Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Science 167 (2004) 471–479
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Knockout of *UBP34* in *Physcomitrella patens* reveals the photoaffinity labeling of another closely related IPR protein

Florent Brun [a], Didier G. Schaefer [b], Michel Laloue [a], Martine Gonneau [a,*]

[a] *Laboratoire de Biologie Cellulaire, INRA-Versailles, route de Saint-Cyr, 78026 Versailles-Cedex, France*
[b] *Laboratoire de Phytogénétique Cellulaire-Institut d'Écologie, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne-Dorigny, Switzerland*

Received 15 September 2003; received in revised form 15 April 2004; accepted 20 April 2004

Available online 18 May 2004

Abstract

UBP34, a soluble 34 kDa protein identified in the moss *Physcomitrella patens* by photoaffinity labeling with an urea-type cytokinin agonist, belonging to the plant intracellular pathogenesis related (IPR) protein family. This class of proteins is ubiquitous in the plant kingdom but their functions are still unknown. Using a reverse genetic approach we generated knockout mutants at the *UBP34* locus by homologous recombination. Knockout plants grown in various conditions do not present any visible phenotypic alteration. However, biochemical and molecular analysis of the knockout transformants reveal the specific photoaffinity labeling of another similar protein.
© 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Allergen; Gene disruption; Intracellular PR proteins; *Physcomitrella patens*

1. Introduction

Using the cytokinin agonist azido-CPPU (azido-1-(2-chloropyrid-4-yl)-3-phenylurea) we specifically photoaffinity labeled a 34 kDa soluble protein in the moss *Physcomitrella patens* [1]. The protein was purified by affinity chromatography, micro-sequenced, and the corresponding cDNA was cloned by screening of a *P. patens* cDNA library. This protein, named UBP34 for urea-type CK-binding protein, is homologous to plant intracellular pathogenesis related (IPR) proteins, known as PR10 proteins [2]. UBP34 is twice the usual size of IPR proteins and consists of two tandemly arranged PR10 units, the highest identity being located in the N-terminal half part of the protein [1]. PR10 proteins were originally characterized at the transcription level, demonstrating increased gene expression in stress situations, such as during microorganism infection. Several PR10 proteins have been described as plant allergens. Among them the Bet V 1 protein in *Betula verrucosa* was recently shown to bind a broad spectrum of physiological ligands including fatty acids, flavonoids, and cytokinins (isopentenyladenine and kinetin) [3]. Other proteins of this family have also been described as cytokinin-binding proteins in *Vigna radiata* [4] or as protein, of which the corresponding gene is transcriptionally activated by cytokinin treatments in *Catharanthus roseus* [5]. However, a role of UBP34 strictly in term of cytokinin perception can be ruled out with regard to previous pharmacological data indicating that N^6-substituted adenine derivatives are poor ligands for this protein [1]. The functional role of the IPR protein family remains to be established as well as the physiologically significant in their cytokinin regulation and binding properties.

In the moss *P. patens*, gene knockout, and allele replacement approaches are feasible as was illustrated by the disruption of various genes involved in different aspects of plant physiology (for a review, see [6]).

We aimed to take advantage of this unique property in the plant kingdom to specify the physiological function of the UBP34 protein in moss. Therefore, we cloned the *UBP34* gene, obtained the corresponding knockout mutants

Abbreviations: IPR, intracellular pathogenesis related; IP, isopentenyladenine; CPPU, 1-(2-chloropyrid-4-yl)-3-phenylurea; BA, benzyladenine

* Corresponding author. Tel.: +33-1-30833046; fax: +33-1-30833099.
E-mail address: gonneau@versailles.inra.fr (M. Gonneau).

0168-9452/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2004.04.013

and analyzed their phenotypes. Under the different growth conditions that we have tested, knockout plants do not show developmental or morphological alteration. However, biochemical and molecular analysis of the knockout mosses reveals the labelling with the azido-CPPU photoaffinity probe of another closely related IPR protein. This suggests a possible phenomenon of functional compensation due to redundancy at the genomic level. In the UBP34-knockout plants, a regulatory mechanism of both proteins could be modified and affect the protein UBP34-like stability and/or activity.

2. Materials and methods

2.1. Plant material and culture conditions

The Gransden Wild-type strain of *P. patens* and growth conditions have been described previously [1].

2.2. Analysis of nucleic acids and genomic library screening

DNA manipulations were performed according to standard procedures [7]. The genomic library of *P. patens* in the bacteriophage lambdaGEM12 was kindly provided by M. Leach (John Innes Institute, UK). The library was built by ligation of *Sau*IIIA partially digested DNA fragments at the *Xho*I site of the lambda phage. The library was infected into *Escherichia coli* host strain LE392. Immobilization of DNA on Hybond–N membrane (Amersham), probe hybridization with *UBP34* cDNA, and isolation of phages of interest were performed according to [7].

2.3. Targeting vector construction

A 2,6 kb *Eco*RI fragment was isolated from the lambda phage containing a 5.5 kb fragment of the *UBP34* locus genomic sequence. This fragment was subcloned into the *Eco*RI site of a modified pBSII–KS$^-$ plasmid (Stratagene) in which the *Xho*I site of the multiple cloning site has been removed. For disruption experiments of the *UBP34* locus, the replacement vector pUBP34–ΔXhoI–NptII was constructed by excision of the genomic internal *Xho*I fragment and replacement by the *Npt*II selectable marker gene under control of the 35S promoter and terminator of the cauliflower mosaic virus [8]. Constructions containing the marker gene in the same orientation as the *UBP34* gene were selected by PCR.

