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# Expression and characterization of the *Arabidopsis thaliana* 11S globulin family

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ABSTRACT

The 11S globulins are the principal seed storage proteins in a variety of major crop species, including members of the legume and mustard families. They are targets for protein engineering studies attempting to alter the physicochemical properties of seed protein extracts (*e.g.* soybean) and to improve the nutritional quality of important agricultural crops. A key factor that has limited the success of this approach to date is insufficient accumulation of the engineered protein variants *in vivo* due to their improper folding and/or reduced stability, compared to the native protein. We have developed the *Arabidopsis thaliana* 11S proglobulins as a model system to enable studies exploring the factors underlying structural stability in this family of proteins. Yields of 1.5–4 mg/L were achieved for the three *A. thaliana* 11S proglobulins expressed in the Origami *Escherichia coli* cell line in super broth media at 20 °C for 16 h and purified *via* immobilized-metal affinity chromatography. We also demonstrate that differential scanning fluorimetry is an effective and accessible technique to facilitate the screening of variants to enable the successful engineering of 11S seed storage proteins. The relative *in vitro* stability of the *A. thaliana* 11S proglobulins (proAtCRU1 > proAtCRU3 > proAtCRU2) is consistent between chemical and thermal denaturation studies.



## 1. Introduction

Seed storage proteins have evolved to act as long-term amino acid reserves in seeds and are the source of nitrogen and sulfur for the germinating embryo [1–3]. These non-enzymatic, secretory proteins are generally classified, based on their solubility, into one of three categories: albumins (soluble in water), globulins (soluble in saline), or prolamines (soluble in aqueous alcohol) [4,5]. Globulins are further classified into the 11S and 7S families and the many alternate designations of each indicate the plant groups from which they have been isolated [6]. For example, the 11S proteins of plant species of the Fabaceae (legume: *e.g.* soybean, chickpea, lentil) and Brassicaceae (mustard: *e.g.* the model organism *Arabidopsis thaliana*) families are often referred to as legumins and cruciferins, respectively. The 11S globulins are encoded by a small gene family and are the predominant storage protein in the seeds of dicots, a large group of flowering plants, including many agriculturally important crops, that is distinguished from the grasses primarily by seed, leaf and pollen structure [1,3,7]. The *A. thaliana* genome contains three 11S globulin loci (in order of predominance in the seed: AT4G28520—AtCRU3, AT5G44120—AtCRU1 and AT1G03880—AtCRU2) [8,9].

The processed, mature 11S globulin is comprised of a pair of cupin domains, a β-barrel followed by a cluster of α-helices (Fig. 1), and assemble to form heterohexamers in the seed [10,11]. The signal peptide of the initial translation product, referred to as the preproglobulin, is removed upon entrance to the endoplasmic reticulum. The assembly of the resulting proglobulins into trimers is primarily mediated by hydrophobic interactions between the α-helices (Fig. 1a and b) [12]. Subsequent proteolytic cleavage, in the protein storage vacuole, at a conserved site between the two cupin domains, generates the disulfide-linked α- and β-chains of the monomer and the resulting conformational shift enables pairs of trimers to associate, producing the mature hexameric 11S globulins [13].

Since 11S globulins are the major seed storage proteins in many agriculturally important crops, they are targets for protein engineering studies aimed at modifying their nutritional and functional properties. Factors including nutritional quality, solubility and emulsification influence downstream food applications, as illustrated by the many food products produced from soybean seed extracts [14,15]. In their comprehensive 2013 review, Galili and Amir note that a limitation of reported protein engineering studies focusing on seed proteins, including 11S globulins, has been the diminished ability of the resulting variant to

*Abbreviations:* His-proAtCRU, N-terminally 6-His-tagged *A. thaliana* procruciferin; SB, super broth media; DSF, differential scanning fluorimetry; TB, terrific broth media; Trp, tryptophan

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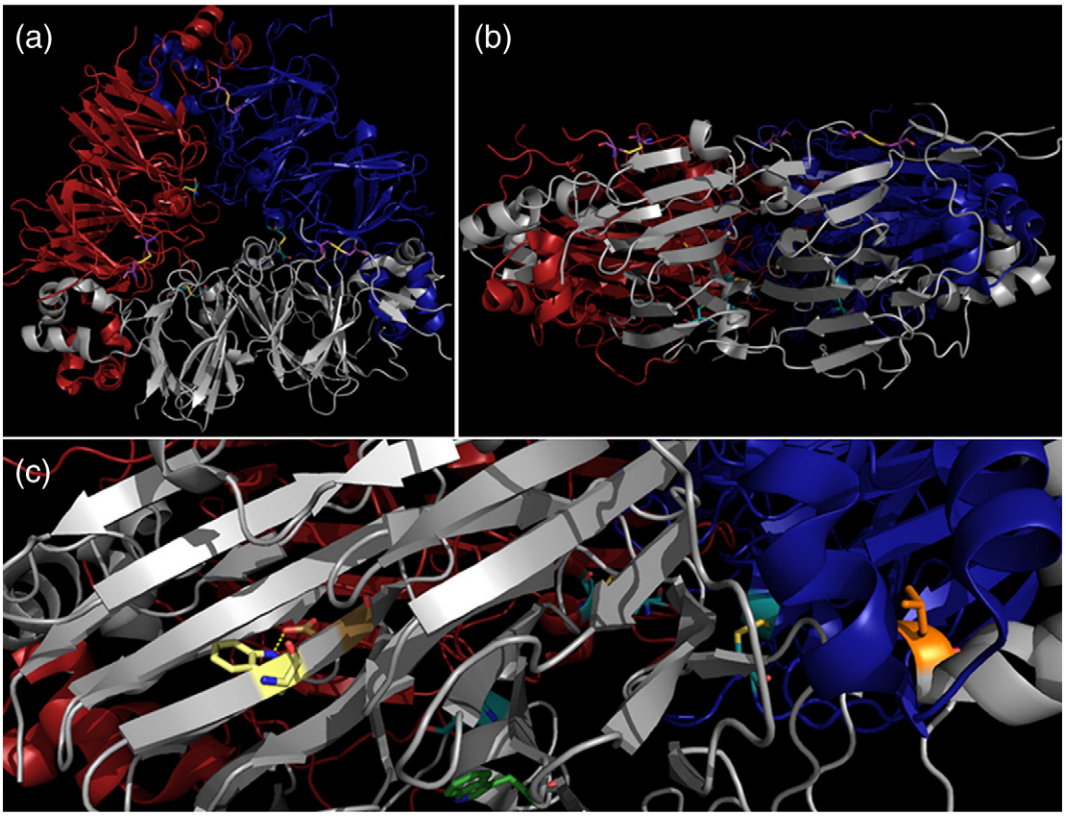


