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Abstract

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

Identifying an arabidopsis sugar transporter interaction network

Plant sugar transport relies on hexose- and sucrose-transport proteins belonging to the major facilitator superfamily (MFS) [1], including **SUT**s/**SUC**s (SUCrose Transporters/Sucrose Carriers), and **MST**s (Monosaccharide Transporters) and a new class of transporters, the **SWEET**s. The first SUTs were identified in the 1990's from plant cDNA libraries (SoSUT1 from *Spinacia oleracea* and StSUT1 from *Solanum tuberosum*) using suppression cloning in a *Saccharomyces cerevisiae* mutant [2,3]. The SUT family is the smallest family of plant-based sugar transporters (**ST**s) and its members are key players in long-distance transport of sugars from source to sink. The MST family is the largest family of plant STs; their locations and cell functions are quite diverse, but the role of the individual members is still poorly characterized [4,5]. SWEETs were recently identified in plants, animals and some fungi [6,7]; they derive from another ancient class of transport proteins already found in *archaea* named Semi-SWEETs [8]. SWEETs are uniporters that mediate in- or efflux of sugars (sucrose, glucose, fructose). SWEETs are involved in many physiological processes including phloem loading, seed filling, nectar secretion, pollen nutrition [9-13], and play crucial roles as susceptibility factors in plant-microbe interactions [14-17].

ST activity is determined by the number of transporters located in the membrane and by their transport rate (number of substrates transported per second), and is tightly controlled at transcriptional, post-transcriptional, translational and post-translational levels [18,19]. Several reports address the regulation of ST activity at the RNA level (*e.g.,* SWEETs) [20] and by post-translational protein modifications [21-25]. In addition, the activity of several STs seems to be regulated via direct protein-protein-interaction (**PPI**) [26-32]. A significant fraction of cellular proteins exists in oligomeric states. Oligomerization may serve a variety of purposes - oligomerization may be advantageous for clustering transporters, for transporter stability in the membrane, may play roles in their delivery to target membranes and endocytosis and may have regulatory roles. A prominent example is the phosphorylation-mediated allosteric regulation, triggered by ammonium in a time- and concentration-dependent manner, of AMT activity [33]. Concerning ST, the importance of oligomerization has previously been demonstrated for SUTs/SUCs [34] and SWEETs [28]. ST interactions could contribute to many biological functions as signaling, sugar homeostasis at the cellular and organism levels and nutrient transfer in plant microbe interactions. Only few

experimental reports about the role of PPI for physiological function have been published; *e.g.* the interaction between a Flowering Locus T-like protein (StSP6A) and a SWEET in potato, linking sugar transport to photoperiodic pathways in the context of the regulation of source-sink relations [35], the interaction of tomato SUT2 (SlSUT2) with proteins involved in brassinosteroid signaling or synthesis that affects arbuscular mycorrhiza formation [36-37]. While experimental techniques such as the two-hybrid system have provided a partial view of ST **interactome** maps [38-47], understanding the ST interactome has a great potential to provide new insights into plant development, plant physiology, plant interactions with their abiotic and biotic environments. To address these questions, we screened the Membrane-based Interaction Network Database (MIND) for *Arabidopsis thaliana* protein interactions [48] to identify candidate ST-interactors potentially involved in the regulation of carbon allocation in a wide range of conditions including abiotic or biotic stress. These candidates are putative interactors, as the MIND is based on a heterologous system and requires in planta validation.

The Membrane-based Interaction Network Database (MIND)

Membrane proteins mediate fundamental roles in many biological processes. Membrane proteins allow for transport of ions and metabolites, and protein trafficking across subcellular membranes. Some of the transporters (called transceptors) detect environmental stimuli and transduce signals into the cells; some catalyze chemical reactions [49]. The regulation of transport activity and the transduction of environmental signals depend to a substantial extent on interactions of membrane proteins with themselves (homodimerization), with other membrane proteins and / or with soluble proteins [50]. The mating-based split-ubiquitin system (mbSUS) paved the way for major advances in the identification of membrane protein interactions [51]. mbSUS identified homo-, hetero-, and oligomeric interactions in Arabidopsis, *e.g.*, among K^+ channels [52], between the Acyl-CoA-binding protein ACBP6 and the plasmodesmata-located protein PDLP8 [53], among subunits of glutamate-like receptors (GLRs) [54], among aquaporins and kinase receptors [55]. Using mbSUS in yeast, 12.102 high-confidence membrane/signaling protein interactions were identified and recorded in MIND (https://associomics.dpb.carnegiescience.edu/Associomics/Home.html) [50,56]. More than

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

Identification of putative sugar transporter interactors

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

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

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

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

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

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

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

Concluding remarks

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

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

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Supplemental information

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Glossary

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

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

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

MST: MonoSaccharide Transporter.

