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Vicilin-like Storage Globulin from Buckwheat (*Fagopyrum esculentum* Moench) Seeds

MIRA DJ. MILISAVLJEVIĆ, GORDANA S. TIMOTIJEVIĆ, SVETLANA R. RADOVIĆ, JELENA M. BRKLJAČIĆ, MIROSLAV M. KONSTANTINOVIĆ, AND VESNA R. MAKSIMOVIĆ*

Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, P.O. Box 23, 11001 Belgrade, Serbia and Montenegro

An 8S storage globulin from buckwheat seed, which resembles the structure and features common to the vicilin-like family of seed storage proteins, was analyzed for this paper. It was found that expression of the 8S globulin gene precedes that of the 13S globulin (the main buckwheat storage protein) and starts from an early stage of buckwheat seed development (9−11 days after flowering), continuing to accumulate throughout seed development to contribute ∼7% of total seed proteins. This protein fraction might be more interesting for biotechnological application than the 13S buckwheat legumin consisting of 23−25 kDa subunits reported to be the major buckwheat allergen. A partial cDNA was also isolated, showing high homology with cDNAs coding for vicilin-like storage proteins from various plant species, and its expression profile throughout seed development as well as in different buckwheat tissues was analyzed.

KEYWORDS: Buckwheat; *Fagopyrum esculentum*; storage proteins; vicilin-like; biosynthesis; expression

INTRODUCTION

Seed storage proteins (SSPs) are a set of proteins that accumulate to high levels in seeds during the late stages of seed development. They are usually classified into four groups: globulins, albumins, prolamins, and glutelins. The globulins, dominantly represented in legumes, are further divided into subgroups: 11S legumin-type and 7−8S vicilin-type (*1*). During seed germination, SSPs are degraded, and the resulting amino acids are utilized by developing seedlings as a nutritional source. SSPs are the major proteins in grains and are the plant proteins most abundantly consumed by humans. Accumulation of SSPs is therefore a closely regulated biosynthetic process of great agronomic and economic importance.

SSPs of most dicotyledonous species are synthesized exclusively in developing embryos, and their biosynthesis represents an excellent system for investigation of tissue specific and developmentally controlled gene expression. SSPs may also be very useful for biotechnological application. Considering attempts to improve seed quality artificially, especially concerning the proportions of essential amino acids, knowledge about variations in naturally occurring seed proteins would be very useful. In that sense, proteins of buckwheat are still insufficiently examined, even though they are generally recognized as the best-known source of high biological value proteins in the plant kingdom, being classified close to proteins from animal sources (*2*, *3*). Published data are mostly related to the 26 kDa subunit of legumin-type buckwheat SSP reported to be lysine/methionine rich (*4*, *5*).

Storage proteins of buckwheat seed are divided into two classes: salt-soluble globulins, forming 70% of total seed proteins, and water-soluble 2S albumins (*6*, *7*). The main storage protein of buckwheat, 13S globulin, resembles legumin-like seed storage proteins of other species. After fractionation of buckwheat seed globulins on a sucrose density gradient, we noticed the existence of a new minor class of globulins with a sedimentation constant of 8S (*6*). Separation on a Sephadex G-200 column revealed that the 8S globulin is a trimer, composed of subunits of M_w 57−58 kDa, a structure common for all vicilin-like storage proteins (*8*).

In this paper, we analyzed the 8S fraction in more detail and confirmed its classification, as a distinct buckwheat seed protein fraction, to the vicilin family. We also isolated a partial cDNA showing high homology with cDNAs coding for vicilin-like storage proteins from various plant species and analyzed its expression profile throughout seed development as well as in different buckwheat tissues.

MATERIALS 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 buckwheat seed was ∼30 days. Different stages of seed development were designated 9−11, 14−17, 19−21, and 23−25 DAF. Seeds were harvested and used immediately or were stored at −70 °C for protein and RNA extraction.

* Author to whom correspondence should be addressed (telephone +381 11 3976658; fax 381 11 3975808; e-mail heljda@sezampro.yu).

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Protein Extraction. The globulin fraction was extracted in 5−10 volumes of buffer A (0.035 M potassium phosphate, pH 7.6, 0.4 M NaCl, 1 mM PMSF) at room temperature for 1 h. The resulting suspension was centrifuged at 10000*g* for 15 min. The supernatant was precipitated with 2 volumes of cold acetone.

