

**A study of functions of plasma membrane-localized
sucrose transporter in plant cells under growth and
aluminum stress conditions**

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Contents

Abbreviations	4
General introduction	5
Chapter I Sucrose transporters in cultured tobacco cell lines.....	7
Introduction.....	7
Materials and Methods.....	14
Results	19
Phylogenetic analysis of sucrose transporters in tobacco	19
Expression analysis of <i>NtSUT1</i> in SL and BY-2 cell lines	20
Subcellular localization of NtSUT1 in onion epidermal cells	23
Discussion.....	23
Chapter II The role of sucrose transporter <i>NtSUT1</i> in growth capacity under normal growth condition	26
Introduction.....	26
Materials and Methods.....	27
Results	32
Construction and expression analysis of over-expressor (OX) and suppressor (RNAi) lines of <i>NtSUT1</i> in BY-2 cells	32
Sucrose uptake rate in BY-2 cell lines (WT, OX, RNAi) during the logarithmic phase of growth in a modified MS medium.....	33
Relationship between soluble sugar content and mass doubling time among BY-2 cell lines (WT, OX, RNAi)	36
Discussion.....	36
Chapter III Effect of auxin (2,4-D) on the expression of <i>NtSUT1</i> and the role of <i>NtSUT1</i> in growth capacity under Al-stress condition	40
Introduction.....	40

Materials and Methods	41
Results	43
Expression analysis of <i>NtSUT1</i> in Al-treatment medium in the absence or presence of 2,4-D	43
Effect of Al on sucrose uptake and soluble sugar content in BY-2 cell lines (WT, OX, RNAi) in the absence or presence of 2,4-D	44
Sucrose uptake in BY-2 cell lines (WT, OX-2, RNAi) during post-Al treatment culture	49
Effect of Al on growth capacity in BY-2 cell lines (WT, OX, RNAi) in the absence or presence of 2,4-D	49
Effect of Al on fresh weight in BY-2 cell lines (WT, OX-2, RNAi-1)	51
Discussion	54
 Chapter IV Expression analysis of sucrose transporters <i>NtSUT3</i> and <i>NtSUT4</i> in BY-2 cells	57
Introduction	57
Materials and Methods	58
Results and Discussion	59
 Chapter V Construction of transgenic tobacco plants of <i>NtSUT1</i>	61
Introduction	61
Materials and Methods	61
Results and Discussion	64
 General discussion	71
 Summary	77
 Acknowledgements	80
 References	81

Abbreviations

Al	Aluminum
BY-2	Bright Yellow-2
CaMV	Cauliflower mosaic virus
CC-SE	Companion-Sieve elements
CLSM	Confocal laser scanning microscopy
2,4-D	2,4-Dichlorophenoxyacetic acid
EGTA	Ethylene glycol tetraacetic acid
FW	Fresh weight
IAA	Indole-3-acetic acid
MS	Murashige Skoog
MES-BTP	2-(<i>N</i> -morpholino) ethanesulfonic acid- <i>Bis-tris propane</i>
ORF	Open reading frame
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
SUT	Sucrose transporter
SUC	Sucrose carrier
WT	Wild type

General introduction

Acid soils are distributed over 30% of the Earth ice free land area, therefore, making the constraints for the production of agricultural crops (Schroeder et al., 2013) and establishment of forests (Godbold et al., 1988). Aluminum (Al) is the third most abundant element and builds 7% of the Earth's crust after oxygen and silicon. Al exists in soil in several forms, but the most toxic form at low pH is Al^{3+} which inhibits root growth and hence decreases yield production of agricultural crops (Kochian et al., 2004; Ma, 2007; Silva, 2012). Al ion causes phytotoxic syndromes in plants at low concentrations within micro molar range at physiological, biochemical and molecular levels.

Since Al ion preferentially binds to carboxyl and phosphate groups, pectin in the cell wall and phospholipids in the plasma membrane have been considered as primary targets of Al. The plasma membrane is the site where transporters for macro, micro nutrients and water are localized. Therefore, direct or indirect effect of Al on these transport proteins can affect nutrient status of plant. Al reduces cell wall extension and changes the polysaccharide components in the cell wall, which can be causes of root elongation inhibition (Chang et al., 1999; Schmohl and Horst, 2000; Ma et al., 2004). The reduction of H^+ -ATPase activity was reported (Kinraide et al., 1992; Ahn et al., 2001). Furthermore, Al can modify the uptake of other cations such as Mg^{2+} , K^+ , NH_4^+ as well (Jan 1991; Mariano and Keltjens, 2005). These changes in ion uptake through the plasma membrane have been proposed as a consequence of direct effect of Al (Pineros and Kochian, 2001). Al also can displace cations (e.g. Ca^{2+}) and cause depolarization of transmembrane potential (Akeson et al., 1989). Furthermore, cytoplasmic Ca^{2+} homeostasis can

be disrupted by Al (Rengel and Zhang, 2003). However, it is still not certain which symptom is primary or secondary cause of Al inhibition of root growth.

