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SEED-SPECIFIC ASPARTIC PROTEINASE FEAP12 FROM BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)

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Abstract –Aspartic proteinase gene (FeAP12) has been isolated from the cDNA library of developing buckwheat seeds. Analysis of its deduced amino acid sequence showed that it resembled the structure and shared high homology with typical plant aspartic proteinases (AP) characterized by the presence of a plant-specific insert (PSI), unique among APs. It was shown that FeAP12 mRNA was not present in the leaves, roots, steam and flowers, but was seed-specifically expressed. Moreover, the highest levels of FeAP12 expression were observed in the early stages of seed development, therefore suggesting its potential role in nucellar degradation.

Keywords: Aspartic proteinase, buckwheat, cDNA, gene expression

Abbreviations: AP, aspartic proteinase; PSI, plant specific insert; DAF, days after flowering; RACE, rapid amplification of cDNA ends

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INTRODUCTION

Proteolytic enzymes are involved in many aspects of plant physiology and development and therefore are the subject of intensive research interest. Proteases are classified into six groups (serine, cysteine, aspartic, threonine, glutamic and metallo-proteases) according to their molecular structure and homology, mechanism of catalytic activity and specific inhibition. It was found that they are most active at acidic pH, are specifically inhibited by pepstatin A and contain two aspartic acid residues crucial for catalytic activity. Aspartic proteinases (EC 3.4.23) are widely distributed, not only in plants but also among vertebrates, yeast, nematode parasites, fungi and viruses (Rawlings and Barret, 1995). According to the MEROPS database (http://merops.sanger.ac.uk/), the majority of plant APs belong to the A1 family, together with pepsin-like enzymes from many origins.

So far, fifty one genes were identified in the *Arabidopsis* genome as coding putative aspartic proteinases. Those sequences were grouped into three categories: typical aspartic proteins, nucellin-like and atypical plant aspartic proteinases (Faro and Gal, 2005). Typical plant APs have been detected or purified from monocotyledonous (Doi et al., 1980; Sarkkinen et al., 1992) and dicotyledonous (Rodrigo et al., 1989) species as well as from gymnosperms (Bourgeous and Malek, 1991). They are typically distinguished from their non-plant homologues by the presence of the so-called plant-specific insert (PSI) in immature forms of the enzymes. Most of the plant APs identified so far are synthesized as a single-chain preproenzyme and are subsequently converted to mature form that can be either a single- or two-chain enzyme (Mutlu and Gal, 1999). Maturation includes the removing of pre and pro segments and is generally accompanied with total or partial excision of PSI (Simões and Faro, 2004).

For the great majority of plant APs biological functions are still hypothetical and represent a challenging field of research. For instance, these enzymes are believed to participate in various proteolytic processes during seed development and germination, including the maturation of storage proteins and the initiation of their hydrolysis (D'Hondt et al., 1993; Guilloteau et al., 2005). Additionaly, APs are associated with protein degradation during organ senescence and cell death (Panavas et al., 1999), autolysis during formation of tracheary elements (Runeberg-Roos and Sarma, 1998), prey digestion in carnivorous plants (An et al., 2002), adhesion-mediated proteolytic mechanisms in pollen recognition and growth (Faro et al., 1999) and degradation of pathogenic proteins (Rodrigo et al., 1991). Besides scientific interest in the clarification of APs structure and function, the research of their properties could be of significance for the food industry, i.e. for milk clothing in the process of cheese production (Roa et al., 1999; Timotijevic et al., 2003).

In this paper we present the isolation and characterization of partial FeAP12 cDNA from buckwheat encoding for typical plant AP. In addition, we analyzed its expression in different buckwheat organs as well as throughout different stages of seed maturation.

MATERIAL AND METHODS

Plant material

Buckwheat (*Fagopyrum esculentum* Moench, cv. Darja) was field-grown in the garden of the Institute of Molecular Genetics and Genetic Engineering, Belgrade. The maturation period of the buckwheat seeds was about 30 days. Seeds from different stages of development were collected and stored at -70°C, or immediately used for RNA isolation. Leaves, roots, steams and flowers were collected and stored at -70°C prior to use.

