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Identification, characterization and expression analysis of sucrose transporters in the model plant *Nicotiana tabacum* cv. petit havana

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Abstract

Aim: Sucrose transporters (SUTs) are transmembrane proteins involved in transport of sucrose along the phloem pathway of plants. *Nicotiana tabacum*, a well-known model plant has been reported to have three sucrose transporters (*NtSUT1*, *NtSUT3* and *NtSUT4*), till date. The present study was undertaken to identify new SUTs from its genome and characterize all the *NtSUT* genes of *Nicotiana tabacum*.

Methodology: In the present study, new putative sucrose transporter genes were identified through extensive searches of the draft genome and transcriptome of tobacco using BLAST. *In-silico* characterization of the *SUT* gene family (*NtSUT1*, *NtSUT2*, *NtSUT3*, *NtSUT4* and *NtSUT5*) of *Nicotiana tabacum* was done using bioinformatics tools. Spatial expression analysis was done for *NtSUT* genes in five different tissues of *Nicotiana tabacum* cv. petit havana using semi-quantitative RT-PCR.

Results: Two new putative sucrose transporters *NtSUT2* and *NtSUT5* were identified from the publicly available tobacco transcriptome data and characterized using *in-silico* tools. Multiple sequence alignment of deduced amino acids of all the five SUTs revealed the presence of highly conserved domains and signature histidine residue in the sugar binding motif. Phylogenetic analysis grouped *NtSUT1* and 3 into group 2, *NtSUT4* and 5 into group 4 and *NtSUT2* into group 3. *NtSUT1* showed higher expression in leaves and flower while *NtSUT2*, 3 and 4 were abundant in root and flower. *NtSUT5* showed a lower expression in all the tissues.

Interpretation: Based on the sequence information and structural similarities, it is clear that the two newly identified putative *SUT* genes (*NtSUT2* and *NtSUT5*) belonged to the *SUT* family of tobacco. This comprehensive study provides a consolidated data on characteristic features of *SUT* family proteins of tobacco.

Key words: Expression analysis, *Nicotiana tabacum* cv. petit havana, Phylogenetic classification, Sucrose transporters

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Introduction

Sugars represent the main energy source in plants. Sucrose is the primary sugar transported from the source (leaves) to the sink (tissues) like young leaves, roots, flowers and seeds through phloem. This transport can be symplastic or apoplastic. Apoplastic transport is mediated by specific membrane proteins, called sucrose transporters (SUTs) which controls sucrose loading into the sieve element-companion cell complex (Zhang *et al.*, 2019). Plant sucrose transporters belong to glycoside pentoside hexuronide (GPH) cation symporter family and a part of major facilitator superfamily. Almost all SUTs have been characterized as sucrose/ H^+ symporters, except a few facilitators (SUFs) which catalyse bidirectional sucrose transport in a pH and energy independent manner (Kühn and Grof, 2010). SUTs are highly hydrophobic proteins with 12 transmembrane helices possessing approximately 500-600 amino acids with an exception of few dicot members that contain an elongated central loop with approximately 60 additional amino acids. SUTs are either located in the plasma membrane of sieve elements or companion cells or in the vacuolar membrane. *SUT* family members, mainly involved in phloem loading are mostly localized in the plasma membrane whereas the vacuolar SUTs are reported to function as sucrose proton symporters by effluxing sucrose from the vacuoles (Hennion *et al.*, 2019).

