

A Pin gene families encoding components of auxin efflux carriers in *Brassica juncea*

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ABSTRACT

Based on the sequence information of Arabidopsis PIN1, two cDNAs encoding PIN homologues from *Brassica juncea*, Bjp1n2 and Bjp1n3, were isolated through cDNA library screening. Bjp1n2 and Bjp1n3 encoded proteins containing 640 and 635 amino acid residues, respectively, which shared 97.5% identities with each other and were highly homologous to Arabidopsis PIN1, PIN2 and other putative PIN proteins. Bjp1n2 and Bjp1n3 had similar structures as AtPIN proteins. Northern blot analysis indicated that Bjp1n2 was expressed in stem, leaf and floral tissues, while Bjp1n3 was expressed predominantly in stem and hypocotyls. Two promoter fragments of pin genes, Bjp1n-X and Bjp1n-Z, were isolated by 'genome walking' technique using primers at 5' -end of pin cDNA. Promoter-gus fusion studies revealed the GUS activities driven by Bjp1n-X were at internal side of xylem and petal; while those driven by Bjp1n-Z were detected at leaf vein, epidermal cell and cortex of stem, vascular tissues and anther. Results of the pin genes with different expression patterns in *B. juncea* suggested the presence of a gene family.

Key words: *Brassica juncea*, polar auxin transport, auxin efflux carrier, promoter.

INTRODUCTION

Auxin is critical for plant embryo development, morphogenesis and especially polar differentiation and growth. Polar auxin transport (PAT), detected in stem and roots of all higher plants, was involved in plant apical dominance, tropic growth, vascular and floral tissue differentiation, plant pattern for-

mation and lateral root development, etc (for reviews, see Ni, et al, 2000[1]; Berleth and Sachs, 2001[2]). Current model of PAT, chemiosmotic hypothesis (Rubery and Sheldrade[3] and Raven[4]), posited the occurrence of PAT through the action of cellular auxin influx and efflux carriers locating on the plasma membrane of transporting cells, and the direction of PAT depends on the basal location of the efflux carriers.

The first molecular support of the model came from the characterization of a candidate auxin influx carrier, AUX1 (auxin resistant1), which was a presumptive membrane protein similar to amino acid permeases[5] and specifically expressed in the root. Further isolation and characterization of two genes, Atpin1 and Atpin2, indicated that the primary func-

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**The nucleotide sequences reported in this paper have been submitted to the GenBankTM / EMBL Data Bank with accession numbers AJ249297 (Bjp1n2 cDNA), AJ249298 (Bjp1n3 cDNA), AJ308229 (Bjp1n-X) and AJ308230 (Bjp1n-Z).

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tion of the AtPIN1 protein was for PAT in the inflorescence axis and AtPIN2 in root tissues[6-10]. AtPIN1 involved in the auxin transport from stem to root tip and was critical for symmetry differentiation of embryo, the initiation and radial position of plant lateral organs, pattern formation of leaf vein [11-13]. AtPIN2 played a role in root gravitropic response together with AUX1[9]. AtPIN1 was a membrane protein locating at the basal end of auxin transport-competent cells in vascular tissues[7]. AtPIN2-expressing yeast cells were resistant to toxic fluorindoles, an IAA analogy[8], and released preloaded IAA more rapidly than control cells demonstrated that AtPIN2 transported IAA out of the yeast cells and indeed a component of auxin efflux carriers[9]. The Arabidopsis mutant pin1 showing normal tropic responses of shoot and root[10] also indicated that there maybe other pin genes controlling the lateral auxin transport pathways. Presence of other cDNAs encoding PIN homologues indicated a multi-gene family in Arabidopsis genome that, however, suggested the specific roles of pin genes in auxin related plant growth and development. Analyses of Atpin1 (widely expressed in all the tissues tested) and Atpin2 (specifically expressed in the root) indicated their expression patterns were closely linked to their physiological functions. As a conclusion, isolation of pin genes, especially studies on their expression patterns will provide informations on functional mechanisms of PAT during plant growth and development, especially polar differentiation.

We are interested in the functions of PAT in plant cells. From *Brassica juncea* we have isolated an auxin efflux carrier (BjPIN1) and performed functional characterization through transgenic approaches of Arabidopsis[14]. Here we report the isolation of two putative pin-gene promoters and two cDNAs encoding PIN homologous of *B. juncea*, as well as the studies on their expression pattern analysis.

