Auxin-binding proteins without KDEL sequence in the moss *Funaria hygrometrica*

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Received: 22 July 2009 / Revised: 4 September 2009 / Accepted: 16 September 2009 / Published online: 2 October 2009
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**Abstract** Whereas the important plant growth regulator auxin has multiple effects in flowering plants, it induces a specific cell differentiation step in the filamentous moss protonema. Here, we analyse the presence of classical auxin-binding protein (ABP1) homologues in the moss *Funaria hygrometrica*. Microsomal membranes isolated from protonemata of *F. hygrometrica* have specific indole acetic acid-binding sites, estimated to be about 3–5 pmol/mg protein with an apparent dissociation constant ($K_d$) between 3 and 5 $\mu$M. Western analyses with anti-ABP1 antiserum detected the canonical endoplasmic reticulum (ER)-localised 22–24 kDa ABP1 in *Zea mays*, but not in *F. hygrometrica*. Instead, polypeptides of 31–33 and 46 kDa were labelled in the moss as well as in maize. In *F. hygrometrica* these proteins were found exclusively in microsomal membrane fractions and were confirmed as ABPs by photo-affinity labelling with 5-azido-[7-3H]-indole-3-acetic acid. Unlike the classical corn ABP1, these moss ABPs did not contain the KDEL ER retention sequence. Consistently, the fully sequenced genome of the moss *Physcomitrella patens*, a close relative of *F. hygrometrica*, encodes an ABP1-homologue without KDEL sequence. Our study suggests the presence of putative ABPs in *F. hygrometrica* that share immunological epitopes with ABP1 and bind auxin but are different from the classical corn ABP1.

**Keywords** ABP1 · Photo-affinity labelling · Phytohormone · *Physcomitrella patens* · Signalling · *Zea mays*

**Abbreviations**

IAA Indole-3-acetic acid

ER Endoplasmic reticulum

**Introduction**

Changes in the development of land plants are usually regulated by phytohormones (Davies 1995). Auxin, as the most prominent one, acts as growth regulator thus being involved in a variety of developmental and physiological processes in a tissue specific manner such as specification of root and shoot, cell elongation and cell division (Weijers and Jurgens 2005; Willemsen and Scheres 2004). Auxin is ubiquitous in heterotrophic and photoautotrophic organisms; besides from seed plants, its presence has been confirmed in algae, bryophytes and ferns (Evans and Trewavas 1991). In algae and...
fungi, auxin is produced and detectable as secondary metabolite, although its function in these organisms is not yet known. The role of auxin as plant hormone in early land plants has been documented starting from the bryophytes (Cooke et al. 2002; Johri 2004, 2008; Paponov et al. 2009; Sztein et al. 1999, 2000). The filamentous, tip-growing protonemata of the closely related mosses Funaria hygrometrica and Physcomitrella patens have been extensively studied to understand the role of phytohormones in cell differentiation (Johri 1974; Decker et al. 2006). The protonema consists of two distinct cell types, the chloronema and the caulonema. Chloronema cells have more chloroplasts and are separated by a straight septum whereas caulonema cells have fewer plastids and oblique cross walls (Johri 1974; Reski 1998). In addition, chloronema cells are mainly arrested in the G2/M phase of the cell cycle, whereas caulonema cells are mainly arrested in G1/S (Schween et al. 2003). Similar to flowering plants, an auxin gradient exists in the moss protonema with maxima in the most actively dividing cells (Bierfreund et al. 2003).

Auxin evokes two responses in Funaria protonema: at a low level it inhibits chloronema proliferation, while at higher levels it enhances secondary caulonema differentiation (Johri and Desai 1973). Both responses are antagonised by the anti-auxin p-chlorophenoxyisobutyric acid (PCIB) (Johri and D’Souza 1990). PCIB reduces polar, basipetal auxin transport in Funaria rhizoids (Rose and Bopp 1983) and was reported to impair the auxin-signalizing pathway by reducing the stability of auxin/indole-3-acetic acid (Aux/IAA) gene transcripts in Arabidopsis roots (Oono et al. 2003). Thus, both basipetal transport and IAA-binding sites seem to be involved during caulonema differentiation and chloronema inhibition (Johri 2004). In addition, cell division is inhibited, if auxin efflux from Physcomitrella proplasts is blocked by naphthylphthalamic acid (NPA) (Bhatla et al. 2002).

