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Evolution of plant sucrose uptake transporters

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In angiosperms, sucrose uptake transporters (SUTs) have important functions especially in vascular tissue. Here we explore the evolutionary origins of SUTs by analysis of angiosperm SUTs and homologous transporters in a vascular early land plant, *Selaginella moellendorffii*, and a non-vascular plant, the bryophyte *Physcomitrella patens*, the charophyte algae *Chlorokybus atmosphyticus*, several red algae and fission yeast, *Schizosaccharomyces pombe*. Plant SUTs cluster into three types by phylogenetic analysis. Previous studies using angiosperms had shown that types I and II are localized to the plasma membrane while type III SUTs are associated with vacuolar membrane. SUT homologs were not found in the chlorophyte algae *Chlamydomonas reinhardtii* and *Volvox carterii*. However, the characean algae *Chlorokybus atmosphyticus* contains a SUT homolog (CaSUT1) and phylogenetic analysis indicated that it is basal to all other streptophyte SUTs analyzed. SUTs are present in both red algae and *S. pombe* but they are less related to plant SUTs than CaSUT1. Both *Selaginella* and *Physcomitrella* encode type II and III SUTs suggesting that both plasma membrane and vacuolar sucrose transporter activities were present in early land plants. It is likely that SUT transporters are important for scavenging sucrose from the environment and intracellular compartments in charophyte and non-vascular plants. Type I SUTs were only found in eudicots and we conclude that they evolved from type III SUTs, possibly through loss of a vacuolar targeting sequence. Eudicots utilize type I SUTs for phloem (vascular tissue) loading while monocots use type II SUTs for phloem loading. We show that HvSUT1 from barley, a type II SUT, reverted the growth defect of the Arabidopsis *atsuc2* (type I) mutant. This indicates that type I and II SUTs evolved similar (and interchangeable) phloem loading transporter capabilities independently.

Keywords: sucrose transporter, SUT, phylogeny, evolution

INTRODUCTION

In angiosperms, H^+-coupled sucrose-uptake transporters (SUTs) are involved in the long-distance transport of sucrose. They function to load sucrose into the phloem (vascular tissue) and in uptake of sucrose into sink tissues such as seeds and flowers. The physiological functions of SUTs have been reviewed recently (Braun and Slewinski, 2009; Kuhn and Grof, 2010; Ayre, 2011). In this paper we focus on the phylogenetic relationship between SUTs in photosynthetic organisms from algae to angiosperms. SUTs are members of the glycoside–pentoside–hexuronide (GPH): cation symporter family which is distantly related to the major facilitator superfamily (Chang et al., 2004). Transporters homologous to SUTs are found in bacteria, fungi, and animals. SUT function in angiosperms predominates in the phloem, and yet SUTs clearly existed prior to evolution of phloem tissue. So it is interesting to investigate SUT sequences in more simple non-vascular land plants and algae to understand the origins of angiosperm SUTs. Analysis of the structure/function of more divergent homologs may also help us understand the SUT transport mechanism.

The SUT homolog SpSUT1 from *Schizosaccharomyces pombe* is a proton-coupled α-glucoside symporter that has a higher affinity for maltose than sucrose (Reinders and Ward, 2001). SUT homologs in animals, including humans, are associated with melanosomes and mutations in the respective genes generally cause hypopigmentation. MATP (Harada et al., 2001) in humans is encoded by *AIM1* or SLC45A2 and mutations result in oculocutaneous albinism type 4 (OCA4; Inagaki et al., 2006). In horse, *MATP* is associated with cream coat color (Mariat et al., 2003). Similarly, in mouse a SUT homolog is encoded by the *underwhite* (*uw*) gene (Newton et al., 2001; Costin et al., 2003), mutations in the AIM1 gene in medaka fish reduce melanin content (Fukamachi et al., 2001) and in birds, plumage color is controlled by alleles of the gene encoding MATP (Gunnarsson et al., 2007). The only animal SUT homolog for which transport activity has been reported is SCRT from *Drosophila*, SCRT is able to transport sucrose and it is localized to subcellular vesicles that resemble melanosomes (Meyer et al., 2011).

In plants, the first SUT was cloned using yeast functional expression (Riesmeier et al., 1992). SUTs are encoded by small gene families in all flowering plants and phylogenetic analysis shows the presence of three groups of SUTs called type I, II, and III (Aoki et al., 2003). Interestingly, type I SUTs are only found in eudicot species. Type I SUTs are necessary for essential functions in eudicots such as phloem loading (Riesmeier et al., 1994; Gottwald et al., 2000) and normal pollen function (Sivitz et al., 2008). All land plant species contain type II and III SUTs. Monocot species utilize type II SUTs for phloem loading (Slewinski et al., 2009). This indicates that evolution of type I SUTs coincided with

monocot and eudicot divergence. Type III SUTs were first cloned from *Arabidopsis*, potato and tomato and characterized as H^+-coupled symporters (Weise et al., 2000). Type III SUTs are localized at the vacuolar membrane (Endler et al., 2006; Reinders et al., 2008) and function in sucrose-uptake into the cytoplasm (Reinders et al., 2008; Schulz et al., 2011).

Advances in genome sequencing allow us for the first time to investigate the origins of angiosperm SUTs. Complete genome sequence is available for representative bryophyte (*Physcomitrella patens*), lycophyte (*Selaginella moellendorffii*), and chlorophytes (*Chlamydomonas reinhardtii* and *Volvox carterii*). In addition, partial sequence is available for the red algae *Galdieria sulphuraria* and *Cyanidioschyzon merolae* and EST sequence is available for several charophyte algae (Timme and Delwiche, 2010). The main questions that we can address by phylogenetic analysis are whether type I SUTs were derived from type II or type III SUTs and whether both type II and III SUTs were represented in the earliest land plants and algae.

