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1 Identification of Putative Interactors of Arabidopsis Sugar Transporters

2

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16

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18

19 Abstract

20 Hexoses and disaccharides are the key carbon sources for essentially all physiological
21 processes across kingdoms. In plants, sucrose, and in some cases raffinose and stachyose,
22 are transported from the site of synthesis in leaves, the sources, to all other organs that
23 depend on import, the sinks. Sugars also play key roles in interactions with beneficial and
24 pathogenic microbes. Sugar transport is mediated by transport proteins that fall into super-
25 families. Sugar transporter (ST) activity is tuned at different levels, including transcriptional
26 and posttranslational levels. Understanding the ST interactome has a great potential to
27 uncover important players in biologically and physiologically relevant processes, including,
28 but not limited to *Arabidopsis thaliana*. Here, we combined ST interactions and co-
29 expression studies to identify potentially relevant interaction networks

30

31

32

33 **Identifying an arabidopsis sugar transporter interaction network**

34 Plant sugar transport relies on hexose- and sucrose-transport proteins belonging to the
35 major facilitator superfamily (MFS) [1], including **SUTs/SUCs** (SUCrose Transporters/Sucrose
36 Carriers), and **MSTs** (Monosaccharide Transporters) and a new class of transporters, the
37 **SWEETs**. The first SUTs were identified in the 1990's from plant cDNA libraries (SoSUT1 from
38 *Spinacia oleracea* and StSUT1 from *Solanum tuberosum*) using suppression cloning in a
39 *Saccharomyces cerevisiae* mutant [2,3]. The SUT family is the smallest family of plant-based
40 sugar transporters (**STs**) and its members are key players in long-distance transport of sugars
41 from source to sink. The MST family is the largest family of plant STs; their locations and cell
42 functions are quite diverse, but the role of the individual members is still poorly
43 characterized [4,5]. SWEETs were recently identified in plants, animals and some fungi [6,7];
44 they derive from another ancient class of transport proteins already found in *archaea*
45 named Semi-SWEETs [8]. SWEETs are uniporters that mediate in- or efflux of sugars
46 (sucrose, glucose, fructose). SWEETs are involved in many physiological processes including
47 phloem loading, seed filling, nectar secretion, pollen nutrition [9-13], and play crucial roles
48 as susceptibility factors in plant-microbe interactions [14-17].
49 ST activity is determined by the number of transporters located in the membrane and by
50 their transport rate (number of substrates transported per second), and is tightly controlled
51 at transcriptional, post-transcriptional, translational and post-translational levels [18,19].
52 Several reports address the regulation of ST activity at the RNA level (*e.g.*, SWEETs) [20] and
53 by post-translational protein modifications [21-25]. In addition, the activity of several STs
54 seems to be regulated via direct protein-protein-interaction (**PPI**) [26-32]. A significant
55 fraction of cellular proteins exists in oligomeric states. Oligomerization may serve a variety
56 of purposes - oligomerization may be advantageous for clustering transporters, for
57 transporter stability in the membrane, may play roles in their delivery to target membranes
58 and endocytosis and may have regulatory roles. A prominent example is the
59 phosphorylation-mediated allosteric regulation, triggered by ammonium in a time- and
60 concentration-dependent manner, of AMT activity [33]. Concerning ST, the importance of
61 oligomerization has previously been demonstrated for SUTs/SUCs [34] and SWEETs [28]. ST
62 interactions could contribute to many biological functions as signaling, sugar homeostasis at
63 the cellular and organism levels and nutrient transfer in plant microbe interactions. Only few

64 experimental reports about the role of PPI for physiological function have been published;
65 *e.g.* the interaction between a Flowering Locus T-like protein (StSP6A) and a SWEET in
66 potato, linking sugar transport to photoperiodic pathways in the context of the regulation of
67 source-sink relations [35], the interaction of tomato SUT2 (SISUT2) with proteins involved in
68 brassinosteroid signaling or synthesis that affects arbuscular mycorrhiza formation [36-37].
69 While experimental techniques such as the two-hybrid system have provided a partial view
70 of ST **interactome** maps [38-47], understanding the ST interactome has a great potential to
71 provide new insights into plant development, plant physiology, plant interactions with their
72 abiotic and biotic environments. To address these questions, we screened the Membrane-
73 based Interaction Network Database (MIND) for *Arabidopsis thaliana* protein interactions
74 [48] to identify candidate ST-interactors potentially involved in the regulation of carbon
75 allocation in a wide range of conditions including abiotic or biotic stress. These candidates
76 are putative interactors, as the MIND is based on a heterologous system and requires in
77 planta validation.