MATERIALS AND METHODS

Materials

Enzymes used for DNA restriction and modification were obtained from Boehringer (Mannheim, Germany). DNA primers for polymerase chain reaction (PCR) and "LAPCR in vitro Cloning" kit used for 'genome walking' were obtained from

TaKaRa Biotechnology (Dalian, China). 5-bromo-4-chloro-3-indolyl b-D-glucuronic acid (X-Gluc) was obtained from Sigma (USA). Radiochemicals [α - 32 P]dCTP was obtained from Yuhui Company (Beijing, China). The leaf lZAPII cDNA library of *Brassica juncea* was kindly provided by Pua E-C. (National University of Singapore).

Bacteria and plants

Escherichia coli strain XL-1 Blue (Stratagene, La Jolla, USA) was used for library screening and DNA cloning. *Agrobacterium* strain LBA4404 was used for plant transformation. *B. juncea* (Indian mustard) and *Nicotiana tabacum* plants were grown in the green house with a 12 h light (26°C) and 12 h dark (20°C) period.

Isolation of the Bjpin cDNAs

Bjpin cDNAs were isolated as described[14]. In short, two reverse degenerated primers were designed based on the nucleotide sequences of Atpin1[8] and two other putative pin sequences and used to amplify the Pin-homologous cDNA fragments from cDNA library of *B. juncea*. Specific primers were then used to isolate the full-length cDNAs through PCR-based screening[14], [15].

DNA sequencing was performed by TaKaRa Biotechnology (Dalian, China). Computational analysis was performed with the help of the programs of the Wisconsin Genetics Computer Group (GCG Package, Version 10.1). The BLAST search programs were used for sequence comparisons on DNA and amino acid sequences in GeneBank, EMBL, dbEST and SwissProt databases.

Northern blot analysis

RNAs were prepared from well-watered *B. juncea* plants according to the protocol of Logemann et al[16] and quantitated spectrophotometrically at 260 nm. RNA (20 μ g per lane) was electrophoretically separated in denaturing agarose gels and blotted onto Hybond-N⁺ membranes (Amersham, USA). RNA was fixed onto the membrane by heating at 80°C for 2 h. PCR products of around 400-bps of the Bjpin2 and Bjpin3 (encoded the amino acid residues at positions 305-441 and 305-436, respectively) were served as [α - 32 P]dCTP-labeled hybridization probe. Membranes were hybridized and washed as described[17]. Blots were exposed to Fuji X- films between intensifying screens for 3-4 d at -70°C.

Isolation of the promoter regions of pin genes

Promoter regions of pin genes were isolated using "LAPCA in vitro cloning" kit. 5 mg genomic DNA was digested with Hind III or EcoR I and then ligated with corresponding adaptors. Ligation products was then denatured at 94°C for 5 min and used as templates for 1st round PCR with primers C1 (5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3') and BjPS1 (5'-TACAGAGCCGTAGGCGAGGATCATAG-3'). PCR was performed as follows: 94oC for 30 sec; 58oC for 90 sec; 72°C for 3 min; 30 cycles. PCR products were then diluted for 10 times and 1 ml was used as templates for 2nd PCR using primers C2 (5'-CGTAATACGACTCACTATAGGGAGA-3') and BjPS2 (5'-

CGAGGATCATAGCGACGTAGAG-3') under same conditions as described above. PCR products were separated on the agarose gel and subcloned into TA vectors. Colonies harbouring PCR products were sequenced (TaKaRa Biotechnology, Dalian) and analyzed with programs of the Wisconsin Genetics Computer Group (GCG Package, Version 10.1) and PLACE.

Plant transformation with binary constructs harbouring promoter regions of pin genes

Promoter regions before ATG of *Bjpin-X* was amplified via PCR using standard primer SP6 and GW1.2A (5' - CTTATTTACGGTGGAAATATAGAGGG-3') with Pfu taq polymerase and then digested with Hind III, resulted DNA fragment was then ligated to pBSK pre-cut with Sma I /Hind III. Resulted plasmid DNA was digested with Hind III / BamH I and DNA fragment was ligated to pBI101.3 cut with same enzymes.

Same cloning strategy was used for *Bjpin-Z*, primers SP6 and *Bjpin1S* (5'-CTTCTTTCTGGTTGATTAGAGAG-3') were used for amplification, restriction enzyme Sal I was used for cloning procedures.

