Cell wall localization of the aspartic proteinase from buckwheat (FeAPL1) over-expressed in tobacco BY-2 cells

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Abstract: The recombinant aspartic proteinase-like protein (FeAPL1-His$_6$) was overexpressed in the tobacco BY-2 cell line and the expected pepstatin A-sensitive enzymatic activity was confirmed at pH 3.0. Immunocytochemistry and protein gel blot analysis of the transformed BY-2 cells and their protoplasts showed extra-cellular localization of rFeAPL1-His$_6$ in the cell wall. Based on the obtained results, potential functions of FeAPL1 are discussed.

Keywords: aspartic proteinase; buckwheat; BY-2 cells; cell wall.

INTRODUCTION

Aspartic proteases (APs) are one of the major classes of proteolytic enzymes and are widely distributed in the whole living world. According to the MEROPS database (http://merops.sanger.ac.uk/), most plant APs belong to the A1 family, together with pepsin-like enzymes from many origins. They are most active at acidic pH values, are specifically inhibited by pepstatin A and contain two catalytic aspartic acid residues. The majority of plant APs are characterized by the presence of the plant specific insert (PSI) sequence, which is removed from most mature APs together with the signal peptide and the autoinhibitory PRO segment.[1] The recently identified new class of plant APs without PSI (AP-like) is represented by only seven members.[2–7] One of those is FeAPL1 (AY536047), the cDNA of which was isolated from mid maturation buckwheat seed.[7]

The biological functions are still hypothetical for most plant APs, including AP-like, and data are generally derived from the analysis of expression in different tissues or under specific conditions, and from co-localization studies with putative substrates. For example, CDR1 is an apoplastic aspartic proteinase, the overexpression of which causes resistance to virulent *Pseudomonas syringae* by

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activating inducible resistance mechanisms.[3] CND41 is the only DNA-binding aspartic proteinase found in chloroplasts and is involved in the degradation of rubisco during leaf senescence.[5,8–10] Extracellular nepenthesins play a role in prey digestion,[4] while barley nucellin may be involved in nucellar cell death.[2]

Most APs are localized in vacuoles but there are several known to be extracellular.[3,4,11,12] The roles of apoplastic proteins in general are unexplored due to technically complicated extraction procedures.[13] In addition, investigation of APs is especially complicated because this class of proteases is a large family comprising more than 70 members most of which are quantitatively underrepresented and with similar biochemical characteristics, which make their separation and detailed characterization difficult.

Therefore, in this study, recombinant FeAPL1 was produced in the BY-2 cell line in order to identify its cellular localization and protease activity, which are the first steps towards elucidation of its physiological role.

EXPERIMENTAL

Preparation of the expression construct FeAPL1-His$_6$

The binary vector pBINAR-EGFP was linearized by double digestion using *Kpn*I/*Sal*I restriction enzymes (Fermentas), which excised the EGFP sequence.

The coding sequence of the FeAPL1 gene was amplified from the FeAPL1 cDNA clone (AY536047)[7] using forward
P9 (5'-ggtaccatgcccacttctctc-3') and P10 (5'-gtcgacttagtgatggtgatggtgatgattttggatcg-3') primers containing *Kpn*I and *Sal*I restriction sites, respectively. The polymerase chain reaction was cycled five times for 30 s at 94 °C, 30 s at 33 °C and 90 s at 68 °C and then twenty times for 30 s at 94 °C, 30 s at 62 °C and 1.5 min at 68 °C. The amplification product was cloned into the pGEM-TEasy vector (Promega), excised by *Kpn*I and *Sal*I restriction enzymes, gel extracted and subcloned into an opened pBINAR-EGFP vector under control of the cauliflower mosaic virus (CaMV) 35S promoter.

The EHA105 strain of *Agrobacterium tumefaciens* was transformed by electroporation (25 µF, 200 Ω, 2.5 kV) on a BIO RAD Gene pulser. The bacterial cells were grown on LB or LA medium[14] containing kanamycin (25 µg ml^{-1}) and rifampicin (10 µg ml^{-1}).