It is known from flowering plants that parts of the multiple auxin responses are mediated by the nuclear auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Dharmasiri et al. 2005; Kepinski and Leyser 2005) which, upon binding of auxin, degrades AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA)-proteins (Dharmasiri and Estelle 2002), releasing the inhibitory effect on auxin response factors (ARFs), transcription factors that in turn regulate auxin responsive gene expression (Quint and Gray 2006; Benjamins and Scheres 2008). It is, however, evident that not all auxin responses are regulated via this nuclear receptor (Badescu and Napier 2006). Thus, another important mediator of auxin action may be ABP1, an auxin-binding protein involved in cell expansion (Jones et al. 1998), and subsequently found in a variety of seed plants by affinity labelling (Christian et al. 2003; Napier et al. 2002). ABP1 is a 22–24 kDa protein from corn which is localised predominantly in the endoplasmic reticulum (ER), as it possesses a C-terminal KDEL ER retention sequence (Henderson et al. 1997; Jones and Herman 1993; Woo et al. 2002). A smaller fraction of this protein is secreted outside and located in the outer leaflet of the plasma membrane (Jones and Herman 1993; Oliver et al. 1995). ABP1 has been shown to mediate the hyperpolarization response and stomatal opening stimulated by auxin (Barbier-Brygoo et al. 1992; Christian et al. 2003, Leblanc et al. 1999; Gehring et al. 1998) and to be involved in cell cycle control (David et al. 2007). Other investigations have proposed a role of ABP1 in directional root growth (Shimomura 2006).

The current study aimed at characterising proteins in the protonema of moss Funaria hygrometrica homologous to ABP1 of Zea mays. To this end a radioactive IAA-binding assay was employed to determine if moss microsomes contained specific auxin-binding sites. To further investigate specific ABPs, biochemical, immunological and photo-affinity labelling approaches were used. The results presented here reveal the presence of specific ABPs in Funaria. Antibodies specific to corn ABP1 could detect polypeptides of 31–33 and 46 kDa, respectively. However, unlike ABP1 of corn, both these polypeptides did not contain epitopes of the classical KDEL ER retention sequence. Similar results were obtained when nucleotide sequences from other mosses homologous to the ABP1 of maize were compared as shown for P. patens and Ceratodon purpureus. We therefore propose that the non-ER localised ABPs of Funaria, and probably in all mosses, play a role in the regulation of auxin responses during the differentiation of moss protonema.

Materials and methods

Plant material

The protonema of Funaria hygrometrica Hedw. (cell line J-2) and dark grown coleoptiles of corn (Z. mays L.) were used in the present study. Chloronema cells of Funaria were grown in liquid suspension cultures as described earlier (Johri 1974). Cells grown in minimal medium supplemented with glucose (MMG) were harvested at a cell density of 4–5 mg/ml and used fresh or kept frozen until used. Hybrid corn seeds (variety MMH 65, from Maharashtra Hybrid Seeds, Mumbai, India) were germinated on moist, autoclaved vermiculite and the coleoptiles (approximately 1.5 cm from the tips) were harvested and used for the experiment.