Besides the inhibition of uptake of cations as mentioned above, Al has been suggested to inhibit the uptake of sugar in different plant species or plant cultured cells (Graham, 2002; Abdalla, 2008; Abdel-Basset et al., 2010). Sucrose transporters have been well characterized and classified in monocots and dicot (Sauer, 2007; Lalonde and Frommer, 2012). Sucrose is synthesized in the chloroplast of mesophyll cells and diffuses via plasmodesmata into phloem parenchyma cells. Sweet proteins facilitate sucrose efflux into the cell wall and then sucrose transporters import sucrose into the Companion cells (CC)- Sieve elements (SE) complex. From SEs, sucrose is transported to sink tissues such as developing tissues, buds, stems, flowers, fruits and roots (Sauer, 2007; Braun, 2012).

Cultured tobacco cell lines (e.g. SL, BY-2) that have been well characterized (Nakamura et al., 1988; Nagata et al., 1992). They are heterotrophic and relatively homogeneous in nature and take all necessary nutrients including sugar from culture medium so that they resemble root system. In our laboratory, a working hypothesis for a mechanism of Al-induced cell elongation inhibition has been proposed as follows: Al inhibits sugar uptake into cells and therefore reduces driving force for water uptake into cell, thereby decreasing cell elongation (Abdel-Basset et al., 2010). Based on this hypothesis, the aim of this study was to examine the roles of the plasma membrane-localized sucrose transporter NtSUT1 in sugar uptake and growth capacity under normal and Al-stress conditions, using BY-2 and its *NtSUT1* transgenic lines. The contribution of NtSUT1 to Al tolerance mechanism is discussed.

Chapter I Sucrose transporters in cultured tobacco cell lines

Introduction

BY-2 cells are non chlorophyllous heterotrophic fast growing plant cells derived from tobacco (*Nicotiana tabacum*) cultivar Bright Yellow-2 (BY-2) (Nagata et al., 1992). The suspension culture system of BY-2 cells has been used as a model system of higher plant cell for physiological, biochemical and molecular studies, due to its high homogeneity and fast growth rate. Therefore, in this study, the role of sucrose transporter under Al stress was studied in this cell line.

Sucrose is non-reducing disaccharide and an energy metabolite which translocates from its site of synthesis by photosynthesis process in mature leaf mesophyll cell to other sink tissues including roots. Due to its non-reducing nature, it can be translocate to sink tissues without degradation. Sucrose can be transported to the sink tissues via sucrose/H⁺ symporters either by apoplastic or symplastic pathway (in angiosperms). Sucrose also acts as a long distance signaling molecule (Rolland et al., 2006), for induction of flowering (King and Ben-Tal, 2001; King, 2012) and for the control of root growth during early seedling development (Kircher and Schopfer, 2012), and interacts with endogenous carbon sensing pathway to trigger increased auxin flux and hypocotyl elongation (Lilley et al., 2012). The interplay between sucrose and folate modulates auxin signaling in *Arabidopsis* (Stokes et al., 2013).

Sucrose transporters (SUTs/SUCs) are sucrose/H⁺ symporters and the members of the glycoside-pentoside-hexuronide (GPH) family which are related to the major facilitator super family (Chang et al., 2004). Plant sucrose transporters are somewhat related to hexose

transporters in bacteria, fungi, plants and animals (Fukamachi et al., 2001; Reinders and Ward, 2001; Abramson et al., 2003) and the animal sucrose transporter (Meyer et al., 2011). In plants, the first sucrose transporter was cloned from spinach (*Spinacea oleracea*), using baker yeast functional expression system (Riesmeier et al., 1992). SUTs/SUCs consist of small gene families within all flowering plants, for example, in *Arabidopsis* there are nine SUCs (*Arabidopsis* Genome, 2000) and in rice five SUTs (Aoki et al., 2003) and five SUTs in tobacco (Lemoine et al., 1999; Schmitt et al., 2008; Okubo-Kurihara et al., 2011; Dominguez et al., 2013). Different classification of SUTs has been proposed by different researchers. SUTs have been classified into three types by Aoki et al. (2003), four groups by Sauer (2007), five clades by Kühn and Grof (2010). Recent classification of SUTs into five groups has been proposed by Braun and Slewinski (2009) and Ayre (2011). Therefore to avoid confusion, the most recent classification by Braun and Slewinski (2009) (Fig. 1) and Ayre (2011) was used in this paper and briefly described as follows:

Group 1 SUTs belong to monocot species which are involved in phloem transport where sucrose is transported into sink tissues through the apoplast (Aoki et al., 2003; Scofield et al., 2007; Braun and Slewinski, 2009; Slewinski et al., 2009). These SUTs seem to be localized in the plasma membrane and have moderate affinity for sucrose, with K_m values ranging from 580 μ M for LpSUT1 from *Lolium perenne* (Berthier et al., 2009), 2 mM and 8.26 mM for ShSUT1 from *Saccharum officinalis* (Rae et al., 2005; Reinders et al., 2006), 3.7 and 6.5 mM for ZmSUT1 from *Zea mays* (Carpaneto et al., 2005; Slewinski et al., 2010) and 7.5 mM for OsSUT1 from *Oryza sativa* (Scofield et al., 2007; Sun et al., 2010).

