Stable expression of maize auxin-binding protein in insect cell lines

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Abstract To achieve continuous expression of the major maize auxin-binding protein (ABP1) in insect cells, the ABP1 gene coding region was placed under control of a baculovirus immediate-early gene promoter and transfected into *Spodoptera frugiperda* Sf9 cells. The ABP1 gene was detected in twelve cell lines, one of which was selected for detailed analysis. Immunolocalisation demonstrated that ABP1 was targeted to and retained in the endoplasmic reticulum (ER), in accordance with its signal peptide and carboxy-terminal KDEL ER-retention signal. We discuss the advantages of stable-transformation over transient expression systems for characterising proteins targeted to the secretory system of insect cells.

Key words: Maize auxin-binding protein (ABP1); Stable expression; Retention signal sequence; Insect cell (Sf9); Immunolocalisation

Introduction

The targeting of proteins to specific organelles and for secretion is of fundamental importance for regulated growth and development of all cells. The machinery for retaining soluble proteins in the lumen of the endoplasmic reticulum (ER) is now well understood and the salvage receptor (the ERD2 protein) for KDEL and HDEL proteins has been identified and sequenced from a number of organisms including *Arabidopsis* [1]. One of the plant proteins targeted with KDEL is the major auxin-binding protein (ABP1) purified from maize membranes. This protein exists as a dimer of 22-kDa subunits, and has its predominant location in the lumen of the ER [2]. Biochemical and electrophysiological evidence suggests it is a good candidate for an auxin receptor, but for this a fraction of ABP1 must reach the plasma membrane [2].

In previous studies using the baculovirus expression system we have demonstrated that ABP1 is processed post-translationally in the endomembrane system of insect cells [3]. However, such studies require careful interpretation because baculovirus infection severely disrupts the host-cell and affects the integrity of the plasma membrane [4]. Stably transformed insect cell lines have been shown to offer significant advantages over transient expression using baculovirus vectors for the characterisation of neurotransmitter receptors by electrophysiological techniques [5]. Here we report expression of a plant protein, maize ABP1, in stably transformed insect cells. These retained the properties associated with non-infected cells, and so provide a system for studying trafficking of proteins in the secretory pathway of the insect cell.

2. Experimental

2.1 Construction of an expression plasmid carrying the maize ABP1 cDNA and production of stable insect cell lines

All DNA manipulations were carried out using standard techniques

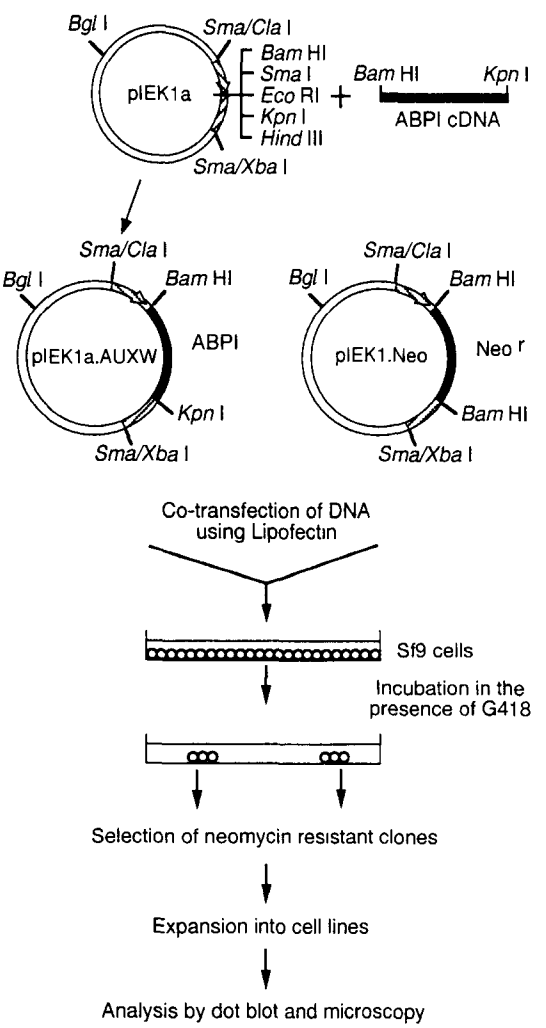

Fig. 1. Protocol for establishing stably-transformed insect cell lines expressing maize ABP1. A cDNA fragment encoding maize ABP1 was inserted between the *te-1* promoter and 5'-leader sequence (P) and terminator region (T) of plasmid pIEK1a. The resultant plasmid, pIEK1a.AUXW, was co-transfected with pIEK1 neo into Sf9 cells