Microsomes for [3H]-IAA binding

Two grams of freshly harvested protonema cells were homogenised in an ice-cold mortar and pestle in the
presence of acid washed sand (0.25 g sand per g cells) for 45 min in a buffer containing 250 mM sucrose, 7 mM citrate buffer pH 5.5 and 5 mM magnesium chloride (MgCl₂) (2.5 vol per gram fresh weight of cells). The crude homogenate was filtered through two layers of nylon cloth and the filtrate was centrifuged at 10,000×g for 30 min using a Kubota RA 400 rotor. The supernatant was then centrifuged at 110,000×g for 45 min using a SW 41 Ti swing-out rotor to obtain the microsomal pellet. The microsomes were resuspended in binding buffer containing 7 mM citrate and 5 mM MgCl₂ pH 5.5 and dialysed overnight against 0.7 mM citrate buffer and 0.5 mM MgCl₂ with four changes of buffer. The dialysed samples were centrifuged at 10,000×g to eliminate any precipitate and used to determine binding of radioactively labelled auxin in the filter assay.

Auxin-binding assay

The assay was performed in the dark at 4°C in a final volume of 160 µl solution containing 7 mM citrate buffer pH 5.5, 5 mM MgCl₂, 83 nM 3H-IAA, 20 µl of microsomal proteins (10 µg). The samples were incubated for 30 min and the bound radioactivity was displaced by incubating with increasing amounts of non-radioactive IAA (1 nM–100 µM) for 30 min on ice. After the incubation, 44 µl of the reaction mixture were filtered through cellulose acetate filters under vacuum. The filters were washed with 20 ml washing buffer (0.7 mM citrate buffer pH 5.5 and 0.5 mM MgCl₂), dried and the radioactivity retained on the filters was determined using PPO (poly p-phenylene oxide), and POPOP [1, 4-bis (5-phenyloxazol-2-yl) benzene], a toluene based scintillant. Each concentration was tested in triplicate and the mean value was plotted against the radioactivity retained on the filter.

Sub-cellular fractionation for immuno-detection of ABPs and electron microscopy analysis

The sub-cellular fractionation of the crude extract was performed under cold conditions. Five grams of frozen moss protonema or corn coleoptiles were homogenised, suspended in grinding buffer [GB: 250 mM sucrose, 20 mM (2-Amino-2-hydroxymethyl-propane-1,3-diol) Tris–HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT)] with a mixture of protease inhibitors, antipain, leupeptin, chymostatin, pepstatin and tosyl phenylalanyl chloromethyl ketone (TPCK) at a final concentration of 50 µM and kept in ice for 15 min. The homogenates were filtered through two layers of nylon cloth and the filtrate was further fractionated by differential centrifugation. Successive centrifugation of the filtrates at 5,000×g for 10 min, 35,000×g for 30 min and 110,000×g for 1 h, resulted in three pellets (P1, P2, P3) and a supernatant (S), respectively. The pellets were washed two times with GB and resuspended in the GB containing 1% sodium cholate and kept for 30 min on ice with occasional vortexing and centrifuged at the respective g forces to eliminate the particulate insoluble material. The resulting supernatant was used in the experiments. To isolate the total solubilised protein, sodium cholate was added dropwise to the filtrate in order to obtain a final concentration of 1%. This suspension was further incubated for 30 min and centrifuged at 35,000×g for 15 min to extract and use the supernatant. The protein content was estimated using the Bradford dye-binding assay, denatured by boiling in Laemmli sample buffer containing 62 mM Tris pH 6.8, 10% Glycerol, 2% SDS, 0.001% Bromophenol blue and 5% β-mercaptoethanol, separated using a 12% Tris–Glycine gel (Laemmli 1970) and transferred to a nitrocellulose membrane. The P2 and P3 sub-cellular pellet fractions were further processed for transmission electron microscope using a method as described in Shanbhag et al. (1995). The P1 fraction was not processed for EM because at that g forces one would enrich nuclei and mitochondria and other broken contaminants.

