Raising anti-PINl polyclonal antibodies for pea

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Introduction

Auxin is a plant hormone and a morphogen and is transported in a polar manner, which regulates organ development in plants. Polar auxin transport establishes a concentration gradient that differentiating cells can perceive and respond to in an appropriate manner depending on their status (1, 2). An auxin gradient has been implicated in directing patterns of morphogenesis in the developing leaf primordium in pea (3, 4), cambial zone of *Pinus* (5), in root organization (6, 7), in the embryo (6, 8, 9) and in the gynoecium (10) of *Arabidopsis.*

Results of recent genetic and biochemical studies in *Arabidopsis* have revealed many aspects of auxin transport. Auxin efflux carriers, the PIN proteins, targeted to specific cell membranes (plasma membranes or ER) bear similarities to amino acid transporters and are able to transport auxin (11). Multiple auxin efflux carriers are specific to organ, tissue or cell types, providing the potential for variable auxin responses (2). PIN1 is the most abundant protein in the gene family.

The recent availability of techniques to localize PIN proteins has provided plant biologists opportunities to visualize routes of auxin transport in developmental processes in *Arabidopsis.* For example, PIN1 is present in the basal membrane of xylem parenchyma and cambial cells in the inflorescence (6, 12). Localization PIN1-4 in the root tips has confirmed the predicted pattern of acropetal auxin transport through the stele to the quiescent center and root cap initial cells, as well as, basipetal transport away from it in the outer cortex/epidermis (13). And changes in PIN1 localization and concentration on the *Arabidopsis* inflorescence apex predict sites of floral meristem initiation (14). The two techniques available for localizing PIN proteins in *Arabidopsis* are (i) raising gene-specific antibodies and doing standard immunolocalization (6, 12, 15) and (ii) transforming plants with a construct expressing a GFP fusion protein under the control of a PIN promoter (14, 16).

Transformation with a visible fusion protein has distinct advantages in *Arabidopsis* since the plants are small and fluorescence is easy to detect and because this process allows real-time observations of changes in localization. However, peas are difficult to transform, plants are large with many cell layers making GFP detection problematic. To provide tools for studying PIN protein localization in pea we decided to make antibodies to its PIN1 protein. This was possible because we previously cloned the gene (17). our goal was to obtain antibodies that could be used for immunolocalization of PIN1 and allow studies to be done in any pea genotype.

Materials and Methods and Results

Expression Vectors

The predicted amino acid sequence of the PSPIN1 coding sequence (AY222857) has the three-domain topology typical of all PIN proteins: 5 N- and 5 C-terminal transmembrane domains and a central hydrophilic region (17). The gene specific, central region is approximately between amino acids 150 and 450. To increase the possibility of success, we designed PCR primers to amplify two products within the central region (aa 203-404 and 155-404) with BamHI and SacI restriction enzyme sites and ligated these products into the pET-28c plasmid (Novagen) using standard protocols. DNA

sequencing was done to confirm that the sequence was in frame. This plasmid was transformed into the expression bacterium Rosetta (DE3) (Invitrogen) using heat shock (18). Mini plasmid preps were done to confirm insert size.

Peptide Expression

LB cultures (5 ml) with 50 |J,M kanamycin and 50 |J,M chloramphenicol were grown overnight at 37° C for each peptide. Afterwards a 1.25 ml inoculation was done into 75 ml of LB (also with antibiotics) and grown until the OD at 600 nm was approximately 0.5, at which point 35 mls were placed in a 50 ml sterile centrifuge tube (uninduced) and 40 |J,l of 1M IPTG were added to induce expression in the remaining culture. These bacteria were returned to the incubator. After 4 hours the tubes were placed in a sterile 50 ml centrifuge tube and spun at 5000 rpm in a Sorval RC-5 centrifuge at 4° C for 10 min. The supernatant was discarded and the cell pellets were frozen in a -80° freezer. Uninduced cultures were also spun down and frozen. Pellets were resuspended, lysed and proteins were extracted using urea and histidine tagged proteins were purified using the Qiagen Ni-NTA Spin Kit protocols.

Protein concentrations of uninduced, induced, soluble and insoluble bacterial total protein and column-purified (elution 1 and 2) preparations were obtained using the Peterson method (19). Protein samples were prepared for and fractionated on 12.5% polyacrylamide mini gels (Hofer Scientific), and stained with Coomassie blue using previously published protocols (20). Distinct bands of the appropriate size for each peptide were obtained in the induced soluble (Fig. 1A, *) as well as, insoluble bacterial protein fractions, and which were not evident in the uninduced protein factions. The induced peptide was the dominant polypeptide in the two column purified fractions (Fig. 1B). These peptides were further purified by fractionation on a 1.5 mm thick preparative, 12.5% polyacrylamide mini gel. The gel was lightly stained with aqueous Coomassie blue, the band was cut out with a razor blade, destained in water and frozen.

