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VvGONST-A and VvGONST-B are Golgi-localised GDP-sugar transporters in grapevine (*Vitis vinifera* L.)

Daniella Utz[a,b], Michael Handford[b,*]

[a] *Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile*
[b] *Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile*

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ABSTRACT

Plant nucleotide-sugar transporters (NSTs) are responsible for the import of nucleotide-sugar substrates into the Golgi lumen, for subsequent use in glycosylation reactions. NSTs are specific for either GDP- or UDP-sugars, and almost all transporters studied to date have been isolated from *Arabidopsis thaliana* L. In order to determine the conservation of the import mechanism in other higher plant species, here we report the identification and characterisation of VvGONST-A and VvGONST-B from grapevine (*Vitis vinifera* L. cv. Thompson Seedless), which are the orthologues of the GDP-sugar transporters GONST3 and GONST4 in *Arabidopsis*. Both grapevine NSTs possess the molecular features characteristic of GDP-sugar transporters, including a GDP-binding domain (GXL/VNK) towards the C-terminal. *VvGONST-A* and *VvGONST-B* expression is highest at berry setting and decreases throughout berry development and ripening. Moreover, we show using green fluorescent protein (GFP) tagged versions and brefeldin A treatments, that both are localised in the Golgi apparatus. Additionally, *in vitro* transport assays after expression of both NSTs in tobacco leaves indicate that VvGONST-A and VvGONST-B are capable of transporting GDP-mannose and GDP-glucose, respectively, but not a range of other UDP- and GDP-sugars. The possible functions of these NSTs in glucomannan synthesis and/or glycosylation of sphingolipids are discussed.

1. Introduction

In plants, the Golgi apparatus is responsible for the synthesis of non-cellulosic polysaccharides of the cell wall and also for the glycosylation of lipids and proteins. Lumenal glycosylation reactions are catalysed by enzymes called glycosyltransferases (GTs), which use sugars activated by the addition of a nucleotide. Most of the enzymes involved in the synthesis of nucleotide-sugars are cytoplasmic, although there is evidence of some interconversion within the lumen of the Golgi [1]. More recently, it has been reported that some Cellulose Synthase Like (CSL) GTs such as CSLA9 have their active site orientated towards the lumen of the Golgi apparatus [2]. CSLA9 synthesises glucomannan, requiring GDP-mannose and GDP-glucose as substrates [3], and it has been proposed that the CSLA family, composed of 9 members in *Arabidopsis thaliana* is responsible for the generation of this non-cellulosic polysaccharide in diverse plant species [4]. Therefore, considering the location of nucleotide-sugar synthesis in the cytosol, and their use as substrates by lumenal-facing enzymes, nucleotide-sugar transporters (NSTs) are required to transport the substrate across the membrane of the Golgi apparatus [5–8].

In plants, several NSTs have been identified and some have been cloned and characterised. UDP-galactose transporters (AtUTr2, AtNST-KT, UDPGalT1 and UDPGalT2 [9–11]), and transporters of UDP-galactose/UDP-glucose (AtUTr7 and OsUGT [12,13]) have been reported. In addition, in *Arabidopsis*, there is a family of NSTs termed GONST1-5 (Golgi Nucleotide Sugar Transporter; At2g13650, Atg07290, At1g76340, At5g19980, At1g21870, respectively [5,6]) that are orthologues of the GDP-mannose transporters VRG4 described in *Saccharomyces cerevisiae* [14], LPG2 from *Leishmania donovani* [15], CgVRG4 from *Candida glabrata* [16] and CaVRG4 from *C. albicans* [17].

NSTs are proteins which are typically approximately 350 amino acids in length, with molecular weights of around 35 kDa [8,18]. Moreover, they are highly hydrophobic, and computer-based algorithms predict that they harbour 6–10 transmembrane domains

Abbreviations: CSL, cellulose synthase-like; ER, endoplasmic reticulum; GDP, guanosine diphosphate; GFP, green fluorescent protein; GONST, Golgi Nucleotide Sugar Transporter; GTs, glycosyltransferases; NDP, nucleotide diphosphate; NSTs, nucleotide-sugar transporters; UDP, uridine diphosphate.

