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Molecular expression of *PsPIN1*, a putative auxin efflux carrier gene from pea (*Pisum sativum* L.)

Rekha Chawla and Darleen A. DeMason*
*Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA; *Author for correspondence (e-mail: demason@ucrac1.ucr.edu; phone: 951-827-3580; fax: 951-827-4437)*

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Abstract

A cDNA coding for a putative auxin efflux carrier was amplified from *Pisum sativum* seedling shoot tips by RT-PCR and the corresponding full-length cDNA, *PsPIN1*, was subsequently obtained by RACE-PCR. The deduced amino acid sequence (599 residues) showed the three domain topology typical of the other PIN proteins. The PsPIN1 protein structure prediction possessed five transmembrane domains at both the N-(7-150) and C-(450-575) termini and a hydrophylic region in the middle. PsPIN1 showed highest similarity to *Medicago*, MtPIN4. Using the Genome Walking technique, a 1511 bp upstream region for *PsPIN1* gene was sequenced. This *PsPIN1* upstream region possessed multiple putative auxin, GA and light regulatory elements. The *PsPIN1* mRNA was ubiquitously expressed throughout the pea plant, especially in growing tissues. Auxin induced *PsPIN1* mRNA in dark grown pea seedling shoot tips. It was induced by 4-chloro-IAA, which is also an active auxin in pea, and by gibberellin (GA_3). Interestingly, the *PsPIN1* mRNA was down-regulated by light treatment, possibly because light negatively regulates auxin and, especially GA levels in pea. Thus PIN1-mediated auxin efflux is a highly regulated process, not only at the level of protein localization, but also at the level of mRNA accumulation.

Abbreviations: ARF – auxin response factor; 4-Cl-IAA – 4-chloro-indole-3-acetic acid; GA_3 – gibberellic acid; IAA – indole-3-acetic acid; m-IAA – methyl 3-indolyl acetate; NPA – *N*-(1-napthyl) phthalamic acid; PAT – polar auxin transport; RT-PCR – reverse transcription polymerase chain reaction; RACE – rapid amplification of cDNA ends; *WT* – wild type

Introduction

Auxins are phytohormones involved in a wide range of normal developmental processes, such as cell division, expansion and differentiation, as well as many plant responses to environmental signals such as phototropism, gravitropism and thigmotropism. The principal form of auxin in higher plants is IAA, which is synthesized in the shoot apex and/or young leaf primordia and is then transported basipetally, from cell to cell towards the roots in a process referred to as polar auxin transport (PAT) (Lomax et al. 1995). The main PAT stream has a velocity of 5–20 mm/h (Lomax et al. 1995). In roots, auxin moves in opposite directions in different cell types, acropetally through the stele and basipetally through the epidermal cells (Jones 1998). Controlled polar transport is an aspect unique to auxin.

PAT is mediated by asymmetric distribution of auxin influx and efflux carriers. Contemporary genetic and molecular techniques have led to the

identification of genes that encode these carriers. These are the *LAX* (*Like-AUX1*, encoding amino acid permease-like proteins) and the *PIN* gene families in *Arabidopsis*, which consist of 4 and 8 gene family members respectively (Bennett et al. 1996; Parry et al. 2001; Friml and Palme 2002). Their orthologs in other plant species are also being isolated and characterized. The *pin1* mutant of *Arabidopsis* has reduced lateral organ numbers and organ fusions throughout development (Okada et al. 1991). The most dramatic effect of the mutation is observed during inflorescence development. In mutants with strong alleles, a naked inflorescence is formed, which occasionally produces flowers with fused organs and has reduced PAT. The *AtPIN1* gene was cloned by transposon tagging and found to encode a 622 amino acid long (67 kDa) transmembrane protein with homologies to transporter proteins in a wide range of organisms. Gälweiler et al. (1998) proposed that the protein might act as a catalytic auxin efflux carrier. Analysis of the *PIN* gene family in *Arabidopsis* has revealed distinct localization patterns for individual proteins. Although AtPIN1 is present in all organs, AtPIN2 is specifically localized to the epidermal, cortical and lateral cap cells of roots, AtPIN3 is present in the stem endodermal layer and in collumella cells of the root cap and is important specifically in lateral auxin transport during tropic movements, and AtPIN4 is localized to the boundaries of the root quiescent center and all surrounding cells and is involved in the sink-driven auxin gradient and root patterning (Chen et al. 1998; Gälweiler et al. 1998; Luschnig et al. 1998; Friml et al. 2002a, b). However, analysis of multiple *pin*-mutants in *Arabidopsis* has shown the functional redundancy between PIN proteins (Benková et al. 2003; Friml et al. 2003).

Among the important functions of auxin in plant development is that of promoting stem elongation in both monocotyledons and dicotyledons. Many of the classical experiments have been done with legumes, especially pea. Although at least four naturally occurring auxins are known in pea, the two most abundant and effective are indole-3-acetic acid (IAA) and 4-chloro-indole-3-acetic acid (4-Cl-IAA)(Law and Davies 1990; Magnus et al. 1997; Ozga and Reinecke 1999). The latter has greater activity in promoting both stem and pod elongation (Reinecke et al. 1995; Karcz and Burdach 2002). IAA is known to interact with the active gibberellic acid, GA_1 to regulate internode elongation in pea. In fact, it has been shown to regulate GA_1 homeostasis as well as GA signaling. It regulates homeostasis by increasing transcription of GA 3-oxidase (GA3ox1), the enzyme that converts inactive GA_{20} to active GA_1 and by reducing transcription of GA 2-oxidase (GA2ox1), which converts GA_1 to inactive GA_{29} (Ross et al. 2000). Stems of GA-deficient dwarf mutant pea plants grown in the light fail to elongate in response to GA treatment unless auxin is present (Brian and Hemming 1958; O'Neill and Ross 2002). Recently it was shown that in *Arabidopsis* the mechanism of this signaling interaction is via auxin destabilization of the DELLA transcription co-factors (Fu and Harberd 2003).

Auxins interact with other phytohormone and light signaling pathways to coordinately regulate many developmental processes including stem elongation (Swarup et al. 2002). In fact a wealth of genetic, physiological and biochemical evidence demonstrates that auxins interact with many light responses. Phototropism is the best example of this. In addition, IAA levels affect phytochrome-mediated growth suppression during de-etiolation (Chory et al. 1996; Kraepiel and Migniac 1997). Dark-grown pea plants exposed to continuous red light have reduced IAA levels in the epidermis. And conversely, the phyB mutant of pea (*lv*) has longer internodes and possesses higher IAA levels in the epidermis (Behringer and Davies 1992; Behringer et al. 1992).

Both physiological and genetic studies have provided evidence that auxin plays a role in leaf primordium initiation, phyllotaxy, lamina expansion and in vascular patterning (Avsian-Kretchmer et al. 2002; Steiger et al. 2002; Aloni et al. 2003; Benková et al. 2003; Mattsson et al. 2003; Reinhardt et al. 2003; DeMason and Chawla 2004a, b; Keller et al. 2004). Many aspects of the *pin1* phenotype are mimicked by inhibitors of auxin transport, such as NPA (Okada et al. 1991). Organ initiation and/or outgrowth are defective because they require the local accumulation of auxin and this process is impaired in the mutant (Reinhardt et al. 2000; Benková et al. 2003). The localization of PIN1 in the meristem flank in *Arabidopsis* suggests it plays a role in this accumulation of auxin at the site of incipient young

leaf primordia and in phyllotaxy determination (Vernoux et al. 2000; Benková et al. 2003; Reinhardt et al. 2003). Differences in timing of auxin gradient dissipation in the axis during leaf development caused by auxin transport inhibitors has a profound effect on general form of pea leaves (DeMason and Chawla 2004a, b). These experiments clearly indicate a significant role for auxins in pea leaf development. Our broader goal is to understand the roles of auxin during leaf morphogenesis in pea. Towards this aim, we need to characterize the PIN genes and their expression profiles. Further comparative studies of genes for which basic function is already known might eventually help in understanding the genetic and molecular basis of variation in leaf morphology, such as simple versus compound and palmate versus pinnate leaves (DeMason and Chawla 2004a, b).