2.4. Protoplast isolation and transformation

For protoplast isolation, protonema tissues were propagated on the minimal medium described by [9] and supplemented with 2.7 mM NH_4 tartrate (Pp-NH_4 medium) and 25 mM glucose. Cultures were grown on cellophane disks in 9 cm Petri dishes on medium solidified with 0.7% Agar (Biomar) at 25 °C, with a light regime of 16 h light/8 h darkness and a quantum irradiance of 80 μE m^{-2} s^{-1}. Protoplasts were isolated from 6-day-old protonemal cultures as described by [8]. Transformation experiments were performed with 300 μl (4×10^5 protoplasts) of a protoplast suspension added to 30 μl of *Eco*RI digested pUBP34–ΔXhoI–NptII plasmid (15 μg) according to the classical protocol [8]. The regenerating colonies were submitted to the paromomycine (DUCHEFA) selection pressure (50 μg ml^{-1}) for 7 days. Stable resistant clones were definitely selected after a second round of growth on non-selective Pp-NH_4 medium followed by transfer on Pp-NH_4 medium containing paromomycine.

2.5. DNA and RNA extractions

P. patens DNA was extracted according to a method described by [10]. Briefly, 200 mg of fresh tissue were ground in liquid nitrogen, mixed with 600 μl extraction buffer (100 mM, Tris–HCl pH 8; 50 mM, EDTA; 500 mM, NaCl; 10 mM, β-mercaptoethanol), and 50 μl 20% SDS and incubated at 65 °C for 10 min. 230 μl 5 M potassium acetate were then added. The mixture was kept on ice for 20 min and centrifuged at 16,000 × *g* for 20 min. DNA was then extracted twice with phenol–chloroform–isoamylalcohol and precipitated with 1.5 volume of isopropanol. The sample was then treated with RNAse-A, extracted again with phenol–chloroform–isoamylalcohol, precipitated in absolute ethanol with 3 M sodium acetate and dissolved in 25 μl Tris–EDTA, pH 8. Total RNA from *P. patens* protonema tissues were extracted using the Qiagen RNeasy Plant extraction kit.

2.6. Southern and Northern analysis

For Southern blot analysis, DNA (10 μg) was digested with the appropriate restriction enzymes. Fragments were separated by electrophoresis in 0.8% (w/v) agarose–Tris Acetate EDTA (TAE)-gels and transferred to nylon membranes (GeneScreen Plus, NEN) in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7).

For Northern blot analysis, 7 μg of total RNA were separated on 1.0% (w/v) agarose gels containing formaldehyde–MOPS and transferred to nylon membranes (GeneScreen Plus, NEN) in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7).

Probes were radiolabelled with 30 μCi [α^{32}P]dCTP by random priming (Prime-a-Gene labeling system, Promega). Hybridisation was conducted in modified Church buffer (7% SDS; 0.25-Na_2HPO_4, pH 7.4; 0.2 mg/ml heparin; 2 mM EDTA; 0.1 mg ml^{-1} denatured DNA of salmon sperm) for 16 h at 65 °C (high stringency) or 55 °C (low stringency). The final washes were in 2 × SSC and then 0.2 × SSC supplemented with 0.1% SDS, at 65 °C or 55 °C, respectively.

2.7. RT-PCR and PCR analysis

The PCR reaction mixture (20 μl) contained 200 μM of each dNTP, 500 nM of each primer, 1.5 mM $MgCl_2$, and one unit of Taq polymerase in PCR buffer (Gibco BRL). The DNA was denatured at 94 °C for 3 min. Amplification was performed for 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C, followed by a 10 min final elongation step at 72 °C. 10 μl of the reaction mixture were analyzed on a 1% TAE–agarose gel.

Specific primers used for the characterization of the *UBP34* genomic locus of the transformant lines were:

U5′A (5′-CGTCTAGGGATACAGGAAGG-3′), L3′A (5′-GACTACATACTGCACCATTG-3′), Uc (5′-TATTTTTGG-AGTAGACAAGCGTGTCGT-3′), UC2 (5′-TAATGTGTG-AGTAGTTCCCAGATAAGG-3′).

For RT–PCR, double-stranded cDNAs were prepared from wild type total RNA by using the SMART–PCR cDNA Synthesis Kit (Clontech). Specific primers used for the amplification of the *UBP34*-like partial cDNA fragment were: U171 (5′-CATTGATCCCCTCCCTCCC-3′) and L173 (5′-TCCCCCGAACACAAAACCC-3′).

2.8. Sequence analysis

Sequence data analysis were performed by Blast search at NCBI against the *P. patens* EST database (http://www.ncbi.nlm.nih.gov/blast/; [11]) and at the NIBB PHYSCObase (http://www.moss.nibb.ac.jp/).

2.9. Protein extraction, photoaffinity labeling, and affinity chromatography

Proteins from *P. patens* wild type and *UBP34*-knockout transformants were extracted and photoaffinity labeled as previously described [1]. Purification of the UBP34-like protein on NH_2CPPU–Sepharose 6B affinity column was achieved as previously described [1].

2.10. Mass spectrometry analysis

Affinity purified proteins were analyzed by SDS-PAGE. After staining with Coomassie blue (Bio-Safe Coomassie stain, Bio-Rad), bands were excised and washed twice in 25 mM ammonium carbonate (pH 8), for 30 min, then twice in 50% acetonitrile, 25 mM ammonium carbonate for 30 min and finally with 100% acetonitrile and dehydrated in a speed-vacuum dryer.