MATERIALS AND METHODS

SUT PROTEIN SEQUENCES

All SUT protein sequences were obtained from the following species in which genome sequence is available: the eudicot *Arabidopsis thaliana*, the monocot rice (*Oryza sativa*), the lycophyte *Selaginella moellendorffii*, and the bryophyte *Physcomitrella patens* using BLAST searches on the Phytozome website[1]. The same database was searched for SUT protein sequences from the chlorophytes *Chlamydomonas reinhardtii* and *Volvox carterii*. Dr. Charles F. Delwiche and Mr. James Thierer, University of Maryland, provided support by searching their algal sequence database (Timme and Delwiche, 2010) for SUT homologs in the charophytes *Chlorokybus atmosphyticus*, *Klebsormidium flaccidum*, *Spirogyra pratensis*, *Coleochaete* sp., *Chaetosphaeridium globosum*, *Penium marinum*, and *Nitella hyalina*. In addition, the genome sequence of the red algae *Galdieria sulphuraria*[2] (Barbier et al., 2005) and *Cyanidioschyzon merolae*[3] were searched for the presence of SUTs. Sequences of *Galdieria sulphuraria* SUTs were provided by Dr. Andreas P. M. Weber, University of Düsseldorf.

PHYLOGENETIC ANALYSIS

Multiple protein sequence alignments were generated with Clustal X (Larkin et al., 2007). The variable length N- and C-terminal regions of the alignment were removed. Percent protein sequence identity is presented, based on the trimmed alignment, as average for each cluster (±SD). Sequences with greater than 90% overall sequence identity were not included in the phylogenetic analysis. Phylogenetic analysis was performed through the iPlant Collaborative website[4]. Maximum likelihood analysis was done using PhyML 3.0 with 100 bootstrap replicates (Guindon and Gascuel, 2003; Guindon et al., 2010). Trees were visualized using the FigTree program[5].

[1]http://phytozome.net
[2]http://genomics.msu.edu/cgi-bin/galdieria/blast.cgi
[3]http://merolae.biol.s.u-tokyo.ac.jp/
[4]http://www.iplantcollaborative.org/
[5]http://tree.bio.ed.ac.uk/software/figtree/

COMPLEMENTATION OF THE *ARABIDOPSIS* atsuc2-1 MUTANT

Constructs for plant transformation contained the AtSUC2 (At1g22710) promoter, coding region of either AtSUC2 or HvSUT1 (CAJ20123.1) cDNAs and the AtSUC2 3′UTR. The AtSUC2 promoter (2 kb) was amplified using the primers 5′ggggac aactttgtatagaaaagttgtaccagatttcggtaaatt and 5′ggggactgctttttttgtaca aacttgaagaaagtaagaaaaaaaagaaatt and cloned into the pDONR P4-P1R vector (Invitrogen) using BP clonase II. The AtSUC2 ORF was amplified using 5′caccggtttgtcaaatatggtcagccatcc and 5′atgaaatcccatagtagctttgaag. The HvSUT1 ORF was amplified using 5′caccggtttgtcaaatatggcgcgcggcggcgg and 5′tcagtgaccgccgccg ctgac. The two ORFs were cloned into pENTR/D/TOPO (Invitrogen). The AtSUC2 3′UTR (500 bp) was amplified using 5′gggg acagctttcttgtacaaagtggattgaattttagcagtggt and 5′ggggacaactttgtataa taaagttgaattaactaaaatagataa and cloned into pDONR P2R–P3 (Invitrogen). Constructs were assembled into the pB7m34GW binary vector (Karimi et al., 2005) by directional multi-fragment recombination cloning using LR Clonase Plus (Invitrogen). *Agrobacterium tumefaciens* strain C58C1 containing these plasmids was used to transform heterozygous *atsuc2-1 Arabidopsis* (WS ecotype) plants (Gottwald et al., 2000) by the floral dipping method (Clough and Bent, 1998). Basta-resistant transformed plants were selected on soil. Homozygous *atsuc2-1* mutants were identified by PCR.

RESULTS

Phylogenetic analysis shows that angiosperm SUTs form three main groups (**Figure 1**). Here, we follow the nomenclature of Aoki et al. (2003) and name these groups type I, II, and III. All SUTs encoded by the eudicot *Arabidopsis thaliana* (seven sequences), the monocot *Oryza sativa* (five), the basal non-vascular moss *Physcomitrella patens* (four), the vascular non-seed spikemoss *Selaginella moellendorffii* (five), and the yeast *Schizosaccharomyces pombe* (one) were included as representatives of those groups where full genome sequence is available. In addition, SUTs that have been functionally characterized were included. In **Figure 1** the commonly used abbreviated names for the transporter genes are listed. In **Table 1** the protein accession number, gene name, protein length, and species are presented and sorted by phylogenetic group. Sequences from single-celled red algae *Cyanidioschyzon merolae* and *Galdieria sulphuraria* that are homologous to SUTs but did not cluster with SUTs encoded by land plants are present in a separate group in **Figure 1** and **Table 1**. Additionally, the fungal sequence SpSUT1 from *Schizosaccharomyces pombe* is homologous (Reinders and Ward, 2001) but did not cluster with plant SUTs. The genome sequence from two chlorophyte green algae, *Chlamydomonas reinhardtii* and *Volvox carteri* is available, however no SUT sequences were identified in these chlorophytes. A single charophyte algal sequence from *Chlorokybus atmosphyticus* (CaSUT1) is present just basal to the plant SUT sequences but does not cluster with type I, II, or II (marked with an asterisk in **Figure 1**).