In recent years, researchers have addressed the mechanism of localization of PIN proteins to understand controls of PAT, however, the regulation of their mRNA levels has not been studied in detail. In order to understand the regulation of *PsPIN1* mRNA levels, our first aim in the present investigation was to clone and sequence the *PsPIN1* gene including the upstream region from pea and compare it with other known PIN proteins. The main goal was to characterize the expression patterns of *PsPIN1* in different plant parts and to study how these patterns are affected by hormones and light that are known to regulate internode elongation in pea. Together these results should provide a better understanding of the regulation of auxin efflux in pea.

Materials and methods

Plant materials and growth conditions

The pea genotype used in this study was line W6 22593, designated as wild-type (*WT*) or *Af St Tl* and it was obtained from the Marx collection, which resides in the USDA-ARS Pacific West Area germplasm collection in Pullman, Washington. This genotype is also homozygous, recessive for the null allele (*le*) of the gene that encodes GA 3-oxidase. Seeds were sown in UC soil mix (Baker 1957) supplemented with slow release fertilizer in 1 gallon pots and plants were grown under standard greenhouse conditions and natural light regimes. For gene expression experiments, pea seeds were sterilized with 10% bleach containing a drop of Tween 20 for 10 min under a vacuum and then washed three times with sterile water and soaked overnight. Seeds were then germinated in sterilized, moist vermiculite at RT in darkness. For the light adaptation experiment, seeds were soaked overnight in complete darkness and sown in sterile, moist vermiculite under green safe lights.

Cloning of PsPIN1 partial cDNA and its sequencing

A partial *PsPIN1* coding sequence was amplified using cDNAs made from *WT* seedling shoot tip mRNA and a set of degenerate primers DEG1F: 5′ TGG TTC CTC TTT ATG TTG CTA TGA THY TNG C 3′ and DEG1R: 5′ CAA TAC CTT GAG GAA GAG CAG CYT GNA CDA TNG C 3′ designed by using the CODEHOP program (Consensus Degenerate Hybrid Oligonucleotide Primers). The CODEHOP program designs a pool of primers containing all possible 11- or 12-mers for the 3′ degenerate core region and having the most probable nucleotide predicted for each position in the 5′ non-degenerate clamp region (Rose et al. 1998). The PCR was done using a gradient of 10 annealing temperatures (around 50 °C) in a final reaction volume of 50 μl containing 5 μl 10 × PCR buffer, 4 μl of each primer, 1 μl of dNTP mix (10 mM each) and 0.5 μl of Taq polymerase (Qiagen, USA). In order to get sufficient product for cloning, a second round of nested PCR was done using another set of degenerate primers, DEG2F: 5′ TTT TTG CTG TTC CTC TTC TTT CTT TYC AYT TYA T 3′ and DEG2R: 5′ GCT TTC TCC AAA CCA TAA TAA GAA TAA NCK NGT CAT NAC 3′. A PCR product of 1.5 kb thus obtained was purified using Microcon PCR (Millipore, USA) and was cloned in the pGEM-T vector (Promega, Madison, WI). In order to obtain the sequence from the middle region of the clone, a set of *PsPIN1* specific internal primers, 5′ CGA AAT CCT ACG CCA AGA G 3′ and 5′ CAT CAG AAA CAG GTG AAG CA 3′ were used and the product was sequenced from both directions until a sequence overlap between the two sequenced fragments was obtained.

Cloning of complete PsPIN1 cDNA and its phylogenetic analysis

RACE-ready cDNA libraries were made from *WT* seedling shoot tip cDNAs with the 5′/3′ RACE kit (Clontech, USA) as per manufacturer's instructions. The 3′ end of the cDNA was obtained by 3′ RACE (rapid amplification of cDNA ends) using a gene specific left primer (5′ CGT GTC TCC AGG AAA AGT GGA CGG TCA 3′) and an adapter primer provided in the kit as the right primer. The upstream region was similarly amplified using the gene specific primer, PsPIN1 5RACE: 5′ CAA TGG AAC CAG CAG TGT CGG GAA A 3′ designed from the 5′ end sequence of the partial cDNA clone.

The phylogenetic tree was generated using the ClustalW program and visualized with NJplot and percentage similarity and identity was calculated using the GCG program. The ClustalW alignment was obtained using the European Bioinformatics Institute web site and SMART (Simple Modular Architecture Research Tool) was used to predict the domains from the deduced PsPIN1 protein.

Cloning, sequencing and in silico analysis of PsPIN1 promoter

The 5′ upstream region of *PsPIN1* was cloned using the Universal GenomeWalker kit (Clontech Laboratories Inc., CA, USA). The DNA was isolated from pea leaves according to Dellaporta et al. (1983). The quality of DNA was checked for high average molecular weight on an agarose gel. For construction of the GenomeWalker libraries, 11 blunt end cutting restriction enzymes (*Dra*I, *Eco*RV, *Hpa*I, *Nae*I, *Nru*I, *Pvu*II, *Sca*I, *Sma*I, *Sna*BI, *Ssp*I and *Stu*I) were used as described previously (Chawla and DeMason 2003). The DNA was purified after digestion and ligated to the adaptors provided in the kit according to manufacturer's protocol. The primers used for obtaining the 5′ upstream region were PIN1-WALK1: 5′ TGC TCC AAA TGA AGA GAA GGG TTA GGA 3′ and PIN1WALK2: 5′ TGG AAT GAG AGA AGT GGA ACA GCG AAA 3′. PCR reactions for primary and secondary walks were performed according to the manufacturer's instructions. The PCR products finally obtained after each walk were gel extracted using the Ultra DNA kit (Millipore) and sequenced and aligned with the help of the GCG program. The promoter and 5′ upstream region (1511 bp) thus obtained was scanned for regulatory elements using the PLACE and PlantCARE programs (Higo et al. 1999; Lescot et al. 2002).

Gene expression in plant parts

For expression pattern analysis, different plant parts such as whole leaves and leaf parts, flower buds, stems and roots of greenhouse grown *WT* plants were harvested and immediately frozen in liquid nitrogen. Whole leaves and leaf parts were obtained from expanding, adult leaves. For observing expression in developing embryos, pods of various stages were collected in the greenhouse and kept on ice. Three stages of embryo development were obtained: young (embryo linear, occupied less than half the ovule's volume and without cotyledon swelling), medium (embryo occupied approximately half the ovule's volume and with swelling cotyledons), mature (embryo filled the ovule's volume but had not yet started drying). In addition, embryo axis from the mature stage was isolated separately. These four samples were then frozen in liquid nitrogen and kept at − 80 °C until RNA extraction.