SUTs are classified into five groups based on their sequences and structural similarities. Group 1 consists of plasma membrane localized monocot SUTs which have moderate affinity for sucrose and catalyses the sucrose uptake into sink tissues. Group 2, being the largest and well-studied group, consists of plasma membrane localized dicot SUTs, which have high affinity for sucrose, involved in phloem loading and these were the first identified SUTs. Group 2 members have a wide range of substrate specificity than group 1. Group 3 consists of both monocots and dicots, most of the members are easily distinguishable from other groups with an extended cytoplasmic loop possessing 15-20% more amino acids and they are proven to possess high affinity for sucrose. Also, studies suggest that some of the members of this group can act as potential sucrose sensors and not as symporters. Group 4 consists of both monocot and dicot SUTs with 10 fold lower affinity for sucrose than group 2. They transport a wide range of substrates but are more specific than group 2. Some of the members under group 4 have been found to be localized on the vacuolar membrane and are involved in the efflux of sucrose. Group 5 is made exclusively of functionally uncharacterized monocot SUTs and they are different from group 1 (Braun and Slewinski, 2009). The only *SUT* which has been characterized from this group is *OsSUT5*, which has high affinity for sucrose and other substrates than group 1 and that explains the separation from group 1 (Sun *et al.*, 2009).

Besides phloem loading, SUTs appears to influence various other processes such as pollen tube growth, retrieval of sucrose along the phloem pathway, flowering and photoperiod dependent induction of tuberization. Since SUTs are essential

proteins in carbon partitioning, their transport mechanisms and metabolism are highly regulated at transcriptional and post transcriptional levels. *SUT* genes are proven to have distinct expression patterns during different developmental stages (Xu *et al.*, 2018). The spatio-temporal expression of many *SUT* genes of various plant species has already been well documented. For instance, in rice, *OsSUT1* shows higher expression in germinating seeds, source leaf sheaths, panicles and developing grains and at lower levels in roots. *OsSUT3* and *OsSUT5* depicts highest expression in sink leaves and low expression in germinating seeds. *OsSUT4* expresses only in sink leaves and *OsSUT2* shows almost equal level of expression in all the tissues (Aoki *et al.*, 2003). Similarly, in tomato, *LeSUT4* expression was higher in young sink leaves, stems, cotyledons, and immature fruits. *LeSUT1* was expressed more in source leaves with lower expression in all other organs. *LeSUT2* expression was low in general but relatively higher in sink leaves and stems (Barker *et al.*, 2000). In addition to organ and tissue specificity, the expression pattern of SUTs vary under nutrient deficit conditions, stress conditions, and at different developmental stages (Shiratake, 2007).

The availability of whole genome information of various plant species has led to the identification and characterization of SUTs in various plant species so far such as *Oryza sativa*, *Solanum lycopersicum*, *Arabidopsis thaliana*, and so on. (Sauer, 2007). So far, there are three *SUT* genes which are reported and characterized for the most widely used model plant and an important cash crop, *Nicotiana tabacum* (tobacco). However, only cDNA information, kinetic properties and expression data of *NtSUT1*, 3 and 4 are available (Bürkle *et al.*, 1998; Lemoine *et al.*, 1999; Okubo-Kurihara *et al.*, 2011). Hence, the present study intended to identify other SUTs from the recently released whole genome information of *Nicotiana tabacum* and to analyse the gene structure, physio-chemical properties, presence of functionally important domains and conserved motifs, and presence of cis-acting elements in the upstream regions of all the *NtSUT* genes, besides documenting spatial expression pattern of *NtSUTs* in the cultivar *Nicotiana tabacum* cv. petit havana.

Materials and Methods

Identification of *SUT* genes in tobacco: Whole genome and transcriptome of three *Nicotiana tabacum* varieties (Basma Xanthi, TN 90 and K326) were carried out recently (Sierro *et al.*, 2014). Transcriptome wide analysis was performed to identify sucrose transporter gene families using stand-alone version of BLASTn and BLASTp with more than 50% homology as cut off. *NtSUT1*, *NTSUT3* and *NtSUT4* cDNA sequences available in the NCBI database were used as query sequences. Amino acid sequences were deduced using NCBI ORF finder. Redundant sequences were removed after aligning the amino acid sequences using CLUSTAL Omega. The cDNA sequences of non-redundant proteins were used as queries to search for their appropriate contiguous gene sequences (contigs) from tobacco genome using BLASTn. Genomic contigs with lower e-values for

SUT genes were retrieved and aligned manually with cDNA sequences for locating the introns. Schematic representation of exon-intron structure was obtained using Exon-Intron graphic maker tool.