SDS-PAGE. Protein samples were analyzed by electrophoresis in 12−15% SDS-PAGE slab gels (*9*). Gels were stained with Coomassie brilliant blue. Gels containing radioactive polypeptides were fixed, dried, and fluorographed. Amersham's Amplify was used for fluorographic enhancement of the signal.

RNA Isolation. RNA was separated as described in a previous paper (*10*). PolyA RNAs were isolated using a Dynabeads mRNA purification kit containing Dynabeads Oligo(dT)25, supermagnetic, polystyrene beads (Dynal).

In Vitro Translation. PolyA RNAs were translated in the wheat germ extract (Promega Wheat Germ Translation System L4181) as suggested by the manufacturer. The final potassium concentration was 125 mmol/L. Polypeptides were labeled with 0.5 μCi of [^{35}S]methionine (1200 Ci/mmol).

In Vivo Labeling. Cotyledons were placed in the labeling solution containing L-[U-^{14}C]amino acid mixture, 50 μCi/mL (Amersham CFB104), 20 μL/cotyledon, and allowed to take up solution for 45 min at 28 °C. After the labeling period, cotyledons were frozen in liquid nitrogen and proteins were isolated as explained.

Preparation of Antiserum. Antibody synthesis was induced by the injection into New Zealand White rabbits of buckwheat polypeptides (57 and 23−25 kDa) separated and purified by SDS-PAGE. The initial injection was 100 μg of protein fraction in Freund's complete adjuvant followed by weekly booster injections of the same amount of the protein in incomplete adjuvant 2 and 3 weeks after initial infection. Rabbits were bled 2 weeks later.

Immunoprecipitation. One-fifth of an in vitro translational reaction mixture was incubated with 10 μL of serum in 2% protein A Sepharose in RIPA buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate, 0.1% SDS) for 1 h on ice. Complex protein A Sepharose-antibody−antigen was collected by centrifugation for 20 s at 12000*g* at 4 °C. Antigen was eluted in buffer (50 mM Tris-HCl, pH 6.8; 100 mM DTT; 2% SDS; 0.1% bromphenol blue; 10% glycerol) by boiling for 3 min. After centrifugation for 20 s at 12000*g*, the supernatant was analyzed by SDS-PAGE.

Western Blot Analysis. For Western blotting, the proteins were transferred to a nitrocellulose membrane (Hybond C) by an electroblotting apparatus in appropriate buffer (39 mM glycine; 48 mM Tris; 0.037% SDS; 20% methanol). After the transfer, the membrane was equilibrated for 2 min in TBST buffer (10 mM Tris, pH 8.0; 150 mM NaCl; 0.05% Tween 20) and incubated for 3 h in blocking solution (1% BSA in TBST buffer). The membrane was incubated for 30 min with the primary antibodies against the 57 kDa or 23−25 kDa polypeptides. After three washes, the membrane was incubated for 30 min with anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was detected by using NBT and BCIP as a substrates.

cDNA Synthesis and Cloning Procedure. cDNA was synthesized according to the method of Chenchik et al. (*11*) using a cDNA synthesis primer (Marathon cDNA Amplification Kit K1802-1, Clontech). Synthesized cDNA was cloned in a pMOS*Blue* vector (Blunt-Ended PCR Cloning Kit RPN5110, Amersham Pharmacia Biotech**)** and sequenced with universal vector primers.

Northern Blot Analysis. For northern blot analysis, 10 μg of the total RNA from buckwheat tissue was blotted on a Hybond-N$^+$ membrane, hybridized with ^{32}P-labeled cDNA clone pFeVIC1 at 65 °C in NaPi/SDS buffer without formamide, and washed as described by Church and Gilbert (*12*). Autoradiography was obtained after overnight exposition of Kodak X-ray film.

Data Analysis. General sequence processing was done with the Sequence Manipulation Suite (*13*). Protein sequences were compared using the BLASTP search program (*14*) and by exploring all of the available sequence databases at the www.ncbi.nlm.nih.gov Web server. Sequences were aligned by the GeneDoc−Multiple Sequence Alignment Editor (www.psc.edu/biomed/gendoc_.

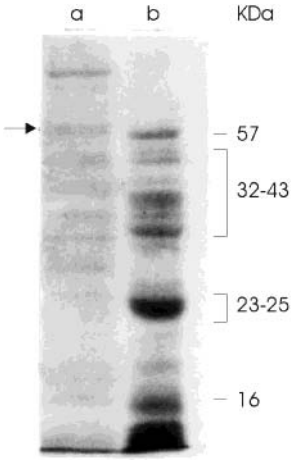

Figure 1. SDS-PAGE of total salt-soluble proteins from (**a**) 9–11 DAF stage and (**b**) mature buckwheat seed. Positions of 13S globulin subunit polypeptides (23–25 and 32–43 kDa) and 8S subunit polypeptide (57 kDa) are indicated.