Two replicate RNA isolations were used for each sample collected. The RNA isolation was done using the RNeasy Plant Miniprep kit (Qiagen, USA). The RNA was DNase treated on the mini spin column according to the manufacturer's instructions to eliminate any contaminating genomic DNA. The RNA was quantified and its quality determined by running on an agarose gel with ethidium bromide staining. The first strand cDNA synthesis was done using 2 μg RNA, 0.5 μg random hexamers, 4 μl of 10 × RT buffer, 500 μM dNTP, 20 U RNase inhibitor and 8 U Omniscript Reverse Transcriptase (Qiagen) at 37 °C for 1h. The cDNAs were then diluted 10 times and 6 μl of each cDNA were used for final PCR. The final PCR for plant parts was done with *PsPIN1* specific primers, PIN1IS1: 5′ ACC GTT TTG TAG CAC TTT TCG 3′ and PIN1IS2: 5′ ACA CAA GAG TAA TGG GCA ATG 3′ using HotStarTaq (Qiagen) in final reaction volume of 50 μl for 50 cycles at an annealing temperature of

58 °C. A single product of 1.4 kb was obtained. β-Actin (X90378) from pea was used as a control gene to demonstrate equal loading. The primers, ActinF: 5′ TGA AGC ACA ATC CAA AAG AGG 3′ and ActinR: 5′ TGA CTA ACA CCA TCC CCA GA 3′ resulted in a product size of 0.6 kb. The PCR was done as for *PsPIN1* except that the annealing temperature used was 60 °C. Ten microliters of each sample were electrophoresed on a 1% agarose gel and then stained with ethidium bromide and photographed with a gel documentation system (BioRad Gel Doc 2000).

Hormone and light treatments

For auxin induction experiments, pea seedlings were grown in the dark at RT. After 7 days, shoot tips were removed under green safe lights and placed in treatments or in water (control) in petri dishes containing 20 ml of solution containing auxin and maintained in the dark with gentle shaking. The sample size for each treatment was 8–10 shoot-tips. The two auxins used were methyl 3-indolylacetate (m-IAA) (Sigma, I-9770) and 4-chloroindolyl 3-acetate (4-Cl-IAA) (obtained from J. Cohen). A 50 mM stock of 4-Cl-IAA was made in isopropanol and a 25 mM stock of m-IAA was made in ethanol. Subsequent dilutions of stocks were made in water. Equivalent amounts of solvents were added to the control samples. The concentration range tested for both auxins was: 1, 10, 25, 50, and 75 μM. The time course tested with 50 μM m-IAA was 1, 2, and 4 h. The reason for choosing m-IAA rather than IAA was that m-IAA diffuses more efficiently through the membranes into the plant cells. Once inside the cell, its methyl group is cleaved and it becomes IAA (J. Cohen, pers. com.). 4-Cl-IAA was used as it is as one of the active forms of auxin in pea (Magnus et al. 1997; Ozga and Reinecke 1999).

For the GA and auxin experiment, plants of similar age were used. 1.5 cm segments of the uppermost internode just below the apical hook were used for treatments with 50 μM GA_3 (Sigma, G-3250), 50 μM m-IAA or both for 4 h. An aqueous stock solution of 25 mM GA_3 was used. Sample size was again 8–10 internode segments. Although GA_3 does not occur naturally in pea, it is still active (Kaur-Sawhney 1986; Wu et al. 1993; Van Huizen et al. 1996).

Shoot tips of continuous light and continuous dark grown 8-day-old seedlings were used for dark versus light expression. For the light-adaptation experiment, plants were grown in complete darkness for 5 days and then transferred to a growth room under long day length conditions (16 h light). The light intensity was 63.5 μmol s^{-1} m^{-2} and the temperature was 24 ± 2 °C. A corresponding set of plants was kept in the dark as controls for each time point. At 0, 2, 4, and 24 h, shoot tips were collected and frozen immediately in liquid nitrogen and stored at –80 °C until RNA isolation.

For semi-quantitative PCR, RNA was isolated, DNase treated and quantified as described above. The same set of primers and conditions were used for *PsPIN1* expression except that the PCR was carried out in a final volume of 30 μl and run for 20 cycles. *DEAD box* (AY167670) was used as the control gene. The *DEAD box* primers were DBF: 5′ TTC TCG TCA TCA ACC TCA CC 3′ and DBR R: 5′ TTC CTA CCA AAC CTT CCA CTA C 3′. The PCR for *DEAD box* was also done for 20 cycles but at an annealing temperature of 64 °C. The product size was 1191 bp. Twenty cycles of PCR was previously determined to be in the linear range of amplification for both *PsPIN1* and *DEAD box*. Twelve microliters of each sample were then electrophoresed on a 1% agarose gel and blotted onto a nylon membrane. After UV crosslinking, the blot was prehybridized and hybridized with a *PsPIN1*-specific internal probe at 42 °C. The probe was 666 bp in size and was PCR amplified using PIN1F1 primer, 5′ GGT AGG ATC CAT GTT ACT GTT AGA AA 3′ and PIN1R primer, 5′ ATT GAG CTC CCT TTC CAT TCC TCT TAT 3′. For *DEAD box* the complete 1191 bp product was used as the probe. The primers used for the pea *Cab15* gene (J01253) were CABexpF: 5′ GGT TGG GAC ACT GCT GGA CT 3′ and CABexpR: 5′ CAC TGG GTC GGC AAG ATG A 3′. The PCR was done at annealing temperature of 69 °C and a product size of 519 bp was obtained. The probe for each gene was made using the Prime A Gene labeling kit (Promega). The blots were washed after 16 hrs of hybridization and quantification of bound probe was done with a Molecular Dynamics Phosphorimager (Typhoon 9410). At least two repeats of each experiment were carried out to observe gene expression. Two separate RNA isolations were

done for each experiment from which cDNA synthesis and PCR were done independently. SigmaPlot 2000 was used to calculate means and standard errors of these two samples. The *t*-test used in the GA experiment was done with MS Excel xp. The images obtained were finally assembled in Adobe Photoshop 6.0.

Results

Structure and phylogeny of the PsPIN1 protein

A *PsPIN1* cDNA was amplified from pea shoot tip cDNA and degenerate primers designed using a CODEHOP program using consensus of *Arabidopsis* and *Brassica PIN* gene families and the codon bias for pea. A second round of nested PCR was done using another set of similarly designed primers, internal to the first set, to further amplify and clone the product. The product was cloned in pGEM-T to facilitate its sequencing. The 5′ and 3′ terminal sequences of the gene were determined by rapid amplification of cDNA ends (RACE) experiments. The predicted amino acid sequence of *PsPIN1* cDNA (AY222857) showed the three-domain topology typical of the other PIN proteins. The modular structure of PsPIN1 showed five N-(7–26, 41–60, 67–89, 99–121, 128–150) and five C-(450–472, 484–506, 516–538, 543–565, 575–597) terminal transmembrane domains and a central hydrophilic region. Further there was a region of low complexity (335–348) within the central region. PsPIN1 was 96.8% similar to MtPIN4, 88.4% to *Populus* PIN1 and 83.3% to AtPIN1 (Figure 1). The PsPIN1 protein was 599 amino acid long. The length of MtPIN4 (AY115839) was 605 aa, *Populus* (AF190881) was 614, rice REH1 (T02876) was 595 aa and that of AtPIN1 (NP_177500) was 622 aa. This variability in length was due to differences in the amino acids in the central region. Alignment of deduced amino acid sequences of PsPIN1 and other similar PIN1 proteins showed that the N- and C-terminus hydrophobic domains were more conserved than the central hydrophilic region, (Figure 1).