Immunological detection of ABPs

The western blotting and detection was done essentially as described by Harlow and Lane (1988), with few modifications. The Western blots were incubated in 5% fat-free milk in a buffer containing 10 mM Tris–HCl pH 7.5, 50 mM sodium chloride (NaCl), 2 mM calcium chloride (CaCl₂) and 0.1% Tween 20 [milk-Tris Buffered Saline-Tween (TBS-T)] for 2 h at room temperature in order to block the non-specific binding sites. The primary polyclonal anti-ABP1 or monoclonal anti-KDEL or anti-HDEL antibodies were incubated in milk-TBS-T for 2 h at room temperature or overnight at 4°C on a shaker. Blots were washed four times, with milk-TBS-T and incubated with biotinylated anti-rabbit IgG (for anti-ABP1) or anti-mouse or rat IgG (for anti-KDEL and anti-HDEL) for 2 h at room temperature. Blots were developed using the ABC kit with di-aminobenzidine as a substrate (Vector laboratories, Burlingame, USA).

Preparation of extracts for photo-affinity labelling of ABPs

The microsomal membranes were prepared from the calcium promoted sedimentation of vesicles as described (Shimomura et al. 1986). The microsomal pellet (30 mg dry acetone powder) was suspended in 425 µl of chilled photo-affinity labelling buffer containing 10 mM citrate buffer pH 5.7, 5 mM MgCl₂, 1 mM phenylmethanesulphonylfluoride
(PMSF) and homogenised for 20 min at 4°C. The suspension was collected in a 1.5 ml polypropylene tube, vortexed vigorously for 3 min and centrifuged at 18,000×g for 5 min. The pellet was again washed with 400 μl of buffer, mixed thoroughly, vortexed for 5 min and used for photo-affinity labelling.

Photo-affinity labelling using 5-azido 7-3H-indole-3-acetic acid

Photo-affinity labelling reaction was carried out as described in Jones and Venis (1989) and Jones et al. (1984), with little modifications. All steps prior to separation of the proteins on the gel were done in dim safe red light in a dark room as described in Jones et al. (1998). The photo-affinity labelling reaction was performed in a final volume of 120 μl. Thirty microlitres of labelling buffer (200 mM citrate pH 5.55, 20 mM MgCl₂ and 1% sodium cholate) and 80 μl of protein suspension were pipetted into a 1.5 ml polypropylene tube and kept at 4°C. Azido-IAA (0.33 mM final) with or without non-radioactive IAA made in water in a final volume of 7 μl was added and incubated for 30 min in dark at 4°C. The mixture was then transferred to a glass cavity slide. The slide was kept on a platform maintained at 4°C, 5 cm below the UV source. The contents of the slides were exposed to the UV source (39254 nm and 29330 nm bulbs, Hofer UV transilluminator from HOFER Scientific) for 30 s. The samples were frozen immediately in liquid nitrogen and kept at −80°C.

Chemicals

Anti-ABP1, anti-KDEL and anti-HDEL antibodies were generous gifts from Dr. Mike A. Venis and Dr. Richard M. Napier, HRI, Warwick, UK. The Avidin–Biotin amplification kit (ABC kit) was purchased from Vector laboratories, Burlingame, USA, while the PPO was obtained from Sisco chemical laboratories, India. 5-Azido 7-3H-IAA with >99% radiochemical purity by radiochromatography was purchased from Dr. Alan M. Jones, North Carolina, Chapel Hill, USA. All other chemicals were obtained from Sigma Chemical Co.

A phylogenetic tree of ABP1 proteins was constructed using Neighbour Joining as implemented in quicktree (Howe et al. 2002) using the ScoreDist distance matrix (Sonnhammer and Hollich 2005), 1,000 bootstrap replicates and setting the root at the longest internal branch. It includes the ABP1 sequences of 13 land plants, which are indicated by a five letter code, as follows: Arath: Arabidopsis thaliana (Genbank: NP_192207), Avesa: Avena sativa (Genbank: BAA25433), Cerpu: C. purpureus (Genbank: AAF37576), Cerri: Ceratopteris richardii, Glyma: Glycine max, Medtr: Medicago truncatula, Orysa: Oryza sativa (Genbank: ABF85613), Phya: P. patens (Cosmoss: Phypa_151546), Poptr: Populus tremula, Selmo: Selaginella moellendorffii, Sorbi: Sorghum bicolor, Vitvi: Vitis vinifera (Genbank: ACG80594), Zeama: Z. mays (Genbank: NP_001105312). Genbank accession numbers can be found on http://www.ncbi.nlm.nih.gov/, while for the other organisms following URLs were used:

