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Expression in *Escherichia coli* and in vitro refolding of the plant transcription factor *Arabidopsis thaliana* RGL3

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Abstract

Recombinant *Arabidopsis thaliana* (At) RGL-3, using two vectors pMAL-c2 and pET 21, was expressed as inclusion bodies in *Escherichia coli* under a range of temperature conditions. Only low levels (8–12% of total protein) of soluble protein were produced. The "soluble" fraction was shown by native PAGE to exist as soluble aggregates of RGL-3. A method was developed, consisting of induction of expression at various temperatures that yielded high levels of refoldable inclusion bodies using the pET vector. (At) RGL-3, as inclusion bodies, was solubilized in 8 M urea and refolding was initiated by 20-fold direct dilution of denaturant. Under optimal conditions, 87% of the denatured protein of inclusion bodies was successfully re-natured. Refolding was monitored by "native" PAGE. Refolded RGL-3 was shown to be present as monomers and dimers. Attempts to further purify His-tagged RGL-3 using Ni/NTA chromatography resulted in the formation of higher polymers.

Keywords: Plant transcriptional factors; DELLA family; Plant proteins; *Arabidopsis thaliana* RGL3; In vitro refolding

The DELLA family of plant proteins are named after the amino acid motif DELLA in the N-terminal domain and are part of a larger family of plant transcription factors named the GRAS family [1] that contain a variable N-domain but a highly conserved C-domain. Five DELLA proteins have been identified in *Arabidopsis thaliana* [2–4]. The DELLA proteins act to depress plant growth by repression of genes required for cell elongation and differentiation [5]. The mechanism by which DELLA proteins repress these genes is not yet known, but it is known that their degradation in response to gibberellin is required. Through use of null mutants it has been established that DELLA genes are partially functionally redundant but do have different tissue expression patterns [6]. RGA and GAI are involved mostly in the repression of juvenile growth and phase change [7,8] and together with minor contribution from RGL-1 and RGL-2 to control the transition of shoot apical meristem to inflorescence meristem [9,10]. These authors also confirmed that RGL-2, together with some contribution from RGA, GAI and RGL-1, is the prominent germination repressor and that RGA and RGL-2 are predominant but RGL-1 has a role in controlling floral organ growth. The role of RGL-3 is not clear. However mutant *Arabidopsis* plants in which all but RGL-3 genes had been deleted had a phenotype that is similar to the wild-type plants and suggests that RGL-3 could function normally by itself in that mutant background (personal communication). All these proteins are expressed at only very low concentrations.

To date all of the research on DELLA proteins has been carried out using genetic approaches to understanding the mechanisms by which they control plant growth. We therefore wished to express (At)[1] RGL-3 as a recombinant protein to prepare antibodies for studying interactions with other proteins and to determine tertiary structure. In this

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[1] *Abbreviations used:* At, *Arabidopsis thaliana*; IPTG, isopropyl-D-thio-galactopyranoside; PMSF, phenyl methyl sulfonyl fluoride.

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report, we describe a strategy for expression and purification of the recombinant DELLA protein (At) RGL-3.

Materials and methods

Materials

pMAL protein expression and purification system was purchased from New England BioLabs. Bacterial strain BL21 (DE3) was purchased from Novagen (Madison, WI, U.S.A.). DNA manipulations were carried out by using standard procedures [11]. Plasmids were isolated with a Qiaprep spin plasmid kit (Qiagen).

Bacterial strains and plasmids construction

Escherichia coli strains DH5α and BL21 (DE3) were used for cloning and overexpression of (At) RGL-3, respectively. Plasmids pET-21 and pMal-c2 were used to construct vectors for overexpression of recombinant (At) RGL-3. The gene encoding the (At) RGL-3 was obtained by RT/PCR (Invitrogen, superscript one step) from mRNA (Dynal, mRNA direct) of *A. thaliana* (*Lansber erecta*). After PCR amplification using polymerase (AmpliTaqGold PCR Master Mix, Roche), T4 DNA ligase (Promega) and appropriate primers, the resulting plasmids pET-21-(At) RGL-3 and pMal-(At) RGL-3 were sequenced and the (At) RGL-3 gene shown to be identical to the corresponding regions of the sequence from GenBank Accession No. NM_121755 [gi:30686387].