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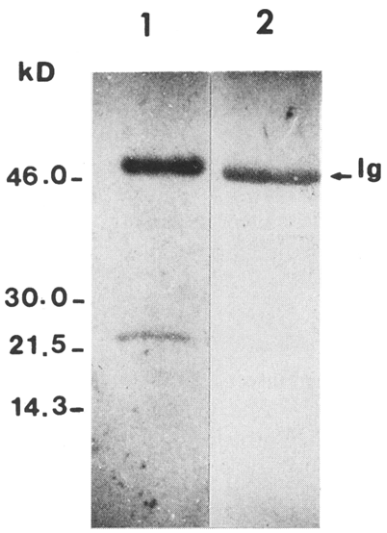


Fig. 2. Expression of maize ABP1 in extracts of Sf9.AUXW cells. Immunoprecipitates from cell lysates were subjected to electrophoresis and blots probed with MAC256. Loadings are equivalent to 1.5×10^6 cells. Lane 1, Sf9.AUXW cells; lane 2, control Sf9 cells.

[6]. The expression vector pIEK1, which has a *Bam*H1 site between the promoter and terminator sequences of the *Autographa californica* polyhedrosis virus *ie-1* gene, has been described previously [5], as has the selection plasmid pIEK1 neo [5] (Fig. 1). pIEK1 was modified by insertion of a polylinker with a single *Bam*H1 site (upper strand: 5′ GATCCCGGGAATTCGGTACCAAGCTTA 3′; lower strand 5′ GATCTAAGCTTGGTACCGAATTCCCGG 3′). Insertion with the *Bam*H1 site adjacent to the *ie-1* promoter yielded pIEK1a (Fig. 1); the opposite orientation yielded pIEK1b (not shown). A *Bam*H1–*Kpn*1 cDNA fragment [7] containing the complete maize ABP1 coding sequence was inserted in the polylinker giving plasmid pIEK1a.AUXW.

Insect cells (*Spodoptera frugiperda*, Sf9) were co-transfected with pIEK1a.AUXW and pIEK.neo using Lipofectin (Gibco Life Technologies) [5]. Stable cell lines were selected as previously described [5] and were passaged at weekly intervals and periodically tested for the continued expression of the maize ABP1.

Fig. 4. The ultrastructure of a conventionally fixed Sf9.AUXW cell embedded in Spurr resin is shown in panel A (Magn. 20K) is contrasted with a ZMABPBacW-infected cell in panel B (Magn. 10K), virus particles in the cell nucleus (N), are indicated by arrows. Immunogold labelling (10 nm gold particles) of recombinant ABP1 in a Sf9 cell infected with ZMABPBacW (C; Magn. 40K), and an Sf9.AUXW cell expressing recombinant ABP1 (D; Magn. 50K). ABP1 is detected in the ER of Sf9.AUXW (D), and in dilations of the ER of virus-infected cells (C) and around the nuclear envelope (indicated by darts). →

2.2. Immunoprecipitation and immunoblotting

For detection of ABP1 in transformed Sf9 cells, ABP1 was precipitated from total cell lysates using polyclonal ABP1 serum [8] and subjected to electrophoresis and electroblotting for detection with monoclonal antibody MAC256 [3].

2.3 Immunolocalisation of ABP1 in transformed SF9 cells

Immunofluorescence microscopy was performed as previously described [3].

For conventional transmission electron microscopy, cells were fixed in 1% paraformaldehyde, 2% glutaraldehyde in 0.1 M PIPES buffer (pH 6.9) for 1 h, washed in buffer and soaked in 1% tannic acid for 1 h. Cells were then post-fixed in 1% aqueous OsO_4 for 1 h, washed in distilled water, dehydrated in a graded series of water/ethanol and finally embedded in Spurr resin [10]. Sections were cut with a Reichert Ultracut E microtome and collected on copper grids. Sections were then stained in uranyl acetate and lead citrate and examined at 100 kV with a JEOL 1200 EXII TEM.

Immunogold labelling was carried out on cells fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) for 1 h at room temperature. Cells were dehydrated in a graded ethanol series and embedded in L R. White resin (London Resin Company) using a progressive low temperature embedding technique [11]. Sections were collected on nickel grids and stained by floatation on droplets of blocking buffer (PBS containing 0.8% BSA, 0.1% fish gelatine and 5% foetal calf serum) for 10 min at room temperature, followed by an overnight incubation at 4°C in anti-ABP1 serum diluted 1:100. After 3×5 min washes in PBS containing foetal calf serum and fish gelatine, cells were stained with 10 nm gold-conjugated anti-rabbit secondary antibody (diluted 1:20 (BioCell)) for 90 min. After four washes in PBS containing fish gelatine and 2×5 min in ultra-pure water, grids were finally post-stained with 2% aqueous uranyl acetate followed by lead citrate.