RNA isolation

RNA isolation was performed as described by Taylor and Powell (1982). Poly (A)+ mRNA was purified using Straight A's mRNA Isolation System (Novagen) and Magnetight™ Oligo (dT) Particles (Novagen).

cDNA synthesis

cDNA synthesis using poly-T primer (5'-TTCTAGAATTCAGCGGCCGC(T)30-N-1N-3' where N-1 is G, A or C, N is G, A, C or T -3') was carried out according to Chenchik et al. (1995) with some modifications (Samardžić et al., 2004).

Isolation of cDNAs encoding for typical aspartic proteinases

Amino acid sequences of nine APs from different plants (X56136, AJ237674, AP002480, AY142687, X80067, AB002695, AB045891, AB069959, AJ313385) were used to design degenerate primers encoding for two highly conserved domains: D T G S S N L W V from the N-terminal and W I L G D V F M G from the C-terminal domain of the mature enzyme. The following degenerate primers were used:

FN1: GAYACIGGVAGYTCHAAYCTNTGGGT (forward primer)

RC1: TGGATHYTKGGIGAYRTNTTTATG (reverse primer)

(Y=C or T, R=A or G, V=C or G or A, H=A or C or T, K=A or G or T, N=C or G or A or T, I = inosine)

The polymerase chain reaction was cycled 30 times for 30s at 94°C, 30 s at 42°C and 3 min at 72°C employing 100 ng cDNA as the template, with 30 pmol of degenerate primers using Advantage™ 2 Polymerase Mix (Clontech). The products of amplification were eluted and cloned into pGEM-T Easy vector (Promega) and then sequenced using ABI PRISM 3100 DNA Sequencer (BMR Servizio Sequenciamento Service, Padova, Italy).

RT PCR gene expression analysis

In order to analyze *FeAP12* gene expression in different buckwheat organs RNA was isolated using a

