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Arabidopsis thaliana AXR4 Recombinant Protein Expression for Polyclonal Antibody Production

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ABSTRACT

The *Arabidopsis AXR4* gene product is involved in polar targeting of AUX1 an auxin influx carrier, to the plasma membrane [1]. The AXR4 gene encodes a protein of 473 amino acids and is predicted as a Type II, integral membrane protein with a short N terminal sequence, a single transmembrane region and a large C terminal domain. The C terminus domain of AtAXR4 referred to as AtAXR4 C, was expressed as a recombinant protein in Escherichia coli and used as an antigen to generate polyclonal antibody. The antibody recognized a protein of approximately 54 kDa in the membrane fractions prepared from Arabidopsis thaliana. The availability of anti-AXR4 antibody will contribute towards elucidation of AXR4 biochemical properties and its molecular function.

INTRODUCTION

The auxin influx carrier, AUX1 polar targeting to the plasma membrane is dependent on the AXR4 gene product [2]. The axr4 mutant was discovered by the Estelle group when screening for mutants with altered root responses to auxin that were resistant to 2,4 D [3]. The axr4 mutation is recessive and was mapped to 750 kb region of chromosome 1. The AXR4 gene was cloned by the Estelle lab [1] based on microarray comparison of wild type and axr4 which showed strong reduced expression of a candidate gene in the region of chromosome 1 of the *Arabidopsis* genome.

The AXR4 gene encodes a protein of 473 amino acids and is predicted as a Type II, integral membrane protein [4] with a short N terminal sequence, a single transmembrane region and a large C terminal domain. Molecular characterization of the AXR4 gene sequence shows that there is only a single copy of the sequence in the *Arabidopsis*, *Oryza sativa* and *Medicago trunculata* genome, suggesting it to be unique and conserved among higher plants [1]. Two main functional motifs have been identified in the AXR4 protein. They are the single membrane spanning domain and a weakly conserved α-ß hydrolase fold which is found in several classes of enzymes including lipid hydrolase/transferases [5].

The molecular mechanisms of AXR4 function still remains unclear and needs to be further characterized. Polyclonal antibodies are routinely generated and used in a variety of assays such as ELISA, Western Blot and radioimmunoassay [6]. Therefore, it was necessary to produce an anti AXR4 antibody for AXR4 immunodetection, biochemistry, topology and protein-protein interaction experiments.

In this study, the AXR4 C-terminal domain that represents amino acids 71-473 was expressed in an E. *coli* system to over express the recombinant AXR4 protein that was used to produce polyclonal AXR4 antisera. The described work provides key reagents for studying the biochemical properties and cellular functions of the AXR4 protein.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana plants were grown in a growth room. Leaves from 4 week old plants were collected, frozen in liquid nitrogen and stored at -80ºC until further use.

Cloning of the AXR4 C terminal cDNA
Primers were designed for amplification of the AXR4

AXR4 forward: 5' GG GTC GAC TCA CCA CAC TTC TCT CAC 3'
AXR4 reverse: 5' GG CTG CAG AGT AGT AGT CCA TTC CTC ACC AAG 3'

The gene was isolated by PCR amplification using *Arabidopsis thaliana* Columbia cDNA as the template and Pfx DNA polymerase. Cycle parameters for PCR included an initial incubation time of 3 min at 95°C, 30 cycles of 30 sec at 94°C, 1 min at 50°C and 1 min at 72°C, followed by a final elongation of 10 min at 72°C. Amplified AXR4 C-terminal domain was analyzed by agarose gel electrophoresis, then digested with *Bgl*II enzyme, an internal AXR4 restriction site and *Sal*1 restriction enzyme site at the 3' end. The digested products were subsequently cloned into pSP73 vectors and single clones. The C-terminal AXR4 was later subcloned into pET28a expression vector. Colony PCR, double digestion and sequencing analysis were carried out again for confirmation.