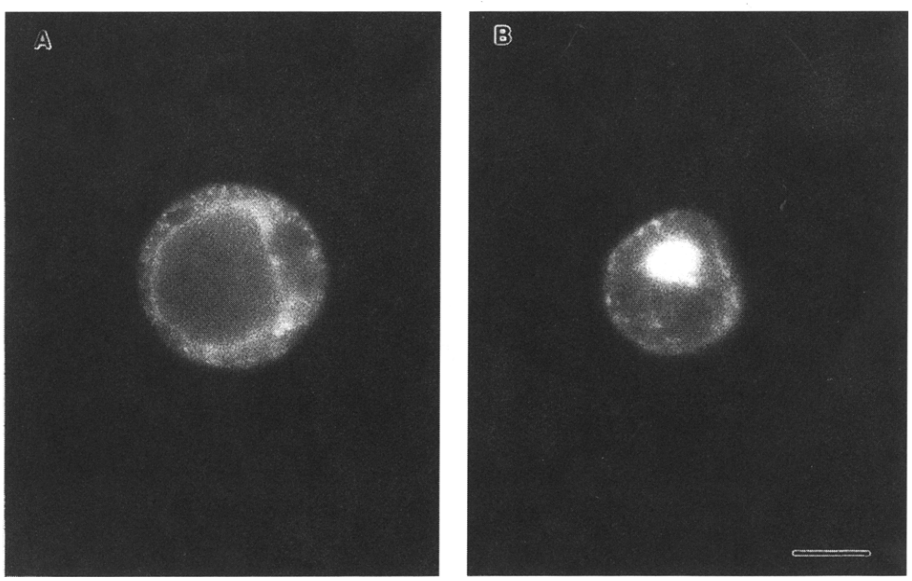
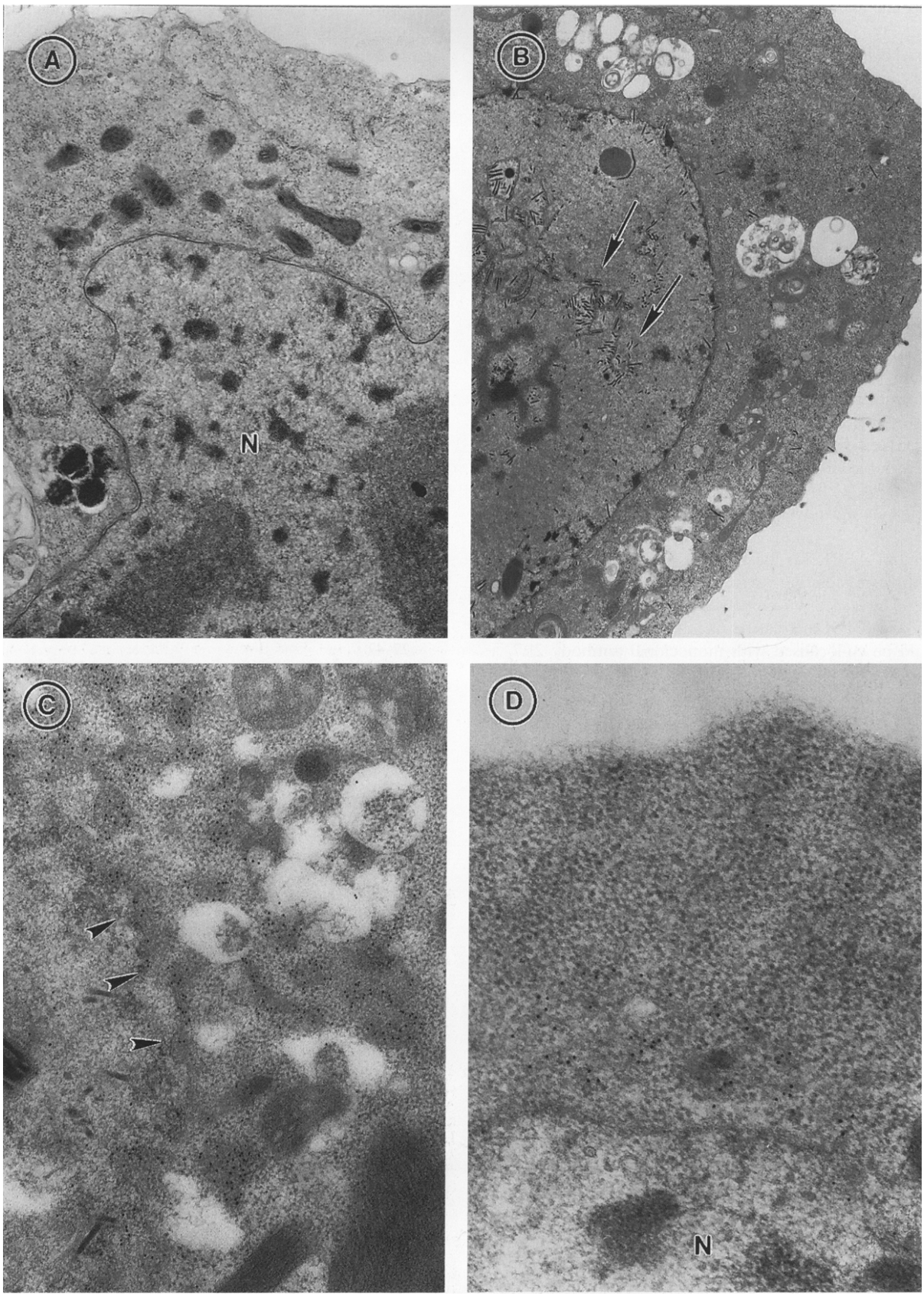