DNA and RNA isolation and cDNA synthesis

RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. DNA was removed with the DNA-free DNase Treatment and Removal Kit (Ambion) according to the manufacturer's instructions.

Using oligohexamers (Applied Biosystems), cDNA was synthesized according to Chenchik *et al.*[15] with some modifications.[16]

The DNeasy Plant Mini Kit (QIAGEN) was used to isolate genomic DNA according to the manufacturer's instructions.

PCR and RT-PCR were performed employing the primers
rtAPL1f (5'-ccaaggagattgaggacagttcg-3') and rtAPL1r (5'-tcggtgacaagtgagaaataatcg-3').
The polymerase chain reactions were cycled 25 times for 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C.

Transformation of BY-2 cells

The tobacco BY-2 cell line was cultured in TSC (Tobacco Suspension Cell) medium in the dark at 26 °C in a 130-rpm shaker.[17] The cells were subcultured once a week by transferring 2.5 ml of the old culture to 25 ml of fresh TSC medium.

The BY-2 cells were transformed according to Gynheung[18] with some modifications. Thus, BY-2 cells were cultured in modified TSC medium, pH 5.3 containing one-tenth the vitamin concentration prior to transformation. The pellet from an overnight culture of *A. tumefaciens* (OD_{600} 1.1–1.2) was resuspended in 5 % sucrose. Four ml of a 3-day old BY-2 cell suspension was inoculated with resuspended bacterial cells in the presence of 300 µM acetosyringone in a Petri dish and incubated at 26 °C for 48 h without shaking. The BY-2 cells were washed twice with fresh TSC medium and once with TSC medium containing kanamycin (100 mg L^{-1}), carbenicilin (500 mg L^{-1}) and cefotaxime (500 mg L^{-1}). The BY-2 cells were plated on TSC agar plates containing kanamycin (200 mg L^{-1}), carbenicilin (250 mg L^{-1}) and cefotaxime (250 mg L^{-1}).

Preparation of BY-2 protoplasts

BY-2 protoplasts were prepared as described in Stoeckel and Takeda[19] with some modifications. A 4-day old BY-2 cell culture was transferred to fresh TSC medium and grown for 3 days. The 3-day old BY-2 cell suspension (2 ml) was then mixed with 6 ml of digestive medium (1 % cellulase "Onozuka R-10", 0.2 % macerozyme R-10, 0.1 % pectolyase Y-23 (Zakult Pharmaceutical Ind. Co., Ltd.) in 0.45 M mannitol, 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 8 mM $CaCl_2$, pH 5.6) and incubated in the dark at 30 °C for 90 min with agitation at 39 rpm. After centrifugation at 80 g for 7 min, the pellet was gently resuspended in 0.35 M sucrose and 5 mM $CaCl_2$ (2 ml) and gently laid over approximately 6 ml of the same solution. After centrifugation at 80 g for 10 min, the purified protoplasts formed a thick layer on the surface. After two washings with 0.5 M mannitol, 20 mM $CaCl_2$, 0.1 % MES, pH 5.8, the protoplasts were ready for protein extraction.

Preparation of protein extracts

Ground buckwheat seeds, pellets of BY-2 cells and protoplasts were resuspended in acidic buffer (1 M NaCl, 1 % Triton X-100, 3 mM $NaHSO_3$) and sonicated on ice three times for 10 s. Concentrated HCl was added until the extracts reached pH 4.0. After centrifugation at 14000 rpm for 15 min at +4 °C, the supernatants were used for further analyses. Protein concentration was determined according to the method of Bradford.[20]

Immunodetection

Polyclonal anti-FeAPL1 antibodies were raised in rabbits against two peptides corresponding to the FeAPL1 sequences: C-IPIYARNKSSTVQS (amino acids 124 to 137) and CLVSHRFDDTPQSGD (amino acids 230 to 244). The affinity-purified antibodies were obtained from the commercial service, BioGenes, Customized Polyclonal Antisera/Antibodies, Berlin, Germany.

Crude seed, BY-2 and protoplast protein extracts were electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in a Fastblot B43 transfer system (Biometra) according to the manufacturer's instructions.