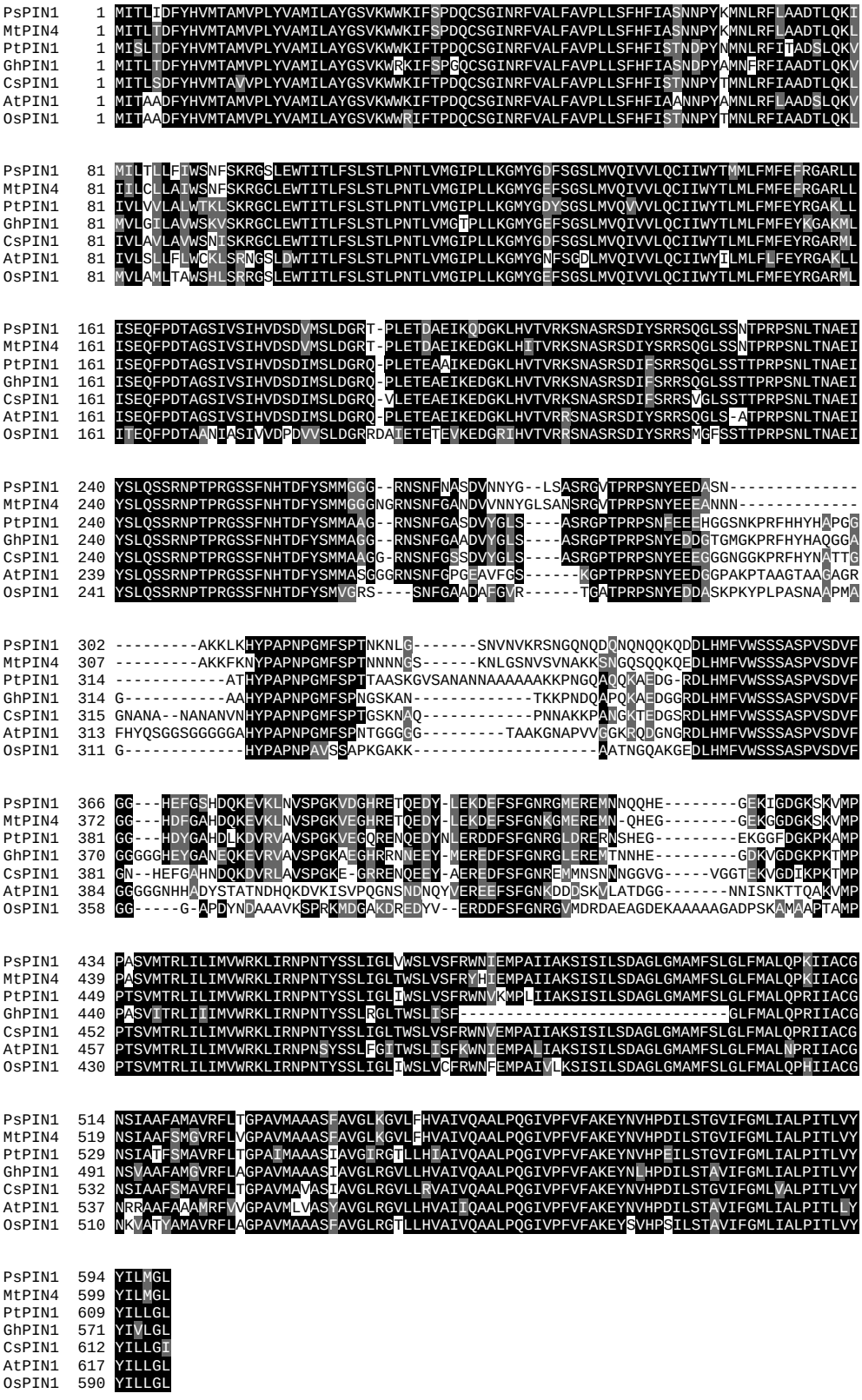
A phylogenetic of tree constructed from the predicted amino acid sequences of PsPIN1, the nine closest genes in the database along with that of PsPIN2 (another PIN gene from pea) and seven other major *Arabidopsis* PIN sequences (Figure 2). For most of the species there exists only one known *PsPIN1*-like gene and the exception was *Momordica charantia*, which had two known genes. These two genes plot closest together because there were only three amino acid differences and they showed closest sequence similarity to the gene in *Cucumis sativus* because these genera are in the same family (Cucurbitaceae). PsPIN1 showed most similarity to PIN4 sequence of *Medicago* because these species are also in the same family. These gene sequences, along with two from cotton and *Populus*, are more similar to that of AtPIN1 than any of the other *Arabidopsis* PIN proteins. Two sequences from wheat and rice, which were quite similar to one another, fell into a clade with AtPIN5, 6 and 8. Finally, PsPIN2 showed most similarity to AtPIN4 and little similarity to PsPIN1.

PsPIN1 promoter: in silico analysis

The promoter region of the *PsPIN* gene (AY656811) was isolated using the genome walker technique (Clontech). Using the 5′ region of the gene to design a probe, we obtained a 832 bp *Eco*RV fragment. Primers were then designed from the sequence of the 5′ end of this fragment and additional screening was done. A final 1511 bp promoter region of *PsPIN1* upstream of the translational start site was obtained and subsequently sequenced. Analysis of this promoter sequence using the PLACE and PlantCARE programs revealed the presence of three auxin-responsive elements including the well known AuxRE (− 761), AuxRR-core (− 457) and ASF-1 element (− 745) (Benfey and Chua 1990; Hagen et al. 1991; Sakai et al. 1996) (Figure 3). The *PsPIN1* promoter also possessed a possible P-box (pyrimidine-box) (− 24) and a TATC-element (− 736), which are gibberellin-response elements

►

Figure 1. ClustalW alignment of predicted PsPIN1 with the most similar PIN1-like proteins. The conserved regions are marked by shaded boxes. Numbers on the sides indicate amino acid positions. PsPIN1, *Pisum sativum* PIN1 (AY222857); MtPIN1 *Medicago truncatula* PIN4 (AY115839); PtPIN1, *Populus tremula* PIN1 (AF190881); *Momordica charantia* PIN1-like McAEC2 (AF247004) and McAEC1(AF246995); GhPIN1, *Gossypium hirsutum* PIN1 (AY148428); CsPIN1, *Cucumis sativus* PIN1 (AB085897); AtPIN1, *Arabidopsis thaliana* PIN1 (NP_177500); and OsPIN1, *Oryza sativa* REH1 (T02876).