The resulted plasmids, containing the promoter-uidA coding region, were transformed into *A. tumefaciens* strain LBA4404 for tobacco transformation. Transformation, using leaf disc as materials for transformation, were performed as described.

Analysis of transgenic plants

Transgenic tobacco plants were confirmed via PCR using primers located in promoter region, for *Bjpin-X*, primers GW1.2S (5' -CTTCTGCTCTTGCATGATCTTCG-3') and W1.2A (see above), for *Bjpin-Z*, primers *Bjpin1S* (see above) and GW07A (5' - TATTATCGCCGATGAGTGGCA-3') were used. PCR was performed as follows: 94°C for 5 min, 1 cycle; 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 35 cycles; with a final extension at 72°C for 10min.

GUS activities were determined using 5-bromo-4-chloro-3-indolyl b-D-glucuronic acid (X-Gluc) as substrate[18].

RESULTS

Isolation of Bjpin2 and Bjpin3 cDNAs from B. juncea

Using degenerated primers, a DNA fragment with a size of 550bp was amplified and sequenced, the deduced peptide sequence showed 75% identi-

ties with isolated AtPIN1 at position 1 ~ 145, indicating a putative PIN-like fragment of *B. juncea*. PCR-based screening of *B. juncea* cDNA library was then performed and two cDNAs, *Bjpin2* and *Bjpin3*, were isolated. There were in-frame stop codons before the translation start code 'ATG' in both cDNAs, which indicated that full-length open reading frames were included. The predicted proteins of BjPIN2 and BjPIN3 contained 640 and 635 amino acid residues respectively, and had a molecular mass of 70 kDa, similar to that of previously reported AtPIN1[6] and AtPIN2/EIR1 [8],[9]. The calculated isoelectric points of BjPIN2 and 3 were around 8.8.

BjPIN2 and BjPIN3 shared 97.5% homologies with each other and were highly homologous to Arabidopsis PIN proteins (Tab 1), with the highest identities (93%) with AtPIN3 (accession No. AF087818).

Structural organization of the BjPIN proteins

Fig 1 shows the alignment of BjPIN2 and BjPIN3 with BjPIN1, AtPIN1, AtPIN2 and other PIN-like proteins in Arabidopsis. Comparison of all the proteins revealed the presence of conserved regions locating at both N- and C-terminus, which are highly hydrophobic. Presence of around 11 predicted transmembrane regions in BjPIN2 and BjPIN3 was similar with that in AtPIN1. A non-conserved central region is predominantly hydrophilic.

Expression pattern analyses of Bjpin2 and Bjpin3

Northern blot analysis was performed to detect the transcription levels of both *Bjpin2* and *Bjpin3*. The DNA fragments encoding the non-conserved (varied) regions of *Bjpin2* and *Bjpin3* were served as hybridization probes. As shown in Fig 3A and Fig 3B, single band of transcripts of approximately 2.5 kb, consistent with the length of both cDNAs, was detected. *Bjpin2* mRNA was detected in stem, leaf, and floral tissues, and the *Bjpin3* mRNA predominantly in stem and hypocotyls. No detectable mRNA was found in root tissues.

Tab 1. Amino acid sequence comparison of BjPIN2 and BjPIN3 to isolated AtPIN1, 2 and other PIN-like proteins of Arabidopsis. Numbers indicated percentages of identical and similar amino acids. Accession numbers for AtPIN1, 2, 3, 4, 6 and 7 refer to Fig 1. I: identities; S: similarities.

I/S	AtPIN 1	AtPIN2	AtPIN3	AtPIN4	AtPIN6	AtPIN7
BiPIN2	64/81	62/77	93/97	82/85	53/72	87/92
BjPIN2	64/82	62/76	93/97	82/85	53/72	87/92