Expression of C Termini Domain of AXR4 in BL21 Cells
BL21 (DE3)[Novagen] competent cells were transformed with recombinant E. *coli* pET28a-C-terminal AXR4 plasmid. The transformed bacteria were selected on LB agar plates containing the appropriate antibiotic. A single colony of the transformed bacteria was inoculated in 5.0 ml LB medium containing the appropriate antibiotic for cultivation at 37°C overnight. Aliquots of the culture were inoculated into 50 ml LB medium with the appropriate antibiotic at 37°C until the OD_{600} reached 0.5. The cultures were then split into six 50 ml Falcon tubes containing 8.0 ml LB and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM or 0.1 mM to induce the expression. For each IPTG concentration, the expression was carried out for three hours at 37°C, for four hours at 25°C and overnight at 20°C. Bacteria was harvested afterwards by centrifugation at 2500 g for 15 minutes. For the purification of the recombinant protein, the bacterial pellet was resuspended in 20 mM Tris-HCl, pH 8.0, and lysed with a sonicator. The soluble and insoluble fractions of the lysate from each expression were analysed by SDS-PAGE.

***E. coli* Cell Disruption**
E. coli cell disruptions were achieved through sonications at 5 to 10 seconds bursts on ice. For cell pellet from large scale culture, a French press was employed. Cells were passed twice through a pressure of 20,000 psi.

Analysis of the Recombinant Proteins
To analyse the recombinant proteins, 10 µg of the soluble and insoluble fractions of the expressed purified proteins were mixed with an equal volume of SDS-PAGE loading buffer containing β-mercaptoethanol and boiled at 80°C for 5 minutes. The denatured proteins were resolved on a 12% SDS-PAGE and visualized by staining in Coomassie brilliant blue solution [7].

Purification from Inclusion Bodies
Following induction of expression by optimized conditions, the E. *coli* cells were spun down at 30, 000 g for 20 minutes and resuspended in 20 ml lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 20% glycerol and 1.0 µM pepstatin, 1.0 µM leupeptin, 2 ug/ml apronitin and 1.0 mM 4({-aminoethyl}-benzensulphonylfluoride protease inhibitors). The suspension was disrupted by 2 passages through French press and then centrifuged to remove cell debris at 10,000g for 10 minutes.The inclusion body was washed three times with 20 ml of Lysis buffer (50 mM Tris pH 7.4 150 mM NaCl, 20% glycerol) containing 0.5% v/v Triton and spun down at 20 000 g for 15 minutes. After the final wash, the resulting pellet which consisted predominantly of inclusion bodies, were solubilized in 5.0 ml of 50 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl containing 8.0 M urea. Aliquots from each wash were then analysed on a 12% SDS-PAGE.

Detection of Antibodies Against C Termini Domain of AXR4

Serums were assayed for the presence of antibodies against the AXR4 antigen by Dot Blot and Western Blotting analysis. To determine the titer to be used for the Western lot analysis, 10 µg of purified AXR4 recombinant samples were spotted on nitrocellulose membrane and cut into individual strips. Different concentration of anti-AXR4 antisera were then tested on each individual strip. Five dilutions of antisera were tested; 1:500, 1:1000, 1:2000, 1:5000 and 1: 10,000. The strips were washed and incubated in anti rabbit-HRP as the secondary antibody.

To test the antibody specificity, total, soluble and microsomal fractions were prepared from the wild type *Arabidopsis* and a*xr4-Arabidopsis* root cultures. Root cultures of *Arabidopsis thaliana* were generated according to the modified protocol of [8]. Microsomal membrane fractions were prepared from the wild type and *axr4 Arabidopsis thaliana* mutant roots by the modified methods of Kjellbom and Larsson [9]. 20 µg of microsmomal protein from each samples were separated by 10 % SDS PAGE and transferred to nitrocellulose membrane before being probed using anti AXR4 antisera concentration that were optimized before. The strips were washed and incubated in anti rabbit-HRP as the secondary antibody. All immunoblot and membrane processing were performed using SuperSignal West Pico (Pierce, Rockford, IL) according to the manufactures instruction.

RESULTS AND DISCUSSION

Cloning of the AXR4 C Terminal cDNA
Polyclonal antibodies were routinely generated and used in variety of assays such as ELISA, Western Blot and radioimmunoassay [6]. In addition to having an AUX1 antibody, it was necessary to produce an anti AXR4 antibody for AXR4 immunodetection and topology experiments. The AXR4 C-terminal domain that represents amino acids 71-473 was expressed in an E. *coli* system to over express recombinant AXR4 protein that was used to produce polyclonal AXR4 antisera. The AXR4 C-terminal domain was chosen because topology studies predicted that the *AXR4* C terminal domain may project into the ER lumen. This domain may play important roles in the interaction with AUX1 based on allele data [1].