In-silico analysis of NtSUT proteins: Physio-chemical properties of five sucrose transporter proteins including two new putative *SUT* proteins of tobacco identified in this study, were predicted using PROTPARAM (Gasteiger *et al.*, 2005). Subcellular localization of these *SUT* proteins was analysed using CELLO server (Yu *et al.*, 2006). Transmembrane helical domains of these proteins were predicted using OCTOPUS (Viklund and Elofsson, 2008). Amino acid sequences of SUTs of various plant species were aligned with SUTs of tobacco using tools CLUSTAL Omega and PRALINE (Bawono and Heringa, 2014) to ascertain the presence of highly conserved motifs among sugar transporters.

Phylogenetic analysis of NtSUTs: Amino acid sequences of sucrose transporters including the newly identified putative sucrose transporter sequences were aligned with those of different plant species using CLUSTAL Omega. Phylogenetic tree was constructed based on alignments using MEGA7 software (Kumar *et al.*, 2016) by maximum likelihood method. To find the groups of identified NtSUTs, the tree was tested using bootstrap analysis with 1000 replicates.

Prediction of cis-regulatory elements using bioinformatics tools: Upstream 1500bp sequence of transcriptional start site of each gene were searched using PLANTCARE database to identify the cis-regulatory elements of NtSUT1-5 genes. Moreover, manual scanning was also done to identify the presence of sugar responsive elements such as sucrose box (NNAATCA) (Chen *et al.*, 2002; Fillion *et al.*, 1999) and pyrimidine box (CCTTTT, TTTTTC) (Washio, 2003).

Plant growth conditions and isolation of total RNA: *N. tabacum* cv. petit havana seeds (obtained from Tamil Nadu Agricultural University, Coimbatore, India) were thoroughly

washed with sterile distilled water and surface sterilized with sodium hypochlorite (2%). The seeds were sown in autoclaved soil and germinated in a plant growth Chamber at 28 °C in 16 hr light and 8 hr dark cycle. Mature leaves, young leaves, stems, roots, and flowers harvested from 6 week-old-plants were cryopreserved. Total RNA was isolated from 100 mg of all the tissues using TRIzol reagent (Thermo scientific, USA) following manufacturer's protocol. The samples were treated with DNase (NEB, England) and the purity and concentration was quantified using Nanodrop spectrophotometer (Thermo scientific, USA). Samples were checked on 1.5% MOPS-formaldehyde gel and stored at -80 °C.

Reverse transcription PCR (RT-PCR): From the quantified RNA samples, 1 µg from each sample was reverse transcribed to cDNA using M-MuLV enzyme (NEB, England) and oligo dT primer. Enzyme, primers and RNA were incubated at 37 °C for 50 min and the reaction was stopped at 70 °C for 15 min. Second strand cDNA was synthesised using gene specific forward and reverse primers and the reaction was normalized with ubiquitin gene as endogenous control. PCR conditions were as follows: initial denaturation at 94 °C, followed by 35 cycles for 30 sec at 94 °C, 30 sec at 54 to 61 °C (for different genes), 25 sec at 72 °C and a final extension of 3 min at 72 °C. The products were analysed on 1.5 % agarose gel.

Results and Discussion

Tobacco is an allotetraploid and a model plant widely used for basic and genetic engineering research in plants. From the transcriptome of tobacco, 28 sequences homologous to *NtSUTs* were retrieved using the *SUT* sequences *NtSUT1*, *NtSUT3* and *NtSUT4* as queries. Of these, 2 non-redundant new sequences with mRNA IDs 116932 and 93499 were filtered. Based on the sequence homology with previously characterised SUTs of other plants, the two new putative *SUT* genes were named as *NtSUT2* and *NtSUT5*. Deduced amino acid sequence of *NtSUT2* showed 92% similarity with the well characterized LeSUT2 and StSUT2. And in the case of *NtSUT5*, it shared 81%