	20	40	60	80	100			
Bjpin2	MISWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Bjpin3	MISWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Bjpin1	MISWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin1	MITAAEYHVMRAM/PLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin2	MITGKMYDVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin3	MISWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin4	MITWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin6	MITGNEEYTVMRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Atpin7	MITWHDYTVLRAVTPLYAMLAYGSVRRWRRIFSPQCQSGINRFVIFAVPLLSFHFISNNPYMNRFFIADTLQKILMIALVLLWANEFRSSLEWSTIFLSLSTLP	100	111					
Bjpin2	NFLVMGIPLLIIMYGEYASGLMVQVVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Bjpin3	NFLVMGIPLLIIMYGEYASGLMVQVVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Bjpin1	NFLVMGIPLLIIMYGEYASGLMVQVVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Atpin1	NFLVMGIPLLIKGMYGNFSDLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFDTAGSIVSIHVDSDIMSLDGRQFLETEEIKEDGKLVIVRKSNAHR...SDIYS	120	140	160	180	200	220	
Atpin2	NFLVMGIPLLIRMYGDFSNLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFETASISIPRVSDVILSNGRPLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Atpin3	NFLVMGIPLLIIMYGEYASGLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Atpin4	NFLVMGIPLLIIMYGTIYASGLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Atpin6	NFLVMGIPLLIQMYGDFCQLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFASLAKIQVDDVILSDGMPLRTEREDVNGRIPLRIRRSVSS...VPDSVMS	120	140	160	180	200	220	
Atpin7	NFLVMGIPLLIIMYGEYASGLMVQIVVLLQIWIYLLFLFPERCAKMLIMEQFFETASIVSFRVESDVVSLDGHFLETDAEIGNDGKLVIVRKSNAHR...RQFC	120	140	160	180	200	220	
Bjpin2	...CPN...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 298	240	260	280	300	320		
Bjpin3	...CPN...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 298	240	260	280	300	320		
Bjpin1	...CPN...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 298	240	260	280	300	320		
Atpin1	RRSQSL...ATPRSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...E...AVFGSKGTPRPSNFEENSAIASPRFG...: 311	240	260	280	300	320		
Atpin2	SHGGLNSSMTPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...GAGFGGQVYSLQSSKGVTPRPSNFEENSAIASPRFG...: 330	240	260	280	300	320		
Atpin3	...CPN...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 298	240	260	280	300	320		
Atpin4	...CPN...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 291	240	260	280	300	320		
Atpin6	P...SLC...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...CNGDLGGFTRPGL...: 302	240	260	280	300	320		
Atpin7	...GG...TPRPSNLTCAEIYSLST...TPRGSNENHSDFYSMMG...FPG...GRLSNFGFA...DMYVQSSSRGTPRPSNFEENSAIASPRFG...: 301	240	260	280	300	320		
Bjpin2	...YYPGGG...GGGYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 393	340	360	380	400	420	440	
Bjpin3	...YYPGGG...GGGYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 393	340	360	380	400	420	440	
Bjpin1	...YYPGGG...GGGYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 393	340	360	380	400	420	440	
Atpin1	RFHYQSGGGGGGGAHPAPNFMESPTNGGGGTAAGNAE...VVGK...RODNGRDLHMFVWSSASPVSD...VFGGGGHHHADYSTATNDHQKD...: 404	340	360	380	400	420	440	
Atpin2	SGELYNNNSVP...SYVPPNEMFTGTSKASGV...KKKESGGGGGGVGVGQSN...KERNMFVWSSASPVSEANAKNAMTRGSSTDV...STD...: 415	340	360	380	400	420	440	
Atpin3	...YYPGGG...GGGYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 393	340	360	380	400	420	440	
Atpin4	...FYNNTNSSVPAAGSYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 383	340	360	380	400	420	440	
Atpin6	...TPRAGNENELDNGNGTPVWMSKSAAGRIYRQSSPK...KMWES...: 352	340	360	380	400	420	440	
Atpin7	...YYPGGG...GGGYPAPNP...EASATATSTANKVSNR...TGCKANNHDSAKKELHMFVWSSNCSVPSVRAGLVDFDAGPS...G...SNNEQ...GAKK...: 385	340	360	380	400	420	440	