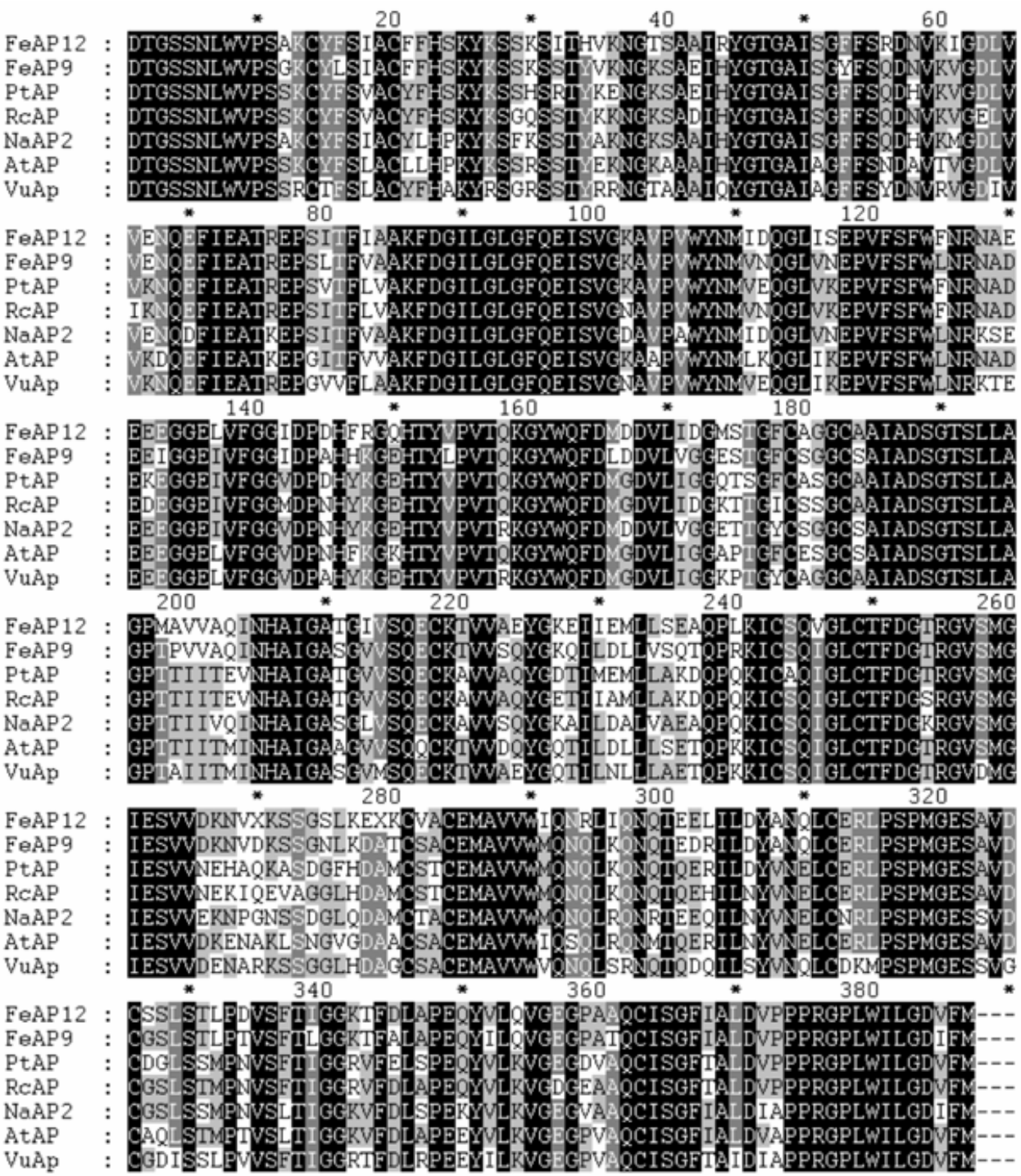

Figure 1. Similarity between FeAP12 and other typical aspartic proteinases. *FeAP9, Fagopyrum esculentum* (AAV84085.2); *PtAP Populus trichocarpa* (XP_002298827.1); *RcAP, Ricinus communis* (EEF32480.1); *NaAP2, Nepenthes alata* (BAB20970.1); *AtAP, Arabidopsis thaliana* (NP_172655.1); *VuAP, Vigna unguiculata* (AAB03843.2).

combination of the modified CTAB mini prep method described by Doyle and Doyle (1987) and the Rneasy® Mini Kit. Plant samples (0.5-1 g) were placed in liquid nitrogen and ground thoroughly with mortar and pestle. Three volumes of extraction buffer (2% CTAB; 100 mM Tris, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 20 mM DTT and 2% PVP) were added to the ground material and the mixture was incubated for 10 min at 65°C. Nucleic acids were purified in 1 volume of SEVAG (chloroform:isoamyl alcohol 1:24). After centrifugation for 10 min at 9000 rpm, the aqueous layer was removed to a fresh tube and 1/10 volumes of 10% CTAB was added. The extraction with SEVAG was repeated and RNA from the aqueous phase was precipitated with 2 M LiCl. The pellet was dried and re-dissolved in 100 μM RLT buffer from the Rneasy® Mini Kit. Further RNA purification was done as described in the Rneasy® Mini Handbook. Prior to cDNA synthesis contaminating DNA was removed from RNA samples using Ambion DNA-free Dnase Treatment and Removal Reagents. First strand cDNA was synthesized from 2 μg of RNA with M-MuLV reverse transcriptase and random hexamer primers (Applied Biosystem) according to the manufacturer's instructions. The cDNA were diluted 1:20 with nuclease-free water. Aliquots of the same cDNA sample were used for RT PCR with primers specifically designed for FeAP12 (AP12f: 5'-TTG TTG AGA ATC AGG AAT TTA TTG AGG-3'/AP12r: 5'-ACC AAA TAC AAG CTC ACC ACC TTC-3') and histone 3 (H3f: 5'-GAA ATT CGC AAG TAC CAG AAG AG-3'/H3r: 5'-CCA ACA AGG ATA TGC CTC AGC-3') as a house-keeping gene. Reactions were done in 25 μL volume containing 500 nM of each primer and AmpliTaq Gold polymerase. RT PCR was performed on the Termocycler (Biometra) using the following cycling conditions: initial denaturation at 94°C for3 min, followed by 20 cycles at 94°C for 15 sec, 60°C for 40 sec and 72°C for 1 min).

