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Buckwheat as a Model Plant in Molecular Biology

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ABSTRACT

Buckwheat (*Fagopyrum esculentum* Moench) is a pseudocereal crop, mostly grown in the Northern Hemisphere. It is desirable for human consumption because buckwheat seeds have a high content of proteins (with high concentrations of essential amino acids) and minerals (e.g. iron, zinc and selenium). Concerning their high nutritive value, buckwheat seed storage proteins (SSPs), and genes that code for them, are of importance to study. Our research focus is the structure and the expression profile of selected buckwheat genes coding for proteins of known functions (such as SSPs), as well as proteins of undefined functions possibly involved in protein degradation/processing, and/or in the stress response (e.g. aspartic proteinases and metallothionein). These genes, their promoters and translational products are important, not only from the aspect of fundamental research, but also in regard to their potential biotechnological application in agriculture and land preservation. In particular, we are interested in the processes taking place during the last stage of buckwheat embryogenesis, especially in the analyses of specific gene expression regulation under normal physiological and/or stress conditions, which is the subject of our present research.

Keywords: aspartic proteinase, biotechnology, metallothioneins, seed storage proteins
Abbreviations: AP, aspartic proteinase; **cDNA**, complementary deoxyribonucleic acid; **CP**, carboxypeptidase; **CPR**, cysteine proteinase; **DAF**, days after flowering; **EYFP**, enhanced yellow fluorescent protein; **FITC**, fluorescein isothiocyanate; **GFP**, green fluorescent protein; **MT**, metallothionein; **MPR**, metalloproteinase; **PB**, protein bodies; **PSI**, plant-specific insert; **rER**, rough endoplasmic reticulum; **SA**, salicylic acid; **SSP**, seed storage protein; **UTR**, untranslated region

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INTRODUCTION

Buckwheat is a dicotyledonous crop with significant use in two important subjects of contemporary interests: providing healthy food/feed and land preservation. Firstly, although not a cereal, buckwheat is used as a cereal (for food and feed) because of its good nutritive characteristics. Secondly, buckwheat is often used as a green manure, a crop for erosion control, as well as a wildlife cover. This use is provided by buckwheat being a short season crop that does well on low-fertility and acidic soils due to its excellent ability to scavenge soil nutrients (e.g. phosphor), and that also makes soil more friable for the next crop (Valenzuela and Smith 2002). Moreover, buckwheat is also known to be an aluminum accumulator (Shen *et al.* 2002), contributing to soil remediation. The acknowledgement of the above stated beneficial characteristics influences growing buckwheat economical importance.

Generally, the term buckwheat refers to a variety of plants in the dicot family *Polygonaceae*. Only two species among them are cultivated: common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn). Common buckwheat is cultivated all over the Northern Hemisphere (China, Japan, Korea, Canada, and northern European countries), with the exception of the South East Asia islands (Hirose and Ujihara 1998). Tartary buckwheat is concentrated to the southern and central parts of China and to the mountainous areas of Himalayan countries (Hirose and Ujihara 1998).

Common buckwheat belongs to the so-called pseudocereals because of the grain-like use of this crop. Plants grow rapidly, producing heart-shaped leaves. Flowering begins about three weeks after planting and proceeds for several weeks. Seeds germinate and the cotyledons emerge fast (in four to five days usually), reaching maturity in about four weeks after pollination. Buckwheat seeds are brown in color, 3-4 times smaller in size than soybean seeds (there is a 5 to 10-fold difference in 1000-seed-weight between the species soybean and buckwheat), irregularly shaped with three triangular surfaces. Because seed proteins

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contain high concentration of all essential amino acids, especially lysine, threonine, tryptophan and the sulphur-containing amino acids, they are one of the best sources of high quality proteins in the plant kingdom (Javornik *et al.* 1981). Seeds also contain iron, zinc and selenium which make buckwheat desirable for human consumption (Wang *et al*. 1995; Wei *et al.* 2003).

During buckwheat seed development, like in other plants, protein reserves accumulate in the form of storage proteins. Among buckwheat storage proteins the most abundant ones are globulins: 13S legumin-like protein and 8S vicilin-like protein. Another abundant protein is 2S albumin that consists of low-molecular mass polypeptides (Radović *et al*. 1999), some of which are potent allergen (Yoshioka *et al.* 2004).

Based on literary data on buckwheat's high nutritive value, we chose buckwheat as a model plant with the idea to study seed storage protein composition and genes that encode them. Seed proteins were fractionated and analyzed, and a cDNA library of mid-maturation seeds (14-19 days after flowering-DAF) was constructed. Among the majority of cDNAs coding for SSPs, several other cDNAs were isolated and characterized, including those encoding metallothioneins and aspartic proteases. This review will present the most important results of our work and discuss the potential application of our findings in plant biotechnology.

SEED STORAGE PROTEINS

Isolation and characterization

Buckwheat seed storage proteins (SSPs) are exclusively synthesized in seeds at specific phases of seed development. The expression of SSP genes is tissue/temporary-specific and therefore it is a good model for studying tissue-specific gene switching mechanisms during late embryogenesis. SSPs are also a promising research object in aspects of molecular evolution, since legumins' comparative serology has shown that SSPs epitopes can be used to differentiate among genera/closely related families (Fischer and Jensen 1990).