PsPIN1 1 MITLIDFYHVMTAMVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIASNNPYKMNLRFLAADTLQKI
MtPIN4 1 MITLTDFYHVMTAMVPLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIASNNPYKMNLRFLAADTLQKL
PtPIN1 1 MISLTDFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFISTNDPYNMNLRFITADSLQKV
GhPIN1 1 MITLTDFYHVMTAMVPLYVAMILAYGSVKWRKIFSPGQCSGINRFVALFAVPLLSFHFIASNDPYAMNFRFIAADTLQKV
CsPIN1 1 MITLSDFYHVMTAVVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFISTNNPYTMNLRFIAADTLQKL
AtPIN1 1 MITAADFYHVMTAMVPLYVAMILAYGSVKWWKIFTPDQCSGINRFVALFAVPLLSFHFIAANNPYAMNLRFLAADSLQKV
OsPIN1 1 MITAADFYHVMTAMVPLYVAMILAYGSVKWWRIFTPDQCSGINRFVALFAVPLLSFHFISTNNPYTMNLRFIAADTLQKL
PsPIN1 81 MILTLLFIWSNFSKRGSLEWTITLFSLSTLPNTLVMGIPLLKGMYGDFSGSLMVQIVVLQCIIWYTMMLFMFEFRGARLL
MtPIN4 81 IILCLLAIWSNFSKRGCLEWTITLFSLSTLPNTLVMGIPLLKGMYGEFSGSLMVQIVVLQCIIWYTLMLFMFEFRGARLL
PtPIN1 81 IVLVVLALWTKLSKRGCLEWTITLFSLSTLPNTLVMGIPLLKGMYGDYSGSLMVQVVVLQCIIWYTLMLFMFEYRGAKLL
GhPIN1 81 MVLGILAVWSKVSKRGCLEWTITLFSLSTLPNTLVMGTPLLKGMYGEFSGSLMVQIVVLQCIIWYTLMLFMFEYKGAKML
CsPIN1 81 IVLAVLAVWSNISKRGCLEWTITLFSLSTLPNTLVMGIPLLKGMYGDFSGSLMVQIVVLQCIIWYTLMLFMFEYRGARML
AtPIN1 81 IVLSLLFLWCKLSRNGSLDWTITLFSLSTLPNTLVMGIPLLKGMYGNFSGDLMVQIVVLQCIIWYILMLFLFEYRGAKLL
OsPIN1 81 MVLAMLTAWSHLSRRGSLEWTITLFSLSTLPNTLVMGIPLLKGMYGEFSGSLMVQIVVLQCIIWYTLMLFMFEYRGARML
PsPIN1 161 ISEQFPDTAGSIVSIHVDSDVMSLDGRT-PLETDAEIKQDGKLHVTVRKSNASRSDIYSRRSQGLSSNTPRPSNLTNAEI
MtPIN4 161 ISEQFPDTAGSIVSIHVDSDVMSLDGRT-PLETDAEIKEDGKLHITVRKSNASRSDIYSRRSQGLSSNTPRPSNLTNAEI
PtPIN1 161 ISEQFPDTAGSIVSIHVDSDIMSLDGRQ-PLETEAAIKEDGKLHVTVRKSNASRSDIFSRRSQGLSSTTPRPSNLTNAEI
GhPIN1 161 ISEQFPDTAGSIVSIHVDSDIMSLDGRQ-PLETEAEIKEDGKLHVTVRKSNASRSDIFSRRSQGLSSTTPRPSNLTNAEI
CsPIN1 161 ISEQFPDTAGSIVSIHVDSDIMSLDGRQ-VLETEAEIKEDGKLHVTVRKSNASRSDIFSRRSVGLSSTTPRPSNLTNAEI
AtPIN1 161 ISEQFPDTAGSIVSIHVDSDIMSLDGRQ-PLETEAEIKEDGKLHVTVRRSNASRSDIYSRRSQGLS-ATPRPSNLTNAEI
OsPIN1 161 ITEQFPDTAANIASIVVDPDVVSLDGRRDAIETETEVKEDGRIHVTVRRSNASRSDIYSRRSMGFSSTTPRPSNLTNAEI
PsPIN1 240 YSLQSSRNPTPRGSSFNHTDFYSMMGGG--RNSNFNASDVNNYG--LSASRGVTPRPSNYEEDASN--------------
MtPIN4 240 YSLQSSRNPTPRGSSFNHTDFYSMMGGGNGRNSNFGANDVVNNYGLSANSRGVTPRPSNYEEEANNN------------
PtPIN1 240 YSLQSSRNPTPRGSSFNHTDFYSMMAAG--RNSNFGASDVYGLS----ASRGPTPRPSNFEEEHGGSNKPRFHHYHAPGG
GhPIN1 240 YSLQSSRNPTPRGSSFNHTDFYSMMAGG--RNSNFGAADVYGLS----ASRGPTPRPSNYEDDGTGMGKPRFHYHAQGGA
CsPIN1 240 YSLQSSRNPTPRGSSFNHTDFYSMMAAGG-RNSNFGSSDVYGLS----ASRGPTPRPSNYEEEGGGNGGKPRFHYNATTG
AtPIN1 239 YSLQSSRNPTPRGSSFNHTDFYSMMASGGGRNSNFGPGEAVFGS------KGPTPRPSNYEEDGGPAKPTAAGTAAGAGR
OsPIN1 241 YSLQSSRNPTPRGSSFNHTDFYSMVGRS----SNFGAADAFGVR------TGATPRPSNYEDDASKPKYPLPASNAAPMA
PsPIN1 302 ---------AKKLKHYPAPNPGMFSPTNKNLG-------SNVNVKRSNGQNQDQNQNQQKQDDLHMFVWSSSASPVSDVF
MtPIN4 307 ---------AKKFKNYPAPNPGMFSPTNNNNGS------KNLGSNVSVNAKKSNGQSQQKQEDLHMFVWSSSASPVSDVF
PtPIN1 314 ------------ATHYPAPNPGMFSPTTAASKGVSANANNAAAAAAKKPNGQAQQKAEDG-RDLHMFVWSSSASPVSDVF
GhPIN1 314 G-----------AAHYPAPNPGMFSPNGSKAN-------------TKKPNDQAPQKAEDGGRDLHMFVWSSSASPVSDVF
CsPIN1 315 GNANA--NANANVNHYPAPNPGMFSPTGSKNAQ-----------PNNAKKPANGKTEDGSRDLHMFVWSSSASPVSDVF
AtPIN1 313 FHYQSGGSGGGGGAHYPAPNPGMFSPNTGGGGG---------TAAKGNAPVVGGKRQDGNGRDLHMFVWSSSASPVSDVF
OsPIN1 311 G-------------HYPAPNPAVSSAPKGAKK-------------------AATNGQAKGEDLHMFVWSSSASPVSDVF
PsPIN1 366 GG---HEFGSHDQKEVKLNVSPGKVDGHRETQEDY-LEKDEFSFGNRGMEREMNNQQHE--------GEKIGDGKSKVMP
MtPIN4 372 GG---HDFGAHDQKEVKLNVSPGKVEGHRETQEDY-LEKDEFSFGNKGMEREMN-QHEG--------GEKGGDGKSKVMP
PtPIN1 381 GG---HDYGAHDLKDVRVAVSPGKVEGQRENQEDYNLERDDFSFGNRGLDRERNSHEG---------EKGGFDGKPKAMP
GhPIN1 370 GGGGGHEYGANEQKEVRVAVSPGKAEGHRRNNEEY-MEREDFSFGNRGLEREMTNNHE---------GDKVGDGKPKTMP
CsPIN1 381 GN--HEFGAHNDQKDVRLAVSPGKE-GRRENQEEY-AEREDFSFGNREMMNSNNGGVG-----VGGTEKVGDIKPKTMP
AtPIN1 384 GGGGGNHHADYSTATNDHQKDVKISVPQGNSNDNQYVEREEFSFGNKDDDSKVLATDGG-------NNISNKTTQAKVMP
OsPIN1 358 GG-----G-APDYNDAAAVKSPRKMDGAKDREDYV--ERDDFSFGNRGVMDRDAEAGDEKAAAAAGADPSKAMAAPTAMP
PsPIN1 434 PASVMTRLILIMVWRKLIRNPNTYSSLIGLVWSLVSFRWNIEMPAIIAKSISILSDAGLGMAMFSLGLFMALQPKIIACG
MtPIN4 439 PASVMTRLILIMVWRKLIRNPNTYSSLIGLTWSLVSFRYHIEMPAIIAKSISILSDAGLGMAMFSLGLFMALQPKIIACG
PtPIN1 449 PTSVMTRLILIMVWRKLIRNPNTYSSLIGLIWSLVSFRWNVKMPLIIAKSISILSDAGLGMAMFSLGLFMALQPRIIACG
GhPIN1 440 PASVITRLIIIMVWRKLIRNPNTYSSLRGLTWSLISF----------------------------GLFMALQPRIIACG
CsPIN1 452 PTSVMTRLILIMVWRKLIRNPNTYSSLIGLTWSLVSFRWNVEMPAIIAKSISILSDAGLGMAMFSLGLFMALQPRIIACG
AtPIN1 457 PTSVMTRLILIMVWRKLIRNPNSYSSLFGITWSLISFKWNIEMPALIAKSISILSDAGLGMAMFSLGLFMALNPRIIACG
OsPIN1 430 PTSVMTRLILIMVWRKLIRNPNTYSSLIGLIWSLVCFRWNFEMPAIVLKSISILSDAGLGMAMFSLGLFMALQPHIIACG
PsPIN1 514 NSIAAFAMAVRFLTGPAVMAAASFAVGLKGVLFHVAIVQAALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPITLVY
MtPIN4 519 NSIAAFSMGVRFLVGPAVMAAASFAVGLKGVLFHVAIVQAALPQGIVPFVFAKEYNVHPDILSTGVIFGMLIALPITLVY
PtPIN1 529 NSIATFSMAVRFLTGPAIMAAASIAVGIRGTLLHIAIVQAALPQGIVPFVFAKEYNVHPEILSTGVIFGMLIALPITLVY
GhPIN1 491 NSVAAFAMGVRFLAGPAVMAAASIAVGLRGVLLHVAIVQAALPQGIVPFVFAKEYNLHPDILSTAVIFGMLIALPITLVY
CsPIN1 532 NSIAAFSMAVRFLTGPAVMAVASIAVGLRGVLLRVAIVQAALPQGIVPFVFAKEYNVHPDILSTGVIFGMLVALPITLVY
AtPIN1 537 NRRAAFAAAMRFVVGPAVMLVASYAVGLRGVLLHVAIIQAALPQGIVPFVFAKEYNVHPDILSTAVIFGMLIALPITLLY
OsPIN1 510 NKVATYAMAVRFLAGPAVMAAASFAVGLRGTLLHVAIVQAALPQGIVPFVFAKEYSVHPSILSTAVIFGMLIALPITLVY
PsPIN1 594 YILMGL
MtPIN4 599 YILMGL
PtPIN1 609 YILLGL
GhPIN1 571 YIVLGL
CsPIN1 612 YILLGI
AtPIN1 617 YILLGL
OsPIN1 590 YILLGL