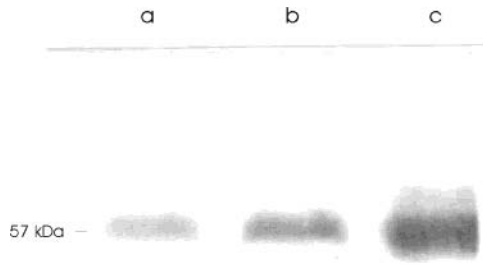

Figure 2. Western blot analysis of salt-soluble proteins from buckwheat seed at different stages of development: (**a**) 9–11 DAF; (**b**) 19–23 DAF; (**c**) mature seed. Antibodies against the 57 kDa subunits of the 8S protein from mature seed were used.

RESULTS

Biosynthetic Pattern of Buckwheat 8S Globulin. In our previous paper (*15*), it was shown that storage protein polypeptides start to dominate the total protein spectrum of buckwheat seed from the 14 DAF stage. From that stage to full maturation, the level of the total storage protein per seed was dramatically increased to >80% of total proteins by the end of development. The progressive accumulation proceeded simultaneously for all storage protein fractions.

To investigate whether the synthesis of 8S storage protein, as a distinct protein fraction, starts very early in development, the protein pattern from buckwheat seed at 9−11 DAF was analyzed and compared to that of mature seeds. **Figure 1**shows that polypeptides with mobility similar to that of 8S subunit polypeptides of 57−58 kDa could be visualized on Coomassie blue stained SDS-PAA gel, even though the total amount of protein per seed in the early stage is 200 times lower than that in mature seed.

The identity of the mentioned polypeptide was confirmed by Western blot analysis with polyclonal antibodies made against the 57 kDa polypeptide from mature seeds (**Figure 2**). On the other hand, none of the polypeptides from the early stage reacted with antibodies made against the 13S basic subunit (data not shown).

In vitro translation experiments were performed to answer the question of whether the observed protein level and composition were correlated with mRNA status throughout buckwheat

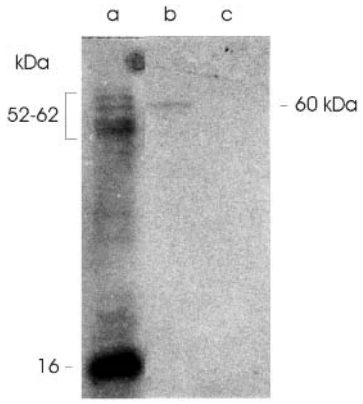

Figure 3. Fluorogram of the ^{35}S-methionine-labeled in vitro translational products of polyA RNA from 9–11 DAF stage seeds: (**a**) total; (**b**) immunoprecipitated with antibodies to the 57 kDa subunits of the 8S protein; (**c**) immunoprecipitated with antibodies to 23–25 kDa subunits of the 13S protein.

seed development. We focused especially on the early stage of buckwheat seed development, when the first appearance of a 57 kDa polypeptide was noticed. Products of in vitro synthesis, labeled with [^{35}S]methionine, were analyzed by SDS-PAGE (**Figure 3**). Four bands in the range from 52 to 62 kDa and a few polypeptides close to 16 kDa were visualized on the fluorogram, but the only polypeptide immunoprecipitated with antibodies to the 8S subunit polypeptide was the 60 kDa polypeptide. None of the in vitro products were immunoprecipitated with antibodies to 13S subunit polypeptides, which confirmed the absence of 13S globulin during the early stage of buckwheat seed development.

We also analyzed the products obtained after intact 14−23 DAF stage cotyledons of buckwheat seed were labeled with a mixture of ^{14}C-labeled amino acids. The in vivo pulse-labeled products were compared on SDS-PAGE to the polypeptides isolated from the same developmental stage. Contrary to the data obtained in vitro, the product labeled in vivo corresponding to the 8S subunit contained no significant extra sequence (data not shown).