TYPE I SUTs

Type I SUTs were only found in eudicots. The 26 type I SUTs analyzed here cluster into a single group with an average of 69% (±5%) identity. The lack of type I SUTs in non-vascular land plants

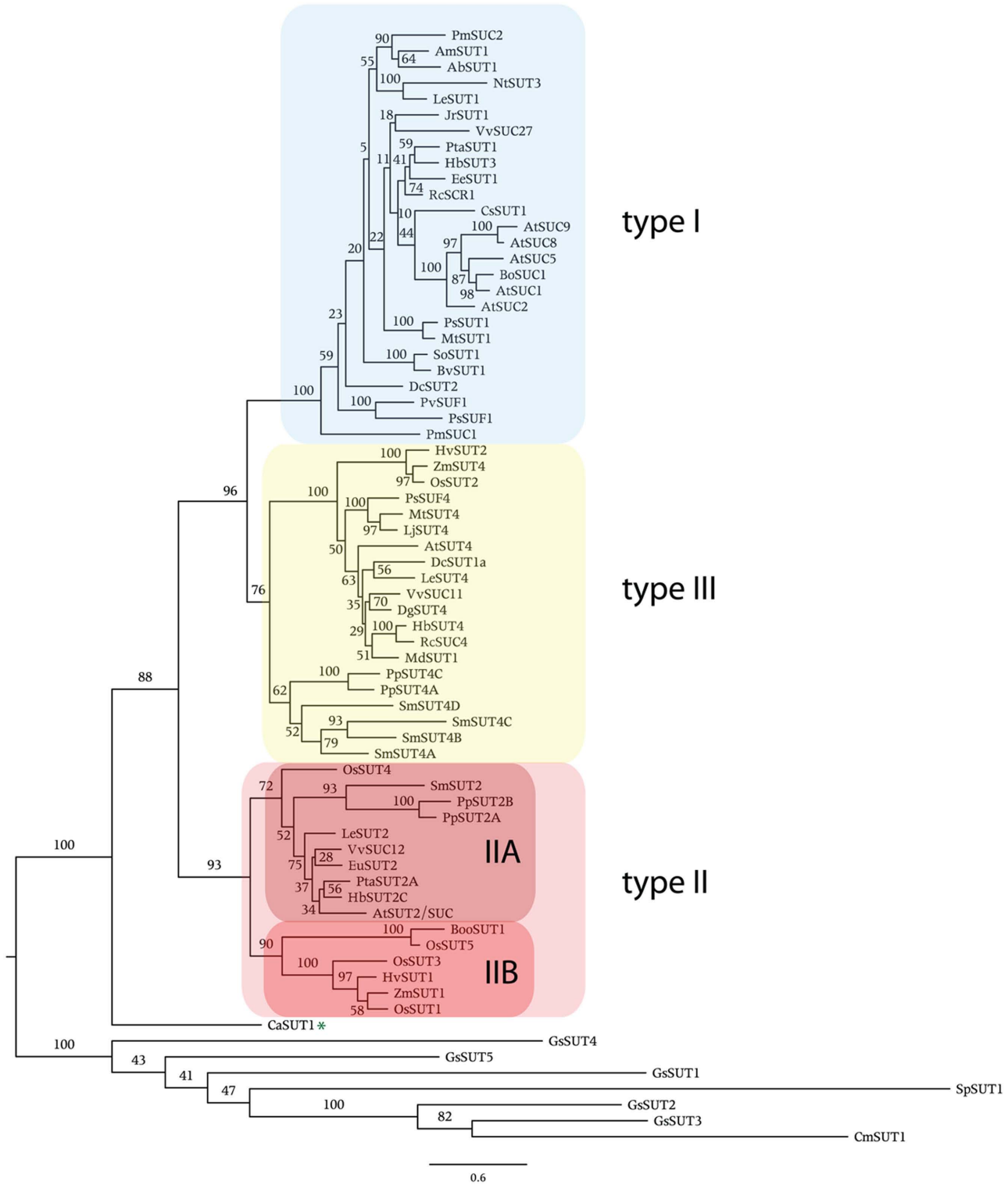

FIGURE 1 | Phylogenetic analysis of plant sucrose transporters and homologs. Protein alignment was done using Clustal X. Sequences with greater than 90% identity were not used in construction of the tree (they are shown in **Table 1**). The variable length N- and C-terminal regions were trimmed from the alignment. The maximum likelihood tree was generated using PhyML 3.0. Numbers indicate percent of 100 bootstrap analyses. Asterisk indicates the single charophyte SUT sequence, CaSUT1, from *Chlorokybus atmosphyticus*.

Table 1 | Sucrose transporter homologs.