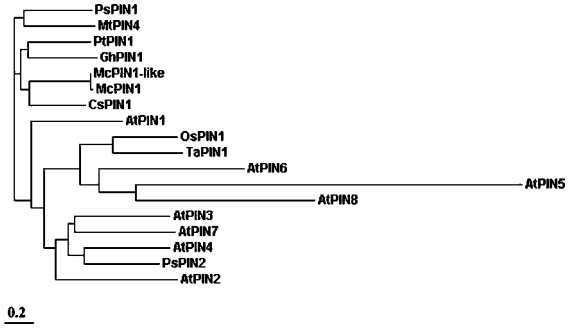


Figure 2. Phylogenetic relationships of PsPIN1, other similar PIN proteins and major PIN proteins known for *Arabidopsis* and pea. The phylogenetic tree was generated using the ClustalW program and visualized using NJplot. AtPIN2 (NP_568848); AtPIN3 (NP_177250); AtPIN4 (NP_849923); AtPIN5 (NP_197014); AtPIN6 (NP_177836); AtPIN7 (NP_849700); AtPIN8 (Q9FFDO); PsPIN2 (AB112364); TaPIN1 (AY496058).

Figure 3. Diagrammatic representation of 1511 bp region upstream of *PsPIN1* (AY656811). ATG: translation start site; CAAT and TATA-box, basic elements of the promoter; P-box, pyrimidine box found in GA responsive α-amylase promoter of rice; AuxRR-core, auxin responsive core found in auxin-induced *parc* gene of tobacco; TATC- element, gibberellin responsive element found in rice storage protein glutelin gene, *Glu B-1*; ASF1 Motif, found in many promoters upregulated by auxin and/salicylic acid/light; AuxRE, found in auxin-responsive *GH3* promoter of soybean.

found in the rice α-amylase promoter (Kim and Wu 1992) (Figure 3).

In silico analysis, of the *PsPIN1* upstream region also revealed the presence of multiple, potential, light-regulatory elements, many of which match those in other light-regulated pea genes. These included the AE-box, G-box, GATA-motif, GT1-motif, I-box and LAMP-element (data not shown). A total of 97 potential light regulatory elements were predicted by the PlantCare program.

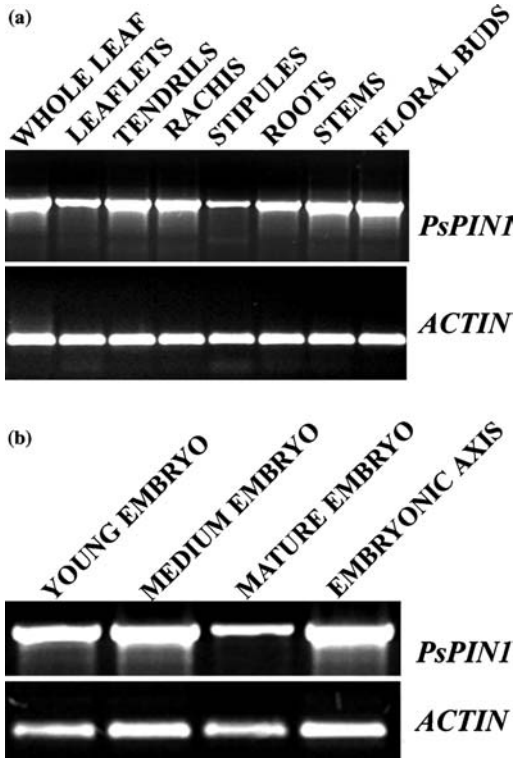


Figure 4. *PsPIN1* expression. (a). Expression of *PsPIN1* mRNA in various plant parts. β-Actin was used as a control for equal cDNA used for RT-PCR and equal loading. (b). *PsPIN1* expression in different stages of developing embryos (shown at the top of each lane). Ten microliters of RT-PCR product were loaded for *PsPIN1* and β-Actin and the gel was stained with ethidium bromide.

Tissue specific expression

A single cDNA of 1.4 kb, was detected in all plant parts (Figure 4a). *PsPIN1* was ubiquitously expressed throughout the pea plant with high expression in growing tendrils, rachis, stem, roots, whole expanding leaves and floral buds (Figure 4a). Although, the technique is not quantitative, the three lateral appendages of the pea leaf (i.e. tendrils, leaflets and stipules) showed differences in *PsPIN1* mRNA levels with expression being highest in tendrils. *PsPIN1* mRNA seemed to be abundant during embryo development, as it was present in all the stages observed (Figure 4b). Expression was mainly confined to the embryonic axis of the mature embryo, as the majority of the whole embryo consisted of cotyledons, which had lower expression at this stage.