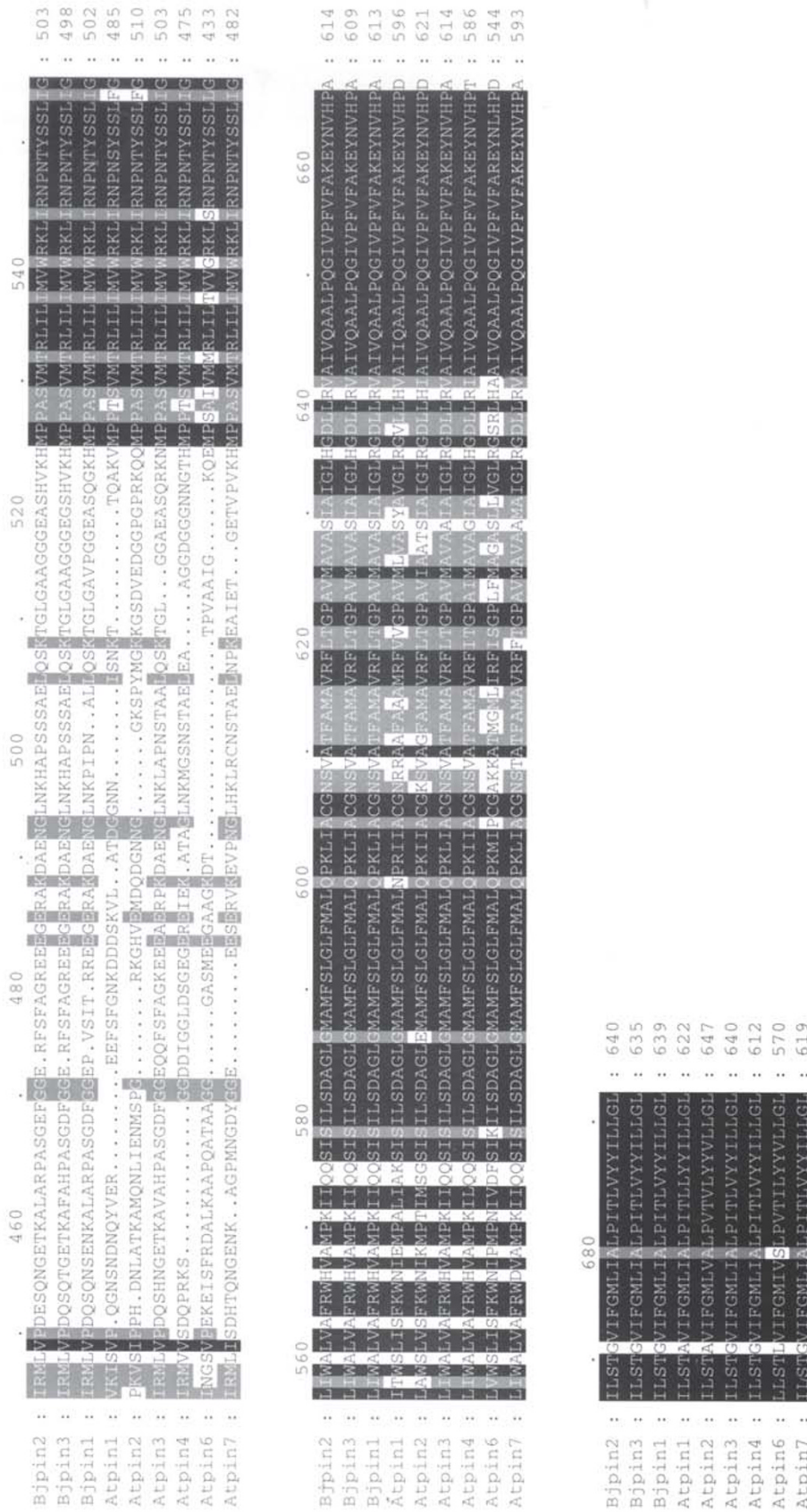


Fig 1. Alignment of BjPIN2 and BjPIN3 with other PIN proteins. Numbers on the top indicate amino acid positions. Accession numbers of cDNAs were as follows: BjPIN1, AJ132363; AtPIN1, AF089084; AtPIN2, AF086906; AtPIN3, AF087818; AtPIN4, AF087016; AtPIN6, AF087819; AtPIN7, AF087820.

Isolation of promoter regions of Bjpin-X and Bjpin-Z, which encoded other two isoforms of pin family from B. juncea

Two promoter fragments, *Bjpin-X* and *Bjpin-Z*, were isolated through genome walking technique based on the cDNA sequence of *Bjpin1*. After amplification using primers within adaptor and specific primers designed at 5' -coding region of *Bjpin1*, two DNA fragments, 1.2 kb (*Bjpin-X*, Fig 2A) and 0.7 kb (*Bjpin-Z*, Fig 2B), respectively, were amplified and sequenced. Comparison analyses showed that the coded peptides by *Bjpin-X* and *Bjpin-Z*, as well as their 5'- untranslated regions shared high homology with isolated *Bjpin1*, *Bjpin2* and *Bjpin3* but still with differences, which indicated that *Bjpin-X* and *Bjpin-Z* were promoter regions of other 2 pin genes from *B. juncea* than *Bjpin1*, *Bjpin2* and *Bjpin3*. Sequence analyses further revealed the presence of basic elements "TAAT" and "CAAT", and of particular interests, two pollen specific elements "AGAAA" in *Bjpin-Z* (Fig 2B).