Table 1: Characteristics of NtSUTs

Gene name	Protein ID	Contig No.	No. of amino acids	No. of Exons	No. of introns	Mol. wt	pI	TMD*	Localization
<i>NtSUT1</i>	BAO47334	AWOJ01093695	507	5	4	54kDa	9.34	12	Plasma membrane
<i>NtSUT2</i>	XP_016433446.1	AYMY01357085	618	14	13	66kDa	6.94	12	Plasma membrane
<i>NtSUT3</i>	AAD34610	AYMY01226069	521	6	5	56kDa	9.10	12	Plasma membrane
<i>NtSUT4</i>	BAI60050	AWOK1098820	501	6	5	54kDa	8.75	12	Plasma membrane
<i>NtSUT5</i>	XP_016438053.1	AWOJ01044985	491	4	3	54kDa	8.70	11	Plasma membrane

*TMD-Transmembrane domain; *Mol.wt – Molecular weight; *pI- Isoelectric point; *kDa- kilo Daltons

Table 2: Classification of plant SUTs into groups

Name of the plant	No.of.SUTs	Sub groups of plant SUTs					References
		Group 1	Group 2	Group 3	Group 4	Group 5	
<i>Nicotiana tabacum</i>	5	-	<i>NtSUT3</i> , <i>NtSUT1</i>	<i>NtSUT2</i>	<i>NtSUT4</i> , <i>NtSUT5</i>	-	Sauer, 2007 Leach <i>et al.</i> , 2017
<i>Arabidopsis thaliana</i>	9	-	<i>AtSUC1</i> , <i>AtSUC2</i> , <i>AtSUC5</i> , <i>AtSUC6</i> , <i>AtSUC7</i> , <i>AtSUC8</i> , <i>AtSUC9</i>	<i>AtSUC3</i>	<i>AtSUC4</i>	-	Sauer, 2007
<i>Oryza sativa</i>	5	<i>OsSUT</i>	-	<i>OsSUT4</i>	<i>OsSUT2</i>	<i>OsSUT5</i>	Aoki <i>et al.</i> , 2003
<i>Zea mays</i>	3	<i>ZmSUT1</i>	-	<i>ZmSUT2</i>	<i>ZmSUT4</i>	-	Braun and Slewinski, 2009

Fig. 1: Exon/intron structure of *NtSUT* genes. Exons are shown in black boxes and introns are represented with grey lines.

similarity with *LeSUT4*, 71% with *AtSUC4* and 81% with *NtSUT4*, which has been already reported as group 4 transporters of tobacco (Leach *et al.*, 2017). However, there were few variations in the number of amino acids and transmembrane domains between *NtSUT4* and *NtSUT5*, which proclaims that *NtSUT5* could be a second member of the same group. Hence, to avoid confusion regarding nomenclature and to maintain consistency, it was designated as *NtSUT5*. Thus, *NtSUT1-5* formed the sucrose transporter gene family in tobacco. The percentage similarities among the *NtSUTs* are predicted in Table 1. So far, the gene structural information of *NtSUT1*, 3 and 4 genes has not been studied in detail. Thus in this study, alignment of all the five cDNA

sequences with their respective genomic contigs were done, which revealed the number and position of introns, exons, start and stop codons. There were 5 introns in *NtSUT3* and *NtSUT4*, 4 introns in *NtSUT1*, 3 introns in *NtSUT5* and 13 introns in *NtSUT2* (Fig. 1). Promoter analysis of five SUTs revealed the presence of sugar responsive element, A-box (TACGTA), which is involved in regulation of sucrose transporters upon addition of sucrose (Kühn, 2011; Osuna *et al.*, 2007). Sugar responsive elements such as sucrose box (NNAATCA) (Chen *et al.*, 2002; Fillion *et al.*, 1999) important for sugar responsive gene expression, pyrimidine box (CCTTTT) (Washio, 2003) partially involved in sugar repression were also observed.