To analyze the SSPs composition and structure at the protein level, buckwheat crude protein extract was fractionated on sucrose density gradient (Radović *et al.* 1996). The results confirmed that the most abundant fraction of buckwheat SSPs is a 13S globulin [a legumin-like hexamer consisted of non-identical subunits, each comprising one larger acidic (32-43 kDa) and one smaller basic polypeptide (23-25 kDa), linked by disulfide bonds]. Also, 8S globulin, a new minor globulin class, and 2S albumin fraction, were identified. Separation on Sephadex G-200 column revealed that 8S globulin is a trimer composed of 57-58 kDa subunits, which is the common structure for vicilin-like storage proteins (Radović *et al.* 1996). The 2S albumin fraction, which makes 25% of total buckwheat seed proteins, was represented with 8-16 kDa single-chain polypeptides (Radović *et al.* 1999) with a high content of essential amino acids (9.2% methionine and 5.6% lysine). Since there are no glutelins among buckwheat SSPs, buckwheat seeds are suitable in a diet of glutelin sensitive (allergic) people.

SSPs biosynthetic pattern analysis showed that polypeptides start to accumulate fully from 14 DAF, and that the total storage proteins level per seed is increasing towards seed maturation, reaching finally > 80% of total proteins (Maksimović *et al.* 1996). Northern blot experiments, using clones *pFeVIC1* (encoding vicilin-like protein) and *pFeLEG643* (encoding legumin-like protein) as probes, confirmed that the biosynthesis of 8S globulin precedes that of 13S globulin. 8S globulin biosynthesis starts from the early stage of buckwheat seed development (9 to 11 DAF) and accumulate throughout seed development until it reaches finally ~7% of total seed proteins (Brkljačić *et al.* 2004; Milisavljević *et al.* 2004). In the mid-maturation period (14-19 DAF), which is characterized by progressive accumulation of storage proteins, expression of 8S globulin parallels that of 13S expression. Also, 8S and 13S globulin mRNAs were not detected among mRNAs isolated from leaf, root or seedlings, which confirms their seed specific expression.

In parallel, with the aim to identify corresponding SSPs genes, we *in vitro* analyzed mRNA presence throughout different phases of buckwheat seed development. These experiments showed that mid-maturation seed stage is the stage that offers a highly abundant SSPs mRNAs, which makes it the right choice for cDNA library construction (Brkljačić *et al.* 1998). From a mid-maturation seed stage cDNA library, made on total mRNAs from the 14-19 DAF stage of buckwheat seed development, we isolated and characterized a full-length cDNAs encoding the 13S legumin-like storage protein *pFeLEG643* (AY256960) and 8S vicilin-like storage protein *FeVIC1* (AY536051). The comparison of 13S legumin-like storage protein deduced amino acid sequence, with those from different representatives of dicots, monocots and gymnosperms, revealed that this specific buckwheat storage polypeptide should be classified as a member of the methionine-rich legumin subfamily (Samardžić *et al.* 2004). This subfamily is present in the angiosperms' lower clades, with the first characterized representative reported in *Magnolia salicifolia* (clone B14) (Fischer *et al.* 1995, 1996). Since in buckwheat methionine-rich legumins and methionine-poor legumins coexist, this finding could imply that B14 ortholog was not lost during the evolution of angiosperms but was protected under pressure of an increased need for sulphur (Fujino *et al.* 2001). Further, using primers designed from characterized cDNA, we have isolated and analyzed its corresponding gene *FeLEG1* (AY359286). The *FeLEG1* genomic sequence of 1946 bp is represented by two-intron gene structure, which occurs infrequently in the modern angiosperms where three-intron gene structure is more frequently found. This was the first buckwheat SSP genomic clone report, also the only one among the methionine-rich legumin group. The obtained *FeLEG1* sequence allowed us to design primers for the next step, in which a 955bp long 5' regulatory region of the legumin gene was isolated. *In silico* analysis of 5' regulatory region revealed the presence of RY repeats, which are known to be involved in tissue-specific expression in seeds, and some other *cis*-regulatory elements (Milisavljević *et al.* 2005).

Beside legumin cDNA, among a few hundred cDNA clones in the cDNA library, we also isolated a clone assigned *pFeVIC1* (AY536051). This clone showed a high homology to the cDNA clones encoding vicilin-like storage globulins from various plant species. It is interesting to notice that the highest homology was found with the cDNA clone that encodes vicilin-like protein in *Sesamum inducum*, which was also the case for buckwheat legumin-like storage protein (Milisavljević *et al*. 2004; Samardžić *et al.* 2004). The list of the "homology top ten" also included representatives of the vicilin-like storage subfamily from gymnosperms (*Picea glauca*) and monocots (*Zea mays*), implying that the evolutionary position of buckwheat is close to the lower angiosperms clades. While the storage function of buckwheat 8S globulin is certain, the homology of buckwheat *FeVIC1* deduced amino-acid sequence with the soybean and pea sucrose binding protein opened the possibility of an additional function for this protein. Deduced amino acid composition analysis of the partial cDNA *FeVIC1* clone revealed favorable content of lysine (5.7%).