Type	Organism	Common name	Gene	Prot ID	Length (aa)	Reference
I	*Alonsoa meridionalis*		AmSUT1	AAF04295	502	Knop et al. (2001)
I	*Arabidopsis thaliana*	Thale cress	AtSUC1 (At1g71880)	CAA53147	513	Sauer and Stolz (1994)
I	*Arabidopsis thaliana*	Thale cress	AtSUC2 (At1g22710)	CAA53150	512	Sauer and Stolz (1994)
I	*Arabidopsis thaliana*	Thale cress	AtSUC5 (At1g71890)	AAG52226	512	Theologis et al. (2000)
I	*Arabidopsis thaliana*	Thale cress	AtSUC8 (At2g14670)	AAC69375	492	Lin et al. (1999)
I	*Arabidopsis thaliana*	Thale cress	AtSUC9 (At5g06170)	BAB09682	491	Tabata et al. (2000)
I	*Asarina barclaiana* (*Maurandya barclaiana*)	Twining snapdragon	AsSUT1	AAF04294	510	Knop et al. (2001)
I	*Beta vulgaris*	Sugar beet	BvSUT1	CAA58730	523	Vaughn et al. (2002)
I	*Brassica oleracea*	Broccoli	BoSUC1	AAL58071	513	Gapper et al. (2005)
I	*Citrus sinensis*	Sweet orange	CsSUT1	AAM29150	528	Li et al. (2003)
I	*Daucus carota*	Carrot	DcSUT2	CAA76369	515	Shakya and Sturm (1998)
I	*Euphorbia esula*	Leafy spurge	EeSUT1	AAF65765	530	
I	*Hevea brasiliensis*	Para rubber tree	HbSUT3/ HbSUT1A	ABK60190	535	Tang et al. (2010)
I	*Juglans regia*	English walnut	JrSUT1	AAU11810	516	Decourteix et al. (2006)
I	*Solanum lycopersicum* (*Lycopersicon esculentum*)	Tomato	LeSUT1	CAA57726	512	Barker et al. (2000)
I	*Medicago truncatula*	Barrel medic	MtSUT1	TC175182, TC184317*	525	http://compbio.dfci.harvard.edu/tgi/
I	*Nicotiana tabacum*	Common tobacco	NtSUT3	AAD34610	521	Lemoine et al. (1999)
I	*Phaseolus vulgaris*	Common bean	PvSUF1	ABB30165	509	Zhou et al. (2007)
I	*Pisum sativum*	Pea	PsSUT1	AAD41024	524	Tegeder et al. (1999)
I	*Pisum sativum*	Pea	PsSUF1	ABB30163	511	Zhou et al. (2007)
I	*Plantago major*	Common plantain	PmSUC1	CAA59113	503	Gahrtz et al. (1996)
I	*Plantago major*	Common plantain	PmSUC2	CAA53390	510	Gahrtz et al. (1996)
I	*Populus trichocarpa*	Black poplar	PtaSUT1/ PtSUT1.2	18221401†	535	Tuskan et al. (2006)
I	*Ricinus communis*	Castor bean	RcSCR1	CAA83436	533	Weig and Komor (1996)
I	*Spinacia oleracea*	Spinach	SoSUT1	CAA47604	526	Riesmeier et al. (1992)
I	*Vitis vinifera*	Grape	VvSUC27	AAF08331	505	Davies et al. (1999)
IIA	*Arabidopsis thaliana*	Thale cress	AtSUT2/AtSUC3 (At2g02860)	CAB92307	595	Meyer et al. (2000), Schulze et al. (2000)
IIA	*Eucommia ulmoides*	Gutta-percha tree	EuSUT2	AAX49396	604	Pang et al. (2008)
IIA	*Hevea brasiliensis*	Para rubber tree	HbSUT2C/ HbSUT2A	CAM33449	539	Dusotoit-Coucaud et al. (2009)
IIA	*Oryza sativa japonica*	Rice	OsSUT4 (Os02g58080)	BAC67164	595	Aoki et al. (2003)
IIA	*Physcomitrella patens*		PpSUT2A	18051919†	635	Rensing et al. (2008)
IIA	*Physcomitrella patens*		PpSUT2B	18064412†	557	Rensing et al. (2008)
IIA	*Populus trichocarpa*	Black poplar	PtaSUT2A	18241865†	602	Tuskan et al. (2006)
IIA	*Selaginella moellendorffii*		SmSUT2	15412113†	521	Banks et al. (2011)
IIA	*Solanum lycopersicum* (*Lycopersicon esculentum*)	Tomato	LeSUT2	AAG12987	605	Barker et al. (2000)
IIA	*Vitis vinifera*	Grape	VvSUC12	AAF08330	612	Davies et al. (1999)

(Continued)

Table 1 | Continued

Type	Organism	Common name	Gene	Prot ID	Length (aa)	Reference
IIB	*Bambusa oldhamii* (*Dendrocalamopsis oldhamii*)	Bamboo	BooSUT1	AAY43226	525	
IIB	*Hordeum vulgare*	Barley	HvSUT1	CAB75882 CAJ20123	523	Weschke et al. (2000), Sivitz et al. (2005)
IIB	*Oryza sativa japonica*	Rice	OsSUT1 (Os03g07480)	BAA24071	537	Hirose et al. (1997)
IIB	*Oryza sativa japonica*	Rice	OsSUT3 (Os10g26740)	BAB68368	506	Aoki et al. (2003)
IIB	*Oryza sativa japonica*	Rice	OsSUT5 (Os02g36700)	BAC67165	535	Aoki et al. (2003)
IIB	*Saccharum hybrid cultivar*	Sugarcane	ShSUT1[#]	AAV41028	517	Rae et al. (2005)
IIB	*Zea mays*	Corn	ZmSUT1	BAA83501	521	Aoki et al. (1999)
III	*Arabidopsis thaliana*	Thale cress	AtSUT4 (At1g09960)	AAL59915	510	Weise et al. (2000)
III	*Datisca glomerata*	Durango root	DgSUT4	CAG70682	498	Schubert et al. (2010)
III	*Daucus carota*	Carrot	DcSUT1a	CAA76367	501	Shakya and Sturm (1998)
III	*Hevea brasiliensis*	Para rubber tree	HbSUT4/ HbSUT4A	ABK60191	498	Tang et al. (2010)
III	*Hordeum vulgare*	Barley	HvSUT2	CAB75881	506	Weschke et al. (2000)
III	*Lotus japonicus*		LjSUT4	CAD61275	511	Flemetakis et al. (2003)
III	*Malus x domestica*	Apple	MdSUT1	AAR17700	499	Fan et al. (2009)
III	*Medicago truncatula*	Barrel medic	MtSUT4	17466537[†]	504	
III	*Oryza sativa japonica*	Rice	OsSUT2 (Os12g44380)	BAC67163	501	Aoki et al. (2003)
III	*Physcomitrella patens*		PpSUT4A	18040351[†]	532	Rensing et al. (2008)
III	*Physcomitrella patens*		PpSUT4B[#]	18037160[†]	500	Rensing et al. (2008)
III	*Physcomitrella patens*		PpSUT4C	18053343[†]	524	Rensing et al. (2008)
III	*Pisum sativum*	Pea	PsSUF4	ABB30162	507	Zhou et al. (2007)
III	*Ricinus communis*	Castor bean	RcSUC4	AAU21439	509	
III	*Selaginella moellendorffii*		SmSUT4A	15419655[†]	514	Banks et al. (2011)
III	*Selaginella moellendorffii*		SmSUT4B	15407332[†]	492	Banks et al. (2011)
III	*Selaginella moellendorffii*		SmSUT4C	15417411[†]	493	Banks et al. (2011)
III	*Selaginella moellendorffii*		SmSUT4D	15402611[†]	531	Banks et al. (2011)
III	*Solanum lycopersicum* (*Lycopersicon esculentum*)	Tomato	LeSUT4	AAG09270	501	Weise et al. (2000)
III	*Vitis vinifera*	Grape	VvSUC11	AAF08329	501	Davies et al. (1999)
III	*Zea mays*	Corn	ZmSUT4	AAT35810	501	
	Chlorokybus atmosphyticus	Soil alga	CaSUT1			
	Cyanidioschyzon merolae		CmSUT1	CMO328C[‡]	502	Matsuzaki et al. (2004)
	Galdieria sulphuraria		GsSUT1	Gs18190[§]	471	Weber et al. (2004), Barbier et al. (2005)
	Galdieria sulphuraria		GsSUT2	Gs34550[§]	546	Weber et al. (2004), Barbier et al. (2005)
	Galdieria sulphuraria		GsSUT3	Gs56570[§]	430	Weber et al. (2004), Barbier et al. (2005)
	Galdieria sulphuraria		GsSUT4	Gs29860[§]	526	Weber et al. (2004), Barbier et al. (2005)
	Galdieria sulphuraria		GsSUT5	Gs08920[§]	638	Weber et al. (2004), Barbier et al. (2005)
	Schizosaccharomyces pombe	Fission yeast	SpSUT1	NP594387	553	Reinders and Ward (2001)