NiSUT1	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	T	P	Y	V	Q	L	L	G	I	P	H	K	F	A	S	F	I	W	L	C	G	P	I	S	G	M	I	V	Q	P	V	V	G	Y	S	D	N	-	-	C	S	S	R	F	G	R	R	R			
NiSUT2	T	V	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	T	P	Y	I	Q	T	L	G	I	E	H	A	F	S	S	F	I	W	L	C	G	P	I	T	G	L	V	V	Q	P	C	V	G	I	W	S	D	K	-	-	C	H	S	K	Y	G	R	R	R		
NiSUT3	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	T	P	Y	L	Q	L	L	G	I	P	H	R	Y	A	S	I	I	W	L	C	G	P	I	S	G	M	I	V	Q	P	A	V	G	Y	L	S	D	N	-	-	L	S	T	F	G	R	R	R			
NiSUT4	S	V	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	W	A	S	I	I	W	L	C	G	P	L	S	G	L	L	V	Q	P	L	V	G	H	M	S	D	K	-	-	C	T	S	R	F	G	R	R	R		
NiSUT5	S	V	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	W	A	S	I	I	W	L	C	G	P	L	S	G	L	L	V	Q	P	L	V	G	H	M	S	D	K	-	-	C	T	S	R	F	G	R	R	R		
AtSUC1	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	I	P	H	K	W	S	S	I	I	W	L	C	G	P	V	S	G	M	I	V	Q	P	I	V	G	F	H	S	D	R	-	-	C	R	S	K	F	G	R	R	R	
AtSUC2	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	I	P	H	K	W	A	S	I	I	W	L	C	G	P	I	S	G	M	L	V	Q	P	I	V	G	Y	H	S	D	R	-	-	C	T	S	R	F	G	R	R	R	
AtSUC3	T	V	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	I	Q	T	L	G	I	P	H	A	F	S	S	F	I	W	L	C	G	P	I	T	G	L	V	V	Q	P	F	V	G	I	W	S	D	K	-	-	C	T	S	K	Y	G	R	R	R	
AtSUC4	S	V	A	C	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	W	A	S	V	I	W	L	C	G	P	L	S	G	L	F	V	Q	P	L	V	G	H	S	S	D	R	-	-	C	T	S	K	Y	G	R	R	R	
AtSUC5	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	I	Q	L	L	G	I	P	H	K	W	S	S	Y	M	W	L	C	G	P	I	S	G	M	I	V	Q	P	I	V	G	Y	H	S	D	R	-	-	C	E	S	R	F	G	R	R	R	
AtSUC6	S	I	A	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	V	P	H	K	W	S	S	F	I	W	L	C	G	P	V	S	G	L	L	V	Q	P	S	V	G	Y	F	S	D	R	-	-	C	K	S	R	F	G	R	R	R	
AtSUC7	S	I	A	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	V	P	H	K	W	P	S	F	I	W	L	C	G	P	V	S	G	L	L	V	Q	P	S	V	G	Y	F	S	D	R	-	-	C	T	S	R	F	G	R	R	R	
AtSUC8	S	I	A	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	V	P	H	K	W	S	S	F	I	W	L	C	G	P	V	S	G	L	L	V	Q	P	S	V	G	Y	F	S	D	R	-	-	C	T	S	R	F	G	R	R	R	