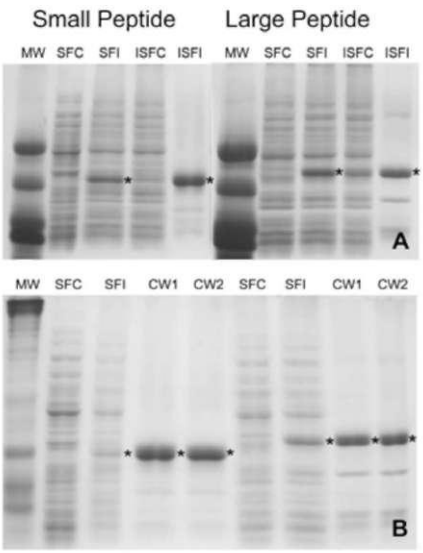

Figure 1. Polyacrylamide gels stained with Coomassie Blue showing polypeptide profiles of the soluble fraction (SF), insoluble fraction (IS) of control (C), induced (I) and first and second Ni column washes (CW1, CW2) fractions of lysed bacteria expressing the small or large peptides of PIN1 from pea MW = molecular weight markers, * = induced PIN1 peptides.

Antibody production and testing

The acrylamide samples were sent to Cocalico Biologicals, Inc. for antibody preparation in rabbits. Two rabbits were used for each peptide. Rabbits were inoculated with a mixture of Freund's adjuvant and peptide for each purified peptide produced. Preimmune serum was obtained from each rabbit beforehand. Three boosts were done, 14, 21 and 49 days after the initial inoculation. Test bleeds were provided for dot blot analysis. Since these were successful after the third boost, the rabbits were exsanguinated and serum was frozen, shipped and stored.

Protein was extracted from shoot tips of one month old *WT* (W6 22593), *tl* (W6 22594), *af* (W6 22597), *aftl* (W6 22598), *uni-tac* (W6 27606) and *uni* (W6 15302) plants using the same protocols as

for the bacteria and prepared for and fractionated on 12.5% polyacrylamine gels as done previously. Proteins were transferred to Immobilon-P membranes (Millipore) using the Hofer TE 22 Mighty Small tank transfer unit and recommended procedures (21). Membranes were air-dried and stored in ziplock bags in the refrigerator until use. For western blotting, membranes were wetted briefly in methanol and blocked in 5% powdered milk in TBS for 2 hrs, treated with a 1:1000 dilution of crude antiserum in TBS for 2 hrs, washed and treated with goat anti-rabbit

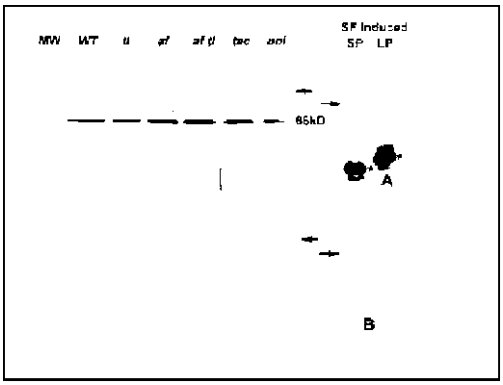

*Figure 2. Western blots of serum treated (A) or pre-immune serum treated (B) samples. Samples are protein extracted from shoot tips of various pea genotypes (10 fig protein loaded) or soluble fraction (SF) of small peptide (SP) or large peptide (LP)(1 fjg protein loaded). The LP control was not done in B. Arrows indicate bands present on both blots, *indicates the expressed small or large*

IgG conjugated to alkaline phosphatase (Sigma) at a 1:5000 dilution in TBS for 2 hrs., rinsed and stained with BCIP/NTB until bands were visible. Total induced bacterial proteins were used as a positive control. No negative control was possible since there are no known *PIN1* mutants of pea. All shoot tips showed a similar major band at the expected size of 66 kD and several minor bands (Fig. 2A). The positive bacterial protein controls also showed staining as expected for each peptide (Fig. 2A,*). Preimmune serum recognized no bands of the estimated size but a faint high molecular weight peptide in the plant extracts and another in the bacterial extract that were also present on the antiserum treated blot (Figs. 2A,B, arrows). Serum from three of the four rabbits recognized the same peptides (data not shown). The high molecular weight band present in the plant extracts was of low abundance and was due to non-specific binding. These results suggested that we should continue to use the anti-serum to attempt immunolocalization.