ftp://ftp.jgi-psf.org/pub/JGI_data/Glycine_max/annotation/Glyma0.1b.pep.fa.gz
http://www.medicago.org/remote/downloads/Mt2/20080103_imgag_proteinMAPPED_NO_OVERLAP.fa
http://www.cosmoss.org
ftp://ftp.jgi-psf.org/pub/JGI_data/Poplar/annotation/v1.1/proteins.Poptr1_1.JamboreeModels.fasta.gz
http://genome.jgi-psf.org/Selmo1/download/Selmo1_GeneModels_FilteredModels2_aa.fasta.gz
ftp://ftp.jgi-psf.org/pub/JGI_data/Sorghum_bicolor/v1.0/Sorbi1_GeneModels_Sbi1_4_aa.fasta.gz

Multiple sequence alignments of all 14 ABP1 sequences were created using MAFFT L-INSI (Katoh et al. 2005) and curated with Jalview (Clamp et al. 2004).

Results

3H-IAA binding to the microsomal membranes

Microsomal membranes from Funaria protonema were isolated and observed to exhibit specific auxin-binding sites. The specifically bound IAA was estimated to be...
between 3 and 5 pmol/mg of protein (Fig. 1). The $^{3}$H-IAA binding was decreased in a dose dependent manner by increasing the amounts of non-radioactive IAA in the incubation medium (Fig. 1). The binding of the $^{3}$H-IAA to the microsomal membranes was improved significantly (15–18-fold) by dialysing the membrane fraction (data not shown). The potential Km for IAA to its binding sites was determined to range in between 3 and 5 μM (Fig. 1).

Detection of Funaria ABPs using anti-ABP1 antibodies

In order to identify the ABP1 homologue in Funaria, anti-ABP1 antiserum raised against the purified corn ABP1 was used. The sodium cholate solubilised protein extracts of moss and corn were separated by SDS-PAGE and Western blots were probed with anti-ABP1 antiserum. In corn, which served as a positive control, the antisera cross-reacted with polypeptides of 22 and 24 kDa, however, no polypeptide of similar molecular mass was detectable in the moss extracts. Strikingly, polypeptides of about 31–33 and 46 kDa were detected in both plants tested (Fig. 2). Furthermore, the 31–33 kDa region appears to contain at least two closely migrating polypeptides (Fig. 3). In order to understand the localisation of these putative ABPs, we performed sub-cellular fractionation using differential centrifugation and probed the Western blots with anti-ABP1 antibodies (Fig. 3). This fractionation led to a significant enrichment of the 31–33 kDa polypeptides which were detected exclusively in the microsomal membrane fraction and resolved into two or three isoforms (Fig. 3). In addition, a 28 kDa putative ABP1 specific to P3 sub-cellular fraction of moss is marked by an arrow.

Fig. 1 Microsomal membranes from the moss Funaria hygrometrica were incubated on ice with 83 nM $^{3}$H IAA for 30 min in dark followed by the addition of increasing concentrations of non-radioactive IAA (0, 6.25, 12.5, 25, 50 and 100 μM) in six different reaction tubes. The reaction mixture was filtered through cellulose acetate filters, washed with binding buffer and the radioactivity is determined. The standard deviation of triplicate filters was plotted as a function of concentration of IAA. The concentration corresponding to ~$K_{m}$ is shown by an arrow. The variation between samples by the filter assay was observed to be <5%.

Fig. 2 Sodium cholate solubilised total proteins from moss (lane 1) and corn (lane 2) were resolved in 12% SDS-PAGE, transferred to nitrocellulose filters and probed with anti-ABP1 antibodies or pre-immune sera at a dilution of 1/1,000.

