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Research paper

Expression, purification and biochemical characterization of AtFUT1, a xyloglucan-specific fucosyltransferase from *Arabidopsis thaliana*

Félix Cicéron, Joana Rocha, Sumaira Kousar, Sara Fasmer Hansen, Valérie Chazalet, Emilie Gillon, Christelle Breton, Olivier Lerouxel*

Univ. Grenoble Alpes, CNRS, CERMAV, 38000 Grenoble, France

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ABSTRACT

Efforts to identify genes and characterize enzymes involved in the biosynthesis of plant cell wall polysaccharides have yet to produce and purify to homogeneity an active plant cell wall synthesizing enzyme suitable for structural studies. In Arabidopsis, the last step of xyloglucan (XG) biosynthesis is catalyzed by fucosyltransferase 1 (AtFUT1), which transfers L-fucose from GDP-β-L-fucose to a specific galactose on the XG core. Here, we describe the production of a soluble form of AtFUT1 ($His\Delta_{68}$-AtFUT1) and its purification to milligram quantities. An active form of AtFUT1 was produced in an insect cell culture medium, using a large-scale expression system, and purified in a two-step protocol. Characterization of purified $His\Delta_{68}$-AtFUT1 revealed that the enzyme behaves as a non-covalent homodimer in solution. A bioluminescent transferase assay confirmed $His\Delta_{68}$-AtFUT1 activity on its substrates, namely GDP-fucose and tamarind XG, with calculated Km values of 42 μM and 0.31 μM, respectively. Moreover, the length of the XG-derived acceptor quantitatively affected fucosyltransferase activity in a size-dependent manner. The affinity of $His\Delta_{68}$-AtFUT1 for tamarind XG and GDP was determined using isothermal titration calorimetry (ITC). Interestingly, ITC data suggest that $His\Delta_{68}$-AtFUT1 undergoes conformational changes in the presence of its first co-substrate (XG or GDP), which then confers greater affinity for the second co-substrate. The procedure described in this study can potentially be transferred to other enzymes involved in plant cell wall synthesis.

1. Introduction

The complex polysaccharides that make up the plant cell wall are synthesized by glycosyltransferases (GTs), a class of enzymes that transfer a sugar residue from an activated donor molecule (usually a nucleotide-sugar) to a specific acceptor molecule, i.e. another sugar residue. Although many GTs are involved in the biosynthesis of the plant cell wall, only a handful have been biochemically characterized to date, mostly from the model plant *Arabidopsis thaliana* [1,2]. Comprehensive knowledge of cell wall biosynthesis can benefit the development of research on biomass deconstruction to sugars, an under-exploited renewable resource [3,4]. The complex polysaccharides synthesized by plants comprise molecules with high potential for various applications (i.e., bioenergy, food industry) [5,6]. However, a current drawback for the use of natural plant cell wall polysaccharides is the batch-to-batch heterogeneity, which greatly hinders the development of high-market-value applications, such as drug delivery [7]. In light of the potential applications of GTs, there is an urgent need for a better understanding of plant GTs, from gene identification to protein expression and purification, but also for the structural study of their reaction mechanisms, still sorely lacking for these enzymes [8]. It is noteworthy that many plant GTs from the CAZY GT1 family (www.cazy.org) have been successfully produced in bacteria for structural studies, but these GTs are soluble cytosolic proteins mostly involved in glycosylation of secondary metabolites in plants [9]. The bacterial system has also been used to produce

Abbreviations: AtFUT1, *Arabidopsis thaliana* fucosyltransferase 1; DLS, dynamic light scattering; EG II, *Trichoderma reseii* endoglucanase; FPLC, fast protein liquid chromatography; GDP, guanosine di-phosphate; GT, glycosyltransferase; IMAC, immobilized metal affinity chromatography; ITC, isothermal titration calorimetry; MOI, multiplicity of infection; PsFUT1, *Pisum sativum* fucosyltransferase 1; SEC, size-exclusion chromatography; XG, xyloglucan; XGO, xyloglucan-derived oligosaccharide.