Computer-Assisted Analysis

Protein sequences were compared using the BLASTP search program and exploring all of the available sequence databases at: www.ncbi.nlm.nih.gov Web server. Sequence analysis was done using the ExPASy program package (www.expasy.org). Sequence alignment was performed by the ClustalW program. Level of expression was estimated by computer analysis of electrophoretogram using Scion Image densitometry program (based on "NIH Image for Macintosh" by Wayne Rasband, National Institute of Health, USA, modified for Windows by Scion Corporation, 1997).

RESULTS AND DISSCUSION

Cloning and sequence analysis of typical AP cDNA

In order to isolate cDNA encoding for buckwheat aspartic proteinase, PCR reaction was performed using degenerated primers and total cDNA from developing buckwheat seeds having in mind that aspartic proteinase activity was found to be the most prominent in that organ (Timotijević et al. 2003). Products of amplification were cloned and two types of PCR products were detected after restriction analysis by EcoRI. Representatives of both were selected and sequenced. Computer analysis using the BLAST algorithm confirmed that one of the selected sequences was FeAP9, (Milisavljević et al., 2008). Another one was 1161 bp long partial cDNA coding for other buckwheat aspartic proteinase, assigned as FeAP12 (acc. no. AY826352). Analysis of the FeAP12 sequence using the BLAST algorithm showed the highest similarity of 93% FeAP12 shares with FeAP9 from buckwheat. Also, high sequence similarity was noticed with aspartic proteinases from other plant species: *Ricinus communis* 90%, *Nepenthes alata* 90%, *Populus trichocarpa* 89%, *Vigna unguiculata* 88% and *Arabidopsis thaliana* 86% (Fig. 1).

Analysis of the FeAP12 deduced amino acid sequence (Fig. 2) showed the presence of the two active site aspartic acid residues, ^{36}Asp and ^{223}Asp, that are essential constituents of all aspartic proteases. Further, two potential N-glycosylation sites at positions 37 (NGTS) and 298 (NQTE) were predicted by the NetNGlyc algorithm (http://www.cbs.dtu.dk/services/NetNGlyc/). Also, the primary structure characteristic for typical plant APs and the presence of 104 amino acid long plant