78

79 **The Membrane-based Interaction Network Database (MIND)**

80 Membrane proteins mediate fundamental roles in many biological processes. Membrane
81 proteins allow for transport of ions and metabolites, and protein trafficking across
82 subcellular membranes. Some of the transporters (called transceptors) detect
83 environmental stimuli and transduce signals into the cells; some catalyze chemical reactions
84 [49]. The regulation of transport activity and the transduction of environmental signals
85 depend to a substantial extent on interactions of membrane proteins with themselves
86 (homodimerization), with other membrane proteins and / or with soluble proteins [50]. The
87 mating-based split-ubiquitin system (mbSUS) paved the way for major advances in the
88 identification of membrane protein interactions [51]. mbSUS identified homo-, hetero-, and
89 oligomeric interactions in *Arabidopsis*, *e.g.*, among K⁺ channels [52], between the Acyl-CoA-
90 binding protein ACBP6 and the plasmodesmata-located protein PDLP8 [53], among subunits
91 of glutamate-like receptors (GLRs) [54], among aquaporins and kinase receptors [55]. Using
92 mbSUS in yeast, 12,102 high-confidence membrane/signaling protein interactions were
93 identified and recorded in MIND
94 (<https://associomics.dpb.carnegiescience.edu/Associomics/Home.html>) [50,56]. More than

95 99% of the putative PPIs identified were previously unknown [56]. MIND data were partially
96 validated in orthogonal *in planta* split-green fluorescent protein interaction assays at a rate
97 of 32%, similar as the confirmation rate obtained for published interactions (38%) [56].
98 MIND also predicted PPIs within the membrane proteome of Arabidopsis roots that were
99 confirmed by Size Exclusion Chromatography - Mass Spectrometry (SEC-MS) [57]. Split GFP,
100 antibody-pulldown assays and Förster resonance energy transfer (FRET) for PPI studies are
101 orthologous assays that can be used to validate candidates present in the MIND database.
102 MIND allowed the identification of several interactions that were further confirmed by
103 orthogonal systems, as for example between the possible cargo-receptor Cornichon and a
104 Golgi-located sodium transporter in rice [58], as well as Cornichon with GLRs in Arabidopsis
105 pollen [59].

106

107 **Identification of putative sugar transporter interactors**

108 The present analysis did not retrieve all of the known interactions among STs [34,60]. This is
109 not surprising as MIND was generated with a subset of the Arabidopsis proteome, and
110 focused on interactions between membrane proteins. MIND thus did not cover all possible
111 interactions (not all STs included, also not all possible interactors included). The total
112 interaction network must thus be substantially larger. Despite the importance of STs in
113 carbon allocation and plant-microbe interactions [61-64], there is a knowledge gap
114 regarding ST activity regulation. Therefore, we used MIND to perform an *in silico* search to
115 identify potential interactors of the 79 Arabidopsis STs (9 AtSUCs, 17 AtSWEETs, and 53
116 AtMSTs; **Figure 1**). We ranked STs based on the number of their potential predicted
117 interactors with 2⁺, 3⁺ or 4⁺ Interaction confidence, respectively. “Interaction confidence”, **F_i**,
118 corresponds to the number of repeats in which a particular interaction tested positively for
119 all three reporter genes (*HIS3*, *ADE2* and *LACZ*) in MIND [56]. For example, 4⁺ Interaction
120 confidence corresponds to the activation of the three reporter genes in two independent
121 biological replicates. A 1⁺ Interaction confidence corresponds to the activation of a single
122 reporter gene; it was not included in our analysis as it can lead to many false positives.