* Corresponding author.
E-mail address: lerouxel@cermav.cnrs.fr (O. Lerouxel).

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an active and soluble form of a plant monogalactosyldiacylglycerol synthase, a monotopic membrane protein, which is located in the inner membrane of the chloroplast envelope [10]. However, the plant GTs responsible for cell wall polysaccharide biosynthesis are membrane proteins, presumably depending on post-translational modifications to acquire proper folding and activity, which makes their production in bacteria particularly challenging. In the past, various heterologous expression systems have been used to produce plant Golgi GTs, from *Pichia pastoris* to insect cells as well as mammalian cells, but most systems only produced minute amounts of the target enzyme and were mainly used to characterize enzyme activity [11–15].

In this study, to gain comprehensive understanding of the interaction of GTs with complex polysaccharide substrates at the molecular level, we developed a procedure for plant GT expression and purification that meets the requirements for biochemical techniques such as isothermal titration calorimetry (ITC) or crystallography. These techniques rely on efficient protein expression and purification procedures that preserve protein quality and activity and recover a sufficient amount of the target protein to go beyond simple characterization of enzymatic activity. The Arabidopsis xyloglucan α1,2-fucosyltransferase 1 (AtFUT1), a Golgi-resident type II membrane protein belonging to the CAZY GT37 family, was selected for this study. It catalyzes the last step of xyloglucan (XG) biosynthesis, transferring a fucose residue from GDP-fucose to a galactosylated side-chain, forming a fucogalactosylated XG molecule [16]. FUT1 proteins from *Pisum sativum* and *Arabidopsis thaliana* have previously been characterized in terms of enzymatic activity and kinetic parameters [11,17,18]. However, the purification procedure developed for PsFUT1 was incomplete and contaminant proteins, such as BiP chaperones, remain after purification [11]. Therefore, we reassessed the whole procedure to produce and purify a soluble form of AtFUT1. We first tested bacterial expression systems, which have numerous advantages in terms of cost efficiency, scale-up production, yield, and batch-to-batch reproducibility. A eukaryotic protein expression system was also tested. The baculovirus-insect cell system is widely used to produce recombinant proteins and is a good compromise between bacterial and mammalian systems [19]. It is easy to use, amenable to scale-up and is less expensive than mammalian systems. It has been successfully used for the production of various fucosyltransferases, ensuring post-translational protein modifications, proper folding and secretion of the enzymes [20,21].

Here, we report the insect-cell expression of a truncated His-tagged form of the AtFUT1 protein and its purification to homogeneity (with regards to the fucosyltransferase activity), as well as the development of low-cost, easy-to-handle, straightforward set-up for protein production, applicable to other plant GTs. We expect that purification of AtFUT1 can be transferred to valuable industrial processes requiring enzymatic fucosylation of tamarind xyloglucan [22]. In addition, we present novel biochemical data on the oligomerization state of AtFUT1 and cooperative binding of its co-substrates.