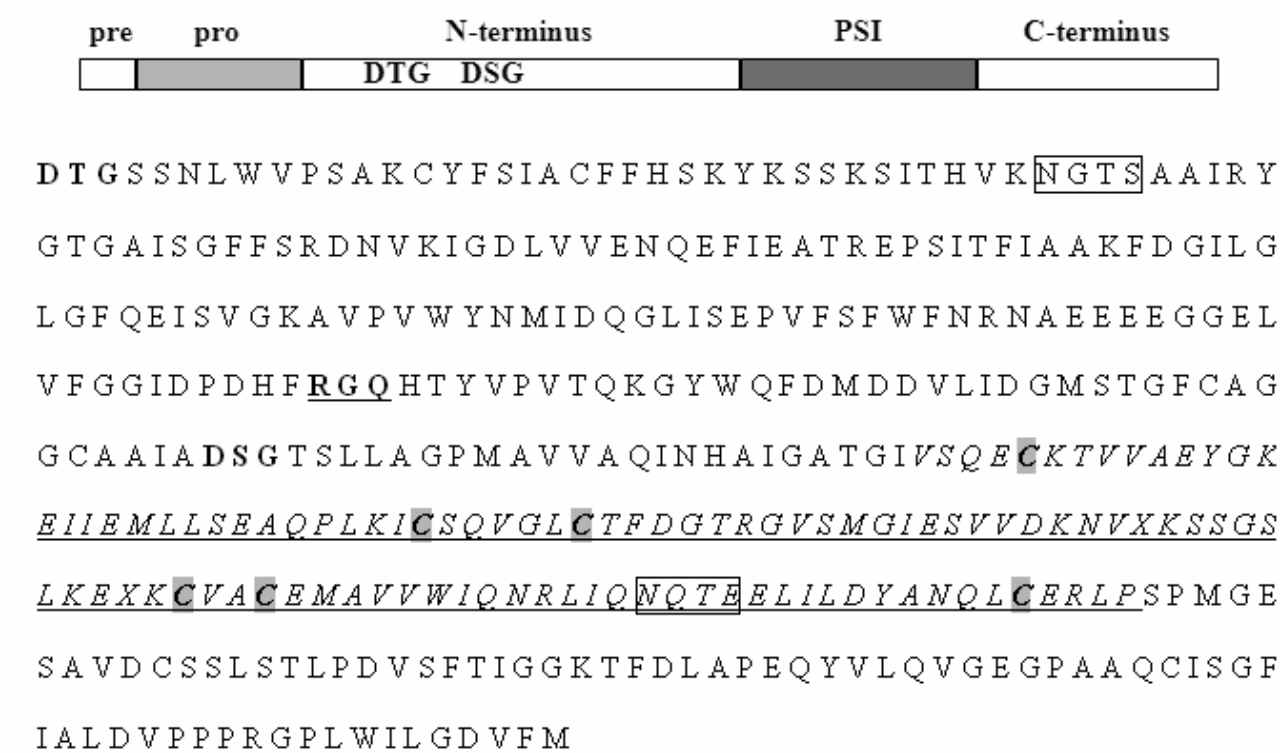

Figure 2. Primary translation product of typical plant AP genes and deduced amino-acid sequence of buckwheat aspartic proteinase FeAP12. DTG/DSG catalytic trieades are bolded, RGQ motif is bolded and underlined, PSI region is underlined and in italic letters and potential N-glycosylation sites are bordered.

specific insert (PSI) were confirmed. Analysis of the PSI region by NCBI conserved domain search showed homology of 41.3% in the N-terminal portion and 40.9 % in the C-terminal portion with two domains of saposin B present in prosaposin (smart00741). Also, 54.1% homology with the saposin-like type B region 2 (pfam03489) and 62.3% homology with the saposin-like type B region 1 (pfam05184) was found. Six conserved cysteins, one invariant tyrosine as well as a putative N-glycosylation site are remarkably conserved between FeAP12 and saposins. It is proposed that lipid binding by regions such as the saposin-like domains founded in the PSI region of FeAP9 are essential for vacuolar targeting of the proteinases (Runeberg-Roos et al., 1994; Guruprasard et al., 1994). On the other hand, it was shown that cardosin B containing PSI has been detected in an extracellular matrix (Vieira et al., 2001). Another as yet unidentified sorting signal may assist together with PSI in vacuolar AP mobilization to this organelle. Possibly PSI is promoting the association to the membrane after translocation into ER, thus preventing unwanted proteolysis during the intracellular transport of the precursor (Vierira et al., 2001). Thus, as the role of PSI remains unclear its presence in FeAP12 does not necessarily mean that this aspartic proteinase has to be localized in vacuoles.

An internal RGQ motif was observed in FeAP12 between residues 246 and 248. In contrast to FeAP12, FeAP9 contains the KGE motif instead of the RGQ motif at the same position. The KGE motif, as well as the RGD motif, found in many other typical plant Aps, are characteristic of