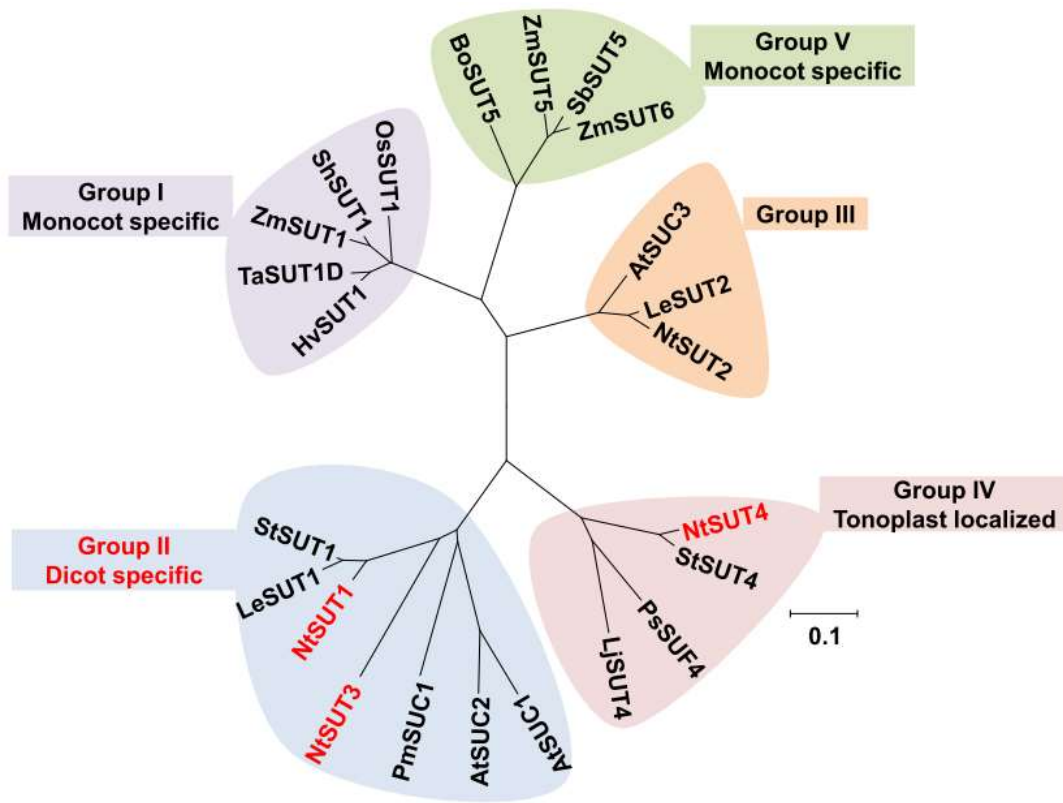


Figure 1. Phylogenetic tree adopted from Braun and Slewinski (2009). Phylogenetic analysis and the relationship between SUTs from monocot and dicot plant species are shown based on amino acid sequences analyzed by neighbor joining method. NtSUT1 is classified into Group 2. Accession numbers are OsSUT1 (AAF90181), HvSUT1 (CAB75882), ZmSUT1 (BAA83501), ShSUT1 (AAV41028), TaSUT1D (AM13410), AtSUC1 (AAV97807), AtSUC2 (AAN31829), PmSUC1 (CAI59556), NtSUT1 (X82276), StSUT1 (CAA48915), LeSUT1 (CAA57726), NtSUT3 (AAD34610), AtSUC3 (AEC05635), LeSUT2 (AAG12987), NtSUT2 (SGN-U423251 for detail please look at page 21), NtSUT4 (BAI60050), StSUT4 (AAG25923), PsSUF4 (DQ221697), LjSUT4 (CAD61275), BoSUT5 (AAY43226), ZmSUT5 (ACF85284), SbSUT5 (Sb04g023860), ZmSUT6 (ACF85673).

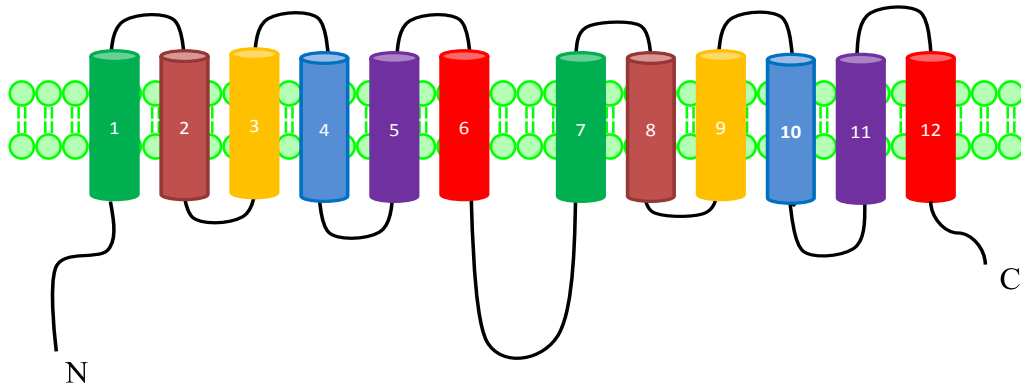


Figure 2. Two dimensional structure of plant sucrose transporter, representing Group 1 (monocot) and Group 2 (dicot), adopted from Sauer (2007). N and C termini are shown to be on the cytoplasmic side. Transmembrane domains are shown in different colors. Similar colors were used for the respective transmembrane helices of the first and the second halves to highlight intra-molecular sequence conservations.