AtSUC9	S	I	A	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	V	P	H	K	W	S	S	F	I	W	L	C	G	P	I	S	G	L	L	V	Q	P	T	V	G	Y	F	S	D	R	-	-	C	K	S	R	F	G	R	R	R	
OsSUT1	M	V	A	G	G	V	Q	Y	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	T	L	G	L	S	H	A	L	T	S	F	M	W	L	C	G	P	I	A	G	M	V	V	Q	P	C	V	G	L	Y	S	D	R	-	-	C	T	S	K	W	R	R	R		
OsSUT2	S	V	A	C	G	V	Q	F	G	W	A	P	Q	L	S	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	F	A	S	L	V	W	L	C	G	P	L	S	G	L	L	V	Q	P	L	V	G	H	L	S	D	R	I	A	P	A	A	S	P	L	G	R	R	R
OsSUT3	M	V	A	G	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	T	L	G	I	P	H	A	L	T	S	V	M	W	L	C	G	P	I	A	G	L	I	V	Q	P	C	V	G	L	Y	S	D	K	-	-	C	T	S	S	L	G	R	R	R	
OsSUT4	M	V	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	I	Q	T	L	G	I	D	H	A	M	A	S	F	I	W	L	C	G	P	I	T	G	F	V	V	Q	P	C	V	G	W	S	D	K	-	-	C	R	S	K	Y	G	R	R	R		
OsSUT5	M	V	A	G	I	Q	Y	G	W	A	L	Q	L	S	L	L	L	L	T	P	Y	S	Q	T	L	G	I	S	H	S	Y	V	S	L	T	W	L	C	G	P	I	A	G	F	V	V	Q	P	I	V	G	Y	S	D	R	-	-	C	T	M	K	M	G	R	R	R		
ZmSUT1	M	V	A	G	G	V	Q	Y	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	T	L	G	L	S	H	A	L	T	S	F	M	W	L	C	G	P	I	A	G	L	V	V	Q	P	L	V	G	L	Y	S	D	R	-	-	C	T	A	R	W	G	R	R	R	
ZmSUT2	M	V	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	L	T	P	Y	I	Q	T	L	G	I	D	H	A	M	A	S	F	I	W	L	C	G	P	I	T	G	F	V	V	Q	P	C	V	G	V	W	S	D	K	-	-	C	R	S	K	Y	G	R	R	R	
ZmSUT4	S	V	A	C	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	F	A	S	L	V	W	L	C	G	P	L	S	G	L	L	V	Q	P	L	V	G	H	L	S	D	R	I	G	P	A	A	S	P	L	G	R	R	R
LeSUT1	S	I	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	I	P	H	R	F	A	S	F	I	W	L	C	G	P	I	S	G	M	I	V	Q	P	V	G	Y	S	D	N	-	-	C	S	S	R	F	G	R	R	R			
LeSUT2	T	V	A	A	G	V	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	I	Q	T	L	G	I	E	H	A	F	S	S	F	I	W	L	C	G	P	I	T	G	L	V	V	Q	P	C	V	G	I	W	S	D	K	-	-	C	H	S	K	Y	G	R	R	R	
LeSUT4	S	V	A	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	L	T	P	Y	V	Q	E	L	G	I	P	H	A	W	A	S	I	I	W	L	C	G	P	L	S	G	L	L	V	Q	P	L	V	G	H	M	S	D	K	-	-	C	T	S	R	F	G	R	R	R	
LjSUT4	S	V	A	S	G	I	Q	F	G	W	A	L	Q	L	S	L	L	L	T	P	Y	V	Q	L	L	G	I	P	H	Q	W	A	S	I	I	W	L	C	G	P	V	S	G	L	F	V	Q	P	L	V	G	H	L	S	D	K	-	-	C	T	S	R	F	G	R	R	R	

