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Ubiquitous aspartic proteinase as an actor in the stress response in buckwheat

Gordana S. Timotijević [a,*], Mira Dj Milisavljević [a], Svetlana R. Radović [b], Miroslav M. Konstantinović [a], Vesna R. Maksimović [a]

[a] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, P.O. Box 23, 11010 Belgrade, Serbia
[b] Faculty of Biology, University of Belgrade, Belgrade, Serbia

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SUMMARY

The aspartic protease (FeAP9) gene from buckwheat resembles the exon–intron structure characteristic for typical aspartic proteinases, including the presence of the leader intron in the 5′-UTR. RT PCR experiments and gel protein blot analysis indicated that FeAP9 was present in all analyzed organs: developing seeds, seedlings, flowers, leaves, roots and stems. Using Real-time PCR, we found that *FeAP9* expression is upregulated in buckwheat leaves under the influence of different abiotic stresses, including dark, drought and UV-B light, as well as wounding and salicylic acid.

Introduction

Proteolytic enzymes occur in all living organisms and are involved in a multitude of physiological functions, from simple protein degradation during food digestion to signal transduction in highly regulated signaling cascades. Historically, proteases have been classified into four groups (serine, cysteine, aspartic and metallo-protease) according to their molecular structure and homology, chemical mechanism of catalytic activity, and specific inhibition (Barrett et al., 1998). Recently, two more types of proteinases were added into general classification: threonine proteases, which are constitutive elements of 26S proteasome (Seemüller et al., 1995), and glutamic proteases uniquely found in filamentous fungi (Sims et al., 2004). Aspartic proteinases (AP) (EC 3.4.23) require an acidic pH for optimal enzymatic activity and they are specifically inhibited by pepstatin A. An important structural feature is the presence of two aspartic acid residues crucial for catalytic activity. APs show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues. Their presence and activity have been increasingly documented in various groups of organisms, including vertebrates, nematode parasites, plants, fungi, bacteria and viruses (Davies, 1990; Rawlings and Barret, 1995). Much of our knowledge about the biological role of APs has emerged from studies on microbial and animal proteases (Davies, 1990; Rawlings and Barret, 1995), whereas data about their functions in plants are still sparse.

Plant APs have been detected or purified from monocotyledonous and dicotyledonous species as well as from gymnosperms. 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 (Runeberg-Roos et al., 1991; Hiraiwa et al., 1997). The majority of plant APs identified to date are synthesized as single-chain preproenzymes, which are subsequently converted to mature forms that can be either single- or two-chain enzymes (Mutlu and Gal, 1999). Maturation includes removal of pre- and pro-segments generally accompanied by total or partial excision of PSI (Simões and Faro, 2004). In addition to typical APs, two more AP groups have been identified and added to AP classification, according to their domain organization and their active site sequence motifs: nucellin-like and atypical plant aspartic proteinases (Faro and Gal, 2005). Thus far, several typical AP gene structures, containing 11 or 12 introns at the same positions and of similar lengths, have been isolated and characterized. Interestingly, the majority of typical APs have a leader intron in the 5′-UTR, but its biological relevance has not yet

Abbreviations: AP, aspartic proteinase; CTAB, cetyltrimethylammonium bromide; DAF, days after flowering; DTT, dithiothreitol; EDTA, ethylenediamine tetra-acetic acid; PSI, plant-specific insert; PVP, polyvinyl-polypyrolidone; PVDF, poly-(vinylidene difluoride); RACE, rapid amplification of cDNA ends; SA, salicylic acid; UTP, untranslated region

* Corresponding author. Tel./fax: +381 11 3976 414.
E-mail address: timotijevic@imgge.bg.ac.rs (G.S. Timotijević).

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been clarified (Chen et al., 2002; Pimentel et al., 2007). Biological functions are still hypothetical for most plant APs and represent a challenging field of research. Data related to the possible functions of APs are derived from analysis of expression in certain tissues or under specific conditions, and from co-localization studies with putative protein substrates. For instance, these enzymes are believed to participate in various proteolytic processes during seed development and germination, including the maturation and hydrolysis of storage proteins (Guilloteau et al., 2005). Also, 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 Saarma, 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). The physiological significance of APs during stress responses is not well documented, although it is widely accepted that plant proteases play key roles in responses to diverse environmental and developmental stimuli (García-Lorenzo et al., 2006). To date, there is little evidence regarding the role of APs under conditions of stress, such as water deficit (Cruz de Carvalho et al., 2001) or metal stress (Pena et al., 2006). In addition to basic scientific interest in the elucidation of AP structure and function, an investigation of their properties could be of significance for the food industry, i.e. for milk clotting (Timotijević et al., 2003), and the pharmaceutical industry, i.e. for extracellular matrix remodeling in surgery (Duarte et al., 2005).