**sequence from DFCI (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago).*

[†] sequence from Phytozome v7.0 (http://www.phytozome.net/).

[#] not included in the phylogenetic analysis (>90% identical to another SUT).

[‡] sequence from Cyanidioschyzon merolae genome project (http://merolae.biol.s.u-tokyo.ac.jp/).

[§] sequence from Galdieria sulphuraria genome project (http://genomics.msu.edu/galdieria/index.html).

(*Physcomitrella*), vascular non-seed land plants (*Selaginella*), and the monocot branch of angiosperms indicates that development of type I SUTs occurred after divergence of monocots and eudicots, around 150 MYR ago (Laroche et al., 1995). Genome sequence representing early diverging eudicots such as *Papaver* sp. (poppy) and *Ranunculus* sp. (buttercup; Soltis et al., 2003) would be useful to more clearly determine the origins of type I SUTs. It is interesting that type I SUT genes were amplified in *Arabidopsis* and acquired specialized functions. *Arabidopsis thaliana* has five genes in this group (**Figure 1**; **Table 1**) and an additional two that have been identified as pseudogenes (Sauer et al., 2004) that were not included in the analysis.

In *Arabidopsis thaliana*, type I SUTs display specialization in both expression and transport function. AtSUC2 is necessary for loading sucrose into the phloem (Gottwald et al., 2000). It has a K_m (affinity) for sucrose of 1.4 mM (Chandran et al., 2003) and a wide substrate specificity for α and β glucosides that is shared with other type I SUTs (**Figure 2**; Chandran et al., 2003). AtSUC1 transport activity is very similar to AtSUC2 but its expression pattern is quite different. AtSUC1 is expressed in trichomes, pollen and roots (Sivitz et al., 2007). AtSUC1 is necessary for normal pollen function (Sivitz et al., 2008). Expression of AtSUC1 in the phloem, under control of the AtSUC2 promoter, has been shown to revert the growth defects of *atsuc2* mutants (Wippel and Sauer, 2011). There are also examples of type I SUTs with modified transport activity. AtSUC9 has a much higher affinity for sucrose compared to other type I SUTs (66 μM; Sivitz et al., 2007) while the substrate specificity is typical of other type I SUTs (**Figure 2**; Sivitz et al., 2007).

TYPE II SUTs

Type II SUT sequences were identified in eudicots, monocots, non-vascular land plants (*Physcomitrella*), and vascular non-seed land plants (*Selaginella*). A total of 16 SUT sequences clustered in the type II group with an average of 62% (±9%) identity. The type II group was divided into two subgroups IIA and IIB. These two subgroups were identified previously (Braun and Slewinski, 2009). There is also a structural difference between type IIA and IIB SUTs. Type IIA proteins have a longer central cytoplasmic loop compared to type IIB SUTs. This is reflected in the average length of proteins in type IIA of 587 amino acids (aa) compared to 523 aa in type IIB (**Table 1**). Each angiosperm genome appears to have one gene in the IIA subgroup. Sequences from *Physcomitrella* (two) and *Selaginella* (one) are also included in the IIA subgroup. PpSUT2A and B from *Physcomitrella* and SmSUT2 contain longer central loops with conserved sequence characteristic of angiosperm type IIA transporters. Overall, this indicates that a type IIA transporter with a longer central loop was an ancestral form of the type II SUTs found in angiosperms.

The type IIB subgroup is monocot specific, rice encodes three type IIB transporters. This group contains the monocot phloem loading SUTs. ZmSUT1 has been shown to be expressed in vascular tissue and to function in phloem loading (Slewinski et al., 2009). Similar to the amplification of type I SUTs in *Arabidopsis*, type IIB SUTs appear to have been amplified in rice. Transport activities of OsSUT1 and OsSUT5 were analyzed by expression in oocytes and electrophysiology. OsSUT5 was found to have a higher affinity for sucrose (2.3 mM) compared to OsSUT1 (7.5 mM) and the activity of OsSUT5 was found to be less pH dependent (Sun et al., 2010).