2. Materials and methods

2.1. Cloning HisΔ_{68}-AtFUT1 and HisΔ_{160}-AtFUT1 constructs for insect cell expression

Oligonucleotides were purchased from Eurofins MWG Operon (Germany), restriction enzymes from Invitrogen (Life technology, France) and DNA polymerase from Qiagen (France). The gene HisΔ_{68}-AtFut1 corresponding to a soluble form of AtFUt1, lacking 68 amino acid residues from the N-terminus, was amplified by PCR using pENTR-AtFUT1 (cloned from TAIR stock U10760) construct DNA as the template and forward (5′-AAACTGCAGTCAAATCGGATTATGGGTTTCG-3′) and reverse (5′-CCGGAATTCTCATACTAGCTTAAGTCCCC-3′) primers. The HisΔ_{68}-AtFUT1 PCR product (1473 bp) was digested with *Pst*I/*Eco*RI enzymes and cloned into the pVT-Bac-His1 transfer vector, generating pVT-Bac-His1-Δ_{68}-AtFUT1. The HisΔ_{160}-AtFUT1 construct was amplified using forward (5′-AAACTGCAGGATCAAGAACATATTGATGGTGATGGTGAATGC-3′) and reverse (5′-CGGGGTACCTCATACTAGCTTAAGTCCCCAGC-3′) primers. The His Δ_{160}-AtFUT1 PCR product (1197 bp) was digested with *Pst*I/*Kpn*I enzymes and cloned into the pVT-Bac-His1 transfer vector, generating pVT-Bac-His1-Δ_{160}-AtFUT1.

2.2. Insect cell culture and transfection

The BTI-TN-5B1-4 cells (High Five™; Invitrogen), derived from *Trichoplusia ni*, were cultured at 27 °C either in monolayer, for titration of the virus stock in Express Five (Exp-5) serum-free medium (Invitrogen) supplemented with 16 mM glutamine (Gibco) and 50 μg mL^{-1} gentamycin, or in suspension, for recombinant HisΔ_{68}-AtFUT1 production in serum-free EX-CELL 405 (Exc-5) medium supplemented with 50 μg mL^{-1} gentamycin (following Pharmingen recommendations). The recombinant baculovirus (Baculogold™; Pharmingen) containing the gene of interest was propagated in Sf9 monolayer cells. The pVT-Bac-His1 transfer vector [33], derived from pVTBac, contains a honeybee melittin-cleavable signal peptide that targets the protein to the secretory pathway [34]. The His-tag sequence fused to the N-terminus is used to purify the protein on a metal chelate affinity column. pVT-Bac-His1-Δ_{68}AtFUT1 DNA (30 ng) was co-transfected with 7 ng of Baculogold™ AcNPV DNA into 4.10^4 Sf9 insect cells coated on a 32 mm^2 tissue culture plate and incubated for 72 h at 27 °C. Recombinant baculoviruses released into the medium were amplified in several rounds at low MOI (~0.1) in insect cells, and a higher titer virus stock (~10^8 pfu/mL) solution was harvested by centrifugation. The titer of the virus was determined by a 50% tissue culture infective dose endpoint dilution assay in High Five™ cells cultured in Exp-5 as described previously [35].

2.3. Recombinant protein expression

HisΔ_{68}-AtFUT1 was expressed as a 6×His- and Xpress-tagged soluble protein secreted in the culture medium (Fig. S1). High Five™ cells were cultured in Erlenmeyer flasks in the serum-free Exc-405 medium, at 27 °C, with shaking at 120 rpm/min. They were maintained up to a final density of 2×10^6/mL before infection, and infected at a MOI of 1 for 4 days. Cultures were then transferred to 1 L centrifuge bottles (Nalgene) and centrifuged at 13,000 × *g* for 30 min to pellet the cells and gross impurities. The supernatant containing the secreted protein was either stored at −80 °C or kept at 4 °C if used within one day. Protein expression was verified by western blot using the anti-Xpress antibody(Invitrogen), specific for “DLYDDDDK” peptide motif, at a 1:5000 dilution with the following procedure: 1 mL of culture medium was incubated for 2 h with 100 μL of nickel-sepharose resin (GE Healthcare) to bind the His-tagged AtFUT1 protein, and supernatant was removed after centrifugation (5 min at 13,000 × *g*). The resin was recovered and boiled in 50 μL of denaturation buffer (50 mM Tris-HCl, pH 6.8, 20% glycerol, 3% SDS, 2% β-mercaptoethanol and 0.01% bromophenol blue) and loaded on a 10% acrylamide gel for SDS-PAGE. Separated proteins were transferred onto a nitrocellulose membrane using a semi-dry western blotting apparatus (2 h at 140 V), and protein content was characterized using the anti-Xpress antibody.