Hormone induction: auxin and GA

Semi-quantitative RT-PCR was done to determine if *PsPIN1* is induced by auxin. The range of 10–50 μm-IAA induced high levels of *PsPIN1* expression, although 50 μM induced the highest expression level in 7-day-old, dark grown shoot tips (Figure 5a). 4-Cl-IAA also induced *PsPIN1* mRNA in a pattern similar to that of m-IAA but with the peak of expression at approximately 25 μM (Figure 5b). *PsPIN1* mRNA was induced within about 1 h of auxin (m-IAA) treatment and a peak was reached within 2 hours, after which time the levels remained mostly steady (Figure 5c).

A 4 h treatment with 50 μM GA_3 also caused induction of *PsPIN1* mRNA in pea internode segments (Figure 6). Levels of *PsPIN1* mRNA were similar when both GA_3 and m-IAA were added together (*t*-test, $p = 0.35$).

De-etiolation and light effects

Since the promoter of *PsPIN1* gene revealed the presence of multiple, potential light-regulatory elements, we looked for light induction of *PsPIN1* mRNA. The steady state levels of *PsPIN1* mRNA

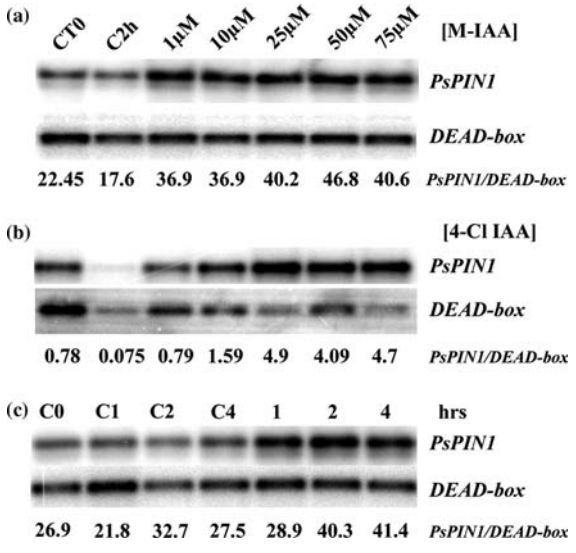


Figure 5. Auxin response of the *PsPIN1* gene. (a). Dose–response curve for hormonal induction of *PsPIN1* gene with m-IAA. (b). Dose–response curve for hormonal induction with 4-CI-IAA. C. Time-course induction by m-IAA. Controls (c). were collected for each time point. RT-PCR was done for 20 cycles. *DEAD box* was used as the control gene.

Figure 6. Regulation of *PsPIN1* expression by GA_3. GA_3, 50 μM GA_3; m-IAA, 50 μM m-IAA; GA_3 + m-IAA, 50 μM GA_3 + 50 μM m-IAA. CTO, the time zero control; C4h, internodes treated with water only. PCR was done for 20 cycles and the gel blotted and probed with *PsPIN1*-specific probe. PCR was done similarly for the control *DEAD box* gene.

were higher in shoot tips of dark-grown rather than in light-grown 8-day-old plants (Figure 7a). Further, 5-day-old dark-grown plants transferred to light showed a consistent decline in *PsPIN1* mRNA levels compared to the control shoot tips from dark-grown plants of the same stage (Figure 7b). This decline occurred between 2 and 4 h after light treatment and levels remained low through 48 h. A light-inducible pea *Cab15* gene, which encodes a chlorophyll *a*/*b* binding protein of photosystem II was used as a positive control for light inducibility. *Cab* genes are expressed at high levels in light-grown green plants and at low levels

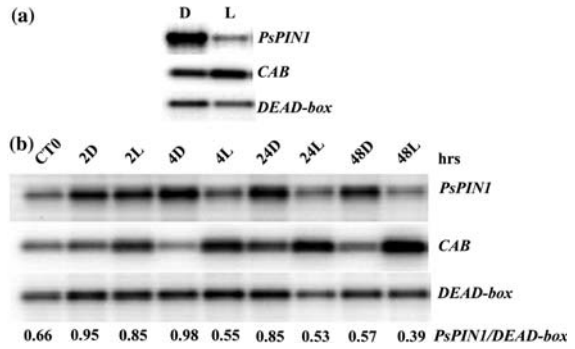


Figure 7. Regulation of *PsPIN1* by light. (a). Steady-state *PsPIN1* mRNA levels in 8-day-old dark (D) and light (L) grown pea shoot tips. (b). Regulation of *PsPIN1* mRNA by de-etiolation. Pea seedlings were grown in complete darkness for 5 days and then exposed to light for 2, 4, 24 and 48 h and their shoot-tips were used for *PsPIN1* expression analysis. Shoot-tips were also collected at the same time from dark-grown seedlings at respective times. *Cab* gene was used as a control for light-induction and *DEAD-box* as a control for equal loading.

in dark-grown plants (Coruzzi et al. 1983). mRNA levels of *Cab15* increased as early as 2 h after light treatment and continued to increase at each sample time through 48 h (Fig. 7b).

Discussion

Pea has been an important model plant for the study of hormonal control of internode elongation (Ockerse and Galston 1967; Yang et al. 1993; Ross et al. 2003a, b). Changes in hormone levels have also been measured in pea during de-etiolation, a process that causes a significant reduction in the rate of shoot elongation (Symons and Reid 2003a). Therefore, in the present study we have analyzed the affect of light and hormones, such as auxins and gibberellins, on *PsPIN1* mRNA accumulation. mRNA expression of this putative auxin efflux carrier may play a role in controlling auxin transport along with the changes in subcellular localization, which has been documented for its *Arabidopsis* ortholog.

PsPIN1 protein and PsPIN1 mRNA expression

More than eight different *PIN* genes have been identified in the *Arabidopsis* genome and multiple family members have been identified in *Brassica*, *Oryza*, *Medicago*, *Cucumis*, *Momordica* and *Gossypium*. Each member of the *PIN* gene family has some functional specificity in *Arabidopsis* but there is a high degree of homology among individual members across species. The topology of the PsPIN1 protein is similar to that of the other members of this transporter protein superfamily with N- and C-terminal hydrophobic regions of transmembrane repeats flanking the central region that is predominantly hydrophilic. The transmembrane domains are highly conserved, whereas the various PIN1 proteins exhibit variability in their lengths due to differences in the amino acids in the central region. However, the *PIN* genes have unique aspects of their central region that presumably provide the functional specificity and allow them to be separated from other gene family members.

PsPIN1 mRNA, like that of *AtPIN1* in *Arabidopsis*, is expressed ubiquitously in all organs and plant parts of pea. Its amino acid sequence is more similar to that of AtPIN1 than to any other known PIN protein in that species. *PsPIN1* mRNA levels are higher in tendrils than in leaflets, which correlate with our previous observations that tendrils transport auxin more efficiently than leaflets do (DeMason and Chawla 2004a, b). We observed that *PsPIN1* mRNA is abundant in growing tissues and organs. For example, young expanding leaves have higher expression levels than mature, fully expanded leaves. Expression is also high in floral buds and in the embryonic axis during all stages of embryo development. Overall, it is clear that *PsPIN1* mRNA is abundant in growing or developing plant parts.