It is interesting to note that monocots and eudicots utilize different SUTs to load sucrose into the phloem. Differences in substrate specificity between type I SUTs such as AtSUC2 that transport sucrose into the phloem in eudicots and type II SUTs such as HvSUT1 that performs the same function in monocots have been identified (Chandran et al., 2003; Sivitz et al., 2005, 2007; Reinders et al., 2006, 2008; Sun et al., 2008). **Figure 2** shows a summary of substrate specificity results for five sucrose transporters. AtSUC2 and AtSUC9 are both type I sucrose transporters and although AtSUC9 has approximately a 20-fold lower $K_{0.5}$ for sucrose (Sivitz et al., 2007) compared to AtSUC2, they have almost

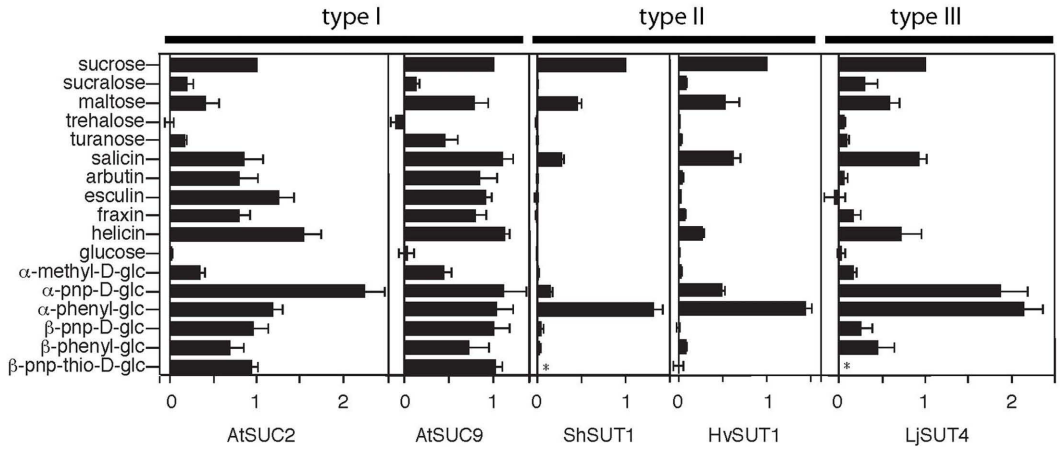

FIGURE 2 | Substrate specificities of type I (AtSUC2, AtSUC9), type II (ShSUT1, HvSUT1), and type III (LjSUT4) plant sucrose transporters. Transport activity was assayed by expression in *Xenopus* oocytes and two-electrode voltage clamping. Oocytes were bathed in sodium Ringer solution containing substrates at concentrations between 0.5 and 25 mM (depending on the transporter affinity and substrate solubility). All currents were normalized to sucrose-dependent currents and are presented as mean ± SE with at least three oocytes per mean. ∗Indicates substrate not tested. Modified with permission from Chandran et al. (2003), Sivitz et al. (2005, 2007), Reinders et al. (2006, 2008).

identical substrate specificities. These type I SUTs transport the plant β-glucosides salicin, arbutin, esculin, fraxin, and helicin. Notably, arbutin, esculin, and fraxin are not transported by the type II transporters ShSUT1 and HvSUT1 (**Figure 2**). Synthetic β phenyl glucosides are also transported by type I and not by type II SUTs (**Figure 2**).

The differences in substrate specificity between type I and type II SUTs might suggest that the specificity of phloem loading in eudicots is different from that in monocots. It is possible that type I SUTs load other glucosides, in addition to sucrose, into the phloem. To begin to address this question we used either AtSUC2 or HvSUT1 to complement the *Arabidopsis atsuc2-1* mutant (Gottwald et al., 2000). The homozygous *atsuc2-1* mutant has greatly reduced growth and accumulates starch in source leaves due to its reduced ability for phloem loading (**Figure 3A**). By comparison, growth of the *atsuc2-1* heterozygous plants is indistinguishable from wild-type (**Figures 3A,B**). As expected, the *atsuc2-1* mutant growth phenotype was complemented by expression of the AtSUC2 gene. Expression of the HvSUT1 coding region driven by the AtSUC2 promoter also resulted in growth that was indistinguishable from wild-type (**Figure 3B**). The type II SUT HvSUT1 appears to revert the growth reduction caused by the loss of AtSUC2 in *Arabidopsis*. This indicates that differences in substrate specificity between type I and II SUTs might not reflect a significant difference in physiological function, although this result is preliminary. Further work is necessary to determine if HvSUT1 fully complements under different growth and stress conditions.

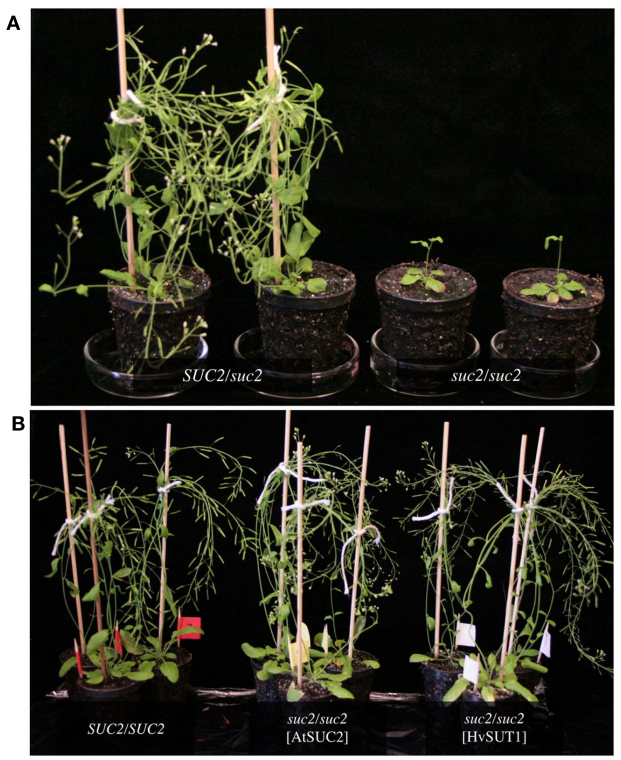

FIGURE 3 | Complementation of *suc2-1* sucrose transporter mutant. **(A)** *Arabidopsis* plants heterozygous for the *suc2-1* insertion left, plants homozygous for the *suc2-1* insertion right. **(B)** Both *AtSUC2* and *HvSUT1* complemented the growth defect of the homozygous *suc2-1* mutant. The WS wild-type (left) is shown for comparison. All plants shown in **(A)** and **(B)** are 8 weeks old.