Immunolocalization

After testing many variations on fixation, embedding and localization procedures, an effective protocol was selected. Shoot and root tips of *WT* pea plants (1 mo old and 1 week old respectively) were fixed in FAA (4% formaldehyde, 5% acetic acid and 50% ethanol), dehydrated through an ethanol series, then a xylene or citrisolve series and embedded in Paraplast Plus (Oxford Labware). Serial sections were cut at 8 thickness on an A/O 820 rotary microtome and adhered to glass slides without adhesive. Slides were deparaffinized in xylene or citrisolve, then in 1:1 ethanol/xylene or citrisolve and rehydrated to water through an ethanol series. Slides were blocked in 5% powdered milk in PBS for 1 hr., washed in PBS with 0.05% tween-20, then in 1% BSA in PBS before treating in a 1/30 dilution of crude serum (or preimmune serum) in PBS for 2 hrs. Three washes with PBS with 0.05% tween-20 were done before secondary antibody treatment. The secondary antibody was goat anti-rabbit IgG conjugated to Alexa Fluor (Invitrogen) and used at a 1/100 dilution for 1 hr. Slides were then washed with PBS and mounted in 50% glycerol for viewing. Sections were viewed on a Zeiss Axioskop fluorescence microscope with an FITC filter set and photographed using a SPOT digital camera (Diagnostic Instruments).

Localization was successful for all plant parts tested. Serum from the three rabbits that recognized polypeptides in the gel fractionations showed similar staining patterns on sectioned material. No significant fluorescence was present on sections treated with preimmune serum instead of antiserum (data not shown). In these preparations, only starch grains showed nonspecific binding to the antibodies. Non-specific binding to carbohydrate, such as cell walls and starch grains is a common artifact in immunolocalization procedures (22). At low magnification, the general intensity of

immunostaining was greatest in the tips of leaf primordia (Figs. 3A,D), pinna primordia (Figs. 3A,B), axillary buds (Fig. 3A), stipule primordium tips (Fig. 3B), inflorescence meristem (Fig. G) and in procambial strands in both shoots (Figs. 3A,B,D) and root tips (Fig. 3C). At high magnification, fluorescence was mainly associated with membranes (Fig. 3F) and asymmetrically associated with procambial strand cell membranes in young growing shoots (Fig. 3E) and roots (Fig. 3H). In the tips of leaf primordia, the strongest fluorescence is present inside the protodermal layer (Fig. 3D). In root tips, the most significant staining was present in the developing stele. In this region of the root tip of 1 wk old seedlings, the staining was present on the upper (proximal) cell membrane, indicating that auxin was being predominantly transferred away from the root meristem upwards through the center.