The cysteine residues were reduced by 45 min treatment at 56 °C, with 10 mM DTT in 100 mM ammonium carbonate (pH 8). The reduced cysteine residues were then alkylated by 55 mM iodoacetamide in 100 mM ammonium carbonate (pH 8), 30 min in the dark, under vigourous agitation. The gel pieces were then washed twice with 100 mM ammonium carbonate (pH 8), acetonitrile (50/50; v/v) and speed-vacuum dried. The gel pieces were re-swollen with 0.25 μg of sequence grade modified bovine trypsin (Roche) in 25 mM ammonium carbonate, pH 8 until the gel was completely re-hydrated and incubated at 37 °C for 18 h.

The tryptic supernatant was then transferred to a clean Eppendorf tube and the gel pieces were covered with 0.1% TFA in 60% acetonitrile and sonicated for 15 min. This operation was repeated twice and the two supernatants were pooled with the first tryptic supernatant. The supernatants were speed-vacuum concentrated.

MALDI-TOF mass spectra were recorded under the reflectron mode with a Reflex-III (Bruker SA, Karlsruhe, Germany) equipped with a nitrogen laser operating at 337 nm (3 ns pulse duration). The accelerating voltage used was 16 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100–250 laser shots. Mass spectra were calibrated with an external standard containing five peptides in the 1000–2500 m/z range.

The matrix solution was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid in 500 μL distilled water, 500 μL acetonitrile, and 1 μL trifluoroacetic acid. One microliter of this solution was placed on the sample plate together with 1 μL of the sample solution and allowed to dry at room temperature. The sample plate was then placed in the instrument.

3. Results

3.1. Characterization of the UBP34 genomic locus and Southern blot analysis

A *P. patens* genomic library was screened by hybridization using the full length *UBP34 cDNA* as a probe. A positive clone was purified and a 5.5 kb *Eco*RV fragment was subcloned in pBS–KS^- and sequenced. This clone contains the entire cDNA sequence plus 1424 bp in 5′ and 1050 bp in 3′ (Fig. 1A). The genomic DNA includes two introns of 295 and 132 bp. A potential site for initiation of the transcription is located 128 bp upstream of the ATG codon (AATATATAGA). The TAA stop codon is located 1297 bp downstream of the ATG codon on the genomic DNA (Fig. 1A).

P. patens genomic DNA was analyzed by Southern blot hybridisation with the internal cDNA *Xho*I fragment as a probe (Fig. 1A). Under high stringency conditions (65 °C), the hybridization pattern indicated a unique *UBP34* copy in the moss genome, with two locus-specific fragments of 2.6 and 3.3 kb, obtained with *Eco*RI and with *Hin*dIII, respectively (Fig. 2A). The double digestion with *Hin*dIII and *Xho*I produced the expected 1.2 kb fragment (Fig. 2A). When hybridization was performed at 55 °C, other weak hybridization signals appear (Fig. 2B) indicating that the genome includes sequences homologous to *UBP34*. Indeed, several overlapping *P. patens* EST sequences which are homologous to *UBP34* can be detected in the Genbank EST database. The resulting EST contig encodes a 290 amino

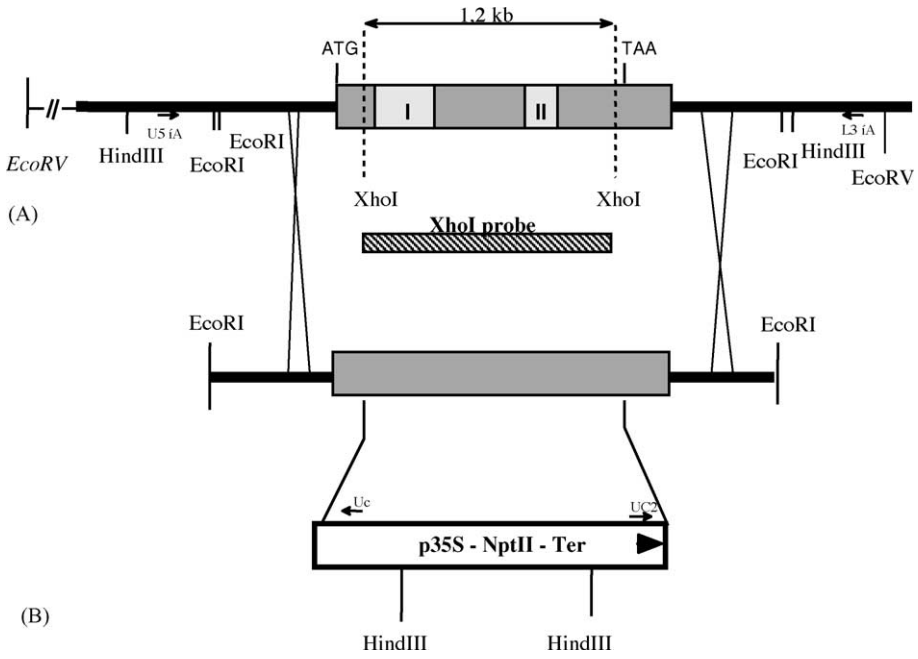

Fig. 1. Structure of the *UBP34* locus (A) and of the replacement vector pUBP34–ΔXhoI–NptII and (B) used for gene disruption. The two introns of the *UBP34* gene are represented by light gray boxes. Location of primers used to screen for targeted transformants is shown by arrows. Hatched box represents the probe used for Southern analysis. (*Eco*RV site on the left belongs to the phage arm).