Expression pattern analyses of Bjpin-X and Bjpin-Z

Both promoter regions of *Bjpin-X* and *Bjpin-Z* were subcloned into binary vector pBI101.3, and the resulted *Bjpin-X*::GUS and *Bjpin-Z*::GUS constructs were used for agrobacterium-mediated tobacco transformation. Confirmation of resistant plants via PCR amplification indicated that both *Bjpin-X*::GUS and *Bjpin-Z*::GUS had been integrated into tobacco genome (Fig 3C). At least 10 independent transgenic tobacco plants were used for GUS activity analyses. GUS activities, driven by *Bjpin-X*, were detected at xylem region (Fig 3D, E) and petal (Fig 3F); while those driven by *Bjpin-Z* were detected in mature anther and pollen (Fig 3G), leaf vein (Fig 3H, I), epidermis, cortex of stem (Fig 3J), and vascular tissues (Fig 3J, K).

DISCUSSION

Till now, at least 8 different *pin-like* genes have been detected in *Arabidopsis* genome and a few homologous genes in rice as well. Presence of multi-isoform auxin carriers, based on the extensive functions of auxin, suggest that some pin genes might share functional redundancy, yet each member of the pin gene family has its distinct functions. On

the basis of distinct expression pattern and functions of AtPIN1 and AtPIN2/EIR1/AGR1 in the shoot and root, respectively[6-9], it is proposed that the tissue- or cell-specific auxin efflux carriers are responsible for multiple auxin transport pathways[19]. Presence of at least 5 pin-like genes in *B. juncea*, as demonstrated by our studies, indicated a pin-gene family in *B. juncea*. Different expression patterns of the pin-like genes in *B. juncea* suggest their different roles during developmental processes. Isolation and expression pattern analysis of pin genes will certainly provide hints on their roles during plant growth and development.

Bjpin2 and *Bjpin3* were relatively high expressed in stem, which, however, suggests that both proteins might participate in the polar auxin transport in stem and play a specific role in stem or vascular tissue development. Recent studies have shown that AtPIN3 was expressed predominantly in endodermal cells with a lateral localization[20]. Since the isolated BjPIN proteins shows highest identity with AtPIN3, it is reasonable to assume that *Bjpin2* and *Bjpin3* might have a similar expression pattern with AtPIN3 in stem, and may play a role in growth that require lateral auxin distribution, such as shade avoidance[21]. Different expression patterns of *Bjpin2* and *Bjpin3* to isolated *Bjpin1*[14] and *Arabidopsis* *Atpin1* and *Atpin2*, together with the difference between themselves, i.e. presence of *Bjpin2* in leaf, floral tissues and stem while *Bjpin3* was relative specific in stem and hypocotyls, also indicated that they might have their distinct functions.

Different expression patterns of *Bjpin-X*, *Bjpin-Z* and isolated *Bjpin1*[14] at xylem (Fig 3E, K, L) indicated their specific roles during vascular development. Further, plant tropism growth was due to the accumulation of auxin at one side epidermis and cortex of stem (response to the stimulations) resulting in the affection of cell growth. Expression of *Bjpin-X* in both epidermis and cortex of stem, *Bjpin-Z* only at partial epidermis and cortex of stem, and isolated *Bjpin1* at epidermis and cortex around stem indicated that *Bjpin-Z* might be involved in the unsymmetrical growth of stem by environmental stimulation such as light and gravity.