Considering globulins' favorable amino acid content they are of interest in human consumption and for potential biotechnological introduction to other crops that do not synthesize them. However, 13S globulin is a major buckwheat allergen for some people and therefore not suitable for general consumption (Urisu *et al*. 1995; Nagata *et al*. 2000). In tartary buckwheat, a 24kDa allergen with high nucleotide sequence similarity with 13S protein from common buckwheat was also found (Zhang *et al*. 2008). That is why we were interested to examine if 8S globulin could be used instead of 13S globulin, since it also has favorable

amino acid content but there are no reports on its allergenic potential. To investigate 8S allergenic potential we tested if there is a cross-reaction between antibodies synthesized against 13 S globulin's 23-25 kDa polypeptides and 8S globulin's 57 kDa polypeptide. Our finding showed there is no cross-reaction between them, which is in agreement with data reported by Urisu *et al.* (1995), and Nair and Adachi (1999), which showed that 57 kDa polypeptide was not recognized by the legumin allergic people's sera. Bharali and Chrungoo (2003) reported an amino acid sequence of the 13S globulin 26 kDa subunit which has high homology (>90%) with 11S storage protein from *Coffea arabica*, and what is more interesting, it did not show any significant homology with amino acid sequences of known allergens.

Experiments on seed storage proteins

A diversity of research is ongoing on buckwheat SSPs and we will mention few of them which involve the study of controlled food processing to preserve/direct their organoleptic characteristics as well as their use in buckwheat genetic diversity assessment and ligand-protein interactions studies.

For instance, the buckwheat seed storage proteins were used to study heat induced proteins conformational changes by Fourier transform infrared (FTIR) spectroscopy an differential scanning calorimetry (DSC) as well as by size-exclusion chromatography (SEC) combined with on-line multiangle laser light scattering (MALLS) and quasielastic light scattering (QELS). Choi and Ma (2005, 2006) reported these techniques could be used to study changes in proteins structure during industrial processing involved in food production in order to learn how to preserve their good characteristics and eventually enable production of food with desired organoleptic features.

Further, SSPs were found useful in assesment of Asian buckwheat genetic diversity (Xia *et al*. 2008). SDS-PAGE proteins profiles were reported to be useful for this purpose (Ohnishi 2000; Tang 2007) and were used to study variations of prolamin and albumin, isolated from 55 accessions of *Fagopyrum tataricum* and 21 accessions of *F. esculentum* collected in 7 Asian countries. Results showed significant interspecific variation in SSPs SDS-PAGE profiles in *F. tataricum* and *F. esculentum.* Their cluster analysis further showed that all accessions could be grouped in three groups and three subgroups, and also that variations could be associated with their geographic origin in some degree. With buckwheat being an important cereal, its genetic variability assessment is important for developing effective breeding programmes to enable production of lines with desired characteristics and SSPs may be useful tool in developing these programmes.

Also, fractions of 13S globulin were used to study ligand-protein interactions (Rapala-Kozik *et al*. 2003). In this work, the polypeptide components of buckwheat seed thiamin-binding protein (BSTBP) were identified and characterized. It is suggested that BSTBP is a fraction of major seed storage protein - 13S legumin. Since thiamin is necessary for seed germination and seedling growth, it is of interest to study the basic characteristic of thiamin-protein interaction and this group showed that BSTBP fraction of 13-S legumin may be involved in these processes.

METALLOTHIONEINS

What are metallothioneins?

Metallothioneins (MTs) represent a cysteine-rich, low molecular mass protein family with strong capacity for metal binding. MTs have been found to be broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes, as well as plants (Hamer 1986). Although plants MT proteins were only isolated from *Arabidopsis thaliana* (Murphy *et al*. 1997), there are numerous data on cDNA clones isolated from different plants found to encode for proteins homologous to animals MT, which were at first named MT-like proteins. These plant MTs are classified into three (Robinson *et al.* 1993) or four types (Rauser 1999), according to the arrangement of CYs residues within theirs domains (Hassinen *et al*. 2010).

Since research on plants' MTs lags for almost 25 years in comparison to research on animals MTs, their functions remained controversial. The first proposed function of plants' MTs was relatively simple – it was thought they sequester the excess amounts of certain metal ions, as a part of a heavy metal detoxification (Kagi and Kojima 1987). Besides detoxification, MTs could take part in gene expression regulation and cell metabolism by donating/accepting metal ions to/from Zn-dependent DNA binding proteins or metalloenzymes (Vallee 1995; Freisinger 2008). Thus, they could be involved in regular processes of growth and differentiation (Liu *et al.* 2002). One hypothesis on MTs family function that is gaining popularity is that MTs can protect against oxidative damage based on finding that yeast and mammalian MTs can functionally substitute for superoxide dismutase (SOD) and provide oxidative stress protection in yeast (Tamai *et al*. 1993). Therefore, based on all collected data for MT family, nowadays is accepted that plant MTs have a great impact on maintaining of metal homeostasis as well as on plant cells redox status balance (Hassinen *et al*. 2010).