Group 2 SUTs belong to dicot plant species which are also apoplastic loaders (Sauer, 2007) and involved in both phloem loading of sucrose for the transport and phloem unloading into sink tissues. These SUTs are localized in the plasma membrane. The SUT1 from *Nicotiana tabacum*, focusing in this study, belongs to this group. These SUTs exhibited relatively higher affinity for sucrose; 0.3 mM for PmSUC1 and 1 mM for PmSUC2 from *Plantago major* (Stadler et al., 1995; Gahrtz et al., 1996), 0.50 mM for AtSUC1 and 0.77 mM for AtSUC2 from *Arabidopsis thaliana* (Sauer and Stolz, 1994), 0.5 mM for DcSUT2 from *Daucus carota* (Shakya and Sturm, 1998), 1 mM for StSUT1 from *Solanum tuberosum* (Kühn et al., 1997), 1 mM for SlSUT1 from *Solanum lycopersicon* (Reinders et al., 2002), 1.4 mM for VfSUT1 from *Vicia faba* (Weber et al., 1997), 1.5 mM for SoSUT1 from *Spinacia oleracea* (Riesmeier et al., 1992), 1.8 mM for AmSUT1 from *Alonsoa meridionalis* (Knop et al., 2004). Besides sucrose transport uptake, they have broader spectrum of the substrate specificity than Group 1. For example AtSUC1 or SUC2 could transport maltose (Sauer and Stolz, 1994), AtSUC9 could transport several natural and synthetic α - and β -glucosides (Sivitz et al., 2007) and AtSUC5 transports biotin (Pommerrenig et al., 2013). Both Group 1 and Group 2 are localized in the plasma membrane and have similar molecular structures (Fig. 2).

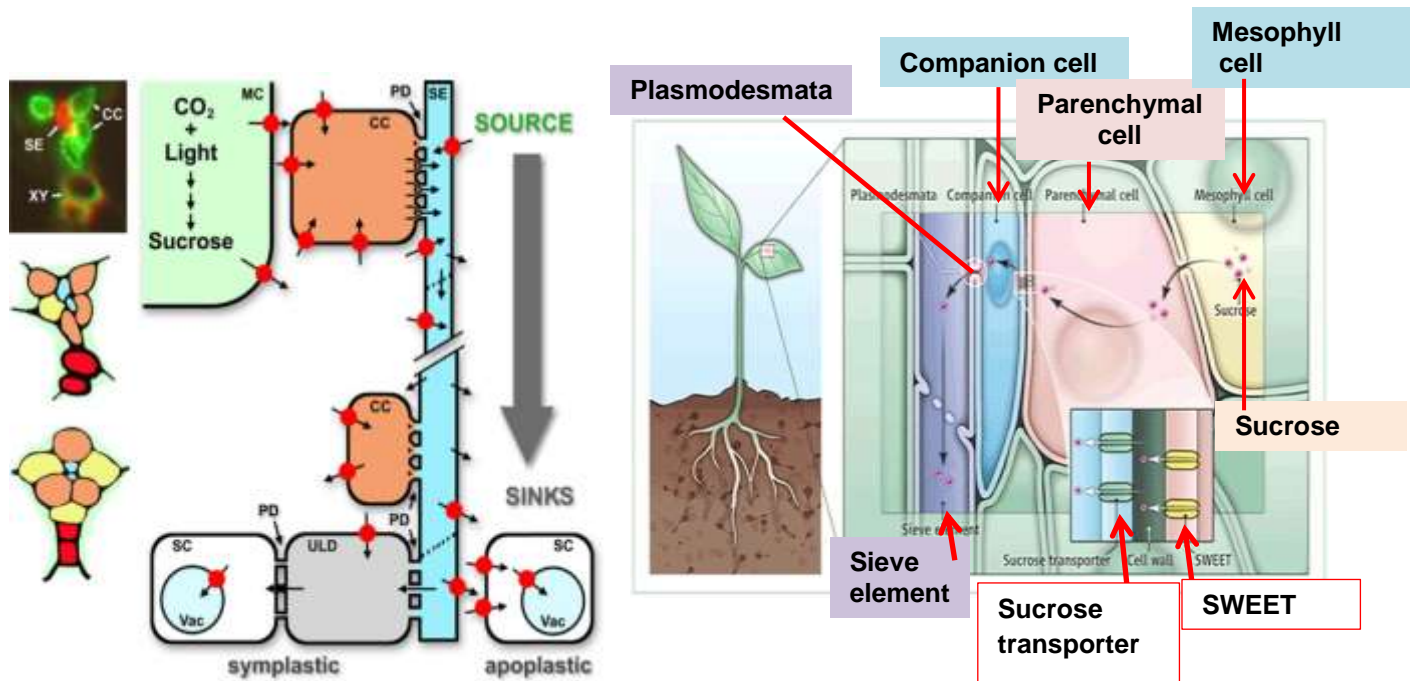
Group 3 SUTs are comprised of both monocots and dicot species. These SUTs have additional amino acids at N and C termini (approximately 30 amino acids) and also in loop 4 (approximately 50 amino acids) which prolong to the cytoplasmic region. The representative members of this group are OsSUT4, SbSUT4, ZmSUT4, AtSUC3, PmSUC3, NtSUT2, LeSUT2 and StSUT2. The N-terminal region of these SUTs has been concluded to have important role in substrate affinity (Schulze et al., 2000; Reinders et al., 2002). Initially these SUTs were proposed