Primers were designed for amplification of the complete AXR4 cDNA with Sal1 restriction enzyme site at the 3'end to facilitate the cloning of the AXR4 gene. The amplified PCR product is shown in Figure 1. AXR4 C- terminal domain was

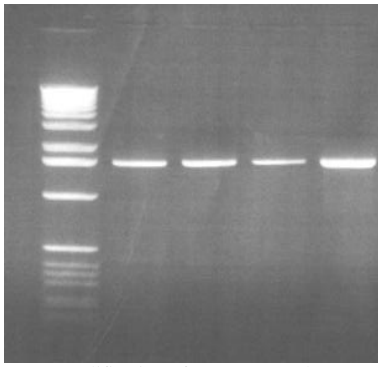

Figure 1: Amplification of *AXR4* cDNA by RT PCR

obtained through digestion of the full length AXR4 cDNA with *Bgl*II enzyme, an internal AXR4 restriction site and *Sal*1 restriction enzyme site at the 3' end primer and ligated overnight at 4°C into pSP73 plasmid (Promega) that was also digested with *Bgl*II and *Sal*I restriction enzyme. The construct was designated pSP73- C-terminal AXR4 and was transformed into E. *coli* DH5α competent cells. Colony PCR was carried out to detect clones with the AXR4 insert. The positive clones were grown on LB-ampicilin media, plasmid isolated using QIAprep Spin Plasmid Miniprep kit and further screened by digestion with *Bgl*II and *Spe*I to release the corresponding insert.

The C terminus of *AXR4* PCR fragment amplified is shown in lane 1-4 was derived from *Arabidopsis thaliana* Columbia cDNA as the template and using *Pfx* DNA polymerase to generate the PCR product. M represents the 1 kb marker. *AXR4* was cloned into pSP73 and the PSP73-AXR4 plasmid was digested with *Bgl*II and *Sal*I restriction enzymes and run on 1% agarose gel (Figure 2). The 1464 bp insert was gel purified and cloned into pET28a vectors. Positive clones were selected by PCR analysis and reconfirmed by restriction enzyme digestion analysis. Sequencing analysis confirmed that the C terminal *AXR4* cDNA was in frame with the coding sequence in the multiple cloning sites.

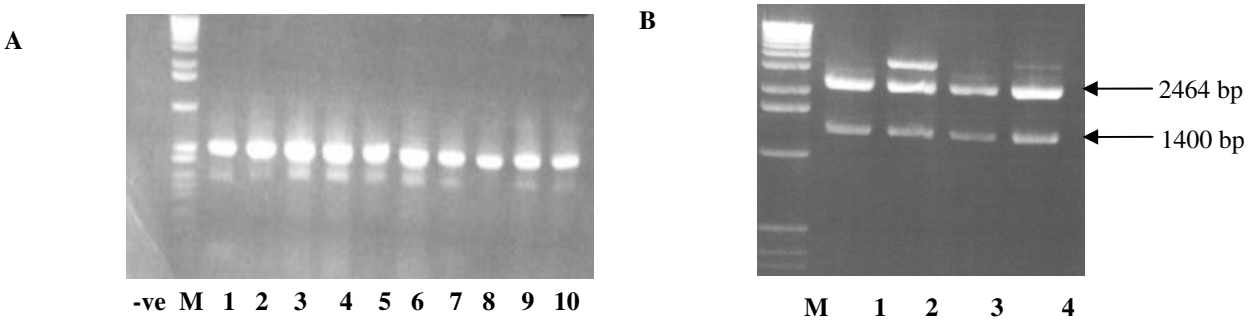

Figure 2: Representation of colony-PCR screening of pET28a *AXR4* colonies and digestion analysis of selected plasmids to confirm the presence of *AXR4* cDNA in the pET28a vector

A: Several putative clones of pET28a-AXR4 were analyzed by PCR colony to identify colonies containing the *AXR4* gene insertion in the pET28a vector. All 10 colonies picked gave positive band withs no detected band at the negative control (PCR mixture without plasmid). This indicated that all clones screened were successfully transformed with *AXR4* cDNA inserts.

B: Plasmids isolated from four independent putative positive colonies from the colony PCR screening (lanes 1-4) was digested with *Bgl*II and *Spe*I. The restriction digestion released the 2464 bp and 1400 bp bands as shown on a 1.0% agarose gel.