123 The resulting ST interactome is a complex scale-free network with a dense central hub
124 where large interaction **nodes** group together (**Figure 1**). Nodes with fewer putative
125 interactors are present in the periphery of the hub (**Figure 1**). Out of the 79 STs, 34 (43%)

126 had at least one interaction (see online **Supplemental Table S1**), revealing a total of 920
127 **interactors** with proteins such as a nitrate transporter (*AtNRT1;1*), AtRBOHD (Respiratory
128 Burst Oxidase Homolog protein D) and the QC-SNARE SFT12 (soluble N-ethylmaleimide-
129 sensitive factor attachment receptor SFT12) (see below) (see online **Supplemental Table S2**).
130 In the SUT family, 4 out of the 9 STs (45%) tested had putative interactors, while in the
131 SWEET family, 10 STs out of 17 (59%) had putative interactors. In the largest family, namely
132 the MSTs, 33 STs out of 53 (38%) could interact with other proteins.

133 Among the largest interaction nodes, we identified AtSWEET5 (112 putative interactors),
134 AtSWEET7 (57 putative interactors), MST At1g54730 (56 putative interactors), AtSUC2 (31
135 putative interactors) and AtSUC4 (30 putative interactors). We detected two new putative
136 interactions between STs not proposed before: between MST At3g05160 and AtSTP4, and
137 between At3g05160 and AtSUC4. At3g05160 is a member of the Early Responsive to
138 Dehydration 6-Like (ERD6 like) sub-family of MSTs. Members of the ERD6-like had been
139 characterized as tonoplasmic glucose exporters [65]. The interaction of At3g05160 with the
140 tonoplasmic sucrose importer AtSUC4 and the monosaccharide plasma membrane STP4 may
141 be involved in the control of cellular sugar homeostasis in response to different stimuli by
142 these interactions. This hypothesis is reinforced by the induction of *AtSTP4* during pathogen
143 infection [66] in order to transport sugars into the host cells and to reduce availability of
144 sugars to the pathogen. All these putative interactions await independent validation (Box 1).

145

146 **Identification of genes co-expressed with sugar transporters and coding for ST interactors**

147 Co-expression network analysis (ATTED-II; <http://atted.jp>) [67] allowed to capture patterns
148 of transcriptome organization whereby gene clusters and co-expression across diverse
149 conditions are identified. Co-expression can indicate that genes are controlled by the same
150 transcriptional regulatory pathway, may be functionally related, or be members of the same
151 pathway or protein complex [68]. Here, within the set of proteins interacting with STs
152 defined with MIND, we identified a small subset of genes encoding proteins that are co-
153 expressed with a ST using the ATTED-II database (**Table 1**). Out of the 34 genes of interacting
154 STs, two did not show co-expression. Eight genes (Table 1) were significantly co-expressed
155 with at least one other gene represented in ATTED-II, and the encoded proteins interact as
156 well (MIND). Functions of proteins encoded by genes with a significant correlation with an ST

157 expression indicated possible crosslinks to ammonium transport, cell trafficking and
158 signaling, and hormone regulation related to sugar transport.

159 mRNA levels of the transmembrane protein gene encoded by At1G27290 were found to
160 correlate with three STs (*AtSTP4*, *AtESL1* and *AtSFP1*). The mRNA levels of the sugar
161 transporter gene At1G67300 correlated with an uncharacterized Xanthine/uracil permease
162 family protein, highlighting a possible crosslink between sugar and nitrogen
163 transport/metabolism. Interestingly, the previously mentioned transmembrane protein
164 (AT1G27290) is predicted to be an interactor of the dual-affinity nitrate transceptor
165 AtNRT1;1 [69] in MIND. In roots, AtNRT1;1 is involved in nitrate uptake from the soil and
166 nitrate signaling, participating in the regulation of primary root growth [70]. In addition to
167 nitrate uptake, AtNRT1;1 functions as a nitrate sensor, regulating the primary nitrate
168 response. In addition, evidence has been provided that AtNRT1.1 is associated with a
169 modification of auxin transport in roots depending on nitrate concentration, defining a
170 mechanism connecting hormone and signaling without any competition. When comparing
171 the root RNA levels of *AtSTP4* (At3G19930) between wild type and the *chl1-5* mutant of
172 *AtNRT1;1*, no RNA was detected in the mutant, indicating a loss of the connection between
173 NRT1;1 and sugar transporter regulation [71]. Beside its role in the acquisition and sensing
174 of nitrate from the soil, *AtNRT1;1* is also expressed in guard cells promoting stomatal
175 opening in the presence of nitrate. Finally, the possible link between Xanthine/uracil
176 permease and the At1G6730 ST, as well as the indirect link of *AtNRT1;1* and key putative
177 sugar transport-related genes such as *AtSTP4*, *AtESL1* and *AtSFP1* through interaction with
178 the transmembrane protein At1G27290 may indicate close regulatory connections between
179 C and N transport and signaling for potential fine-tuning of the C/N ratio [72,73].