After transfer, the membranes were washed in PBS-T buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1 % Tween-20, pH 7.5) and incubated in blocking buffer (5 % non-fat dry milk in PBS-T buffer) overnight at +4 °C. The further immunodetection steps were performed at room temperature. The membranes were incubated with anti-FeAPL1 antibodies (1:100 dilution) in PBS-T buffer for 1 h. After washing with PBS-T buffer three times

for 5 min each, the membranes were incubated with anti-rabbit IgG peroxidase conjugate (Sigma) (1:10000 dilution) in PBS-T buffer and again washed three times for 5 min.

Chemiluminescence was detected on an ECL-Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer's recommendations.

Immunocytochemistry

BY-2 cells were immunostained according to Bogre *et al.*[21] with some modifications. The untransformed and transformed 7-day old BY-2 cell suspensions (500 µl) were washed in PBS buffer (140 mM NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) and then fixed in 3 % paraformaldehyde in PBS for 1 h at room temperature. After washing in 0.1 M glycine in PBS and PBS for 5 min each, the cells were digested with 1 % cellulase and 0.5 % macerozyme in PBS for 15 min. The cells washed in PBS were attached to slides treated with poly-L-Lys and air dried. The cells were permeabilized in 1 % Triton X-100 in PBS for 10 min, washed in PBS, and blocked in 1 % BSA in PBS for 10 min. Following incubation with anti-FeAPL1 antibodies (1:20) for 1h in a humid chamber, washing in PBS and incubation with secondary anti-rabbit FITC-conjugated antibodies (Sigma) at 1:200 dilution, the BY-2 cells were washed in PBS and H_2O. The nuclei were stained with DAPI (0.5 mg mL^{-1}, Qbiogene). Fluorescence was detected using an Olympus BX51 fluorescence microscope.

RESULTS AND DISCUSSION

Expression of FeAPL1-His$_6$ in BY-2 cells

Stable integration of the FeAPL1-His$_6$ transgene and its expression in transformed BY-2 cells were confirmed by polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR), respectively, using gene-specific primers (Fig. 1). In addition, a single polypeptide with an approximate size of 55 kDa was detected by affinity-purified anti-FeAPL1 antibodies. A band of the same length was detected in the protein extract of buckwheat mid-maturation seeds (Fig. 2). The difference in protein size of the FeAPL1 produced in *Escherichia coli*,[22] from that in BY-2 plant cells is probably due to post-translational modifications, such as glycosylation.
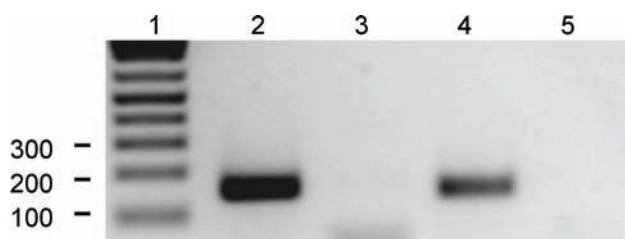

Fig. 1. PCR of gDNA and RT-PCR of cDNA from transformed (lanes 2 and 4, respectively) and untransformed BY-2 cells (lanes 3 and 5, respectively) using rtAPLf/rtAPLr primers. Lane 1 is a "1kb+" DNA ladder (Fermentas) with the marked band sizes.

FeAPL1-His$_6$ showed pepstatin A-sensitive protease activity against BSA in 0.1 M Na-citrate buffer, pH 3.0. The same activity profile was previously shown for recombinant His$_6$-FeAPL1 produced in the Rosetta-gami strain of *E. coli*.[22]
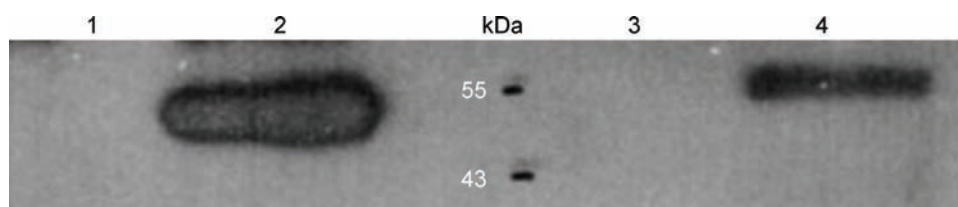
Fig. 2. Protein gel blot analysis of crude protein extracts of untransformed (lane 1), transformed BY-2 cells (lane 2), protoplasts from transformed BY-2 cells (lane 3) and mid-maturation buckwheat seed (lane 4) with anti-FeAPL1 antibodies. Page Ruler Protein Ladder (Fermentas) bands are marked. Extract containing 40 µg of protein was loaded per lane.