Recombinant Protein Expression

The C terminal domain of the *AXR4* gene was cloned into the pET28a vector which was under the control of IPTG inducible phage T7 or Tac promoter. The T7 and Tac promoters are well characterized promoters that produce high level but leaky expression [10]. Positive clones were transformed into E. *coli* BL21 λDE3 and the transformants were selected on LB agar plate containing appropriate antibiotics. E. *coli* BL21 λDE3 host cells provided the phage T7 RNA polymerase for expression of heterogonous genes [11]. Small scale experiments were carried put to obtain optimized conditions for recombinant protein expression. Single colony bacteria representing transformants from each constructs were inoculated into 5ml test tube culture and allowed to grow at 37°C in a shaker at 220 rpm until mid logarithmic phase (OD_{600} 0.5) before induction with IPTG at 0.5 mM final concentration. However, there was no observable difference in the solubility of the protein in pET28a (His tagged recombinant protein) using different temperature, different concentrations of IPTG and lengths of expression (data not shown).

Since the fusion proteins do not encode a secreted protein, cells harbouring the recombinant expression plasmids were disrupted either by sonication or french press and cell debris were removed through centrifugation. Analysis of the recombinant protein on a 12% SDS-PAGE confirmed the correct size of the C terminal *AXR4* fusion protein.

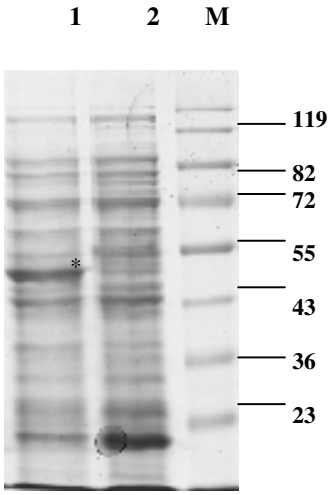

Figure 3: Recombinant C Terminal AXR4 protein expression in *E.coli*.

The C terminal domain of the *AXR4* gene were cloned in the pET28a vector and expressed in E.*coli.* 10 µg of lysate from each expression were separated on 12% SDS PAGE and stained by Coomassie staining. Asterisks indicate the expected recombinant protein. Lane M is protein marker, lane 1 is the expressed C terminal AXR4 protein and lane 2 is uninduced sample as negative control.

The C terminal AXR4 protein was not detected in uninduced E. *coli* BL21 λDE3 cells transformed with one of the constructs (Figure 3). From the expression analysis, insoluble protein or inclusion bodies were obtained in pET28a- AXR4 protein expression.

Inclusion Bodies Purification

Soluble and active proteins are often difficult to obtain by E. *coli* recombinant expression [12]. Instead, insoluble aggregates composed of a set of partially folded intermediates known as inclusion bodies normally occur when recombinant protein is expressed at high levels [13]. This was the case observed with AXR4 recombinant expression constructs in E. *coli*. After the optimization of the expression conditions, AXR4 recombinant proteins were not expressed in soluble form but in the form of inclusion bodies. The formation of AXR4 inclusion bodies may be due to the presence of insufficient chaperone or folding enzymes, lack of PTM enzymes or simply due to high recombinant protein concentration that leads to non-specific interactions [12].

AXR4 inclusion bodies were purified by repeated centrifugation and Triton-X 100 wash method. Triton-X100 washes were incorporated in the purification to reduce contaminants in order to increase yield and to refine the required recombinant AXR4 protein during the purification steps [14]. As the AXR4 inclusion bodies are very dense, they tend to sediment and can be separated easily from the lighter membrane components and soluble contaminants under the influence of a centrifugal force. Following the extensive washes, the purified AXR4 inclussion bodies were examined in 12% SDS PAGE as in Figure 4.

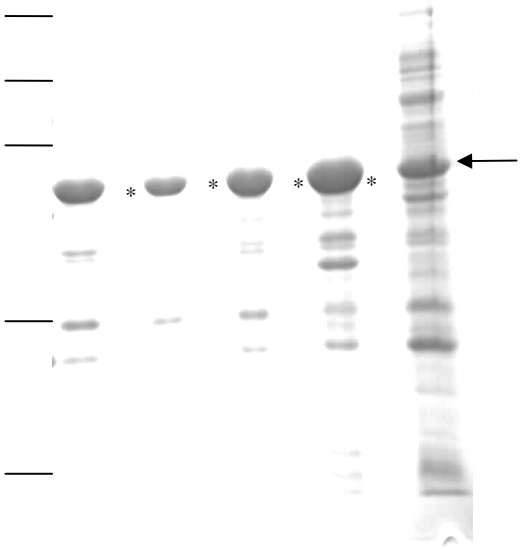

Figure 4: Inclusion bodies purification of Pet28a AXR4 His6 from BL21 λDE3.