acids protein which will be referred as UBP34-like (Fig. 3). UBP34 and UBP34-like share 76% identity at the amino acid level with each other. The alignment of a duplicated tandem arrangement of *Pinus monticola* PR10 protein, one of the most closely related higher plant IPR, with UBP34 and UBP34-like (Fig. 3) emphasizes the imperfect duplication of the *P. patens* IPR proteins amino acid sequences [1]. A cDNA fragment, covering all the open reading frame of the *UBP34*-like gene, was amplified by RT–PCR using specific primers and wild-type total RNA. This PCR fragment was used as a probe for low stringency Southern blot (Fig. 2D). It appears that two weak signals, of 2 and 2.2 kb, resulting from *Hin*dIII digestion, and detected at low stringency with the *UBP34* probe (Fig. 2B), correspond to the *UBP34*-like locus. However, other weak signals can not be attributed either to the *UBP34* or to the *UBP34*-like genes (Fig. 2B

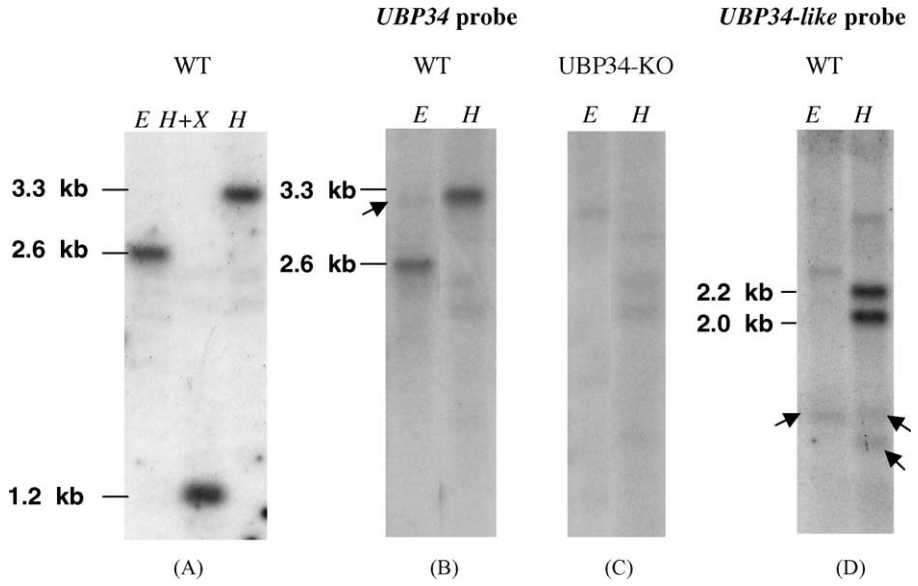

Fig. 2. Southern analysis of wild type (A, B and D) and *UBP34*-knockout plants (C). Hybridization at high stringency (65 °C, A) or at low stringency (55 °C, B–D). Hybridization with the *UBP34 Xho*I probe (A–C) and the *UBP34*-like cDNA probe (D). Restriction digest: H: *Hin*dIII; E: *Eco*RI, and X: *Xho*I. Arrows indicate signals detected at low stringency, with one probe or the other, which can be attributed neither to the *UBP34* locus nor to the *UBP34*-like locus.

Fig. 3. Alignment of UBP34,UBP34-like peptide sequence (deduced from a contig of overlapping EST (**BJ180871**, **BJ186774**, **BJ178893**, **BI487420**, **AW599666**, **BJ170059**, **BJ175657**)) and a duplicated tandem arrangement of *Pinus monticola* PR10 protein, one of the most closely related higher plant IPR (**AAL5006**, from valine (V)11 to glutamic acid (E) 150). Alignment was achieved with the ClustalW program.

and D), indicating that at least another homologous gene exists in the *P. patens* genome.

A phylogenetic analysis was performed using 14 IPR related proteins: **P43185** the major birch pollen allergen (*Betula pendula*); **P52779.1** from yellow lupine which crystal structure has been established [12], the Arabidopsis peptide **At1g24020** with the highest identity to IPR proteins; **P80890** from *Panax ginseng* which possess putative RNase activity; **AAB09084.1** a 17 kDa isolated from Asparagus, a monocotyledonous species and induced by thiocarbamate [13]; **AAD26553** from *Malus domestica*, this protein has been photoffinity labelled using the azido-CPPU probe (P. Simoneau, personal communication); **P19418**, PcPR1 from *Petroselium crispum* the first isolated PR-10 protein; **T10059** a protein from Madagascar periwinckle induced by cytokinin treatment; **BAA74451** from *Vigna radiata* a cytokinin-specific binding protein; **AAF12810** and **AAL50006**, IPR-like from conifers (*Picea glauca, Pinus monticola*); **BJ197020** a single EST from *P. patens*, the first 150 N-terminal aa of UBP34 and UBP34-like proteins where the highest identity with IPR proteins is located (UBP34-like protein is also twice the size of higher plants IPR protein). The resulting tree indicates that BetV1 and MalD1 are very narrow and that *UBP34* and *UBP34*-like genes do not cluster away from IPR of higher plants species (Fig. 4). No IPR homologous sequences for rice or maize are currently available.