Fig. 3 Cell free extracts from moss (M) and corn (C) were fractionated by differential centrifugation to obtain various pellets (P1–P3) and post-microsomal supernatant (Sup) as described in “Materials and methods”. Proteins from the pellets were solubilised with 1% sodium cholate and 15 μg of solubilised proteins from each sample were separated using 12% SDS-PAGE, transferred to nitrocellulose filters and probed with anti-ABP1 antibodies. A 28 kDa putative ABP1 specific to P3 sub-cellular fraction of moss is marked by an arrow.
These vesicles were more prominent in the P2 fraction compared to the P3. These results suggest that the putative ABPs may be associated with membranes and/or vesicles.

Immuno-precipitation using anti-KDEL monoclonal antibodies and detection using anti-ABP1 antiserum

Since the classical corn ABP1 is known to possess a C-terminal KDEL sequence and to be retained in the ER, it was investigated if any of the moss polypeptides sharing the immunological epitopes of ABP1 also possesses a KDEL epitope. Monoclonal antibodies raised against the KDEL sequence were used to immuno-precipitate proteins from the P2 sub-cellular fractions as described previously. Equal amounts of protein from the immuno-precipitated samples of moss and corn were either silver stained or transferred to a polyvinylidene fluoride (PVDF) membrane and probed with anti-ABP1 antiserum (Fig. 5). None of the 31–33 or 46 kDa polypeptides was detected by the monoclonal antibody, while the 22–24 kDa ABP1 of corn was clearly detected (Fig. 5). These results suggest that the identified putative moss ABPs do not include a KDEL signal peptide sequence. It indicates further that the putative ABPs of higher molecular mass identified in corn and Funaria by Western blotting do not possess the ER retention signal, thus not being a mere finding in lower plants.
Photo-affinity labelling using 5-azido 7-[^3]H-indole acetic acid

In order to determine if the putative moss ABP1 homologues with a size of 31–33 and 46 kDa identified in this study are capable of binding auxin, photo-affinity labelling using 5-azido-7-[^3]H-IAA was carried out with the microsomal membrane fractions under the conditions described in “Materials and methods”. Binding of the radioactive azido-auxin to the microsomal fractions of moss also revealed the presence of two major polypeptides at 31–33 and 46 kDa (Fig. 6). The specificity of this binding was evaluated by increasing the amounts of non-radioactive IAA in the reaction mixture prior to UV cross-linking as by such means the high-affinity binding sites would be out-competed. The proteins were separated under denaturing conditions and signals from the gel were detected by fluorography. Strikingly, polypeptides of identical molecular masses, i.e. 31–33 and 46 kDa, were intensively labelled by azido-auxin, although with different affinities (Fig. 6, lane 1). Upon competition with increasing amounts of non-radioactive IAA (5–10 μM) in the reaction mixture (lanes 2–5) and prior to UV cross-linking, there was a complete disappearance of the azido-IAA labelling (Fig. 6, lanes 4, 5).

Phylogenetic analysis and sequence comparison of ABP1 in land plants

The topology of the phylogenetic tree for the 14 ABP1 proteins from 13 land plants (Fig. 7) confirms the evolutionary conservation of the protein sequence among mosses, as the ABP1 from P. patens and the one from C. purpureus cluster together. These species lack the classical C-terminal KDEL ER retention sequence that is found in all other land plants, neither do the moss sequences encode a derived ER retention signal [Prosite pattern PS00014, according to Andres et al. (1990)] (Fig. 8). The sequences for the ABP1 proteins from S. moellendorffii, as well as one from P. tremula (Poptr_ABPin–2) and from G. max are likely to be fragmentary which might explain why they do not depict the KDEL sequence.