Buckwheat metallothionein

Buckwheat cDNA clone pBM 290 (AF056203), encoding a 59-amino acid-long MT-like protein was isolated from the developing buckwheat seed cDNA library (Brkljačić *et al.* 1999). *In silico* analysis of the deduced amino acid sequence showed the highest homology to the MT3-like protein from *Arabidopsis* (Murphy *et al.* 1997). Using primers designed from pBM 290, a genomic fragment of the buckwheat MT3 gene (*gFeMT* 4.1) comprising 3 exons and 2 introns that are preceded by a 640-bp long sequence (placed upstream of the first ATG codon) has been isolated. In the 640-bp long sequence, a 569-bp long promoter region and 71-bp long 5′ UTR were detected. The promoter region *in silico* analysis, using overlapping data from three different databases, showed the existence of regulatory sequences which could be involved in the different hormonal and external stimuli responses (ERE, heat shock-HSE, light and stress-GT-1, I-box, GATA, G-box, metal-MRE), as well as the presence of plant-specific transcription factors putative binding sites (Dof1, NtBBF1, Athb-1). The more detailed investigation involved proximal and distal promoter region, which comprise most of the putative regulatory sequences. The studies included analysis of interactions of these DNA fragments with the purified Dof1ΔC domain of Dof1 and the HD-Zip-1 domain of Athb-1 transcription factors, as well as interactions with buckwheat nuclear extract. The results confirmed the predicted specificity of putative Dof1- and Athb1-binding sites located in proximal promoter region. Furthermore, there was a competition for complex formation among both protein factors and buckwheat seed/leaf nuclear protein(s). Analysis of the distal promoter region also showed binding ability to leaf nuclear proteins, indicating an interaction trough predicted G- and I-boxes, which are proposed to be involved in light- and/or stress-regulated *MT3* gene expression (Brkljačić *et al*. 2004, 2005).

Functional promoter analysis was performed with a complete 5'-regulatory region and two deletion variants, employing stably transformed tobacco plants. Histochemical GUS assay of transgenic tobacco lines detected the strongest signals in vascular elements of leaves and in pollen grains, while somewhat weaker staining was observed in the roots. In a simulation of a complex stress situation (composed of several synergistically related stress stimuli) where leaves treated with Cu^{2+} and Cd^{2+} were submerged for a prolonged period of time in liquid MS medium containing sucrose, quantitative GUS assay showed strong up-regulation for all of the three promoter-constructs (propor-

tional to the length of the regulatory region) (Bratić *et al*. 2009).

The effects of heavy metal treatment and different abiotic stresses were monitored in buckwheat leaves employing Real-time PCR technology. Buckwheat plants were exposed to various metals, drought, oxidative stress, darkness and mechanical injuries. ROS (reactive oxygen species) production is a common consequence of most abiotic stresses, and increased expression of *FeMT3* during the stress could be connected with its ROS protection function. These ROS and heavy metal protection abilities of *FeMT3* were confirmed in three different systems subjected to heavy metals and peroxide: *E. coli, S. cerevisiae* and transiently transformed leaves of *N. debneyii*. The applied toxic metal and peroxide concentrations were found to cause less damage in cells and tissues expressing *FeMT3* in comparison to untreated controls (Nikolić *et al*. 2010; Samardžić *et al*. 2010). *FeMT3* transcripts were present in the root vascular system, the vascular system, mesophyl and guard cells in leaf, stem, flower and embryo-tissues of developing seeds (Samardžić *et al.* 2010).

The cytoplasmic localization of FeMT3-GFP (green fluorescent protein - GFP) fusion and FeMT3-EYFP (enhanced yellow fluorescent protein - EYFP), detected in transformed tobacco leaves, remained unchanged under heavy metal stress, suggesting a different defense mechanism against heavy metals deleterious effects of metallothioneins in comparison to phytochelatins (**Fig. 1**) (Nikolić *et al*. 2010). These results support the high promoter inducibility and increased transcript level upon Cu^{2+} and Cd^{2+} exposure, which strongly indicate that *FeMT3* may play an important role in buckwheat's heavy metal tolerance and hyperaccumulation.

ASPARTIC PROTEINASE

Synthesis/degradation of seed storage proteins are under tight control, and mechanisms regulating these processes are a subject of numereous investigations. In buckwheat, storage proteins are synthesized in cotyledons and embryonic axis during middle stage of seed maturation (11 to 23 DAF). Proteins are protected against proteolytic attack during seed maturation/dormancy, and mobilized during seed germination and subsequent seedling growth. The degradation of storage protein starts during germination, which indicates that the protective mechanisms have been overcome. Generally, the protein degradation is mediated by proteinases, that could either be synthesized during seed development and stored in the form of inactive precursors or *de novo* synthesized during germination (Müntz *et al.* 2001). Inactive proteinases precursors are synthesized on the rough endoplasmic reticulum (rER) and transported into the protein bodies (PB), where they are activated during germination. Among proteases present in a dry buckwheat cotyledons, a metalloproteinase (MPR), an aspartic proteinase (AP) and a carboxypeptidase (CP) were detected (Belozerski and Dunaevsky 1995).

Protein bodies in buckwheat cotyledons are found to contain stored Zn-metalloproteinase, which is proposed to be responsible for the initiation of globulin breakdown (Dunaevsky and Belozersky 1989a, 1989b, 1993). This enzyme has narrow substrate specificity, in contrast to other known metalloproteinases (MPRs), and it is limited on buckwheat SSPs. Zn-MPR mediates a 13S globulin limited proteolysis, causing a protein conformational change. Also, a papain-like cysteine proteinase (CPR), which is synthesized in buckwheat cotyledons during germination, is involved in buckwheat 13S globulin hydrolisis, but only when it has previoulsy been modified by MPR. Another proteinase, a serine proteinase, has also been detected in buckwheat seeds, but it did not hydrolize SSPs (Dunaevsky and Belozersky 1998).

Further, in proteins extracted from developing, mature and germinating buckwheat seeds, a pepstatin A-sensitive proteolytic activity has been detected (Timotijević *et al*. 2003). This activity is attributed to an aspartic proteinase (AP). Indeed, three forms of APs (molecular masses of 47, 40 and 28 kDa) were purified from mature seeds, while two forms (47 and 28 kDa) were detected in developing seeds (Timotijević *et al*. 2006). A 47 kDa AP form is localized in membrane fraction. It is composed of two subunits: 31 and 16 kDa polypeptides and is accumulated during seed maturation. A 47 kDa AP form is also present at the beginning of seedling germination. Interestingly, it was found that this enzyme has the ability to clot milk, a useful characteristic that could be utilized as discussed in the Conclusion section.