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

SUT: SUcrose Transporter. Also called SUC: SUcrose Carriers

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

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

Figure Legend

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

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

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

Box 1. What is next?

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

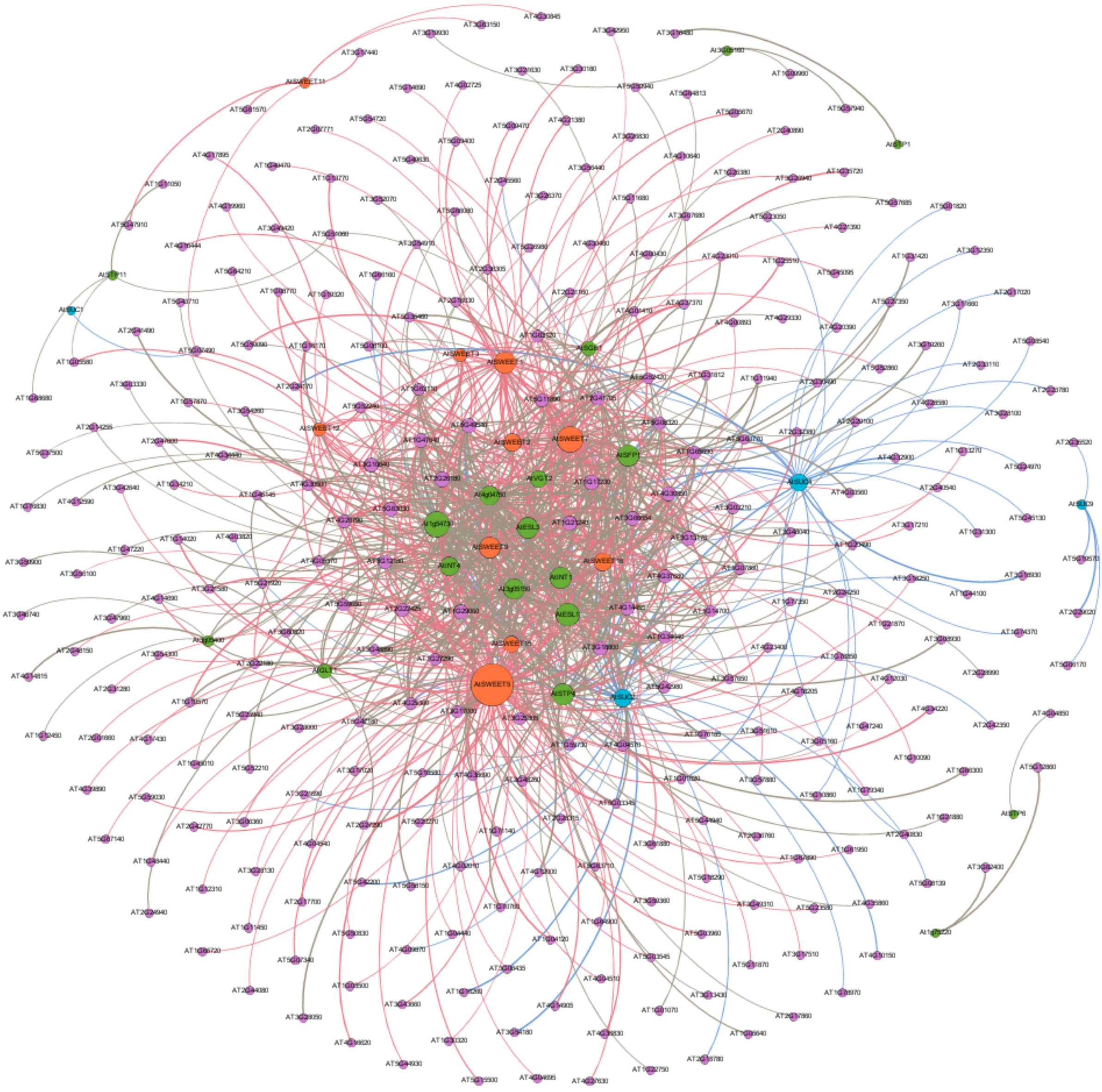
583 **Table 1.** Arabidopsis sugar transporters and candidates that are interactors[a] of and co-
584 expressed[b] with a given sugar transporter.

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 [a]according to MIND Database

586 [b] according to ATTED-II Database

587


AtSWEET11
AtSWEET13
AtSWEET1
AtSWEET12
AtSWEET2
AtSWEET17
AtSFP1
AtVGT2
At4g34750
At1g54730
AtESL3
AtSWEET9
AtSWEET16
AtINT4
AtINT1
At3g05150
AtESL1
AtSWEET15
AtGLT1
AtSWEET5
AtSTP4
AtSUC2
AtSTP11
AtSUC1
AtSTP1
AtSUC4
AtSUC3
AtSTP6
AtSUC9