Hormone regulation: auxin and gibberellin

According to Sach's hypothesis (Sachs 1991, 1992), canalization of auxin flow during vascular development results from a positive feedback mechanism that enhances the conductivity of cells functioning as auxin conduits. In support of Sach's hypothesis we find that auxin induces accumulation of *PsPIN1* mRNA and that this provides a mechanism for his proposed feedback response. Further support of Sach's hypothesis is provided by the observation that *PIN1* mRNA is abundant in the provascular tissues of auxin-induced leaf primordia of *Arabidopsis* (Reinhardt et al. 2003). Peer et al. (2004) conclude that *AtPIN1* and *AtPIN2* are auxin responsive at the level of gene expression and AtPIN1 and AtPIN4 are auxin responsive at the level of protein localization. Also, auxin regulates *PIN1* mRNA in stems of cultured *Populus* plantlets and in the transition zone between hypocotyl and root of *Cucumis* seedlings (Kamada et al. 2003; Schrader et al. 2003). In addition, auxin regulates protein stability of *PIN2*/*EIR1* of *Arabidopsis* (Sieberer et al. 2000). Therefore, PIN expression is complex and regulation occurs at multiple levels depending on species, tissue and organ.

The direct induction of *PsPIN1* transcription by auxin in pea is also supported by the fact that *PsPIN1* promoter harbors multiple auxin-responsive elements, including AuxRE, AuxRR-core and ASF-1CaMV. All these elements are present within 1000 bp of ATG. AuxRE is a common element present in many early auxin-responsive genes and is recognized by the transcription factor called ARF (auxin-response factor) (Guilfoyle

et al. 1998; Kepinski and Leyser 2002). The AuxRR core has been reported in promoters of *PsIAA4/5* from pea, *parB* and *parC* from tobacco and *SAUR* genes from soybean (Takahashi and Nagata 1992; Ballas et al. 1993; Li et al. 1994). The ASF-1 motif has been observed in promoters responsive to auxin/salicylic acid and/or light (Ockerse and Galston 1967; Terzaghi and Cashmore 1995). The multiple auxin-inducible elements within the *GH3* promoter of soybean have been shown to contribute incrementally to the overall level of auxin induction observed with this promoter (Liu et al. 1994).

Response of another active auxin, 4-Cl-IAA, which promotes internode and pod elongation in pea (Magnus et al. 1997; Ozga et al. 2003) was compared with that of IAA. 4-Cl-IAA, but not IAA, restores pericarp elongation when applied to deseeded ovaries (Reinecke 1999). The size and lipophilicity of 4-Cl-IAA are correlated with its growth promoting activity in pea (Reinecke 1999). Further, 4-Cl-IAA has been reported to be more active than IAA in stimulating growth of maize and *Avena* coleoptile segments (Katayama et al. 2000; Karcz and Burdach 2002) and in stimulating transcription of *PsGA20ox* and *PsGA3ox1*, two enzymes involved in the biosynthesis of GA_1 in pea (Ngo et al. 2002; Ozga et al. 2003). We find that 4-Cl-IAA also increases the levels of *PsPIN1* mRNA in shoot tips. m-IAA shows an induction maximum at 50 μM while that of 4-Cl-IAA is at 25 μM and is therefore more effective than IAA in regulating *PsPIN1* mRNA levels.

Auxins and gibberellins are classical plant hormones and are known to coordinately regulate several developmental programs such as stem elongation in pea. *PsPIN1* mRNA levels increase in etiolated pea epicotyls in response to GA_3 treatment. The *PsPIN1* promoter harbors two putative GA-responsive elements, the P-box and a TATC-element. Both a P-box and a TATC-element are required for GA-responsiveness of the rice glutelin gene, *GluB-1* (Washida et al. 1999). A P-box is also present in the rice α-amylase promoter (Kim et al. 1992).

There have been many early reports of interaction of auxin and GA. Results of experiments involving decapitation in combination with application of IAA or IAA transport inhibitors in pea clearly suggest that IAA derived from shoot apex is required to maintain normal levels of bioactive GA in elongating stem tissues (Ross 1998). *PsGA3ox1* (Mendel's *LE* gene), whose product catalyses the conversion of GA_{20} to GA_1 is positively regulated by auxin (Lester et al. 1997). Therefore, auxin promotes GA biosynthesis. Since auxin regulates GA levels in pea, it is often difficult to tease apart the roles of each hormone individually. The *le* mutant has a 10-to 20-fold reduction in GA levels and is more responsive to applied GA and less responsive to applied auxin with respect to internode elongation than *WT* plants (Ross et al. 2003b). *le* is considered to be a 'leaky' mutant because GA is still present in *le* plants and often they are still somewhat responsive to auxin. We used a *le* genotype in this study, so that mediation by GA of the auxin response is reduced. Our experiments show that the levels of *PsPIN1* mRNA increase in response to both auxin and GA treatment. This is further supported by the fact that *PsPIN1* promoter harbors elements responsive to both these hormones. However, no additive or synergistic response was observed when the two hormones were added together for our genotype.

Negative regulation by light

In silico analysis of the *PsPIN1* promoter shows the presence of multiple light-regulatory elements, such as AE-box, G-box, GATA-motif, GT1-motif, I-box and LAMP-element. Most of these elements are present in genes up-regulated by light. However, we found that *PsPIN1* mRNA is more abundant in the shoot tips of dark grown rather than light grown seedlings. Further, *PsPIN1* mRNA decreases during de-etiolation. In *Arabidopsis*, the *AGR1/AtPIN2* mRNA has been shown to be present in root, hypocotyl and cotyledon of etiolated seedlings but is absent in the shoot of young seedlings grown in the light (Chen et al. 1998). Plant hormones have also been implicated in the regulation of de-etiolation (Chory and Li 1997; Kraepiel and Migniac 1997; Neff et al. 2000; Alabadí, et al. 2004). In pea, GA levels decrease rapidly for the first 4 h after light treatment and remain low through 48 h (Symons and Reid 2003a). This is due to down-regulation of *PsGA3ox1* and up-regulation of *PsGA2ox2* (Symons and Reid 2003b). Although it is possible that the rate of GA reduction is regulated by light intensity, this timeframe appears to parallel the

reductions in *PsPIN1* mRNA levels that we observe. Further, IAA levels also decrease during de-etiolation in pea and maize (Jones et al. 1991; Behringer and Davies 1992; Behringer et al. 1992). In pea, auxin levels drop slightly during the first 4 h after transfer to light, but then increase gradually over the next 20 h (Symons and Reid 2003a). Together, these results provide the likely mechanism by which light mediates the reduction in shoot elongation during de-etiolation. Our observation that *PsPIN1* mRNA declines after light treatment can be explained mainly by decreases in GA levels in response to light since the timing is similar and we also demonstrate that this hormone positively regulates its mRNA levels.

In conclusion, PsPIN1 is structurally quite similar to other known PIN proteins with strong sequence conservation in the transmembrane domains. Its sequence is more similar to that of AtPIN1 than to the other known AtPINs. *PsPIN1* mRNA is expressed ubiquitously in all growing plant parts from the developing embryo onwards and is probably one of the most abundant and extensively expressed *PIN* genes in pea. Further, it is upregulated by treatments that increase stem growth rates, such as high levels of GA and auxin, including 4-Cl-IAA and darkness.

Acknowledgments

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