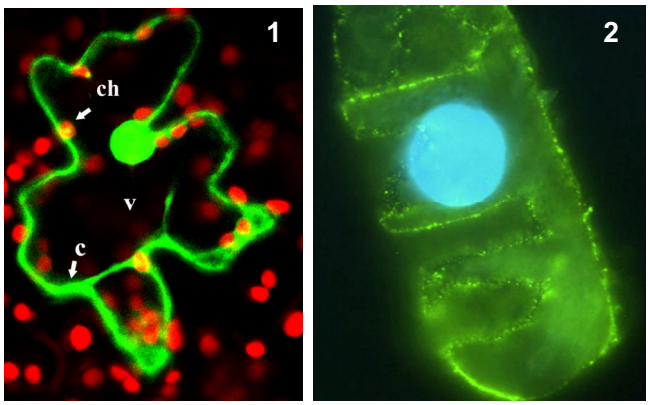

Fig. 1 Cytosolic localization of FeMT3-EYFP (enhanced yellow fluorescent protein) in transiently transformed epidermal cells of *N. debneyii* leaves (green signal). Cells are with large vacuole (no signal). Red signal originated from chloroplasts autofluorescence. (v - vacuole, c - cytosol, ch - chloroplast)

Fig. 2 Cell wall localization of overexpressed FeAPL1-His$_6$ protease in BY-2 cells detected using FITC-labelled secondary antibodies (green spots). Reprinted with kind permission from **Milisavljević M, Timotijević G, Nikolić D, Samardžić J, Maksimović V** (2011) Cell wall localization of the aspartic proteinase from buckwheat (FeAPL1) over-expressed in tobacco BY-2 cells. *Journal of Serbian Chemical Society,* DOI: 10.2298/JSC110120106M. With kind permision from Serbian Chemical Society.

The cDNAs encoding two types of buckwheat AP (typical and atypical) have been isolated from a cDNA library. While typical plant APs are easily distinguished by their structure from their non-plant homologs there are exceptions from this rule that include several enzymes, often called atypical, AP-like or APs novel class (Milisavljević *et al*. 2007). Typical APs have so-called plant-specific insert (PSI) in their primary structure. When they enter further processing, the PSI will be removed in most cases, creating mature APs without PSI. However, atypical APs lack PSI domain in their primary structure. The identified buckwheat APs genes, a cDNA encoding *FeAP9* (AY826351) resembled the structure and shared high homology with typical plant APs, while the other cDNA, *FeAPL1* (AY536047) encoded for an AP-like protein without PSI. Sequences of *FeAP9* and *FeAPL1* cDNAs allowed identification of corresponding genes from buckwheat genomic DNA.

FeAP9 gene containes 12 introns and 13 exons, which are typical plant AP structural characteristics, including the presence of the leader intron in the 5′-UTR (Timotijević *et al*. 2010). In contrast to *FeAP9*, the other identified genomic fragment g*FeAPL1* was found to be identical in length to the cDNA sequence, indicating that the *FeAPL1* gene does not contain introns (Milisavljević *et al*. 2008), which is the structural feature of atypical AP genes. Also, in a *FeAPL1* gene a 5′-regulatory region was identified and found to be rich in potential *cis*-elements that could influence stress-induced and seed-specific expression. Further, *FeAP9* BLAST analysis showed highest homology to *Oryza sativa* orizasin gene, supporting the hypothesis that differences among typical APs (with respect to intron number and arrangement) appeared after the divergence of monocotyledonous and dicotyledonous plants (Asakura *et al*. 1995). In addition, nucleotide sequence analysis allowed

insight into one of the aspects of molecular phylogenetic relations between the typical and atypical APs (Milisavljević *et al*. 2008). Analysis suggested that AP and AP-like genes diversification most likely occured independently of the presence of the PSI segment.

Expression analysis showed *FeAP9* and *FeAPL1* have a different expression pattern. While *FeAP9* mRNA was present in developing seeds and seedlings, in leaves, roots and flowers, *FeAPL1* mRNA was detected in seeds only. Also, expression profile was more similar to 13S storage protein genes expression profile in the case of *FeAP9* as compared to *FeAPL1*, which may suggest possible involvement of *FeAP9* in the seed storage globulin processing. Also, a high level of *FeAP9* expression detected in senescent leaves may imply its possible involvement in protein degradation during senescence. It is also noticed that under the influence of different abiotic stresses (drought, UV-B lihgt, wounding, darkness) as well as salycilic acid (SA) treatment, expression of *FeAP9* is upregulated in buckwheat leaves (Timotijević *et al.* 2010).

The recombinant aspartic proteinase-like protein (FeAPL1-His_6) was overexpressed in the tobbaco BY-2 cell line, and immunocytochemistry and protein gel-blot analysis of the transformed cells and their protoplasts showed extracellular localization of rFeAPL1- His_6 in the cell wall (**Fig. 2**). According to these data, it could be proposed that FeAPL1 modifies the internal structure of the cell wall, trough functionally affecting some proteins connected with polysaccharide matrix (Milisavljevic *et al.* 2011). In addition, it cannot be excluded that FeAPL1 may act on pathogens proteins as well, taking in mind that 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, W box) (Milisavljevic *et al.* 2007).