Discussion

From an evolutionary viewpoint bryophytes (mosses and liverworts) are considered to be the earliest known land plants (Qiu et al. 1998, 1999; Lang et al. 2008). The study of hormone signalling in these organisms might hence be gaining attention in the scientific community as research on them might be the key to understand the possible origin of a primitive auxin sensing machinery (Joehl 2004; Paponov et al. 2009). Protonema cells of Funaria were chosen in this study because they are highly responsive to physiologically relevant concentrations of exogenously applied auxin. In moss both developmental steps, chloronema proliferation and the differentiation to caulonema are under the regulation of auxin. Hence, as part of these processes basipetal transport as well as auxin-binding sites might be involved (Reski 1998). The present investigation of ABPs in Funaria using microsomal membrane fractions of this moss showed the presence of specific auxin-binding sites. The derived Km of IAA to its binding sites in Funaria and corn was comparable being in a physiological range of 3–5 μM (Hertel et al. 1972).

There are several ABPs described in different plants. The classical ER localised ABP1 of corn has drawn far more attention over the years. A small fraction of ABP1 (ca. ~2%) is localised to the plasma membrane where it has been shown to function in one of the early effects of auxin, i.e. hyper-polarisation of the membrane (Henderson et al. 1997; Barbier-Brygoo et al. 1989). Several lines of evidence is accumulating that suggest ABP1 might function in the plasma membrane and control a variety of events such as cell polarity, cell cycle, ion channels and sense cytoplasmic auxin gradients. These assign ABP1 as a possible candidate for a bonafide auxin receptor (Kramer 2009).

Among mosses, the C. purpureus ABP1 homologue possesses most of the conserved domains, however, it lacks the C-terminal ER retention sequence (Napier et al. 2002). The analysis of sequence data from the moss P. patens also leads to the identification of an ABP1 (Phyta_151546) although it does also not contain any ER retention sequence as well. The use of standard computational prediction
programmes [i.e. TargetP (Emanuelsson et al. 2000), SherLoc (Shatkay et al. 2007), MultiLoc (Hoglund et al. 2006), WoLF PSORT (Horton et al. 2007)] to localise this moss ABP1 homologous protein suggested that it is not localised to the ER (data not shown). It therefore seems possible that a true homologue of ABP1 with a classical KDEL ER retention sequence may not be present in mosses. Attempts were also made to investigate if the putative ABP1 homologues use HDEL as a signal sequence for its retention in the ER. The HDEL retention sequence is sufficient for secretory plant proteins to be retained in the ER while promoting vascular targeting of the proteins that escapes ER (Gomord et al. 1997). None of the putative ABPs were detected with the respective antibody, although as a positive control a 80 kDa protein (most likely to be a Bip, binding protein, a member of HSP70 family and a major chaperone of ER) was detected in corn extracts (data not shown). There is a report from animal systems describing signal sequences such as DKEL, RDEL, KNEL to be used as the ER retention signal (Andres et al. 1990). However, neither the P. patens nor the C. purpureus ABP1 nucleotide sequences contain such alternative retention signals. Moreover, there is a remarkable conservation between regulatory as well as signal sequences between moss and mammals (Schaaf et al. 2005; Gitzlinger et al. 2009), making it unlikely that mosses contain hitherto unknown ER retention signals.

Therefore, it is tempting to suggest that the ER retention sequence of ABP1 in higher plants could have evolved independently and at a later stage along with the needs for a specialised auxin signalling during the evolution of higher land plants, as was recently described for the evolution of nuclear auxin signalling (Paponov et al. 2009). It is also possible that these proteins are functional homologues of higher plant ABP1 and control essential non-nuclear processes such as cell elongation, polarity and control of ion channels, fundamental to plant development. Not only in the moss Funaria, but also in the extracts of corn and sorghum (data not shown) higher molecular mass putative ABPs were detected in the current study by