as sucrose sensors (Lalonde et al., 1999; Barker et al., 2000), but later these SUTs were reported to be involved in active uptake of sucrose in AtSUT2 (Schulze et al., 2000) and PmSUC3 (Barth et al., 2003). Therefore the putative function of sucrose sensing no longer exists. These SUTs are characterized as low affinity (Ayre, 2011).

Group 4 SUTs are characterized as transporters localized mainly in tonoplast but in some plant species also localized both in tonoplast and the plasma membrane, and characterized as low substrate affinity (Chincinska et al., 2013). The function of these SUTs was demonstrated as sucrose efflux transporters from vacuoles (Neuhaus, 2007; Reinders et al., 2008). Solanaceous SUTs, StSUT4 and NtSUT4 are characterized as localized in tonoplast and also plasma membrane, since the complementation of *NtSUT4* in sucrose uptake deficient yeast mutant requires a small fraction of NtSUT4 to be localized also in the plasma membrane (Okubo-Kurihara et al., 2011; Chincinska et al., 2013).

Group 5 SUTs are characterized as monocot specific high affinity sucrose transporters which were originally in the Group 1 but later divided into two separate groups. Representative SUTs in this group are OsSUT5, SbSUT5, ZmSUT5 and BmSUT5. These SUTs seem to be less pH dependent and demonstrate broad substrate specificity (Sun et al., 2010).

Another subfamily of SWEET sucrose efflux transporters has been recently discovered (Chen et al., 2012). By the discovery of this important sucrose effluxers, the pathway of sucrose transport from mesophyll cells to the SUTs localized in the CC-SE complex has been completed (Fig. 3) (Braun, 2012). There are two main pathways for loading of phloem with sucrose by sucrose transporters: (1) the transport of photoassimilates through cell membrane before loading into phloem is called apoplast loading (Schmitt et al., 2008). Since the concentration of photoassimilates is higher in phloem than in the photosynthetic cells, the concentration gradient



➡ **Sucrose-H⁺ symporter**
(Sauer, 2007) FEBS Lett.

David (2012) Science

Figure 3. A pathway of sucrose transport in plants adopted from Sauer (2007) and David (2012). Sucrose is synthesized in leaf mesophyll cells and diffuses through plasmodesmata into phloem parenchyma cells. SWEET proteins facilitate sucrose efflux into the cell wall (apoplast). Sucrose transporters import sucrose into companion cells and/or sieve elements. Sucrose is transported through sieve elements out of leaves to non-photosynthetic tissues, such as roots, stem, and fruits (David, 2012).

requires active transport of sucrose through sucrose/H⁺ symporters which is energized by H⁺-ATPase (Bush, 1993; Gaxiola et al., 2007). Most of the plant species including solanaceous are apoplastic loaders (Sauer, 2007; Schmitt et al., 2008). (2) Symplastic pathway is facilitated for the transport of photoassimilates through plasmodesmata, and this pathway is passive therefore does not require energy. *Quercus rubrum* and *Fragaria* sp. are the symplastic loaders (Rennie and Turgeon, 2009) so that the down-regulation of *VpSUT1* does not inhibit phloem loading (Zhang and Turgeon, 2009).

In this chapter, the *NtSUT1* gene was cloned from tobacco cell line SL, in order to investigate the role of *NtSUT1* (Group 2 SUT) in growth capacity in tobacco cultured cells under normal (chapter II) and Al stress (chapter III) conditions. Phylogenetic and expression analyses of the *NtSUT1* were also performed.

Materials and methods

Tobacco cell lines and growth conditions

Two non-chlorophyllous tobacco cell lines were used as wild-type (WT): a cell line SL derived from *Nicotiana tabacum* L. cv. Samsun (Nakamura et al., 1988) and cell line BY-2 derived from cv. Bright Yellow-2 (Nagata et al., 1992). Cells were cultured on a rotary shaker operated at 100 rpm at 25°C in the dark. The nutrient medium employed for cell growth was a modified version of Murashige-Skoog (MS) medium containing 3% (88 mM) sucrose and 1.5 µM 2,4-D as described previously (Yamamoto et al., 1994). The cell lines were maintained by sub culturing one mL or 0.3 mL aliquot of cells of SL or BY-2 respectively into 15 mL of the modified MS medium every seven days.