For most plants APs biological functions are still hypothetical and represent an interesting and provocative field of investigation. Several functions for typical APs have been proposed, including processing and degradation of seed storage proteins (as already discussed), protein degradation during organ senescence and cell death, adhesion-mediated proteolytic mechanisms in pollen recognition and growth. For AP-like enzymes, proposed functions also include proteins degradation during leaf senescence and cell death, and prey digestion in carnivorous plants. Possible involvement/ role of AP-like enzymes in biosynthesis and degradation of seed storage proteins is still unknown.

CONCLUSIONS

Analysis of specific gene expression regulation is an important element in understanding the regulation of basic plant cells mechanisms. Also, it enables elucidation of the possible functions of proteins involved in different physiological processes under normal/stress conditions. Moreover, the structural and functional analyses of identified genes and their promoters could provide the molecular basis for their potential biotechnological use in agriculture and land preservation.

As discussed in previous sections, buckwheat displays several features beneficial for human consumption and land preservation, offering a potential for biotechnological use. Nowadays plant biotechnology researches are focused primarily on two main fields: a) developing transgenic plants which will be used as bioreactors for production of heterologous proteins that are of high biological value and b) producing genetically modified plants with improved qualities or new characteristics important for agricultural application.

Concerning improvement of seed quality in agriculturally important crops the knowledge on variations in naturally occurring seed proteins could be very useful. In that sense, buckwheat SSPs, recognized as an excellent source of minerals and high biological value proteins (rich in essential amino acids), are still insufficiently exploited. In particular, buckwheat 8S globulin (vicilin-like storage protein), being nutritive rich and non-allergenic, could be a potential candidate for gene transfer into cereals with limited essential amino acids content (like wheat, corn and soybean), in order to improve their nutritive quality.

When considering modified plants production for land preservation (biosensing and phytoremediation), than the gene that encodes buckwheat's MT protein (involved in heavy-metal detoxification) would be a suitable candidate for biotechnological use. With plant biotechnology as well interested in using transgenic plants as bioreactors, a choice of inserted promoter becomes very important for final heterologous protein yield. Strong and constitutive promoters (such as CaMV 35S), are not ideal for obtaining a high accumulation of heterologous proteins due to the "silencing" phenomenon (characteristic for highly expressed genes). Therefore non-constitutive promoters, like tissue specific or external stimuli inducible promoters, become a very attractive choice. In that sense, the promoters of genes encoding buckwheat storage proteins, as well as the promoter of buckwheat MT gene, could be suitable candidates.

One more biotechnological potential of buckwheat involves the use of buckwheat aspartic proteinase in a milk-clotting process, for instance trough introducing AP gene into lactic acid bacteria that are used for cheese production.

Our further investigations will involve analyses of the possible physiological function(s) of buckwheat MT, as well as aspartic proteinases, under different physiological (normal/stress) conditions.

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REFERENCES

Asakura T, Watanabe H, Abe K, Arai S (1995) Rice aspartic proteinase, oryzasin, expressed during seed ripening and germination, has a gene organization distinct from those of animal and microbial aspartic proteinase. *European Journal of Biochemistry* **232**, 77-83

Belozersky MA, Dunaevsky YE (1995) Utilization of the main storage protein in germinating seeds of buckwheat: Mechanisms and regulation. In: *Current Advances in Buckwheat Research: Proceedings of the 6th International Symposium on Buckwheat*, August 24-29, Shinshu, Shinshu University Press, Shinshu, Japan, pp 749-752

Bharali S, Chrungoo NK (2003) Amino acid sequence of the 26 kDa subunit of the legumin-type seed storage protein of common buckwheat (*Fagopyrum esculentum* Moench): Molecular characterization and phylogenetic analysis. *Phytochemistry* **63**, 1-5

Bratić A, Majić D, Samardžić J, Maksimović V (2009) Functional analysis of the buckwheat metallothionein promoter: Tissue specificity pattern and up-regulation under complex stress stimuli. *Journal of Plant Physiology* **166**, 996-1000

Brkljačić J, Majić D, Miljuš-Djukić J, Bratić A, Konstantinović M, Milisavljević M, Maksimović V (2005) The metallothionein-like gene from buckwheat: Structural and functional analysis of the promoter region. *International Journal of Plant Sciences* **166**, 889-900

Brkljačić J, Maksimović V, Radović S, Savić A (1998) Identification of legumin-like storage protein cDNA clone from cDNA library of developing buckwheat seed. *Archive of Biological Sciences* **3**, 15-20

Brkljačić J, Maksimović V, Radović S, Savić A (1999) Isolation of metallothionein-like cDNA clone from buckwheat. *Journal of Plant Physiology* **154**, 802-804

Brkljačić J, Samardžić J, Timotijević G, Maksimović V (2004) Expression analysis of buckwheat (*Fagopyrum esculentum* Moench) metallothionein-like gene (MT3) under different stress and physiological conditions. *Journal of Plant Physiology* **161**, 741-746

Choi SM, Ma CY (2005) Conformational study of globulin from common buckwheat (*Fagopyrum esculentum* Moench) by Fourier transform infrared spectroscopy and different scanning calorimetry. *Journal of Agricultural and Food Chemistry* **53**, 8046-8053