Expression of His-tag (At) RGL-3 and MBP-(At) RGL-3 fusion protein

Escherichia coli strain BL21 (DE3) expressing *His-tag* (At) RGL-3 or MBP-(At) RGL-3, was grown in LB medium containing ampicillin (100 μg/ml) to an A_{600} of 0.3–0.4 at 37 °C. Expression was induced by addition of IPTG (isopropyl-D-thiogalactopyranoside) (0.3, 0.5 and 1 mM) followed by 3 h incubation at 37 °C. Cells were collected by centrifugation and resuspended in a lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM EDTA, and 0.5 mM dithiothreitol) and frozen at −20 °C. The bacteria were thawed and lysed by sonication on ice. The bacterial lysates were subjected to centrifugation to analyze for solubility of the fusion protein. For purification of the MBP-(At) RGL-3, the soluble fraction containing MBP-(At) RGL-3 fusion protein was loaded on an amylose affinity column, washed with three column volumes of 20 mM Tris, pH 7.4, and 100 mM NaCl. Elution was performed with the same buffer supplemented with 10 mM maltose.

For the refolding experiment, four 2-l flasks containing 300 ml of Terrific Broth [11] supplemented with ampicillin (100 μg/ml) were inoculated with 3 ml of overnight culture of *E. coli* BL21 (DE3) containing the pET 21:(At) RGL-3 construct. The cells were grown at 37 °C, with shaking (250 rpm) to an absorbance (600 nm) of 0.2–0.3. Expression was induced with 0.3 mM IPTG. After addition of IPTG, one culture was incubated at 37 °C for 3 h with shaking. The second culture was shifted to room temperature and incubated overnight. The third culture was shifted to 4 °C and incubated overnight. The fourth culture was treated as follows: after exposure to cold at 4 °C, for 1 h, the culture was shifted to room temperature (18–20 °C) for 3 h incubation (no shaking), to 4 °C for 1/2 h and finally to overnight incubation at room temperature (no shaking) (Absorbance at 600 nm = OD 0.9–1.2). This experiment was repeated twice. Cells were collected by centrifugation at 6000 rpm for 15 min (Sorval RC5B) and the pellets were stored at −20 °C. The pellets were thawed on ice and resuspended in 40 ml of Tris–HCl buffer pH 8.4 (20 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF). The cells were disrupted by sonication on ice (10 × 10 s). Cell lysates were centrifuged at 19,000 rpm for 20 min, the supernatants collected on ice, and examined for soluble protein by SDS–PAGE. Inclusion body pellets were resuspended in washing buffer (20 mM Tris–HCl, pH 8.4, 5 mM EDTA, 500 mM NaCl, 2 M urea, 2% Triton-X) and centrifuged at 15,000 rpm (Sorval RC5B) for 30 min. The washing step was repeated three times. The pellets were resuspended and solubilised in 10 ml of buffer A (8 M urea, 100 mM Tris–HCl, 5 mM EDTA, 10 mM dithiothreitol, 1.5 mM reduced glutathione, 0.2 mM oxidized glutathione, pH 8.4) and placed on ice. Refolding of (At) RGL-3-His-tag was initiated at 4 °C by 20-fold dilution of the urea-denatured proteins of inclusion bodies with buffer B (20 mM Tris–HCl, 50 mM NaCl, 2 mM EDTA–$(Na)_2$, pH 8.4) and placed in ice-bath. The mixture was then sonicated in an ultrasonic bath for 10 min (Samophone, Ultrasonic Industries Pvt., Ltd.). The mixture was then centrifuged (19,000 rpm for 1 h at 4 °C). The supernatants were aspirated and concentrated 10 times by ultra filtration (20 kDa type 20 Diafilter Pall Filtron Technology Corporation) at 4 °C. The (At) RGL-3 was further purified by employing Ni–NTA chromatography. The concentrate was loaded onto a 5-ml Ni–NTA column equilibrated with buffer B plus 0.4 M urea and 5 mM imidazole. The column was washed with the same buffer containing 0.4 M urea and 30 mM imidazole. The His-tagged (At) RGL-3 was eluted from the column with buffer B containing 0.4 M urea and 300 mM imidazole. Fractions containing (At) RGL-3 were pooled and concentrated by ultrafiltration as above.

Electrophoresis and Western blot analysis

Protein samples were analyzed for purity and checked for degradation using SDS and native polyacrylamide gels. Samples were resolved on a 7.5% denaturing and native gel. Protein bands were either stained with Coomassie blue R or electro-transferred from an unstained gel onto a nitrocellulose membrane (Trans-Blot Transfer, Bio-Rad Laboratories, CA, USA) for Western blot analysis. The membrane was blocked in 0.5% I-block (Tropix, Bedfod, MA, USA) in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 2 h at