3.2. Molecular analysis of UBP34-knockout transformants

The replacement vector consists of a 3.2 kb *Eco*RI fragment with two 750 bp and 760 bp genomic fragments flanking the *Npt*II cassette which replaces a 1200 bp genomic *Xho*I fragment (Fig. 1B). Transformation experiments performed according to the classical method resulted in 2×10^6 regenerating protoplasts. Independent and stable transformed lines have been isolated with a relative transformation frequency of 4.6×10^{-3}. First, 75 stable transgenic lines were screened by PCR for targeted integration of the replacement vector at the *UBP34* locus. PCR analysis with primers U5′A/UC and L3′A/UC2 reveals 30% of gene replacement events among the 75 stable transformants. The sequences of the amplified fragments are in agreement with the corresponding sequences of the pUBP34–ΔXhoI–NptII resulting from the replacement of the genomic *Xho*I fragment by the *Npt*II cassette. Nine knockout transformants were further characterized by Southern blot analysis. The restriction digests of genomic DNA using the *Xho*I fragment as a probe, show that the wild type major signals are lost in the knockout line (Fig. 2C). However, the weak signals visible only under low stringency conditions and corresponding to *UBP34*-like and other homolog(s), are identical in wild type and knockout lines.

In Northern blot analysis with the *Xho*I probe, the *UBP34* transcript of approximately 1.2 kb detected in wild type is absent in the knockout transformant, confirming that a null mutant for the *UBP34* gene was obtained (Fig. 5 A-1). Moreover the *UBP34* transcript does not appear up-regulated upon IP treatment (Fig. 5 A-1).

3.3. Phenotypic analysis of the knockout transformants

Knockout transformants do not exhibit visible morphological alterations throughout the developmental cycle compared to wild type under standard culture conditions (liquid or solid medium). At that time, we could not visualize any overt difference in term of color or density of the tissues, growth rate, and number of gametophore on a protonema

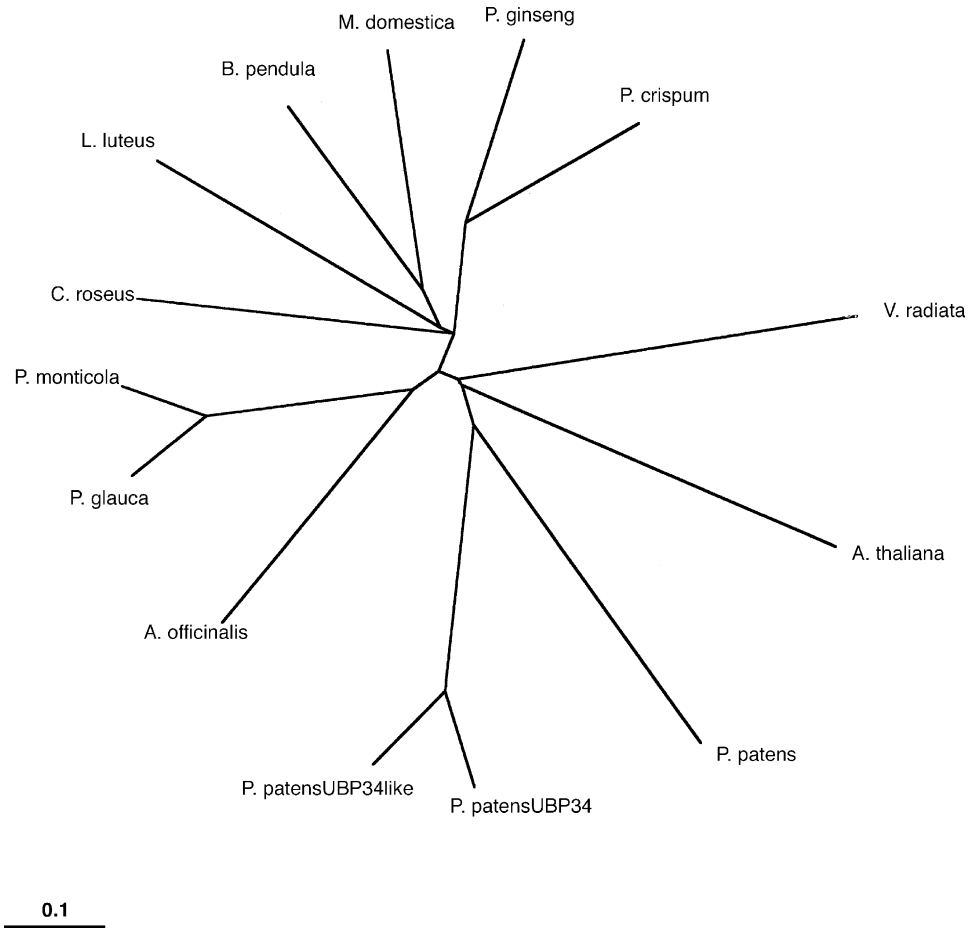

Fig. 4. Distance analysis of the IPR family using the full length amino-acid sequence of 14 IPR proteins, the first 150 N-terminal aa of UBP34 and UBP34-like proteins and the sequence of another related *P patens* protein corresponding to a single EST (Arabidopsis, **At1g24020**; *Lupinus luteus*, **P52779**; *Panax ginseng*, **P80890**; *Betula pendula*, **P43185**; *Asparagus officinalis*, **AAB09084**.1; *Malus domestica*, **AAD26553**; *Petroselium crispum*, **P19418**; *Catharanthus roseus*, **T10059**; *Vigna radiata*, **BAA74451**; *Picea glauca*, **AAF12810**; *Pinus monticola*, **AAL50006**; *Physcomitrella patens* **BJ197020** (single EST)).The sequences were aligned using ClustalX under Phylip format and the phylogenetic tree was drawn using the Treeview software.

base, etc. In order to reveal conditional phenotypes related to the *UBP34* loss-of-function, we tested the growth of the transformant under different conditions.