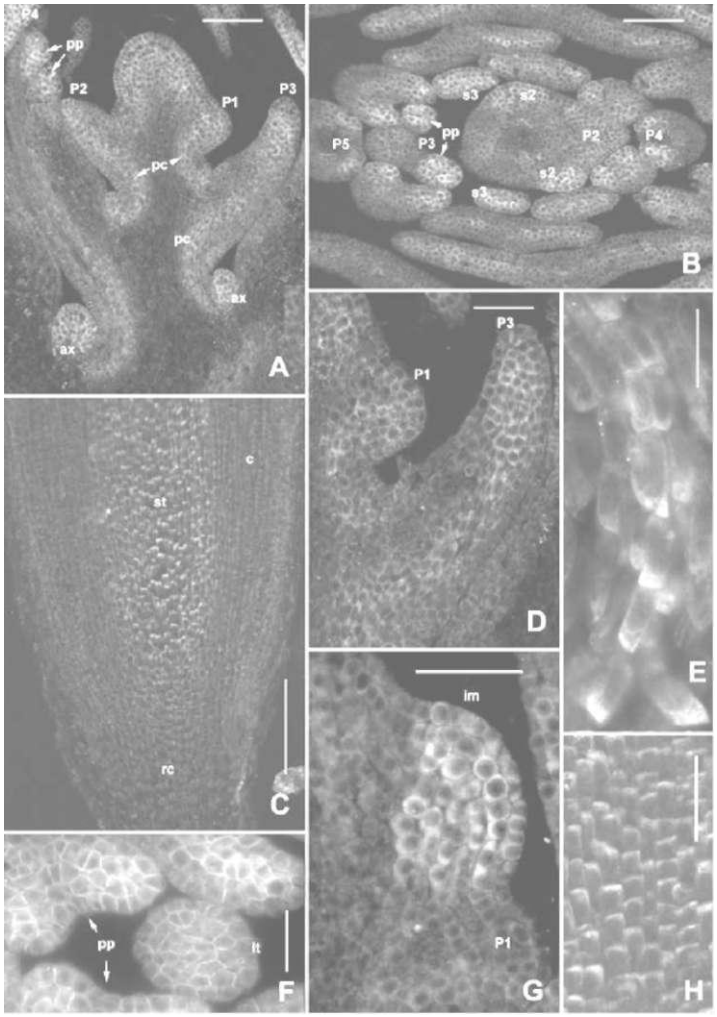

Figure 3. Immunolocalization on sectioned shoot and root tips of pea. A. Median longitudinal section through the shoot tip. B. Transverse section through a shoot tip through the node of second youngest leaf primordium (P2). C. Longitudinal section through a root tip. D. Longitudinal section through a shoot tip showing the youngest (P1) and third youngest leaf primordium (P3). E. Longitudinal section through a procambial strand at the base of a leaf primordium. F. Transverse section through a shoot tip showing a section through a leaf primordium tip (P3) and pinna primordia associated with an older leaf primordium (P5). G. Longitudinal section through an initiating inflorescence meristem in the axil of a P1 leaf primordium. H. Longitudinal section through a root primordium showing staining in the developing stele. ax- axilliary bud, c-cortex, im-inflorescence meristem, lt-leaf primordium tip, cp-procambial strand, pp-pinna primordium, s2-3-stipules of leaf primordia P2 and P3. Scale bars = 100 fm in A,B,C; 50fm in D,G; 20

Discussion

Antibodies are useful tools for many laboratory procedures including immunoblotting, immunoaffinity purification, immunoassays and immunolocalizations of important cellular proteins. The use of antibodies combined with the auxin reporter *DR5::GUS* has greatly facilitated the study of auxin-directed development in *Arabidopsis.* Until recently, these tools have not been available for pea or other legumes. *DR5::GUS* peas are now available (23) and in this publication we report the successful production of antibodies raised against the PIN1 protein of pea.

Our main objective for raising anti-PIN antibodies was to use them in immunolocalization studies. Peptides expressed in bacteria (non-eukaryotes) and purified from urea and SDS treated purification procedures often do not possess the same epitopes present in fixed, dehydrated, paraffin-embedded and sectioned material of the native protein. The standard methodologies for testing newly made antibodies is to test them with western blotting using the same treatments used for purification of the peptide and then to test them in localization procedures. Typically, a much more concentrated serum preparation is needed for immunolocalization than for immunoblotting. These need to be determined empirically.

Based on recognition of polypeptides of the appropriate size in our western blots of both bacterial protein fractions and protein extracted from pea tissues, we were successful in raising polyclonal antibodies to the expressed peptide of PSPIN1. Some staining was present in minor higher molecular weight bands, which is probably due to cross-reactivity with other members of the PIN family. Further, preimmune serum treated blots were mainly blank. Faint staining of a minor, high molecular weight band in plant preparations is likely due to non-specific binding.

Our criteria for determining specific immunolocalization were (i) to compare with a pre-immune serum control, (ii) to compare the localization to that of published reports for *Arabidopsis* PIN1 localization and *(iii)* to look for abundance in areas of presumed high auxin content/response, since both transcription and translation of PIN is regulated by auxin (14, 17, 24). Our immunolocalization procedures produced specific and reproducible patterns of localization which were not present in the preimmune serum controls. Strong signal occurred in most regions where GUS is expressed in *DR5::GUS* plants, including tips of leaf primordia, axillary buds, procambial strands and tips of stipule and pinna primordia on developing leaves. These are all presumably regions of high auxin concentration. Further, PIN1 is known to be localized to procambial strands in leaves (16), in membranes within meristems and initiating lateral primordia (1, 14) and asymmetrically in membranes of procambial strands in shoots and roots (1, 2, 16) in *Arabidopsis* and we see the same localizations in pea with our antibodies. One difference in localization involves the position of membrane localization in the developing stele of the root. In *Arabidopsis* PIN1 is specifically localized to the basal (distal) membrane, whereas it is in the apical (proximal) membrane in pea roots, which suggests that auxin transport is in opposite directions in the central stele of these two species. Basipetal transport of auxin from pea root tips has been physiologically demonstrated recently (25). Therefore, the role of auxin in root apex development may be variable in different angiosperms and as more work is done on "nonmodel" species a true assessment of how much variability there is may be realized.

In conclusion, we have successfully raised polyclonal antibodies to the pea PIN1 protein, which can be used in future studies of plant development for pea and possibly other legume species. A limited amount of immune serum will be distributed upon request.

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