In this study, the gene of the buckwheat aspartic proteinase FeAP9 was isolated and its structure analyzed. In addition, we investigated the presence of AP9 mRNK by RT PCR, and AP9 protein using antibodies raised against an oligopeptide from the N-terminus in different buckwheat organs and tissues. We also examined *FeAP9* expression during different abiotic stresses.

Material and methods

Plant material and treatments

Buckwheat (*Fagopyrum esculentum* Moench, cv. Darja) was grown in the greenhouse of the Institute of Molecular Genetics and Genetic Engineering, Belgrade. The maturation period of buckwheat seeds was about 30 d. Seeds from different stages of development were collected and stored at −70 °C, or immediately used for RNA or protein isolation. Leaves, roots, stems and flowers were collected and stored at −70 °C prior to use. For dark-induced senescence, plants were kept in continuous darkness and fully mature leaves were collected from a number of buckwheat plants after 1, 2, 3, 5, 7, 9 and 11 d. Drought stress was induced by withholding watering for 13 d from completely developed plants. Fully expanded leaves were collected after 3, 5, 7, 9, 11, 13 and 15 d of drought. Wounding was performed by puncturing several holes (approximately 10 per leaf) using a syringe needle ($\varnothing$ 0.45 × 12 mm, 26G × 1/2″). Wounded leaves were collected after 1, 2 and 3 d. For UV experiments, plants were treated with supplemented UV-B light (radiation 290–320 nm) for 1, 2 and 24 h as described by Jovanović et al. (2006). In addition, buckwheat leaves were incubated with medium containing SA according to Wen et al. (2005) with minor modifications. The leaves were sliced into small 1 cm^2 squares and immediately immersed in the equilibrium buffer. Experimental leaf slices were then placed in a Petri dish with 20 mL of incubation buffer containing equilibrium buffer and 200 μM SA. As a control, the same weight of leaves was placed in incubation buffer with distilled water instead of SA solution. Experimental and control leaves were taken out of the buffers after 1, 3 and 5 h.

DNA isolation

Genomic DNA from buckwheat buds was isolated as described by Dellaporta et al. (1983). Plasmid DNA was isolated using the Qiagen MiniPrep Kit according to the manufacturer's instructions (Qiagen).

RNA isolation

RNA was isolated using a 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 a mortar and a 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 were added. The extraction with SEVAG was repeated, and RNA from the aqueous phase was precipitated with 2 M LiCl. The pellet was dried and redissolved in 100 μM RLT buffer from the Rneasy® Mini Kit. Further RNA purification was performed as described in the Rneasy® Mini Handbook.

Protein extract preparation

Buckwheat organs were ground to a fine powder with a mortar and pestle in liquid nitrogen. An acidic protein extract from buckwheat seed powder was prepared by homogenization in three volumes of acidic buffer (1 M NaCl; 1% Triton X-100; 3 mM $NaHSO_3$) with a supplement of PVP, adjusted to pH 4.0 with HCl as described in Sarkkinen et al. (1992). Protein concentration was determined according to the method of Bradford (1976).

cDNA synthesis and Real-time RT PCR

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 was diluted 1:20 with nuclease-free water. Aliquots of the same cDNA sample were used for Real-time PCR with primers designed for *FeAP9* (Uap1/Uap2) and histone 3 (H3f/H3r) as a house-keeping gene (Table 1). Buckwheat 26S rRNA (26Sf/26Sr) was used as an additional reference gene in RT PCR experiments. Reactions were performed in a 25 μL volume

Table 1
Sequences of the oligonucleotides used in this work.

Name	Sequence (5′ **to 3′**)	Name	Sequence (5′ **to 3′**)
APK1	atgaccaacatttctcggatttcgt	If	attggctctttgcatcatcc
P408	gcgattgagccagaatgaaaatac-	Ir	ccaaggccatacttctgcat
P284	agtttgacgggatacttgggcttg-	Uap1	aaaccgccgtgcaaattc
P952	tgcattcctggctcactacacc	Uap2	tgaaagcagcaccacgacg
P916	gcaattggtgcctctggtgtag	H3f	gaaattcgcaagtaccagaagag
APK2	ctaagcagcttcagcaaacccgact	H3r	ccaacaaggtatgcctcagc
AP1	ccatcctaatacgactcactatagggc	26Sf	attcccaaacaacccgactc
P429	caatagcaatctcaccgaagtattgg	26Sr	gccgtccgaattgtagtctg
AP2	actcactatagggctcgagcggc	APSHn1	tgacccgggaataatgatttggtcagag
P269	ttctcttctttaggcccactctgacc'	APSHn2	tataagcttagcagcttcagcaaacccg