UBP34 was initially identified as a urea-type cytokinin binding protein. Therefore, the response to cytokinin of *UBP34*-knockout mosses was investigated. Moss transformants resulting from germination of spores had similar phenotypes compared to wild type when grown on medium supplemented with 0.2 μM isopentenyladenine (IP). The response to urea type cytokinin was compared to the

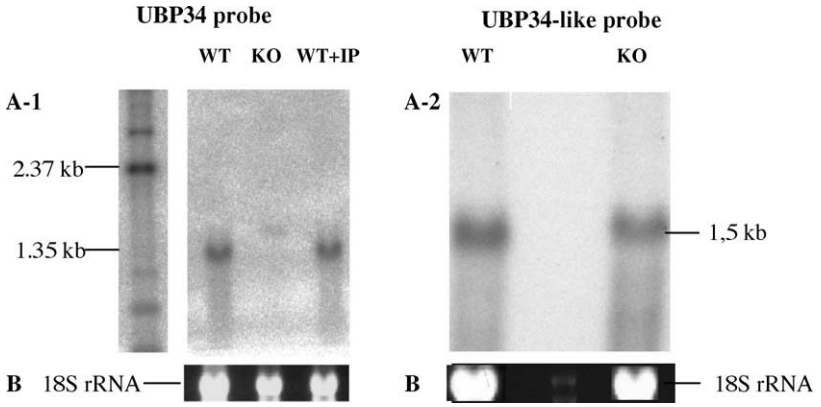

Fig. 5. Northern analysis of wild type (WT) and *UBP34*-knockout plants (KO). Total RNA from wild-type plants (treated or not with 0.2 μM IP for 5 h) and from KO transformants were hybridized with the *UBP34 Xho*I probe (A-1) or *UBP34*-like cDNA probe (A-2). As control, 18S rRNA, with ethidium bromide (B).

response to adenine type cytokinin on 6-day-old vertically dark-grown protonema. Dark-grown mosses are negatively gravitropic [14], and such growth conditions offer the advantage to obtain many unidirectional filaments. Tissues were treated with 0, 30, and 100 nM benzyladenine (BA) or CPPU for 72 h and buds formed on caulonemata files were counted. The cytokinin-induced bud formation of knockout lines was similar to that of wild type.

Considering that UBP34 belongs to a family of proteins involved in stress responses in higher plants, the growth of wild type and knockout plants was compared under osmotic (50 and 350 mM mannitol) or saline (50 and 350 mM NaCl) stress situations. No visible difference was observed in these conditions between wild type and *UBP34*-knockout transformants. High salt concentrations were toxic and killed the colonies and 350 mM mannitol resulted in reduced growth.

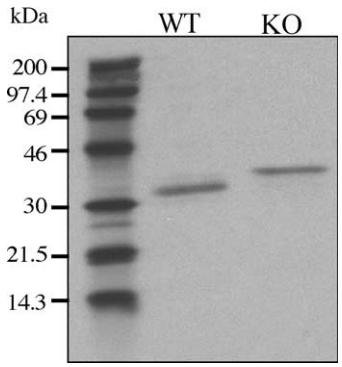

Fig. 6. Photoaffinity labeling with [^{3}H]azido-CPPU of soluble protein extracts of wild type (WT) and *UBP34*-knockout (KO) *P. patens* protonema (25 μg of protein are loaded in each lane). Molecular weights (kDa) of the size markers are indicated on the left.

3.4. Photoaffinity labeling and purification of the UBP34-like protein in the knockout lines

Photoaffinity labeling of soluble proteins from wild type and knockout lines confirmed the absence of the signal in the knockout lines. However, a new photoaffinity labeled protein with an apparent molecular weight of 36 kDa appears in the knockout (Fig. 6). This protein was purified on NH_2CPPU–Sepharose 6B affinity column using soluble proteins from a knockout line, as previously described for purifying the UBP34 protein [1]. Thereafter MALDI-TOF peptide mass fingerprint was realized after tryptic digestion of the 36 kDa purified protein. Eight peptide ions matched masses of predicted peptides resulting from tryptic digestion