180 Transpiration and water movement are affected both by stomatal aperture and hydraulic
181 conductance. Previous studies implicated sucrose/hexoses in the regulation of aquaporin
182 genes, which encoded water channels, in plant hydraulic conductivity and stomatal closure
183 [74]. For instance, glucose addition reduced the movement of water from the xylem into the
184 mesophyll, coordinating transpirational water loss via the regulation of several aquaporins
185 [75]. Among STs, *At1G54730* mRNA levels were highly correlated with aquaporin *AtPIP1;5*
186 *transcript levels*, consistent with a role of both plasma membrane (PM) transporters in the
187 need for parallel transport of sugar and water according to the Münch hypothesis.

188 The uncharacterized Xanthine/uracil permease family protein (At1G27290) is also predicted
189 to be an interactor of AtSFT12 (At1g29060) in MIND, which can interact directly with a large
190 number of STs (see online **Supplemental Table S2**). SFT12 belongs to the SNARE family,
191 proteins that play critical roles in the fusion of endomembranes. More specifically, SFT12 is a
192 Qc-SNARE localized in the Golgi apparatus [76]. Trafficking-related proteins were found as
193 high-degree hubs (proteins with many interactions) involved in a regulatory association with
194 receptors [56]. SFT12 interacted directly with two AtSUCs (SUC2 and SUC4), eight AtSWEETs
195 (SWEET1, 3, 5, 7, 9, 12, 15 and 16) and 9 AtMSTs (STP4, INT1, INT4, VGT2, GLT1, SGB1, ESL1,
196 At3g05400 and At4g04750), with 4⁺, 3⁺ or 2⁺ interaction confidence. One may argue that STs
197 need this Qc-SNARE at the beginning of the secretory pathway *en route* to their final location
198 by **membrane trafficking**. For example, SUC2/SUT1 is targeted to the plasma membrane
199 [77], while AtSWEET16 is addressed to the tonoplast membrane [78,79]. Thus, the SNARE
200 may interact with the transporters to help them get to their correct destination. Qc-SNAREs
201 are specifically involved in vesicular transport during salt and osmotic stress responses and
202 influence Na⁺ accumulation in vacuoles [76]. Since mRNAs of several *ST* genes are increased
203 in response to environmental cues, thereby possibly impacting carbohydrate allocation
204 [80,81], one can hypothesize that PPI between Qc-SNARE and STs could contribute to
205 effective vesicular targeting of STs, or to subcellular dynamics of STs and recycling *e.g.* at the
206 plasma membrane [82-84] to adjust the composition of vacuolar and plasma membrane and
207 sugar flux to adjust osmolality.

208 Another interesting result was that the Phosphoinositide phospholipase C (PLC) -like
209 phosphodiesterase superfamily protein (At4G38690), which was co-expressed and was an
210 interactor of *AtINT1/AtINT1*, also interacted with AtRBOHD in MIND. AtRBOHD is a
211 membrane NADPH oxidase producing reactive oxygen species, for example during pathogen
212 infection [85]. AtRBOHD interacted with AtSWEET11 (3⁺ interaction confidence), as well as
213 with several proteins involved in the regulation of intracellular trafficking that interact also
214 with several STs (4⁺ interaction confidence). Interestingly, some of the STs interacted with
215 proteins involved in the regulation of intracellular trafficking. For example, the early
216 endosome marker (the small GTPase Rab5, gene accession *At5g49540*) appeared to directly
217 interact with 16 STs, and the prenylated Rab acceptor PRA1.E (gene accession *At1g08770*)
218 had two direct interactions with two STs. Rab5 and PRA1.E interacted with each other, and