Finally, the grouping of moss type II SUTs can give us a few more clues about the evolution and function of these ancestral type SUTs. The type II moss and spikemoss sequences cluster with type IIA and contain longer central loops. Both *Physcomitrella* and *Selaginella* lack type I and type IIB SUTs. If early vascular plants such as *Selaginella* have SUTs that function in phloem loading, those transporters are likely to be type IIA such as SmSUT2 and are different from those used by monocots and eudicots. Also, type IIA SUTs in angiosperms do not compensate for loss of the main phloem loading SUT as evidenced by mutant phenotypes of *atsuc2* (Gottwald et al., 2000) and *zmsut1* (Slewinski et al., 2009) mutants.

TYPE III SUTs

The first type III SUTs were isolated from *Arabidopsis*, tomato, potato, and barley and named AtSUT4, LeSUT4, StSUT4, and HvSUT2, respectively (Weise et al., 2000; Weschke et al., 2000). AtSUT4 from *Arabidopsis* and HvSUT2 from barley (Endler et al., 2006), LjSUT4 from *Lotus japonicus* (Reinders et al., 2008), and OsSUT2 from rice (Eom et al., 2011) were demonstrated to localize to the vacuole membrane. Twenty type III SUT sequences were included in this study (**Figure 1**; **Table 1**) and these have an average of 65% (±8%) identity. Each angiosperm genome appears to contain a single type III SUT gene. Both *Selaginella* and *Physcomitrella* contain multiple type III SUT genes. No type III SUT homologs have been identified in green algae.

Transport activity has been characterized in detail for type III SUT LjSUT4 (Reinders et al., 2008). The substrate specificity of LjSUT4 is intermediate between type I and II SUTs (**Figure 2**). Like other type III SUTs (Weise et al., 2000; Weschke et al., 2000) LjSUT4 functions as a H^+-coupled sucrose-uptake transporter. This indicates that its physiological function in the vacuolar membrane is sucrose-uptake into the cytoplasm from the vacuolar lumen. This activity for AtSUT4 has been demonstrated in *Arabidopsis* vacuoles (Schulz et al., 2011).

SUTS IN *CHLOROKYBUS ATMOSPHYTICUS, GALDIERIA SULPHURARIA, CYANIDIOSCHYZON MEROLAE*, AND *SCHIZOSACCHAROMYCES POMBE*

No SUT sequences were found in chlorophytes *Chlamydomonas reinhardtii* and *Volvox carteri*. Charophyte green algae are considered to represent ancestors of land plants. A single SUT sequence was found in the charophyte *Chlorokybus atmosphyticus* (CaSUT1). It did not cluster with type I, II, or III SUTs from land plants but appears to be basal to these clades (**Figure 1**). Since a complete genome sequence of a charophyte is not yet available it remains to be determined whether additional SUTs are present in charophyte genomes. The central loop of CaSUT1 is not extended as in type IIA SUTs. Also, the N-terminal sequence for CaSUT1 is not available so we could not determine if the putative vacuole targeting sequence is present (see Discussion).

Galdieria sulphuraria and *Cyanidioschyzon merolae* are closely related, unicellular red microalgae. While *G. sulphuraria* can grow

on 27 different sugars and sugar alcohols (Gross and Schnarrenberger, 1995), *C. merolae* can not grow heterotrophically (Matsuzaki et al., 2004). Five SUT homologs were identified in *G. sulphuraria* (GsSUT1-5) and one, CmSUT1, was identified in the *C. merolae* genome (**Figure 1**; **Table 1**). This is consistent with the larger number of genes encoding transporters and enzymes involved in carbohydrate metabolism identified in *G. sulphuraria* compared to *C. merolae* (Barbier et al., 2005).

DISCUSSION

THE ORIGIN OF PLANT SUTs IN CHAROPHYTE ALGAE

SUTs function as H^+-coupled cellular sucrose uptake transporters. In angiosperms, type I and II SUTs are localized to the plasma membrane while type III SUTs are localized to the vacuole membrane. They are important for the long-distance transport of sucrose in apoplastic phloem loaders (requiring transmembrane transport). Another important function for SUTs in angiosperms is in sucrose-uptake into sinks that are symplastically isolated such as seeds and pollen. The availability of bryophyte (non-vascular), lycophyte (early vascular), and algal genome sequences allows us to begin to analyze the origins of SUTs in land plants. The presence of CaSUT1 in the charophyte alga *Chlorokybus atmosphyticus* as well as the absence of SUTs in chlorophyte algae (*Chlamydomonas reinhardtii* and *Volvox carterii*) is consistent with the hypothesis that charophyte algae are ancestral to land plants (McCourt et al., 2004).

The physiological function of SUT homologs in *Chlorokybus*, which exists as small clusters of cells and in the unicellular red algae *Galdieria* and *Cyanidioschyzon* is currently unknown but will depend on their membrane localization. They are likely to function as H^+-coupled symporters for glucoside uptake into the cytosol whether they are localized to the plasma membrane or an internal membrane. Interestingly, *Cyanidioschyzon* lacks a central vacuole (Barbier et al., 2005), so it is more likely that CmSUT1 is a plasma membrane transporter. Bryophytes lack true vascular tissue yet *Physcomitrella* contains both type IIA and type III SUTs. In angiosperms, type IIA SUTs are localized to the plasma membrane (Barker et al., 2000; Meyer et al., 2000) while type III SUTs are vacuolar (Endler et al., 2006; Reinders et al., 2008). Therefore, it is likely that *Physcomitrella* contains both plasma membrane and vacuolar SUTs but this will need to be determined experimentally. Long-distance transport of photosynthate in mosses involves leptoid cells and the mechanism appears to be symplasmic, involving plasmodesmata not transmembrane transport (Raven, 2003). Therefore, if SUTs are localized to the plasma membrane in bryophytes their function is not in phloem loading but may be involved in recovery of sucrose that is released to the apoplast. Although leptoid cells evolved independently of phloem, many groups of angiosperms that utilize a similar passive mechanism for phloem loading (Rennie and Turgeon, 2009) also encode SUTs. The function of type III SUTs in bryophytes is likely to be the same as in angiosperms. Sucrose is transiently stored in the vacuole in angiosperms and type III SUTs function in the vacuole membrane to return sucrose from the vacuole lumen to the cytoplasm (Reinders et al., 2008; Schulz et al., 2011). The more recent development of type I SUTs in eudicots and type IIB SUTs in monocots is likely to be linked to the evolution of active phloem loading requiring energy and transmembrane transport.