Fig. 3. Immunofluorescence detection of maize ABP1 in Sf9.AUXW cells with a polyclonal anti-ABP1 serum (A), and DNA staining in the nucleus of the same cell with Hoescht 33342 (B). Scale bar = 10 μm.


A
N
B
C
D
N

3. Results and discussion

3.1. Production of stable insect cell lines synthesising the maize ABP1

Transformation of Sf9 cells was achieved by co-transfection with the expression plasmid pIEK1a.AUXW and the selection plasmid pIEK1.neo (Fig. 1). Clones resistant to G418 were selected and amplified before selective pressure was relaxed. DNA dot-blot hybridisation demonstrated that 12 out of 20 G418-resistant clones had incorporated the ABP1 cDNA (data not shown). One cell line, Sf9.AUXW, was amplified further and analysed by immunocytochemistry for detection of expressed ABP1. These cells have been frozen in liquid nitrogen and successfully revived by standard techniques.

Immunoprecipitation using anti-ABP1 serum followed by immunoblotting using a monoclonal antibody MAC 256, detected a polypeptide of the expected size (22 kDa) in cell lysates of transformed cells but not in the control Sf9 cells (Fig. 2). The band just above the 46-kDa marker in both tracks is due to rabbit IgG heavy chains from the precipitin complex cross-reacting with the anti-rat IgG secondary antibody on blotting.

3.2. Immunolocalisation of maize ABP1 in transformed insect cells

Staining of ABP1 was located in a fine reticulate structure in the cytoplasm radiating out from the nucleus through the cell (Fig. 3A). The nuclear envelope was also stained, but no staining was observed at the plasma membrane. This staining pattern could be co-localised with monoclonal antibody 2E7, a marker for the ER [3] (data not shown). Fig. 3B shows the same cell stained with Hoescht 33342 which defines the nucleus.

The ultrastructure of Sf9.AUXW cells (Fig. 4A) and ZMABPBacW-infected Sf9 cells [3] (Fig. 4B) were compared using conventional electron microscopic analysis of osmium tetroxide-fixed cells. Additionally, ultrathin sections of insect cells infected with ZMABPBacW expressing recombinant ABP1 (Fig. 4C) and Sf9.AUXW cells (Fig. 4D) were probed with anti-ABP1 antibodies and indirectly labelled with anti-rabbit IgG coupled to colloidal gold. In baculovirus-infected insect cells, colloidal gold particles were seen in dilations of the ER. In Sf9.AUXW cells, gold label was also found in the ER, but which was not dilated. It is unclear whether these dilations are the consequence of massive synthesis of ABP1, or whether they are induced by virus infection. Immunological control reactions using preimmune serum were devoid of labelling for both cell types (data not shown). Levels of expression achieved in the cell line system were low compared with baculovirus infection. Others have reported similarly low yields of protein [12]. One reason for this is that the copy number of the expression cassette is likely to be low. More importantly, the baculovirus *ie-1* promoter is weak relative to the polyhedrin promoter, but it was necessary to use a promoter that would function independently of virus infection. A number of different promoters are currently being assessed for their ability to direct high level gene expression in insect cells.

Baculovirus infection allows higher expression levels of foreign genes in insect cells, however the stable cell line system provides a way in which we can gain a more comprehensive understanding of the secretory pathway in insect cell lines and how it might be compromised in baculovirus-infected cells. Furthermore, we have shown that ABP1 when expressed in insect cells is located in the ER and there is no evidence of secretion to the cell surface. These results suggest that some specific mechanism exists in plant cells to transport an active fraction of ABP1 from the ER to the cell surface [2].

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