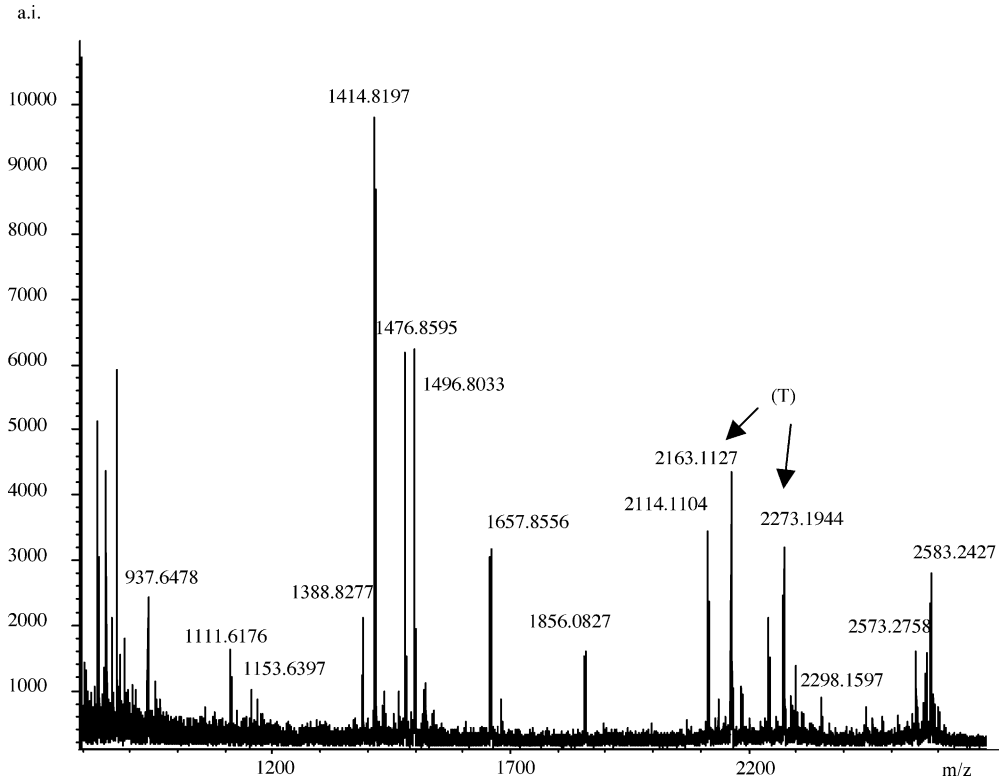

Fig. 7. MALDI-TOF peptide mass fingerprint spectrum of a peptide mixture from in-gel tryptic digestion of the 36 kDa photolabelled protein. Eight peptide ions matched with predicted peptide ions masses expected from the tryptic digestion of UBP34-like protein (Peptide Mass; http://www.expasy.org/cgi-bin/peptide-mass.pl) and covering 41% of the protein sequence; their theorical masses (m/z) are: 1388.7824 (VLPDLLPEFFAK), 1414.7172 (TEILEGDGGPGTLR), 1476.8595 (VLHFGPAIPQAGAAK), 2298.0805 (LDTVDDATMTLSYTVVEGDPR), 1657.8431 (YVNVTGVVSFASTGEK), 1496.7379 (YDVVGEAGPPEHVK), 937.5175 (NITALMFK), and 2114.0400 (TATHTETLDASPDAIWSAVK). Peptide ions 1856.0827 (HSDKVLPDLLPEFFAK), 2583.2427 (ERLDTVDDATMTLSYTVVEGDPR), and 2573.2758 (MGPAIPDAGELVEQVDVFDDAEKK) result from one missed cleavage. Two peptides ions are identified as a result from trypsin (T) autolytic digestion (2163.1127 and 2273.1944).

of the UBP34-like protein, covering 41% of this protein (Fig. 7).

The predicted molecular weights for the two proteins are very similar (UBP34: 31103.23 and UBP34-like: 31115.31) and slightly distant from the apparent molecular mass. It may be related to different post-translational modifications and/or to peculiar three-dimensional folding.

The UBP34-like cDNA fragment, previously used for Southern blot, was used as a probe for Northern blot analysis of total RNA in both wild-type and knockout lines. A corresponding transcript of 1.5 kb, in good correlation with the predicted cDNA size, is present at the same level in wild type and in knockout lines (Fig. 5 A-2). The light signal observed on the Northern blot of KO transformants (Fig. 5 A-1) most probably corresponds to a cross hybridisation of the UBP34-probe with the UBP34-like transcript.

4. Discussion

In the moss *P. patens*, we identified a soluble 34 kDa protein that was specifically labeled by photoaffinity with the phenylurea-derived cytokinin agonist, azido-CPPU. The UBP34 protein is homologous to plant IPR of unknown function. The *UBP34* gene belongs to a multigene family, among which the *UBP34*-like gene could be identified in the EST database. The open reading frames of *UBP34* and *UBP34*-like share 76% identity at the amino acid level with each other. We took advantage of the powerful property of *P. patens* to knockout genes by homologous recombination, to analyze the phenotype of *UBP34* loss of function mutants. In *P. patens*, the requirement for strict sequence homology in gene targeting experiment was illustrated with the highly conserved chlorophyll a/b binding (*cab*) protein multigene family. Although sequence homology between 11 members of the cab multigene family is as high as 86–94% at the nucleotide level, successful targeting of a specific member, the *ZLAB1* locus, has occurred in 30% of the transgenic plants analyzed [15].

The *UBP34* locus was inactivated using a replacement vector containing a *UBP34* truncated genomic fragment (85% of the ORF has been removed and replaced by the *NptII* cassette as a selectable marker). In our study, specific gene replacement at the *UBP34* locus occured in 30% of integrative transformants. The insertion of such a replacement vector in the genome results in a null mutation.

UBP34-knockout plants cultivated on various media, in response to cytokinin and in stress conditions do not display any phenotypic modifications. Even if old publications described interaction of mosses with mycorrhizal fungus and soil organisms as quoted in [16], little is known about the responses of *P. patens* to pathogenic microorganisms. Tomato spotted wilt virus has been reported to infect wild type *P. patens* (Kellmann J., 16. Tagung Molekularbiologie der Pflanzen, Dabringhause 2002), but viruses able to infect specifically bryophytes have not been described so far [17]. Biological assays based on moss behavior in response to higher plant pathogens or elicitors would be very useful to go further in the phenotypic characterization of the *UBP34*-knockout lines.