219 also with AtSWEET7 (4⁺ interaction confidence). The calcium-binding Annexin 1 (gene
220 accession *At1g35720*) interacted with AtRBOHD and AtSWEET7. Annexins are described as
221 regulators of membrane trafficking [86], and Annexin 1 is also involved in the response to
222 salt stress and plant immunity [87]. These examples of ST-interacting proteins, which are
223 related to membrane trafficking, signaling and environmental constraints, provide possible
224 clues about the regulation of STs by PPIs. Localization of ST in mutants for genes encoding
225 these ST-interacting proteins, would be of interest as it could highlight failures in the
226 assembly of ST complexes in the endoplasmic reticulum or in their help for trafficking
227 toward membranes.

228

229 **Concluding remarks**

230 Our findings highlight the importance of combining interactome and co-expression studies
231 to detect potential crossroads of biological functions. Some STs appear to be crucial nodes
232 and their functional characterization (**Box 1**) could help to improve our knowledge of their
233 cellular interactions. Understanding these interactions is crucial to follow the sugar trade
234 from cells to organs for plant nutrition and plant-microbe interactions. Interactome and co-
235 expression studies also reveal many targets that have so far not been linked to sugars. Here,
236 we observed that some STs form large nodes of putative interactions while others interact
237 only with few membrane proteins and/or at the periphery of membranes. We identified a
238 large set of 920 candidate proteins interacting with 34 STs with various biological functions.
239 Although a large proportion of these proteins has no known biological function to date,
240 several are involved either in transport or in cellular processes as trafficking or signaling.
241 Since MIND represents only a subset of all possible interactions, the network must be
242 substantially larger. The MIND database is based on a library of 3233 membrane proteins
243 and soluble signaling proteins, whereas at least several thousands of other membrane
244 proteins exist and that many membrane proteins will interact with soluble proteins. It thus
245 seems pivotal to generate both a complete interactome of membrane proteins, with
246 membrane proteins and soluble proteins, and to link it with soluble protein interactome as
247 developed by Trig and collaborators [88]. Such studies could then be further expanded by
248 large-scale interactomes integrating responses to multiple conditions, and in diverse
249 arabidopsis genetic backgrounds to gain a deeper knowledge in functional relationships and

250 potential network differences in arabidopsis, and by extent or comparative phylogenomics,
251 in crops (see also outstanding questions).

252

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262 **Supplemental information**

263 Supplemental information associated with this article can be found at doi:XXXXXXX'

264

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520

521 **Glossary**

522 **F_i**: Confidence of the interaction according to MIND (2⁺, 3⁺ and 4⁺), with 4⁺ the strongest
523 interaction between two partners [53]. The 1⁺ Interaction confidence was excluded from the
524 present analysis.

525 **Interactant**: defined as Boolean; “True” if the connected node is a ST, “False” if the
526 connected node is an NonST-PROT.

527 **Interactome**: biological networks/interactions formed by and between molecules within a
528 cell.

529 **MST**: MonoSaccharide Transporter.

530 **Node:** A connection point that participates in a network. Here, it could be ST-type and
531 NonST-PROT.

532 **SUT:** SUCrose Transporter. Also called SUC: SUCrose Carriers

533 **SWEET:** *Sugars Will Eventually be Exported Transporter.*

534 **Membrane trafficking:** Process by which proteins and other macromolecules are distributed
535 throughout cell organelles, and released to or internalized from the extracellular space,
536 using membrane-bound vesicles.

537

538 **Figure Legend**

539 **Figure 1. Global Arabidopsis interactome of 34 sugar transporters and 296 interacting**
540 **proteins.** To identify a network of proteins interacting with sugar transporters (see online
541 **Supplemental Table S2**), the protein sequences encoding 9 AtSUTs, 17 AtSWEETs and 53
542 AtMSTs (see online **Supplemental Table S1**) were used to interrogate the MIND Database
543 (<https://associomics.dpb.carnegiescience.edu/Associomics/Home.html>).