**Fig. 1.** The structure of a *B. napus* procruciferin (PDB ID: 3KGL). The three subunits (in white, red and blue) are shown from the (a) IE face of the trimer and (b) side of the trimer with the IE face on the top and the IA face on the bottom. The IA and IE disulfide bonds are teal and purple, respectively. (c) The residues corresponding to the candidates for the tryptophan residues that dominate the fluorescence spectrum of the *A. thaliana* procruciferins are shown in stick representation to illustrate their relative positions: W31 (green), W397 (pale yellow) and L220 (orange).

appropriately accumulate *in vivo* [14]. This is likely due to reduced stability or improper folding of the engineered protein as previous studies have generally not assessed the thermodynamic impact of the modifications in a systematic manner. The 11S globulins from several plant species including *Glycine max*, *Amaranthus hypochondriacus* and *Brassica napus* have been successfully purified from source as well as, *via* heterologous expression, from *Escherichia coli* [16–21]. Crystal structures of these 11S globulins purified from *E. coli* demonstrate that the homotrimers resulting from heterologous expression can be employed to investigate the factors underlying structural stability in this group of proteins [19,22]. The three cruciferins of *A. thaliana* provide a useful system for these studies as the genetics and physiology of this model plant species have been thoroughly characterized. This study reports the first purification and comparative characterization of the *A. thaliana* 11S globulins and provides the basis for development of the tools required to facilitate the engineering of seed storage proteins.

## 2. Materials and methods

### 2.1. Reagents

Isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin were purchased from BioShop; tetracycline and lysozyme from Sigma; Ni-nitrilotriacetic acid (Ni-NTA) resin from Qiagen; and EDTA-free protease inhibitor tablets from Roche. All enzymes and the ER1821 cell line were acquired from New England Biolabs (NEB), oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT) and coding sequences were sequenced by BioBasic. The Origami *E. coli* strain was obtained from Novagen.

### 2.2. Amplification of A. thaliana procruciferin coding sequences

Total RNA was extracted from *A. thaliana* siliques using the RNeasy mini kit (Qiagen), with the DNaseI treatment, and cDNA was synthesized from 1 μg of RNA using an oligo($dT_{18}$) primer and 200 U M-MuLV reverse transcriptase (NEB). The coding sequences of the three *A. thaliana* procruciferins (*proAtCRU1–3*) were amplified from cDNA and inserted between the *NdeI* and *Bam*HI sites of the pTrc-99aAF expression vector [23], which encodes an N-terminal His-tag. A silent mutation was introduced to remove the internal *Bam*HI site of *proAtCRU3* to facilitate insertion into pTrc-99aAF.

### 2.3. Optimization of expression and purification conditions for the affinity-tagged procruciferins

Batch purification of 1 L cultures, grown in 2.8 L baffled Fernbach flasks, was employed to determine the optimal expression, growth and purification conditions for the three N-terminally 6-His-tagged *A. thaliana* procruciferins (His-proAtCRU). The host ER1821 (NEB) or Origami (Novagen) cells containing the pTrc-99aAF/proAtCRU1, pTrc-99aAF/proAtCRU2 or pTrc-99aAF/proAtCRU3 plasmids were cultured at 37 °C in LB medium containing either 0.17 M or 0.5 M NaCl, or super broth medium (SB: 12 g/L tryptone, 24 g/L yeast extract, 12.5 g/L $K_2HPO_4$, 3.8 g/L $KH_2PO_4$, 5.1 mL/L glycerol, and Vogel–Bonner salts) [24,25]. Expression was induced during log phase of growth with 0.15 mM IPTG and growth proceeded for 16 or 60 h at 20 or 25 °C. Cells were harvested by centrifugation at 7000 rpm for 10 min at 4 °C, washed with 0.85% NaCl and stored at −80 °C.

All purifications were carried out at 4 °C. Frozen cells were resuspended in ~20 mL buffer A (50 mM potassium phosphate, pH 8,