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 metallothioneins). 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...
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, SSPs 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 allergens (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 1996). The 2S albumin fraction, among the methionine-rich legumin group. The obtained FeLEG1 gene 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, from a few hundred cDNA clones in the cDNA library, we have isolated and analyzed its corresponding gene FeVIC1 (AY536051). The compari-son 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). In buckwheat, 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 FeVIC1 (AY536051). This clone showed a high homology to the cDNA clones encoding vicilin-like storage globulins from various plant species. It is interesting to note that the highest homology was found in the clone that encodes vicilin-like protein in Sesamum indicum, 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 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 Urusu et al. (1995), and Nair and Adachi (1999), which showed that 57 kDa polypeptide was not recognized by the legumin allergic people’s sera. Bharalai 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 multilayer 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 assessment 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 their 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 plant MTs was alleviating the heavy metal toxicity by sequestering 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, NiBBF1, 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 Dof1AC 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 legumin 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. A positive silical 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 Cu2+ and Cd2+ were submerged for a prolonged period of time in liquid MS medium containing sucrose, qualitative GUS assay showed strong up-regulation for all of the three promoter-constructs (propor-
tional to the length of the regulatory region) (Bratíc 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, mesophyll 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 Cu²⁺ and Cd²⁺ 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 numerous 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 activated during dormancy (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 metallocproteinase (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-metallocproteinase, 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 metallocproteinases (MPRs), and it is limited on buckwheat SSPs. Zn-MPR mediates a 135 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 135 globulin hydrolysis, but only when it has previously 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.

The CDNs 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 contains 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 occurred independently of the presence of the PSI segment.

Expression analysis showed FeAP9 and FeAPL1 have a different expression pattern. While FeAP9 mRNA was pre- sent in developing seeds and seedlings, in leaves, roots and florescence region of the FeAPL1 gene contains some pathogen proteins as well, taking in mind that the promoter condition, it cannot be excluded that FeAPL1 may act on 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 seeds which will be used as bioreactors for production of hetero- logous 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 (viciin-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 heterogeneous 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, 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 like in a milk-clotting process, for instance trough introducing AP gene into bovine gene 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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