Isolation of cDNA Clone Coding for Vicilin-like Globulin. Among a few hundred cDNA clones isolated from the cDNA library made on total mRNA from the 14−19 DAF stage of buckwheat seed development, one clone, assigned as pFeVIC1 (accession no. AY536051), showed high homology to the cDNA clones coding for vicilin-like storage globulins from various plant species. The highest homology of the deduced amino acid sequence was found for vicilin-like globulin from sesame seeds (46% identity**)** followed by those from *Zea mays*, *Picea glauca*, and *Juglans nigra* (**Figure 4**). Besides homology to the vicilin-like cDNAs, the deduced amino acid sequence of *FeVIC1* showed homology to the sucrose-binding protein from *Glycine max* (AAF05723), *Pisum sativum* (CAA72090), and *Vicia faba* (CAC27161**)**.

Analysis of the deduced amino acid composition of the partial cDNA *FeVIC1* revealed that the content of lysine is 5.7 mol %, a characteristic that could be of biotechnological importance.

Northern Blot Analysis of Buckwheat Vicilin-like Subunit Expression. The clone, pFeVIC1, was used as a probe to analyze tissue-specific expression of corresponding mRNA throughout seed development (**Figure 5**). Specific mRNAs were identified in the RNA isolated from buckwheat seed at the 9−11 DAF stage and continued to accumulate throughout buckwheat

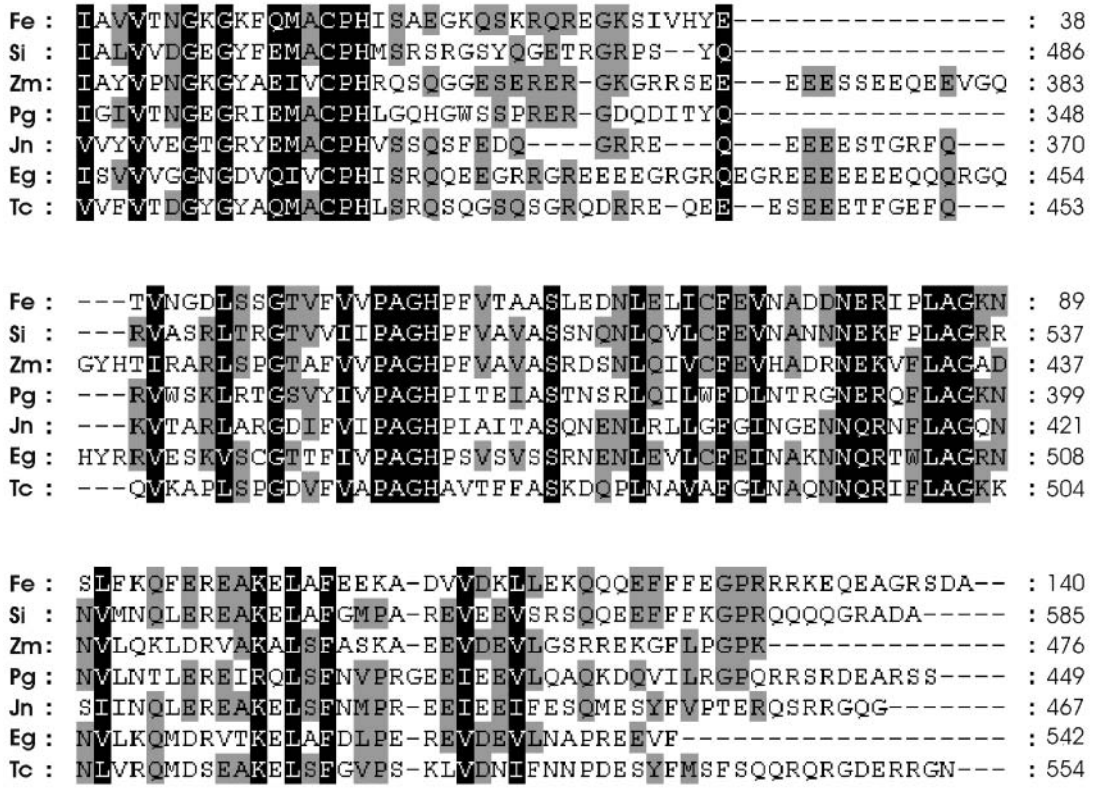

Figure 4. Alignment of deduced amino acid sequence of *FeVIC1* and representatives of various plant species: Fe (*Fagopyrum esculentum*, AY536051), Si (*Sesamum indicum*, AAK15089), Zm (*Zea mays*, S21825) Pg (*Picea glauca*, AAB01554), Jn (*Juglans nigra*, AAM54366), Eg (*Elaeis guineensis*, AAK28402), Tc (*Theobroma cacao*, S22477). As pFeVIC1 is a partial cDNA clone, 609 bp long (consisting of 420bp of coding sequence and 189 bp 3′ untranslated region), numbering for that clone corresponds to the available C terminus of the complete cDNA sequence. Identical and similar residues are shown in reverse type shaded boxes.