* Corresponding author at: Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile. Tel.: +56 2 29787263; fax: +56 2 22712983.
E-mail addresses: dani.utz@gmail.com (D. Utz), mhandfor@uchile.cl (M. Handford).

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(TMDs), consistent with their location embedded in a membrane. It has also been determined that the protein sequence of GONST1-4 and other GDP-sugar transporters [5,6,16,17] contain a conserved GX(L/V)NK motif, which in the case of VRG4 from *S. cerevisiae* has been shown to bind the guanosine diphosphate portion of GDP-sugars [19]. After the GDP-sugars have been used by lumenal facing GTs, GMP is produced by the action of an NDPase [8]. It has been postulated that an NK motif located towards the amino-terminal of these NSTs facilitates the export of the nucleoside-monophosphate, in exchange for the import of the GDP-sugar(s) [6].

Although the vast majority of the characterised plant NSTs has been identified in *Arabidopsis*, it has been proposed that the mechanism of delivery of nucleotide-sugars is conserved, given that other higher plant species also harbour NSTs in their genomes [8]. To date, this hypothesis has been tested for UDP-sugar transport [13], but not for GDP-sugar transport. However, it is not possible to predict the functions and subcellular localisation of possible NSTs based only on sequence similarities. For example, although many NSTs are closely related at the primary sequence level, their specificity of transport may differ [12]. In addition, not all NSTs are localised in the Golgi membrane; AtUTr1 and AtUTr3 are present in the ER where they are involved in the process of protein folding through the calnexin/calreticulin cycle [20,21]. Therefore, experimental functional characterisation of all predicted genes is necessary to determine their specificity and localisation *in planta*.

In this study, we report the cloning and characterisation of two NSTs from grapevine (*Vitis vinifera* L.). Grapevine is one of the most cultivated fruit crops in the world, and grape berries are commercialised as table grapes and dried fruits, and processed in wine making, with Chile being a leading producer and exporter of these products. Grape berries contain polysaccharides, and glycoproteins decorated with sugars which originate from GDP-sugars, implicating the necessity of NSTs for their import into the Golgi apparatus. We called these grapevine NSTs VvGONST-A and VvGONST-B, the most-closely related orthologues of GONST3 and GONST4, respectively. *VvGONST-A* and *VvGONST-B* are widely expressed throughout berry development, and by heterologous expression in tobacco leaves, we show that both NSTs are Golgi-localised and capable of transporting different GDP-sugars.

2. Materials and methods

2.1. Plant material

To characterise VvGONST-A and VvGONST-B, three grapevine plants (*V. vinifera* L. cv. Thompson Seedless) were selected from a field located in INIA La Platina (Chile), during the 2012 growing season. Samples for RNA extraction were frozen in liquid nitrogen and stored at −80 °C.

2.2. Cloning of VvGONST-A and VvGONST-B and vector construction

The nucleotide sequences of *A. thaliana GONST3* and *GONST4* (accession numbers At1g76340 and At5g19980, respectively) were used in a tBLASTn search to identify orthologous gene models in the Grape Genome Browser (version 12X) designed by Genoscope (the French National Sequencing Center; http://www.genoscope.cns.fr/vitis).

VvGONST-A and *VvGONST-B* cDNA were amplified from *V. vinifera* cv. Thompson Seedless leaf cDNA using *Pfu* polymerase (Fermentas) and primers VvGONST-A-F (5′-ATGTCTAATG ATGAGGAGAATC-3′) and VvGONST-A-R (5′-TTTCCCCTCTTGTGAATT-3′), and VvGONST-B-F (5′-ATGTCTTCGACTAGATTTGATT-3′) and VvGONST-B-R (5′-TACTGC AGCAAGTTTACCC-3′). Both primers flank the coding region and the PCR product was cloned into pCR8 (Invitrogen) forming pCR8-VvGONST-A and pCR8-VvGONST-B and then homologously recombined into the Gateway-compatible vector, pGWB5 for the addition of a C-terminal GFP tag [22].