544 *Building the sugar transporters – protein interaction network.* The sugar transporters –
545 membrane proteins (Interactant, INTPROT) interaction network was built from a list of
546 interaction tuples (ST_i , $INTPROT_i$, F_i). The interaction network is an unoriented graph that
547 includes nodes and edges with attributes computed using the *NetworkX Python package* of
548 Python 3 [89]. The attribute definitions have been summarized in the Glossary and examples
549 of attributes of a network of interactions between sugar transporters and interacting
550 proteins are presented in Figure 1. Visualization of the interaction network was carried out
551 via the Gephi software [90] using the *Fruchterman Reingold* algorithm. The node sizes are
552 proportional to their regular degree, and the color of the edges depends on their interaction
553 confidence.

554 *Calculations and presentations.* All calculations were performed with Jupyter Notebooks [91]
555 and rendered with the Bokeh Python library [92]. SUT, SWEET and MST family members are
556 respectively represented by blue, orange and green dots. The larger the size of the node, the
557 higher the number of interactants with the sugar transporter. The 4⁺ interaction confidence
558 is represented by red lines, the 3⁺ interaction confidence is represented by blue lines and the
559 2⁺ interaction confidence by green lines.

560

561

562 **Box 1. What is next?**

563 Investigating experimentally membrane protein—protein interactions is a challenge, not
564 least because of the partial hydrophobicity of membrane proteins. This explains why only a
565 small number of membrane protein interactions are known. After identifying protein—
566 protein interactions of high interest through the combined MIND-ATTED approach, several
567 genetic, biochemical and *in-silico* techniques could be used and/or combined to study
568 specific interactions in Eukaryotes, for example: Atomic Force Microscopy (AFM) [93], Blue
569 Native/SDS PolyAcrylamide Gel Electrophoresis (BN/SDS PAGE) [94,95], Co-
570 immunoprecipitation (co-IP) [96], Developing further *In-silico* tools as for example large-
571 scale interactomes integrating responses to multiple conditions, and in diverse Arabidopsis
572 genetic backgrounds, DihydroFolate Reductase (DHFR) [97], Förster Resonance Energy
573 Transfer (FRET) [98], Membrane Strep—Protein INTeraction experiment (SPINE) [99], Phage
574 display [100], Protein chips [101], Protein Interaction Reporter (PIR) [102], PUPylation-based
575 InTeraction tagging (PUP-IT) [103], Reconstitution of membrane proteins [104], Reverse Ras
576 recruitment System (reverse RRS) [105], Site-directed chemical cross-linking [106], Site-
577 Directed Spin Labeling (SDSL) Electron Paramagnetic Resonance (EPR) spectroscopy [107],
578 Split-ubiquitin yeast two-hybrid system [50,108], SPOT-analysis [109], Surface Plasmon
579 Resonance (SPR) [110], Tandem affinity purification (TAP) [111-113], and X-ray
580 crystallography of protein complexes [114].

581

582

583 **Table 1.** Arabidopsis sugar transporters and candidates that are interactors^a of and co-
 584 expressed^b with a given sugar transporter.

Sugar transporters		Candidates that are interactors of and co-expressed with a given sugar transporter	
Name	Accession numbers	Protein identity	Gene accession number
AtSWEET1	At1G21460	Peptidase	At1G34640
AtSTP4	At3G19930	RING/U-box superfamily protein	At3G13430
		At1G27290	Transmembrane protein
AtINT1	At2G43330	PLC-like phosphodiesterases superfamily protein	At4G38690
		Transmembrane protein	At1G27290
AtSGB1	At1G79820	IQD6 – IQ-domain 6	At2G26180
		Peptidase	At1G47640
At1G67300	At1G67300	Xanthine/uracil permease	At5G49990
AtESL1	At1G08920	Transmembrane protein	At1G27290
		NHL3 – NDR1/HIN1-like 3	At5G06320
		AMP-dependent synthetase and ligase family protein	At1G20490
At1G54730	At1G54730	Plasma membrane intrinsic protein 1;5	At4G23400
		GPI transamidase subunit PIG-U	At1G63110
AtSFP1	At5G27350	Transmembrane protein	At1G27290

585 ^aaccording to MIND Database

586 ^b according to ATTED-II Database

587