A.G1Q2

GWALQLSLLTPY1Q.LG1.H

WLCGP1

G

VQP

G

SD

GRRR

A.G1Q2 GWALQLSLLTPY1Q.LG1.H WL CGP1 G . . VQP . G . . SD GRRR

Fig. 2: Multiple sequence alignment of amino acids of sucrose transporters from various plant species. CLUSTAL omega alignment of amino acid sequences shows the conserved regions among plant SUTs. The highly conserved histidine residue (His-65) is shown underlined and the other motifs are highlighted in grey. RXGRRR, a consensus signature motif present in all plant SUTs is also shown.

Besides, various *cis*-acting elements associated with important physiological processes such as light responsiveness (ACE, Box I, Spl, and Box 4), stress responsiveness (MBS, HSE, LTR, TC-rich repeats (ATTCTCTAAC), and EIRE) hormone responsiveness (GARE, ABRE, ARE, TCA and Me-JA motif), tissue specific expression (GCN4 motif, CAT, as-2-box, Skn-1-motif), and metabolism (Ibraheem *et al.*, 2010) were also observed. These results propound that these sucrose transporter proteins might be playing role in regulating the photoperiodic control during flowering, regulating biotic and abiotic stresses.

Furthermore, to ascertain that the two newly identified putative sequences *NtSUT2* and *NtSUT5* belong to the *SUT* family of tobacco, they should have the characteristic features of plant *SUT* proteins. Also, previous studies have reported only the cDNA sequences for *NtSUT1*, *NtSUT3* and *NtSUT4* genes while their basic protein characteristics and their homology with other plant SUTs were not highlighted (Bürkle *et al.*, 1998; Lemoine *et al.*, 1999; Okubo-Kurihara *et al.*, 2011). Hence, in this study, a detailed prediction of the characteristic features of all five *NtSUT* proteins has been well documented using bioinformatics tools and tabulated in Table 1. *NtSUT2* is the largest of all SUTs (618 aa) and possess additional amino acids in its N-terminus and central cytoplasmic loop. It carries 40 amino acid extension at the N-terminus and an extended central cytoplasmic loop with insertion of 52 additional amino acids between transmembrane helices VI and VII, a feature distinct for group 3 transporters (*AtSUC3*, *LeSUT2*, and *PmSUC3*) (Sauer, 2007). *NtSUT4* and *NtSUT5* have shorter C-terminus, which is a proven feature of the members of group 4 sucrose transporters that usually have the shortest polypeptide sequences when compared to other family members (Sauer, 2007). The isoelectric point of all *NtSUT* proteins ranged from 8.70-9.30, except *NtSUT2* which was only 6.94, again a typical characteristic of group 3 transporters. All five proteins were predicted to be localized in the plasma membrane with 11 or 12 transmembrane domains. *NtSUT1*, *NtSUT2*, *NtSUT3* and *NtSUT4* possessed 12 transmembrane helical domains (TMD) whereas *NtSUT5* possessed only 11 transmembrane helical domains (Table 1).

Multiple sequence alignment of the amino acid sequences of *NtSUTs* with other known functional plant SUTs depicted the presence of consensus sequence pattern derived from the highly conserved region of plant SUTs: QFGWALQLSLLTPY (TMD I); IWLCGP (TMD II); RXGRRR (between TMD II and TMD III) domains (Fig. 2). Amino acid sequence alignment also revealed the presence of highly conserved histidine residue (His-65), a signature for the identification of plant sucrose transporters (Lu and Bush, 1998). Based on their sequence information, structure, function and other biochemical properties, SUT proteins of various plant species can be classified into 5 different groups (Braun and Slewinski, 2009). A phylogenetic classification was done for 35 SUT proteins including tobacco using their amino acid sequences. A rooted phylogenetic tree was constructed by

Fig. 3: Phylogenetic analysis of sucrose transporter family in tobacco. Maximum likelihood tree was generated by MEGA7 software with the amino acid sequences aligned using CLUSTAL omega. Following are the sequences used for the construction of phylogenetic tree: *NtSUT1* (X82276.1), *NtSUT3* (AF149981.1), *NtSUT4* (BA160050), *NtSUT2* (XP_016433446.1), *NtSUT5* (XP_016438053.1); *Arabidopsis thaliana* *AtSUC1* (At1g71880), *AtSUC2* (At1g22710), *AtSUC3* (At2g02860), *AtSUC4* (At1g09960), *AtSUC5* (At1g71890), *AtSUC6* (At5g43610), *AtSUC7* (At1g66570), *AtSUC8* (At2g14670), *AtSUC9* (At5g06170); *Oryza sativa* *OsSUT1* (Os03g0170900), *OsSUT2* (Os12g0641400), *OsSUT3* (Os10g0404500), *OsSUT4* (Os12g44380), *OsSUT5* (Os02g0576600); *Vitis vinifera* *VvSUC11/VvSUT1* (HQ323256), *VvSUC12* (HQ323257), *VvSUT27* (HQ323258), *Solanum tuberosum* *StSUT1* (CAA48915), *StSUT4* (AAG25923.2); *Lycopersicon esculentum* *LeSUT1* (X82275), *LeSUT2* (AF166498); *LeSUT4* (AF176950); *Lotus japonicas*, *LjSUT4* (CAD61275); *Hordeum vulgare* *HvSUT1* (CAB75882), *HvSUT2* (CAB75881); *Zea mays*, *ZmSUT1* (BAA83501), *ZmSUT2* (AAS91375), *ZmSUT4* (AAT51689); *Spinacea oleracea* *SoSUT1* (Q03411); *Schizosaccharomyces pombe*, *SpSUT1* was included as an outgroup.