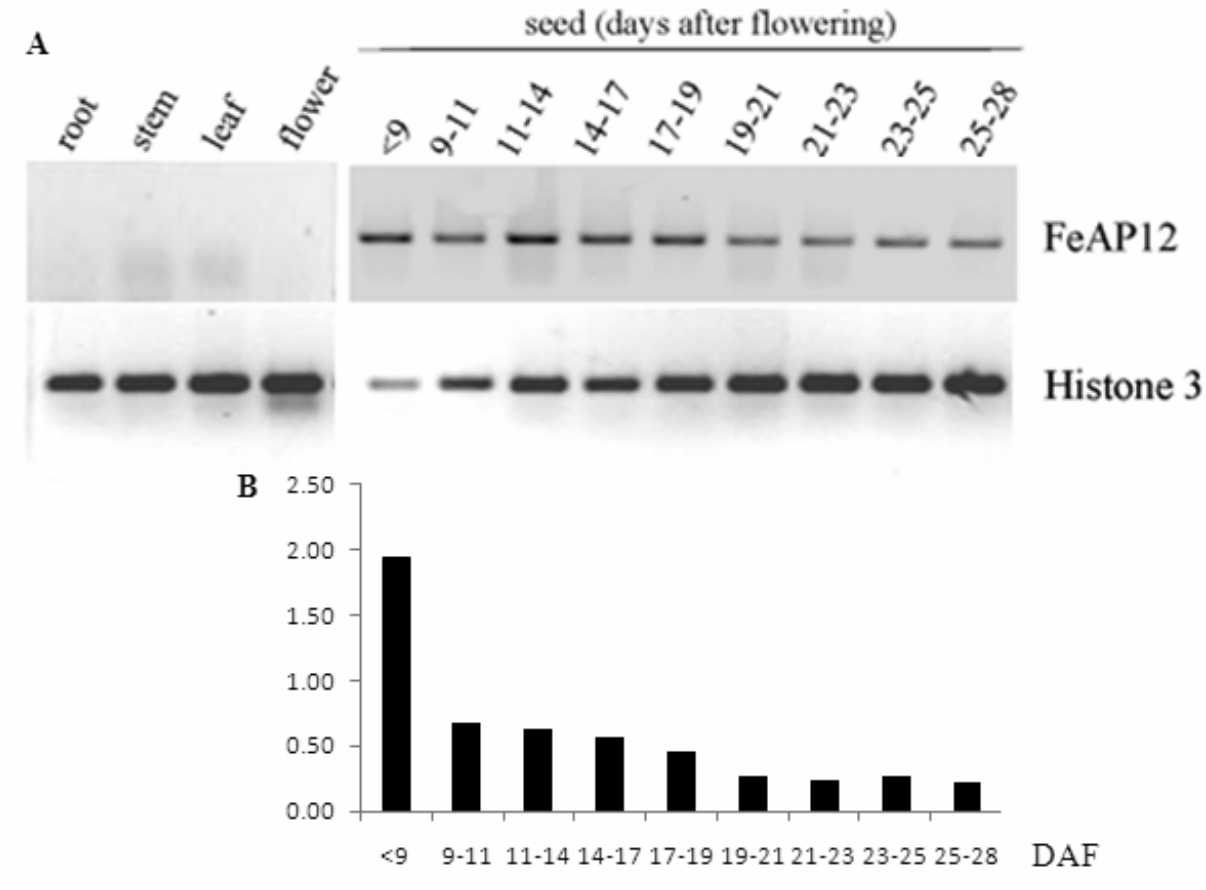

Figure 3. Expression of FeAP12 gene in several buckwheat organs and during seed development. RT-PCR analysis obtained using the FeAP9 gene specific primers. Histone 3 gene was used as the amplification positive control.

integrin-binding proteins. Recently, Simões et al. (2005) showed that KGE in cardosin A had a role in the interaction between proteinase and phospholipase Dα in pollen-pistil recognition. Even though RGQ residing in the FeAP12 sequence is not structurally or charge-wise similar to KGE or RGD, analysis of the predicted tertiary structure of FeAP12 (data not shown) indicated that this sequence is located on the molecular surface and that it may possibly be responsible for some other interaction or function of this particular protein.