Cloning of *NtSUT1-L* and *-S* from SL cell line

The cloning of *NtSUT1-L* from cell line SL was performed by reverse transcription (RT)-PCR. Total RNA was isolated from actively growing cells at the logarithmic phase of growth of SL cells and first strand cDNA was prepared by standard method. Forward and reverse primers were designed from *NtSUT1a* sequence in the EMBL database (accession number X82276), together with restriction sites *Sal* I and *Eco* RV (see Table 1) and were used to amplify *NtSUT1*. PCR was performed using high fidelity enzyme Primer STAR GXL DNA polymerase (Takara Bio, Osaka, Japan). The PCR products were cloned into T vector, pGEM-T easy (Promega, Madison, WI, USA), and sequenced. Two types of *NtSUT1* sequence were obtained with slightly different nucleotide sequences. The sequence of one clone (named as *NtSUT1-L*; DDBJ accession number AB823663) was close to *NtSUT1a* (X82276), while another clone (named as *NtSUT1-S*; DDBJ accession number AB823664) was close to *NtSUT1y* (FM164640) (Schmitt *et al.* 2008) but not identical. Therefore, the clones were named as *NtSUT1-L* and *NtSUT1-S*. The specific primer sets to detect the *NtSUT1-L* and the *NtSUT1-S* expression respectively were designed (Fig. 4, Table 1). For normalization during quantitative real-time PCR, *Actin 9* of tobacco (Volkov *et al.*, 2003) was used.

Phylogenetic analysis of sucrose transporters in tobacco

Full length amino acid sequences of previously reported tobacco sucrose transporters (*NtSUT1x*, *NtSUT1y*, *NtSUT1a*, *NtSUT2*, *NtSUT3* and *NtSUT4*) and *NtSUT1-L* and *NtSUT1-S* cloned from SL cell line in this study were aligned using Clustal W (Chenna *et al.*, 2003) and the phylogenetic tree was generated using GENETYX-TREE 1.1.1 (GENETYX Corp., Tokyo). The amino acid sequences of two well-known potato sucrose/H⁺ symporters *StSUT1* and *StSUT4*,

AtSUC3 and LeSUT2 (Riesmeier et al., 1993; Barker et al., 2000; Meyer et al., 2004; Chincinska et al., 2008) were also aligned as an outgroup.

Real time RT-PCR

Cells were harvested and ground in liquid nitrogen. Total RNA was extracted using RNeasy Qiagen mini plant kit and then treated with RNase-free DNase (Qiagen Sciences, Gaithersburg, MD, USA). Total RNA (1µg) was used for the first strand cDNA synthesis using Super Script II kit (Life Technologies) and oligo (dT) primer set. For real-time RT-PCR, gene specific primer sets for *NtSUT1-L*, *-S*, *NtSUT3* and *NtSUT4* used are listed in Table 1. For normalization during quantitative real-time PCR, *Actin 9* of tobacco (Volkov *et al.*, 2003) was used. Reaction conditions for thermal cycling were as described previously with minor modifications (Sasaki *et al.* 2004). For real time PCR: 95°C for 60 sec; 40 cycles at 95°C for one sec, 60°C for 5 sec and 72°C for 45 sec to amplify the *NtSUT1* using a Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) using LightCycler (Roche, Penzberg, Germany).

Subcellular localization of NtSUT1 in onion epidermal cells

To determine the subcellular localization of NtSUT1, the plasmid for transient expression of NtSUT1-L fused with GFP was constructed. Briefly, the NtSUT1-L::GFP fusion was constructed as follows: PCR was performed using the primer sets described in Table 1. The products were introduced into the pTH2 vector which includes sGFP (S65T), kindly provided by Dr. Yasuo Niwa (Chiu et al., 1996). The resulting construct contained CaMV 35 promoter::NtSUT1-L::GFP::NOS terminator. The particle bombardment (PDS-1000, Bio-Rad Laboratories, Hercules, CA, USA) for transient expression of the NtSUT1-L::GFP fusion and the GFP only



Figure 4. *NtSUT1* nucleotide sequences used to design gene specific primers to distinguish *NtSUT1-L* (or a) and *NtSUT1-S* (or y) expression levels. There are 3 single nucleotide polymorphism (SNPs) in sense strands and 2 SNPs in antisense strands.