However, biochemical and molecular analysis of the *UBP34*-knockout lines are indicative of a possible compensation mechanism. A new protein appears specifically photoaffinity labeled in the *UBP34*-knockout lines. This protein could be purified on NH_2CPPU–Sepharose 6B affinity column from soluble proteins of knockout lines, whereas it was not previously purified from wild type strain. Mass finger printing allows the identification of this protein as the UBP34-like protein. In addition, Northern blot analysis indicates that the *UBP34*-like transcript is as abundant in wild type strain as in knockout lines. If the frequency of ESTs in cDNA library are generally consistent with mRNA abundance and can constitute an indicator of gene expression, then the expression pattern of the two genes, deduced from the NIBB-*PHYSCObase*, may be under developmental control. Numerous EST sequences for both *UBP34* and *UBP34*-like are listed in the *P.patens* databases and although *UBP34* EST are present in the three cDNA libraries (auxin- and cytokinin-treated gametophytes and gametophytes that were grown without exogenous plant hormones) the *UBP34*-like transcript is not found in the auxin treated library and over-represented in the cytokinin-treated library [18]. This developmental control may be affected in an UBP34–KO genetic background. At the protein level competition for CPPU between UBP34 and UBP34-like cannot account for the impossibility to photolabel and purify UBP34-like in wild type strain. CPPU in photolabeling experiment is indeed in large excess; moreover a photolabeling experiment with a mixture of proteins from wild type and knockout line, resulted in photolabeling of both UBP34 and UBP34-like (data not shown). Therefore, photoaffinity labeling and affinity chromatography in knockout lines indicates that UBP34-like is either less abundant in wild type strains than in knockout lines or may be present in wild type under a non functional form at least considering CPPU binding properties. Quantification of UBP34-like in both genetic backgrounds, with specific antibodies, could indicate whether this protein is affected in its translation/stability. CPPU binding properties to UBP34-like could also be examined. However, the definitive role of these IPR proteins in moss could be established only when a specific functional assay is available. Host pathogen in moss is still a black box and increased knowledge in this field will enable use of knockout facilities in *P. patens* for functional analysis of this class of IPR protein.

Acknowledgements

We thank Dr. F. Nogué, Dr. H. Barbier-Brygoo and Dr. H. Höfte for stimulating and helpful discussions. We thank

Mr. C. Malosse and Dr. J-J Einhorn for mass spectrometry analysis.

References

[1] M. Gonneau, S. Pagant, F. Brun, M. Laloue, Photoaffinity-labelling with the cytokinin agonist azido-CPPU of a 34 kDa peptide of the intracellular pathogenesis-related protein family in the moss *Physcomitrella patens*, Plant. Mol. Biol. 46 (2001) 539–548.

[2] K. Hoffmann-Sommergruber, M. Vanek-Krebitz, C. Radauer, J. Wen, F. Ferreira, O. Scheiner, H. Breitender, Genomic characterization of members of the Bet V 1 family: genes coding for allergens and pathogenesis-related proteins share intron positions, Gene 197 (1997) 91–100.

[3] J. Mogensen, W. Reinhard, J. Larsen, M. Spangfort, D. Otzen, The major birch allergen, Bet V 1, shows affinity for a broad spectrum of physiological ligands, J. Biol. Chem. 277 (2002) 23684–23692.

[4] Y. Fujimoto, R. Nagata, H. Fukasawa, K. Yano, M. Azuma, A. Iida, S. Sugimoto, K. Shudo, Y. Hashimoto, Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*), Eur. J. Biochem. 258 (1998) 794–802.

[5] S. Carpin, S. Laffer, F. Schoentgen, R. Valenta, J.C. Chénieux, M. Rideau, S. Hamdi, Molecular characterization of a cytokinin-inducible periwinkle protein showing sequence homology with pathogenesis-related proteins and the Bet V 1 allergen family, Plant Mol. Biol. 36 (1998) 791–798.

[6] D.G. Schaefer, Gene targeting in *Physcomitrella patens*, Curr. Opin. Plant Biol. 4 (2001) 143–150.

[7] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

[8] D.G. Schaefer, J.P. Zrÿd, Efficient gene targeting in the moss *Physcomitrella patens*, Plant J. 11 (1997) 1195–1206.

[9] N. Ashton, N. Grimsley, D. Cove, Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants, Planta 144 (1979) 427–435.

[10] S.L. Dellaporta, J. Wood, J.P. Hicks, A plant DNA minipreparation, Version II, Plant Mol. Biol. Reporter 1 (1983) 19–21.

[11] S. Altschul, T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[12] J. Biesiadka, G. Bujacz, M.M. Sikorski, M. Jaskolski, Crystal structures of two homologous pathogenesis-related proteins from yellow lupine, J. Mol. Biol. 319 (2002) 1223–1234.

[13] D. Yeo, T. Abe, H. Abe, A. Sakurai, K. Takio, N. Dohmae, N. Takahashi, S. Yoshida, Partial characterization of a 17 kDa acidic protein, EFP, induced by in the early flowering phase in Asparagus seedlings, Plant Cell Physiol. 37 (1996) 935–940.

[14] C. Knight, T. Futers, D. Cove, Genetic analysis of a mutant class of *Physcomitrella patens* in which the polarity of gravitropism is reversed, Mol. Gen. Genet. 230 (1991) 12–16.

[15] A.H. Hofmann, A.C. Codon, C. Ivascu, V.E. Russo, C. Knight, D. Cove, D.G. Schaefer, M. Chakhparonian, J.P. Zrÿd, A specific member of the Cab multigene family can be efficiently targeted and disrupted in the moss *Physcomitrella patens*, Mol. Gen. Genet. 261 (1999) 92–99.

[16] D.G. Schaefer, J.P. Zrÿd, The moss *Physcomitrella patens* now and then, Plant Physiol. 127 (2001) 1430–1438.

[17] Matthew's, Plant Virology, in: Roger Hull (Ed.), fourth ed., Academic Press, 2002, pp. 796.

[18] T. Nishiyama, T. Fujita, T. Shin-I, M. Seki, H. Nishide, I. Uchiyama, A. Kamiya, P. Carninci, Y. Hayashizaki, K. Shinozaki, Y. Kohara, M. Hasebe, Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: Implication for land plant evolution, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 8007–8012.