help uncover the mapping and functional overlap of PIN interaction pathways.

Another particular detail to be further investigated is PIN6 relation to cell division. It is now known that PIN6 is involved in meristematic processes that require active dividing cells, but how this activity is regulated, and whether PIN6-transported auxin induces cell division or cell division induces *PIN6* expression, is not yet clear. This should be resolved by crossing *pin6* mutants with *CYCB::GUS* or *35S:Histone-GFP* markers for cell division, by analysing mutants impaired in cell division, or using a *DR5::VenusGFP* marker, a line with GFP expression confined to cell nuclei, thus making it possible to follow cell division and cell lineage formation.

The approaches mentioned will provide helpful insights into further characterization of PIN6. Soon, it will be possible to establish how PIN6 provides the direct link between auxin transport, by maintaining certain levels of auxin concentration and distribution in meristems, and the coordination of developmental processes such as phase transition, new lateral organ emergence and response to gravity.

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