PUTATIVE VACUOLAR TARGETING MOTIF IN TYPE III SUTs

Recently, a dileucine-like motif (LXXXLL) in the N-terminal cytoplasmic domain of the *Arabidopsis* monosaccharide transporter ESL1was shown to be necessary for localization of the transporter to the vacuole membrane (Yamada et al., 2010). Dileucine-like motifs are recognized by a clathrin-associated, heterotetrameric adaptor protein (AP-3) complex and function in sorting of vacuole membrane proteins in yeast (Vowels and Payne, 1998). Similar dileucine motifs contain an acidic residue spaced several residues prior to the leucine pair with a consensus of DXXLL or [DE]XXXL[LI] (Braulke and Bonifacino, 2009). The AP-3 complex has been shown to be necessary for normal vacuole function in *Arabidopsis* (Zwiewka et al., 2011). An LXXLL motif is found in the cytoplasmic N-terminus of type III SUTs (**Figure 4**) but is lacking in type I and II SUTs. All of the angiosperm type III SUTs contain a perfect LXXLL motif with the exception of AtSUT4 that has the sequence KRVLL (**Figure 4**). AtSUT4 has been demonstrated to localize to the vacuole membrane (Endler et al., 2006) so it is likely that the first leucine of the motif is not strictly required. Recently, localization of AtSUT4 to the vacuole membrane in *Arabidopsis* was shown to be dependent on AP-3 (Wolfenstetter et al., 2012). None of the *Physcomitrella* or *Selaginella* type III SUTs contain a

Type	Name	Sequence
I	AtSUC1	28SPLRKIISVASIAAGV43
	PsSUT1	33SPLRKIMVVASIAAGV48
II	AtSUT2/SUC3	58CSLVTLVLSCTVAAGV73
	OsSUT1	47ISLGRLILSGMVAGGV62
III	SmSUT4A	15VP**L**RS**L**ARVACVAAGV30
	SmSUT4B	15VP**L**KA**L**ARVASVAAGV30
	SmSUT4C	22VP**L**RG**L**ARVASVALGV37
	PpSUT4A	12VPIRA**L**IQVASVAAGV27
	PpSUT4C	12VPIRA**L**IQVASVAAGV27
	SmSUT4D	26IRQRQ**L**FRVSSVAAGI41
	DcSUT1a	25VS**L**RL**LL**RVASVACGI40
	LeSUT4	24VP**L**RL**LL**RVASVAGGI39
	DgSUT4	21VS**L**RK**LL**RVSSVACGI36
	MdSUT1	21VP**L**RQ**LL**RVASVACGI36
	VvSUC11	25VP**L**RR**LL**RVASVACGI40
	RcSUC4	32VS**L**RK**LL**RVTSIAGGI47
	HbSUT4	21VP**L**RQ**LL**RVTSVAGGI36
	AtSUT4	38VSKRV**LL**RVASVACGI53
	LjSUT4	36VP**L**RQ**LL**RVASVASGI51
	MtSUT4	33TP**L**RQ**LL**RVASVASGI48
	PsSUF4	32VP**L**TK**LL**RVASVAGGI47
	OsSUT2	22VP**L**RK**LL**RAASVACGV37
	ZmSUT4	17VP**L**RK**LL**RAASVACGV32
	HvSUT2	25VP**L**RS**LL**RAASVACGV40
	Motif	**..LXXLL.....**

FIGURE 4 | Putative dileucine-like vacuolar targeting sequence in type III SUTs. A part of the multiple protein alignment of sucrose transporters is shown. All type III SUTs and selected type I and II SUTs are shown for comparison. Numbers indicate the amino acid positions for each protein. Amino acid positions that conform to the dileucine-like motif LXXLL are shown in bold.

complete LXXLL motif and it is unknown whether they localize to the vacuole membrane.

THE ORIGIN OF TYPE I SUTs

Type I SUTs are localized to the plasma membrane in eudicots. Based on phylogeny (**Figure 1**) and substrate specificity (**Figure 2**) they are more similar to type III SUTs than to type II SUTs. Since type III SUTs are present in bryophytes and lycophytes, we suggest that type I SUTs are derived from vacuolar-type III SUTs. This would likely involve mutation of the vacuolar targeting information resulting in localization to the plasma membrane, the default targeting pathway for membrane proteins in plants. We hypothesize that the LXXLL motif found in type III SUTs serves as the vacuolar targeting domain but this needs to be tested directly.

CONCLUSION

Angiosperm SUTs clustered into three groups, type I, II, and III. Type I SUTs, only found in eudicots appear to have evolved from vacuolar-type III SUTs which were found in all land plants from bryophytes to angiosperms. Type II SUTs were divided into an ancestral form, type IIA, that exist in all land plants and have an extended central loop. Type IIB SUTs only exist in monocots and include the phloem loading transporters in those species. Here we identify an algal SUT (CaSUT1) from the charophyte *Chlorokybus atmosphyticus*. Based on phylogenetic analysis, CaSUT1 appears basal to three types of land plant SUTs and this is consistent with the hypothesis that charophytes are ancestral to land plants.

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