1A

CGTAATACGACTCACTATAGGGAGAAGCTTATTCCATGGTGCAGCCTTACACA
 TGTTTTGAAAATTTGTGCCTCCATCACATAACACCTTTAGATCACTCATCTCT
 TCGTATGGCAGCTTGTACCAAATTCGTTGTTTGTACATATCCACTATTTTCAGC
 CGAAATAAGGTTACTAACAGACTGGGAGCCGCAGATTGCAAATAATGGCTTT
 TCCCTCTACATAGCAAGTTGTTCCCTTCTTTTAGCTCAAACCTCCCTCATAG
 TTTACAATGAAAGTGACGTGCATATCACCCTGAAACATTACAAAACAACATC
 AGTTTGACACAGGAACACAAAAAAACTTCATTGACACATTTAATCGAACAC
 CTTATGTGCATTATATTTTTTTTCCAACTTTTTTCCAATTAATCTCAATTATTC
 TTGTCCCAACACTCTAATAGCGTCACTTTAACATTCTTAACAAGAATTCCCA
 ACTCATAAACCGCGAACCTTATTTATATGAGTTTCATACCCATTTCTCTGTT
 GGTGATTTAGAAAGAGAGGTACCTCATCTCGTAGATCTCAAATCGATGTCCT
 CTCCTCTGATTATATCTTTTGCTTTCAATTTCTTCTAATTATATCTTCTGATT
 ACTTCCCAAAGTTTCTTCTGCTCTTGCATGATCTTCGTCCCAACAACCTTAG
 AGAATCTTCGAGTGTGGAAAGGTAAGAGAAACAATTTTTTTTTAACTTTGTT
 GAGAAACGACGTCGTCTAGTTTAAGGGTTTATTATATTTTTTTAAACAATAA
 AGTGACAAAATATTTAAGAAAAGCCACATAATCAATTCAAATGAACAGCTCA
 GCTTCCGTCACGGCTGAATAACGGAAAATGGGTTTATGTATGATAAGACTATT
 GTTTGTGTGTTGGTGTATGAAATTTAAATAAATGATGTGTTGGTGTATGTTTT
 CGCACAGCTAACGTGTTGATGTACTGATCGGGACCGCAGTCTCTGTGATGT
 ATGAATTAGCCGATTTCCCTCTTCTTACATACCCCAACACCGTTTACAACTTT
 ACCCTCTATATTCCACCGTAAATAAGATG▶

1B

GAATTATTATTTTTATTATTGTCTAGGGTACTTATATAAATACTGAAATTCGT
 CATTTAATCATTATACGAAAATGGATTAGTTAGAGAATGTAAAAGAAAAGGTG
 ATGTGAACTTATACCAAAGCAAAGAGGAGGGACAGAAGAGAAAAGGTAT
 GTCTGCGATGCCACATGTAGACCTTCTTTTAAAGATCTAACTCTATTTTTGCA
 CATGTAAACTATTGCAGACTACTTCTGTTTTACATAAGTGTCCCCAGATAT
 ATTTTCATCGGCAAGTTTATTTGTATGCTAATTTATTCTTGTATTATAACTA
 TACCCTAAAATATTCCATTTATTTACAAATTAATTTTTTGGTCAAATTAGCTA
 TATAGTCATTTAACCAAACAAATGAAACACTATAGTTACTTACCAAATGACCC
 AATACACGCGGTATGGTTATTCTACGAGAAGGACAATGACCCTGTTAAATACG
 ATACTCAATTTTCAAAGAATAAGAAAATAAATAAATGAATGAGTAAAAAGA
 AACTTTGGGTTGTCAAGTATTTGCTGTATAAAAAGAGGTGTAAGGTGGCTATC
 ACTCCTTAAATTATATCCTCAGAGCCAAGGTTGCTCACTCCTTCCACACATGC
 TCCAAGTCCCGCCTTCTTCTTCTTTCAGTCTCCTTTACATACCTCCACACC
 ATTGCTTAACGTTTTTCTCTCTAATCAACCAGAAAGAAGATG▶

Fig 2. Promoter regions of Bpin-X and Bpin-Z

A: Bpin-X; B: Bpin-Z. **ATG**: translation start site; CAAT and TAAT: basic elements of promoter region; **AGAAA**: element for pollen-specific expression.