Choi SM, Ma CY (2006) Study of thermal aggregation of globulin from common buckwheat (*Fagopyrum esculentum* Moench) by size-exclusion chromatography and laser light scattering. *Journal of Agricultural and Food Chemistry* **54**, 554-561

Dunaevsky YE, Belozersky MA (1989a) The role of cysteine and carboxypepetidase in the breakdown of storage proteins in buckwheat seeds. *Planta* **179**, 316-322

Dunaevsky YE, Belozersky MA (1989b) Proteolysis of the main storage protein of buckwheat seeds at the early stage of germination. *Physiologia Plantarum* **75**, 424-428

Dunaevsky YE, Belozersky MA (1993) Effects of the embryonic axis and phytohormones on proteolysis of the storage protein in buckwheat seed. *Physiologia Plantarum* **88**, 60-66

Dunaevsky YE, Belozersky MA (1998) Protease of limited specificity in buckwheat seeds. In: *Advances in Buckwheat Research. Proceedings of the 7th International Symposium on Buckwheat*, August 12-14, Winnipeg, Organizing Committee of the Seventh International Symposium on Buckwheat under the auspices of the International Buckwheat Research Association, Winnipeg, Manitoba, Canada, pp III-72-III-77

Fischer H, Jensen U (1990) Phytoserological investigation of the tribe *Cardueae* s.l. (*Compositae*). *Plant Systematics and Evolution* **4 (Suppl.)**, 99-111

Ficher H, Chen L, Wallisch S (1996) The evolution of angiosperm seed proteins: A methionine-rich legumin subfamily present in lower angiosperm clades. *Journal of Molecular Evolution* **43**, 399-404

Fischer H, Haake V, Horstmann C, Jensen U (1995) Characterisation and evolutionary relationship of *Magnolia* legumin-encoding cDNAs representing two divergent gene subfamilies. *European Journal of Biochemistry* **229**, 645-650

Freisinger E (2008) Plant MTs – long neglected members of the metallothionein superfamily. *Dalton Transactions*, pp 6663-6675

Fujino K, Funatsuki H, Inada M, Shimono Y, Kikuta Y (2001) Expression, cloning, and immunological analysis of buckwheat (*Fagopyrum esculentum* Moench) seed storage proteins. *Journal of Agricultural and Food chemistry* **266**, 608-13

Hamer DH (1986) Metallothionein. *Annual Review of Biochemistry* **55**, 913-951

Hassinen VH, Tervahauta AI, Schat H, Kärenlampi SO (2010) Plant metallothioneins – metal chelators with ROS scavenging activity? *Plant Biology* **13**, 225-232

Hirose T, Ujihara A (1998) Buckwheat flower pictorial. *Fagopyrum* **15**, 71-82

Javornik B, Eggum BO, Kreft I (1981) Studies on protein fractions and protein quality of buckwheat. *Genetika* **13**, 115-121

Kagi JHR, Kojima Y (1987) Chemistry and biochemistry of metallothionei. In: Kagi JHR, KojimaY (Eds) *Metallothionein II*, Birkhauser-Verlag, Basel, pp 25-62

Liu P, Goh CJ, Loh CS, Pua EC (2002) Differential expression and characterisation of three metallothionein-like genes in Cavendish banana (*Musa acuminata*). *Physiologia Plantarum* **114**, 241-250

Maksimović V, Varkonji-Gašić E, Radović S, Savić A (1996) Biosynthesis of 13S buckwheat seed storage protein. *Journal of Plant Physiology* **147**, 759-761

Milisavljević M, Timotijević G, Nikolić D, Samardžić J, Maksimović V (2011) Cell wall localization of the aspartic proteinase from buckwheat (FeAPL1) over-expressed in tobacco BY-2 cells. *Journal of Serbian Chemical Society* **76 (9)**, 1229-1236

Milisavljević M, Konstantinović M, Brkljačić J, Maksimović V (2005) Isolation and computer analysis of the 5'-regulatory region of the seed storage protein gene from buckwheat (*Fagopyrum esculentum* Moench). *Journal of Agricultural and Food Chemistry* **53**, 2076-2080

Milisavljević M, Timotijević G, Radović S, Brkljačić J, Konstantinović M, Maksimović V (2004) Vicilin-like storage globulin from buckwheat (*Fagopyrum esculentum*) seeds. *Journal of Agricultural and Food Chemistry* **52**, 5258-5262

Milisavljević M, Timotijević G, Radović S, Konstantinović M, Maksimović V (2008) Two types of aspartic proteinases from buckwheat seed – Gene structure and expression analysis. *Journal of Plant Physiology* **165**, 983-990

Milisavljević M, Timotijević G, Radović S, Konstantinović M, Maksimović V (2007) Isolation and structural analysis of a gene coding for a novel type of aspartic proteinase from buckwheat seed (*Fagopyrum esculentum* Moench). *Archives of Biological Sciences* **59**, 119-124

Müntz K, Belozersky MA, Dunaevsky YE, Schlereth A, Tiedeman J (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *Journal of Experimental Botany* **52**, 1741-1752

Murphy A, Zhou J, Goldsbrough PB, Taiz L (1997) Purification and imunological identification of metallothioneins 1 and 2 from *Arabidopsis thaliana*. *Plant Physiology* **113**, 1293-1302

Nagata I, Fujino K, Hashiguchi S, Abe N, Zaima Y, Ito Y, Takahashi Y, Maeda K, Sugimura K (2000) Molecular characterization of buckwheat major immunoglobulin E-reactive proteins in allergic patients. *Allergology International* **49**, 117-124