Profile of FeAP12 gene expression

In order to understand the possible function of the FeAP12 described in this paper, RT PCR analysis was carried out to examine the specificity of its expression in several buckwheat organs. RT PCR was performed using as a template cDNAs obtained from different buckwheat organs such as leaf, root, flower and stem as well as from seeds in different stages of development. For this purpose, gene specific primers for FeAP12 were designed and consequently a specific PCR-amplified fragment of about 150 bp was obtained. Primers designed according to the sequence of the tobacco histon 3 gene were used for internal control amplification. This experiment showed that FeAP12 gene expression was restricted to seeds only as FeAP12 mRNAs were not detected in most of the tested organs: leaves, roots, stems and flowers (Fig. 3A). The highest FeAP12 expression was detected in

seeds younger than 9 DAF and after that stage its expression decreased. Even though we detected a lower level of expression in the last stages of seed development, the expression of FeAP12 was still significant (Fig. 3B)

Interestingly, the profile of FeAP12 expression in developing buckwheat seeds was different in comparison to the FeAP9 expression pattern (Timotijevic et al., 2009). Namely, FeAP12 mRNA was clearly present already in the early stage of seed development, when FeAP9 mRNA was hardly detectable. Also, the expression profile of FeAP9 was similar to the profile of the 13S storage protein (SP) gene expression (Milisavljević et al., 2008), while FeAP12 did not follow this storage protein gene expression profile. Several studies have suggested a role for plant APs in proteolytic processing and maturation of storage proteins based on *in vitro* data (D'Hondt et al., 1993). Correspondingly, the positive correlation between FeAP9 and 13S SP implies that FeAP9 is involved in some processing mechanisms interacting with storage proteins directly, or via some other types of proteolytic enzymes, the activity of which is FeAP9-dependent. On the other hand, the absence of correlation of FeAP12 and 13S SP expression indicates that possibly FeAP12 is not involved in this particular storage protein maturation in developing seeds. Nonetheless, we presume that FeAP12 could have some implication in storage protein processing and modification as it is expressed in all the investigated stages of seed development. The abundant accumulation of FeAP12 mRNA in the early stages of seed development clearly suggests that this AP plays an important role in this phase of the process. Also, there is a possibility that FeAP12 is expressed at earlier stages of embryogenesis that were not investigated in this study. The pattern of FeAP12 expression during seed development suggests that it could be involved in nucellar degradation, as it was previously reported that caspase-like proteases, which had major role in this process, had the highest activity in the early stages of seed development (Lombardi et al., 2007). It is well known that the nucellus is the central portion of the ovule inside the integuments and that it plays a very important role in the early stages of seed development as it feeds the embryo. After rapid growth, the nucellus degrades by means of a regulated programmed cell death. Subsequently, the endosperm as the main seed storage organ expands and accumulates reserves at the expense of the nucellus (Chen and Foolad, 1997). Thus, we hypothesize that FeAP12 could be involved in PCD of nucellar cells.

In conclusion, we identify here a new typical aspartic proteinase from buckwheat, named FeAP12. Its distinctive pattern of expression and structural specificities in comparison to previously characterized FeAP9 indicate that this enzyme might have distinctive functional features. Further investigation is needed in order to determine specific functions, substrate specificity and intracellular localization of the two different APs coexisting in developing buckwheat seeds. Specifically, in future experiments we intend to obtain full-length cDNA encoding for FeAP12 by the 5'- and 3'-RACE method and also to specify the localization of this AP by *in situ* hybridization, as well as to produce recombinant proteins in order to analyze their proteolitic activities.

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АСПАРТИЧНА ПРОТЕИНАЗА (FeAP12) ЈЕ СПЕЦИФИЧНО ЕКСПРИМИРАНА У СЕМЕНУ ХЕЉДЕ (*FAGOPYRUM ESCULENTUM* MOENCH)

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Ген за аспартичну протеиназу (FeAP12) је изолован из eDNA библиотеке семена хељде у развићу. Анализа изведене амино киселинске секвенце FeAP12 гена указује на њену високу хомологију са осталим типичним биљним аспартичним протеиназама (AP) које се одликују присуством биљно специфичног инсерта (plant specific insert PSI), јединственог међу AP. Показано је да ген FeAP12 није експримиран у листу, корену, стаблу и цвету, већ да је iRNA за *FeAP12* присутна само у семену. Највећи ниво експресије овог гена је уочен у раним фазама развића семена, што указује на његову могућу улогу у деградацији нуцелуса.