Table 1. Primer sets used for cloning and expression analyses of *NtSUT* genes

Gene	Primer sets	Reference
<i>NtSUT1</i> (for cloning)	Forward primer 5'-GCAGATCTGTCGACACCATGGTACCAAAAACTT-3' (underline: <i>SalI</i> site) Reverse primer 5'-GCGATATCTCAATGGAAACCGCCCATTGTGGTG-3' (underline: <i>EcoRV</i> site)	This work
<i>NtSUT1-L</i> (expression)	Forward primer 5'-CGTCTTCCTTCTACTCAGCTTAACAATCT-3' Reverse primer 5'-CCAAAACTCCACACTTAGCGACATA-3'	This work
<i>NtSUT1-S</i> (expression)	Forward primer 5'-GTCTTCCTTCTCCTCAGCTTAACAACCC-3' Reverse primer 5'-CAAAAACTCCACACTCAGCGACATG-3'	This work
<i>NtSUT3</i> (expression)	Forward primer 5'-GCAAAGAGGTATATGGGGGC-3' Reverse primer 5'-GGACTGCTAGAGGAATTCCCG-3'	This work
<i>NtSUT4</i> (expression)	Forward primer 5'-AGCCTCTAGATCCCAATCATTGCTC-3' Reverse primer 5'-CACCATAAATTCTCGACCAAACCA-3'	Okubo-Kurihara E <i>et al.</i> (2011)
<i>NtSUT1</i> (expression in RNAi line)	Forward primer 5'-AGAGAGGCTACCTTTACAAGTG-3' Reverse primer 5'-TCTTAATCTCCCTTCACCAA CC-3'	This work
<i>Actin9</i> (expression, internal control)	Forward primer 5'-CTATTCTCCGCTTTGGACTTGGCA-3' Reverse primer 5'-AGGACCTCAGGACAACGGAAACG-3'	Volkov RA <i>et al.</i> (2003)
<i>NtSUT1-L</i> (for <i>NtSUT1-L::GFP</i> construct)	Forward primer 5'-GCAGATCTGTCGACACCATGGAGAATGGTACCAAAAACTT-3' (underline: <i>BglII</i> site) Reverse primer 5'-GCAGATCTTCCTCCGCCACCATGGAAACCGCCCATTGTGGTGGCTG-3' (underline: <i>BglII</i> site)	This work

(control) was performed, using onion epidermal cell layer put onto the filter paper immersed in a nutrient medium containing 1.5 μ M 2,4-D during bombardment. Post bombardment incubation was extended for 18 h at 25°C. The confocal microscopy was performed as described previously (Yamaguchi et al., 2005, Sasaki et al., 2010), using the 488 nm excitation line (LP 560) of an argon laser and a 505-530 nm band pass filter in a Zeiss microscope (Axioplan II) coupled with a confocal module (LSM 510, Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analyses were performed, using Microsoft Excel. Data showing significance was subjected to LSD test to show the significant differences among mean values at $P < 0.05$.

Results

Phylogenetic analysis of sucrose transporters in tobacco

Tobacco is an amphidiploid plant species. Therefore, it has two parental genes corresponding to sucrose transporters localized at the plasma membrane, *NtSUT1x* and *NtSUT1y* (Schmitt et al., 2008). From different tobacco plant cultivars, two genes (*NtSUT1a*, *NtSUT1b*) were reported (Bürkle et al., 1998). Consistent with the previous reports, two clones with nearly identical sequences (97% homology) were obtained at 2 to 1 ratio from cell line SL (see Materials and Methods), and named *NtSUT1-L* (large amounts) and *NtSUT1-S* (small amounts), respectively. Phylogenetic analysis was performed using NtSUT1 sequences (NtSUT1-L, S, x, y, a) reported to date from different tobacco cultivars, and among NtSUTs (NtSUT1, NtSUT2, NtSUT3, NtSUT4), together with well characterized StSUT1 and StSUT4 in potato and also AtSUC3, LeSUT2 for comparison (Fig. 5). NtSUT1-L clustered with NtSUT1x and NtSUT1a, while

NtSUT1-S was close to NtSUTy, and NtSUT3 in the Group 2 which belongs to dicot plant species. NtSUT2 is in the Group 3 which comprises of SUTs from monocot and dicot plant species. NtSUT4 belong to Group 4 which consists of mainly tonoplast SUTs.

Expression analyses of *NtSUT1* in SL and BY-2 cell lines

Previously, the mechanisms of toxicity and tolerance of Al ion was investigated, using a cultured tobacco cell line SL (Nakamura et al., 1988), and it was proposed that the inhibition of sucrose uptake could be a target mechanism whereby Al inhibits the cell elongation (Abdel-Basset et al., 2010). To evaluate this hypothesis, we focused on the *NtSUT1* gene encoding a sucrose/ H⁺-symporter localized in the plasma membrane (Bürkle et al., 1998). To compare the expression levels of *NtSUT1-L* and *S*, the gene-specific primer sets were designed at the same *NtSUT1* region with a few single nucleotide polymorphisms (SNPs) (Fig. 4). The sequences of primers for *NtSUT-L* and *NtSUT-S* are identical to those of *NtSUTa* and *y*, respectively. The results of real-time RT-PCR analysis suggested that *NtSUT1-L* is dominantly expressed over *NtSUT1-S* (Fig. 6) in SL cells. Subsequently, another tobacco cell line BY-2 was used which derived from cv. Bright Yellow-2, and the expression levels of *NtSUT1-L* (or *a*) and *-S* (or *y*) were estimated with the primer sets for *NtSUT1-L* (or *a*) and *NtSUT1-S* (or *y*) designed based on *NtSUT1* sequences of SL cell line (see above). In BY-2 cells at the logarithmic phase of growth, both *NtSUT1-L* (or *a*) and *NtSUT1-S* (or *y*) were expressed at comparable levels (Fig. 6).