Fig. 7 Phylogenetic tree of ABP1 proteins among 13 land plants. The ABP1 sequences for each organism are indicated by a five letter code, as follows: Arath: Arabidopsis thaliana, Avesa: Avena sativa, Cerpu: Ceratodon purpureus, Cerri: Ceratopteris richardii, Glyma: Glycine max, Medtr: Medicago truncatula, Orysa: Oryza sativa, Phypa: Physcomitrella patens, Poptr: Populus tremula, Selmo: Selaginella moellendorffii, Sorbi: Sorghum bicolor, Vitvi: Vitis vinifera, Zeama: Zea mays. The phylogenetic tree was constructed using Neighbour Joining as implemented in quicktree (Howe et al. 2002) using the ScoreDist distance matrix (Sonnhammer and Hollich 2005), 1,000 bootstrap replicates and rooted at the longest internal branch.
anti-ABP1 antibodies, nicely confirming earlier findings on corn (Jones and Venis 1989). This result suggests that there are other ABP1-related proteins or proteins sharing epitopes with ABP1 and which are present not only in *Funaria* but also in higher land plants. These proteins might be part of the auxin metabolising enzymes or transporters presumably being conserved across species, like the GH3 proteins (Ludwig-Müller et al. 2009). Based on these results, it is also tempting to speculate that these proteins may be the molecular imprint of a primitive auxin sensing machinery. Retention of these proteins in the ER of vascular plants may have evolved much later as a safeguard.
storage mechanism in order to ensure a continuous supply of this to the plasma membrane. This suggestion is supported by the observation that ABP1 does not bind auxin in the lumen of the ER despite this being the predominant sub-cellular location (Tian et al. 1995).

It is also interesting in this respect to note that two distinct signalling pathways of auxin action have been described in the epidermal cells of pea (*Pisum sativum*): the ABP1-dependent pathway, which has higher sensitivity to NAA and is independent of extracellular Ca2+ and the ABP1-independent pathway, which shows higher sensitivity to IAA and is dependent on extracellular Ca2+ (Yamagami et al. 2004). Since *Funaria* responds to IAA much better than NAA and grows better in calcium rich soil, it is tempting to suggest that the ABP1-independent mechanism seems to have evolved earlier than the ABP1-dependent one. The plasma membrane bound ABPs have been found in plants earlier with the ones of Zucchini displaying a molecular mass of 40–42 kDa (Hicks et al. 1993) and showing to be photo-affinity labelled by azido- auxin (Hicks et al. 1989). Therefore, the putative ABPs detected in the microsomal fraction appear to be associated with the plasma membrane (Fig. 4b, d). However, more work, like in vivo targeting of GFP-fusion proteins (Schaaf et al. 2004), is required to establish their precise localisation as it is also conceivable that plants have variable amounts of classical ABP1 proteins. The lack of detection of the classical 22 kDa ABP1 protein with an ER retention signal in *Funaria* does not appear to be a result of lower expression or abundance. Even after loading about 15–20 times more protein from moss than from corn, and even after several fold enrichment by subcellular fractionation, a polypeptide comparable to ABP1 was not detectable in our recent study. The 28 kDa polypeptide detected in the P3 sub-cellular fraction of *Funaria* (Fig. 3 marked with an arrow) could be most likely the result of protein degradation specific to P3 fraction.

In summary, evidence is provided here for the presence of additional immunologically related ABP1 homologues in the moss *Funaria* that do not show the presence of the classical ER retention KDEL signal sequence. As sequence comparisons with other mosses confirm the lack of the retention signal in the ABP1 of mosses it is postulated that such a signal might have evolved during later stages of evolution in land plants, a feature that has recently been described for nuclear auxin signalling (Paponov et al. 2009) and for gibberellin signalling (Vandenbussche et al. 2007).

Acknowledgments We thank Prof. Mike A. Venis and Dr. Richard M. Napier, HRI, UK for providing the anti-ABP1, anti-KDEL and anti-HDEL antibodies. Additionally, acknowledge Dr. Karen Hicks and Dr. Amaury de Montaigu for discussions on the results. Moreover, we are thankful to Dr. Jacinta S. D’Souza for maintaining the cell line and Dr. Mark von Stackelberg for providing some moss *F. hygrometrica*. Special thanks to Prof. Eberhard Schaefer and Prof. George Coupland for their continued support. This research was supported by Deutsche Forschungsgemeinschaft (grant Re837/10) and by the Excellence Initiative of the German Federal and State Governments (EXC 294). Substantial work for this publication was carried out by K. P. as graduate student in the Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India.

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