Cellular localization of FeAPL1-His$_6$ in BY-2 cells

Based on the presence of the N-terminal signal sequence,[7] it could be expected that FeAPL1 may be extracellular. Recombinant protein localization was determined by immunocytochemistry with FITC-conjugated secondary antibodies. Fluorescence was detected only on the surface of the transformed cells, as discrete fluorescent spots along the continuum of the cell wall invaginations and regularly shaped part of the cell wall (Fig. 3). To confirm this, intact protoplasts were prepared in a saccharose gradient from transformed BY-2 cells by enzymatic removal of the cell wall. The isolated protoplasts were checked under a light microscope for viability and analysis in a polyacrylamide gel showed that the extracted proteins were not degraded. Protein gel blot analysis of the crude protein extract from protoplasts was performed. The FeAPL1-His$_6$ protein was absent, confirming the localization of the FeAPL1-His$_6$ protein in the cell wall (Fig. 2).
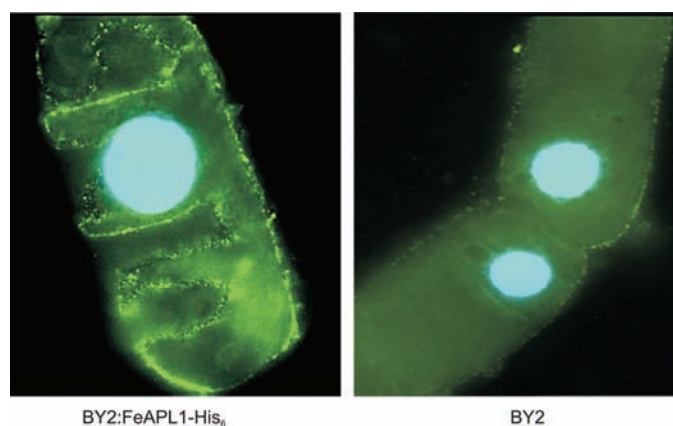

BY2:FeAPL1-His$_6$ BY2

Fig. 3. Localization of FeAPL1-His$_6$ protease overexpressed in BY-2 cells using FITC-labelled secondary antibodies (green spots). The nuclei are DAPI-stained (blue).

The presence and content of plant cell wall proteases as well as their role are unexplored due to technical complications in their extraction.[13] The best studied

proteins are those functionally associated with the polysaccharide matrix (cellulose synthase, hydrolases, esterases, expansins, transglycosylase, *etc*.). Among cell wall proteases, two serine endopeptidases, SDD1 and ALE1; a serine carboxypeptidase, BRS1; cysteine proteases, RCR3, NbCathB, and aspartic protease, CDR1, are relatively well described at the phenotypic level. All of them play strikingly diverse roles in plant development as well as in the response to biotic stress.[23]

Regarding cell wall APs, several mechanisms of action have been proposed. For instance, two extracellular typical APs (StAP1 and StAP3) from potato may be involved in pathogen defence, by direct interaction with the pathogen spore surface, causing membrane permeabilization and death of the cells.[12] Cell wall APs from yeasts, yapsins (GPI-anchored proteases), are involved in cell wall formation and/or remodelling, probably by participation in the regulation of the activation of β-glucan synthesis.[24] Moreover, *Candida albicans* yapsin-like APs, SAP9 and SAP10, which are crucial for the infection process, may be involved in the maintenance of cell surface integrity, separation of the cell wall during budding and adhesion to host epithelial cells.[25]

Taken together, the results obtained here and previous ones related to promoter and tissue expression analysis of the FeAPL1 gene[7,26] offer a contour of its possible functions.