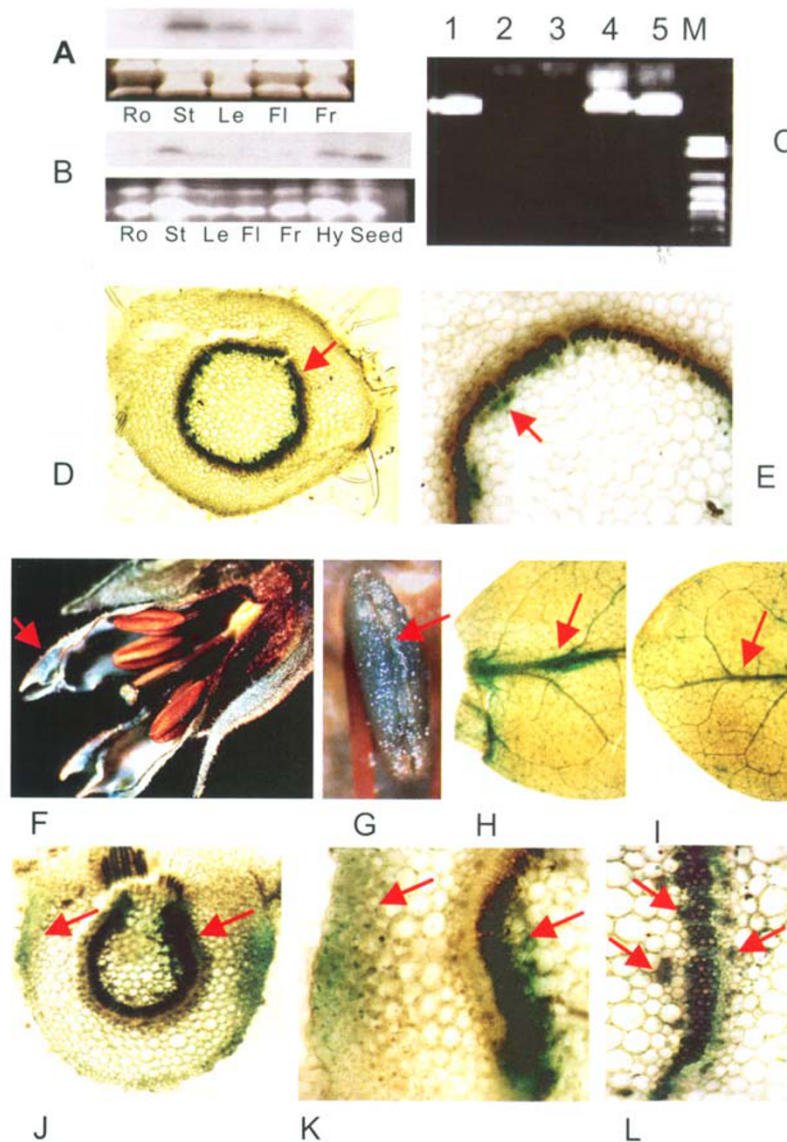


Fig 3. Expression pattern analyses of Bjpın genes

Northern blot analysis of Bjpın2 and Bjpın3 transcription levels. A: Bjpın2; B: Bjpın3. Plant tissues were as follows: Ro-root; St-stem; Le-leaf; Fl-flower; Fr-fruit; Hy-hypocotyl; seed. Equal amounts of RNA (shown in bottom lines) were used for hybridization. C: Confirmation of transgenic tobacco plants harbouring Bjpın-Z::GUS construct via PCR. Transformed vector (Lane 1), genomic DNA of untransformed (Lane 2) and transformed plants (Lane 3-5) were used as templates for PCR. M: DNA ladder.

GUS expression detection in transgenic tobacco plants. GUS activities driven by Bjpın-X were detected in stem (D), vascular tissues (E) and petal (F); those driven by Bjpın-Z were detected in anther (G), leaf vein (H, I), epidermis and cortex of stem (J), stem vascular tissues (K); and driven by Bjpın1 were in stem vascular tissues (L). Arrows showed the positions of GUS expressions.

Expression of *Bjpın-Z* in anther and mature pollen indicated its role in reproductive growth. The relatively high level of auxin in plant anther is critical for anther development, while source of auxin, i. e. the biosynthesis site and transport in anther is still unclear. Plant floral tissues originate from leaf

and auxin can be synthesized at edge of young leaf then transported to stem through vascular tissues [22],[23]. Recent studies showed that stigma may synthesize auxin and the auxin can be transported to floral base through polar transport resulting in the auxin gradient from stigma to receptacle and

controlling of morphogenesis of pistil[24]. Expression of *Bjpin-Z* in anther may suggest the auxin synthesis capacity of pollen, even the role of *Bjpin-Z* during pollen germination and fertilization processes.

The differences of the expression patterns of *Bjpin-Z* and *Bjpin1* in leaves, i.e. *Bjpin-Z* was restricted at vascular tissues while *Bjpin1* was mostly at edge of leaves, suggested their different roles in leaf vascular development. Further expression of *Bjpin1* at leaf trace indicated its role in auxin transport from leaf to stem.

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