Fig. 4: Expression profile of *NtSUTs* in tissues of *Nicotiana tabacum* cv. petit havana. 1 µg of DNase treated total RNA, isolated from mature and young leaves, stem, root and flower of tobacco plants were used for cDNA synthesis. Expression pattern was analysed using semi-quantitative reverse transcription PCR. Ubiquitin was used as endogenous control.

maximum likelihood method: bootstrap values with 1000 replicates using MEGA7.0 (Kumar *et al.*, 2016) to examine the phylogenetic relationship between the sucrose transporters of tobacco and various plant species (Fig. 3). As earlier reports on phylogenetic analysis of plant sucrose transporters suggests, the three previously reported SUTs of tobacco belong to 2 different groups: group 2 and group 4. *NtSUT1* fall under group 2 transporters as it is closely related to LeSUT1 by sharing 78% identity. *NtSUT3* is a second member of same group by sharing 67% sequence identity with *NtSUT1*. Similarly, *NtSUT4* fall into group 4 transporters depicting close relationship with LeSUT4. On these terms, among the two newly identified *NtSUT* genes in this study, *NtSUT5* becomes a second member of group 4 transporter by sharing 81% identity with LeSUT4 and *NtSUT4* whereas *NtSUT2* falls into group 3 transporters, showing close relationship with LeSUT2 by sharing 88% sequence identity.

These results depicts that the *SUT* gene family (5 genes) of tobacco belong to 3 different groups (Table 2).

Expression of plant sucrose transporters is proven to be differentially and developmentally regulated. In this study, the spatial expression pattern of *NtSUT* transcripts has been recorded using semi-quantitative reverse transcription PCR (RT-PCR) (Fig. 4). Expression of *NtSUT1* was high in the shoot tissues and flowers as compared to root. The transcript level was comparatively high in the source leaf tissues than stem. This observation is in concordance with the previous report that the expression of *NtSUT1* was low in the sink tissues and high in the source tissues (Bürkle *et al.*, 1998). However, in the present study, *NtSUT1* expression was high in the flower, which is a sink organ. Higher expression of *SUT1* in the sink tissues is attributed to its functions such as retrieval of sucrose along the translocation

and phloem unloading (Barker *et al.*, 2000). *SUT2* and *SUT4* type transporters are proven to be expressed high in sink leaves and stems than source leaves (Barker *et al.*, 2000). In a similar fashion, *NtSUT2* also showed high expression in the stem and root, reduced expression in the leaf tissues and showed no expression in the flower. Similarly, in the case of *NtSUT4*, the expression was moderate in the sink tissues such as stem and roots while there was no expression in the mature source leaves and young leaves which is concurrent with the previous reports on *SUT4* of various plant species (Weise *et al.*, 2000). However, in case of *NtSUT3*, which has already been proved as a pollen specific transporter (Lemoine *et al.*, 1999) shows high expression in the flower and a comparatively lower expression in the root tissue of *Nicotiana tabacum* cv. petit havana cultivar. There is hardly any expression seen in other tissues such as leaves and stem. However, the exact role of *NtSUT3* in the roots is yet to be elucidated. *NtSUT5* showed similar low level expression in all the tissues suggesting a role in sucrose uptake in all the tissues.

The amino acid sequence composition, sequence similarities, structural similarities, phylogenetic analysis, conserved domains and motifs of the two newly identified transporters (*NtSUT2* and *NtSUT5*) highly recommends them as putative sucrose transporter like proteins of tobacco. The present study, thus, propounds that there are five genes which comprises the SUT gene family in *Nicotiana tabacum*.

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