BY-2 cells producing FeAPL1-His$_6$ showed the same viability as untransformed cells in growth medium, but a difference was observed during washing these cells with PBS. Unlike the viable untransformed cells, the transformed cells were prone to lysis and aggregation. It is possible that FeAPL1 modifies the internal structure of the cell wall, affecting some proteins functionally connected with the polysaccharide matrix. In addition, it cannot be excluded that FeAPL1 may act on proteins originating from pathogens. Indeed, the promoter region of the FeAPL1 gene contains some *cis*-elements involved in regulation of gene expression under biotic and abiotic stress (ABRE, ERE, Gbox, RITA1 and W box).[26]

FeAPL1 is expressed during the 9–23 DAF stages of seed development, with a maximum of expression in the 14–17 DAF stage.[7] In these phases, the endosperm is cellular, embryogenesis is complete and the embryo is growing by cell elongation. FeAPL1 activity in the cell wall can be regulated by a shift in the redox status of the seed cells in these developmental stages, which triggers proper disulphide bond formation and protein folding, thus leading to activation of the enzyme. This mechanism was proposed for another AP-like enzyme, CDR1 from *Arabidopsis*, which is also localized in the cell wall and contains twelve conserved Cys residues, as does FeAPL1. CDR1 synthesis is induced by pathogen attack and it is suggested that the oxidative signals generated in response to biotic stress may have an effect on the structurally important intra- or intermolecular disulphide bonds and be part of the regulation of CDR1 activity.[27]

For elucidating the function of FeAPL1, it would be crucial to determine tissue localization as well as to confirm cell wall localization in the buckwheat seeds, which will be the subject of future work.

CONCLUSIONS

Production of proteins in the BY-2 cell line enables the investigation of those that are very difficult to purify in sufficient amounts from plant tissue. In addition, the BY-2 cell line is a more "natural" host for plant proteins than heterologous systems such as bacterial, yeast and insect cells. Production of FeAPL1 in BY-2 cells enabled the determination of its cellular localization – in the cell wall, as well as the confirmation that it retains its pepstatin-A sensitive proteolytic activity at acidic pH values in this biological system, supporting previous findings with recombinant FeAPL1 from *E. coli*.

These results, together with analyses of seed tissue expression, as well as expression under different environmental stress conditions are necessary for revealing the physiological function of FeAPL1. Bearing in mind that the functions of plant aspartic proteases are elusive, this is a significant contribution to research efforts to solve this biological puzzle.

ABBREVIATIONS

ALE1, abnormal leaf shape 1; AP, aspartic proteinase; BRS1, Bri1 suppressor; BSA, bovine serum albumin; BY-2, Bright Yellow; CaMV, cauliflower mosaic virus; CDR1, constitutive disease resistance; DAF, days after flowering; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; NbCathB, *Nicotiana benthamiana* cathepsin B; PCR, polymerase chain reaction; PSI, plant-specific insert; RCR3, required for *Cladosporium* resistance 3; SDD1, stomata density and distribution 1.

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ЋЕЛИЈСКА ЛОКАЛИЗАЦИЈА НОВОГ ТИПА АСПАРТАТНЕ ПРОТЕИНАЗЕ ХЕЉДЕ (FEAPL1) У ТРАНСФОРМИСАНИМ BY-2 ЋЕЛИЈАМА ДУВАНА

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Инсійшійуій за молекуларну ґенеійику и ґенеійичко инжењерсійво, Универзийей у Беоґраду, Војводе Сійейе 444а, й. йр. 23, 11010 Беоґрад

BY-2, ћелијска линија дувана, употребљена је за продукцију новог типа аспартатне протеиназе хељде – FeAPL1-His$_6$. Потврђена је очекивана пепстатин-А сензитивна ензимска активност овог протеина на pH 3,0. Локализација протеина FeAPL1-His$_6$ је одређена имуноцитохемијски и упоредном анализом Western blot трансформисаних ћелија BY-2 и њихових протопласта. Овим методама је утврђено да испитивани протеин има екстрацелуларну локализацију у ћелијском зиду. На основу добијених резултата разматрана је потенцијална функција протеазе FeAPL1.

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