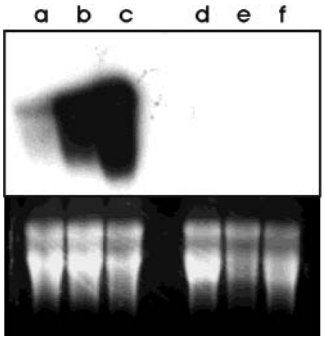

Figure 5. Autoradiograph of the northern hybridization of pFeVIC1 with RNAs from different buckwheat tissues. Buckwheat seeds were collected at 9–11 DAF (**a**), 14–17 DAF (**b**), and 19–23 DAF stages (**c**), as well as leaves (**d**), seedlings (**e**), and root (**f**). In each line 10 μg of total RNA was loaded. The corresponding EtBr-gel representing rRNA is shown at the bottom.

seed development. Expression was not detected for RNA isolated from either leaf, root, or seedlings.

DISCUSSION

Our interest in buckwheat seed storage proteins was aroused by two facts. The high biological value of those proteins makes their genes potential candidates for transfer to cereal or legumes in order to improve the nutritional quality of those commonly used crops. On the other hand, the regulation of gene expression of storage proteins is particularly important in fundamental research, as the intricate mechanism of gene switching during late embryogenesis as well as tissue specificity can be studied on those particular genes.

In our previous papers we analyzed the complexity of subunit composition of buckwheat storage proteins (*6*, *7*), as well as biosynthetic events specifically related to the main storage protein of the buckwheat 13S globulin (*15*). In this paper we concentrated on the analysis of the 8S globulin fraction from buckwheat seed.

We found that biosynthesis of the 8S globulin precedes that of the 13S globulin. 8S subunits (57 kDa polypeptides) were detected in the early stage of buckwheat seed development, as early as 9−11 DAF. In the midmaturation period, which is characterized by progressive accumulation of storage proteins, synthesis of 8S globulin parallels that of 13S.

The product of in vitro translation of mRNA corresponding to the subunit polypeptide of 8S globulin was ∼3 kDa larger than that in the mature 8S globulin. This finding suggests the existence of a signal peptide, which directs the translocation of the nascent polypeptide into the lumen of the RER. The signal peptide was found in all investigated storage proteins (*16*), as well as in other plant and animal proteins that were transported (*17*). The product pulse-labeled in vivo had no extra sequence, indicating that the signal peptide is cotranslationally removed.

Isolation of cDNA showing high homology to the other cDNAs coding for vicilin-like storage polypeptides from various plant species also confirmed the existence of that specific storage protein in buckwheat seeds. It is interesting to note that homology was highest with the vicilin-like protein from *Sesamum inducum* (*18*), as was also found for buckwheat legumin-like storage polypeptide (*19*). The list of the "homology top 10" also included representatives of the vicilin-like storage subfamily from gymnosperms (*Picea glauca*) and monocots (*Zea mays*), confirming that the evolutionary position of buckwheat is close to the lower evolutionary clades. A storage function for buckwheat 8S globulin is certain, but homology of the deduced amino acid sequence of buckwheat *FeVIC1* with the sucrose-binding protein from soybean (*20*) and pea opened a new research puzzle relating to the possibility of an additional function for this protein fraction. This finding could be also discussed regarding the evolutionary pathway of seed proteins analyzed by Shutov et al. (*21*) in more detail.

Taking everything into account, the 8S storage globulin of buckwheat seed can be classified as a distinct protein fraction belonging to the vicilin-like family, making up 7% of total buckwheat seed proteins. This protein fraction could be more interesting for biotechnological application than the 13S buckwheat legumin consisting of 23−25 kDa subunits, which was reported to be the major buckwheat allergen (*22*−*25*). In our experiments, antibodies made against 23−25 kDa polypeptides did not cross-react with the 57 kDa polypeptide, which is a subunit of the 8S globulin. This finding is in agreement with data reported by Urisu (*23*) and Nair (*25*), who found that the 57 kDa polypeptide was not recognized by sera from allergic patients.

In our further experiments, we intend to isolate a full length of buckwheat vicilin cDNA and to analyze its possible biotechnological potential according to its dedeuced amino acid composition.

ABBREVIATIONS USED

DAF, days after flowering; SSPs, seed storage proteins; VSPs, vegetative storage proteins; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

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