2.3. Computational analysis

Protein sequences were aligned using Clustal-OMEGA. The phylogenetic analysis was carried out using the Mega 6.06 software package [23]. Hydrophobicity analysis was performed using a Kyte-Doolittle hydrophobicity plot. The number of putative TMDs was estimated using the web-based algorithms TMHMM, SOSUI, TOPRED, HMMTOP and PRODIV-TMHMM.

2.4. RNA isolation and quantitative expression analysis of VvGONST-A and VvGONST-B in V. vinifera

Total RNA from berry stages E-L27, 29, 30, 31, 35 and 38 [24], and mature leaves was isolated as described by Reid et al. [25]. RNA concentration and integrity were measured after DNase I (Fermentas) treatment with a NanoDrop3300 Fluorospectrometer (ThermoScientific) and agarose gel electrophoresis under denaturing conditions. By use of a common oligo AP primer (5′-CGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′), 0.5 μg of DNAse I-treated total RNA were heated (70 °C, 5 min) and then subjected to a reverse transcription (RT) reaction using Improm-II reagents (Promega). Quantitative polymerase chain reactions (qPCR) were performed with an Mx3000P Real-Time PCR System (Stratagene), using the SensiMix SYBRkit (Bioline) to monitor dsDNA synthesis. The following standard thermal profile was used for two-step cycling qPCR reactions: 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 58 °C for 1 min. No-template controls were included for each primer pair and each PCR reaction was performed in triplicate. Dissociation curves for each amplicon were then analysed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. Data were analysed using MxPro system software (Stratagene). The expression of three reference genes, *Actin* (Genbank accession: EC969944), *EF1-α* (Genbank accession: EC959059) and *GAPDH* (Genbank accession: ECCB973647) [25] was monitored and *GAPDH* was selected using NormFinder [26]. *VvGONST-A* and *VvGONST-B* transcript levels were normalised to the expression of *GAPDH* using the ΔCt method [27]. The sequences of the primer pairs used were: *VvGONST-A* (5′-TTTGTGGGGACAGTTGGGCT TTT-3′ and 5′-CTCTCTTGCACTTTCACGTCCGTT-3′), *VvGONST-B* (5′-AGGAAGGCAATC TCTGCCAC-3′ and 5′-AGGAGGCAAACC-AACCCAAA-3′), *Actin* (5′-CTTGCATCCCTCAG CACCTT-3′ and 5′-TCCTGTGGACAATGGATGGA-3′), *EF1-α* (5′-GAACTGGGTGCTTGA TAGGC-3′ and 5′-AACCAAAATATCCGGAGTAAAAGA-3′) and *GAPDH* (5′-TTCTCGTTG AGGGCTATTCCA-3′ and 5′-CCACAGACTTCA-TCGGTGACA-3′). All qPCR data represent the average of three biological pools of samples and three technical replicates. *In silico* analyses were performed using data provided in www.plexdb.org.

2.5. Subcellular localisation of VvGONST-A and VvGONST-B

Leaves of tobacco (*Nicotiana tabacum*) were used for transient expression after *Agrobacterium tumefaciens*-mediated transformation (strain GV3101) with pGWB5-VvGONST-A-GFP or pGWB5-VvGONST-B-GFP by syringe infiltration as described by previously [28]. Lower epidermal peels of transformed leaves were analysed 4 d after infiltration with a bacterial culture ($OD_{600\,nm}$ 0.5–0.7). In some cases, samples were incubated in 100 μg mL^{-1} Brefeldin A solution for 2 h. Some samples were agroinfiltrated using the Golgi marker (G-rk CD3-967) or the ER marker (ER-rk CD3-959) [29]. Images were captured with a confocal microscope (LSM510 Meta

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