Nair A, Adachi T (1999) Immunodetection and characterization of allergenic proteins in common buckwheat (*Fagopyrum esculentum*). *Plant Biotechnology* **16**, 219-223

Nikolić D, Samardžić J, Bratić A, Radin I, Gavrilović S, Rausch T, Maksimović V (2010) Buckwheat (*Fagopyrum esculentum* Moench) FeMT3 gene in heavy metal stress: protective role of the protein and inducibility of the promoter region under Cu2þ and Cd2þTreatments. *Journal of Agricultural and Food Chemistry* **58 (6)**, 3488-3494

Ohnishi O (2000) Geographical distribution of allozymes in natural populations of wild tartaty buckwheat. *Fagopyrum* **17**, 29-34

Radović S, Maksimović V, Brkljačić J, Varkonji-Gašić E, Savić A (1999) 2S albumin from buckwheat (*Fagopyrum esculentum* Moench) seeds. *Journal of Agricultural and Food Chemistry* **47**, 1467-1470

Radović S, Maksimović V, Varkonji E (1996) Characterisation of buckwheat seed storage proteins. *Journal of Agricultural and Food Chemistry* **44**, 972-975

Rapala-Kozik M, Ostrowska K, Bednarczyk K, Dulinski R, Kozil A (2003) Polypeptide components of oligomeric legumin-like thiamin-binding protein from buckwheat seeds characterized by partial amino acid sequencing and photoaffinity labeling. *Journal of Protein Chemistry* **22**, 167-175

Rauser EW (1999) Structure and function of metal chelators produced by plants. *Cell Biochemistry and Biophysics* **31**, 19-48

Robinson NJ, Tommey AM, Kuske C, Jackson PJ (1993) Plant metallothioneins. *Biochemical Journal* **295**, 1-10

Samardžić J, Milisavljević M, Brkljačić J, Konstantinović M, Maksimović V (2004) Characterization and evolutionary relationship of methionine-rich legumin-like protein from buckwheat. *Plant Physiology and Biochemistry* **2**, 157-163

Samardžić J, Nikolić D, Timotijević G, Jovanović Z, Milisavljević M, Maksimović V (2010) Tissue expression analysis of FeMT3, a drought and oxidative stress related metallothionein gene from buckwheat (*Fagopyrum esculentum*). *Journal of Plant Physiology* **167**, 1407-1411

Shen R, Ma JF, Kyo M, Washita T (2002) Compartmentation of aluminium in leaves of an Al-accumulator, *Fagopyrum esculentum* Moench. *Planta* **215**, 394-398

Tamai KT, Gralla EB, Ellerby LM, Valentine JS, Thiele DJ (1993) Yeast and mammalian matallothioneims functionally substitute for yeast copper-zinc superoxide and hydroxyl radicals. *Proceedings of the Nationail Academy of Sciences USA* **90**, 8013-8017

Tang CH (2007) Functional properties and *in vitro* digestibility of buckwheat protein products: Influence of processing. *Journal of Food Processing* **82**, 568-576

Timotijević G, Milisavljević M, Radović S, Konstantinović M, Maksimović V (2010) Ubiquitous aspartic proteinase as an actor in the stress response in buckwheat. *Journal of Plant Physiology* **167**, 61-68

Timotijević G, Radović S, Maksimović V (2003) Characterization of an aspartic proteinase activity in buckwheat (*Fagopyrum esculentum* Moench) seeds. *Journal of Agricultural and Food Chemistry* **51**, 2100-2104

Timotijević G, Radović S, Maksimović V (2006) Aspartic proteinase from buckwheat (*Fagopyrum esculentum* Moench) seeds – purification and properties of the 47 kDa enzyme. *Archives of Biological Sciences* **58**, 171-177

Urisu A, Kondo Y, Morita Y, Yagi E, Tsuruta M, Yasaki T, Yamada K, Kuzuya H, Suzuki M, Titani K, Kurosawa K (1995) Isolation and characterization of a major allergen in buckwheat seeds. In: *Current Advances in Buckwheat Research*, Shinshu University Press, Matsumoto, Japan, pp 965-974

Valenzuela H and Smith J (2002) Cowpea, Sustainable Agriculture, Green Manure Crops CTAHR. Available online: http://www.ctahr.hawaii.edu/oc/freepubs/pdf/GreenManureCrops/cowpea.pdf

Vallee BL (1995) The function of metallothionein. *Neurochemistry International* **27**, 23-33

Wang L-M, Fan M-Y, Wang D-J (1995) The mineral element contents in Chinese buckweat. In: *Current Advances in Buckwheat Research*, pp 765-771

Wei Y, Hu X, Zhang G, Ouyang S (2003) Studies on the amino acid and mineral content of buckwheat protein. *Molecular Nutrition and Food Research* **47**, 114-116

Xia YY, Wei E, Liang ZL, Ting YW, Rong CQ (2008) Genetic diversity of storage proteins in cultivated buckwheat. *Pakistan Journal of Biological Sciences* **11**, 1662-1668

Yoshioka H, Yasueda N, Adachi T (2004) Molecular cloning and expression of a major allergenic protein Fag e 2 from buckwheat. *Fagopyrum* **21**, 35-38

Zhang X, Yuan JM, Cui XD, Wang ZH (2008) Molecular cloning, recombinant expression, and immunological characterization of a novel allergen from Tartary buckwheat. *Journal of Agricultural and Food Chemistry* **56**, 10947-10953