Pet28a AXR4 HIS6 from BL21 λDE3 inclusion bodies were purified by four times centrifugation and 0.5% v/v Triton-X100 washes. Asterisks indicate the expected AXR4 protein. Arrow denotes the size of expected band. Lane M is the protein Marker, lane 1 is the 1st wash inclusion bodies, lane 2 is the 2nd wash inclusion bodies, lane 3 is the 3rd wash inclusion bodies, lane 4 is the 4th washes inclusion bodies and lane 5 is the total protein before the washes.

Solubilisation of the AXR4 inclusion bodies was achieved by using 8 M urea as denaturants. After treatment with urea, the solubilised AXR4 lysate was transferred to prepared dialysis tube with MWT cut off of 8.0 kDa and dialyzed against 500 ml PBS at 4°C for 16 hour. This process was carried out to remove the urea and to promote AXR4 refolding into its native and active conformation. AXR4 dialyzed material was cleared by centrifugation and the protein content in the samples were determined using the spectrophotometer. The samples were then sent to Genosphere Biotechnologies (Paris, France) to raise polyclonal antibodies against the E.*coli* recombinant AXR4 protein The inclusion bodies obtained from pET28 AXR4 expression were used to immunise AXR4 rabbits because of the high yield of protein obtained, the small tag (His6) attached to it and also the availability of technique to purify it from other contaminating protein.

AXR4 Polyclonal Antibody Analysis

The antisera obtained was first tested first using dot blot techniques to determine the titre to be used in western blottings. From the results of the titre of the anti-AXR4 antisera, it was

concluded that the 1:2000 dilution was the best dilution to be used in a Western blot experiment (data not shown). Polyclonal antibody raised against the C terminal domain of AXR4 was tested through immunoblotting techniques on the wild type *Arabidopsis* and a*xr4-2* microsomes protein to test its specificity and reliability. Western blot analysis detected a 55 kDa protein in Columbia microsomes which was not detected in *axr4* mutant microsomes (Figure 5). From the western analysis, it was concluded that the antisera generated was successful in detecting the AXR4 proteins in the wild type *Arabidopsis* membrane fractions but not in the *axr4-2* negative control membrane fractions or any soluble fractions which gave the evidence of the AXR4 protein as an integral membrane protein. Even though the background signal was detected, the antibodies generated are capable of detecting the AXR4 proteins and can be used for other analysis.

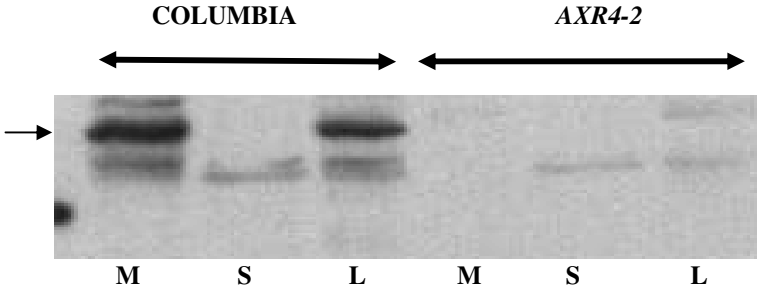

Figure 5: Western detection of AXR4 in Columbia (WT) and *axr4-2* microsomes. Columbia (WT) and *axr4-2* microsomes were isolated and separated on a 12% SDS-PAGE at 20 ug of protein per lane, then blotted onto PVDF membrane. The blot was probed with anti AXR4 antisera at 1: 2000 final dilutions. The *At*AXR4 protein is indicated by the arrow on the left. M represents microsomes; S represents supernatant and L represents lysate.

CONCLUSION

This paper describes recombinant AXR4 expression and purification in the *E. coli* system. C terminal AXR4 partial fragment was successfully cloned into pET28a expression vector and expressed in the expression host *E.coli* BL21. The protein was expressed as an insoluble protein. The insoluble protein was denatured using urea; semi purified by dialysis and was further used to obtain the antibodies against the AXR4 protein. The AXR4 antisera generated was successful in detecting the AXR4 proteins using Western Blot analysis.

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