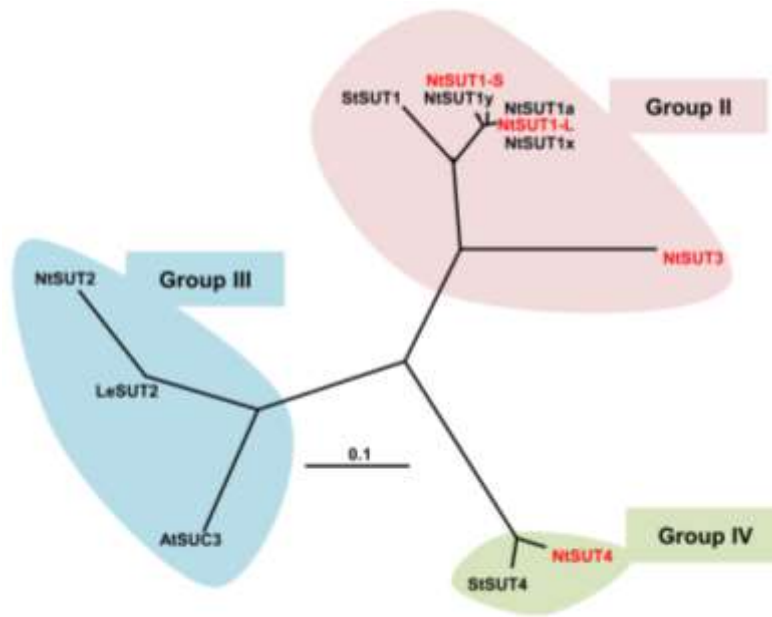


Figure 5. Phylogenetic analysis of NtSUT1-L and S cloned from cultured tobacco cell line SL. Phylogenetic analysis of the newly isolated NtSUT1 (L, S) was performed together with the previously reported SUTs by neighbor joining method. Potato StSUT1 and StSUT4 were used as outgroups for phylogenetic analysis. Accession numbers of sucrose transporter amino acid sequences are as follows: NtSUT1-L (AB823663), NtSUT1-S (AB823664), NtSUT1a (CAA57727), NtSUT1x (CAM33257), NtSUT1y (CAQ58421), NtSUT3 (AAD34610), NtSUT4 (BAI60050), StSUT1 (CAA48915), StSUT4 (AAG25923), NtSUT2 (SGN-U423251) protein sequence was not available from the NCBI database therefore partial gene sequence (from Sol Genome Network http://solgenomics.net/search/unigene.pl?unigene_id=SGN-U423251 was online translated by web.expasy.org/translate, LeSUT2 (AAG12987), AtSUC3 (AEC05635). Group classification is based on phylogenetic tree adapted from (Braun and Slewinski, 2009).

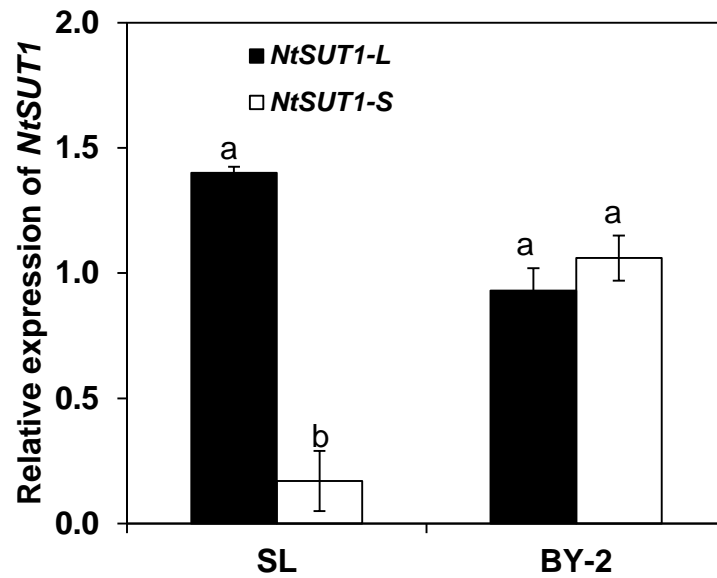


Figure 6. The expression levels of *NtSUT1-L* and *NtSUT1-S* were examined in tobacco cell lines (SL, BY-2) at the logarithmic phase of growth.

Quantitative real-time RT-PCR was performed, using primer sets specific for *NtSUT1-L* (identical to *NtSUTa*) or *NtSUT-S* (identical to *NtSUTy*), respectively. Relative expression levels were normalized to the values of the *Actin9* transcripts. Each value represents the mean \pm SE of three samples from three independent experiments. For each cell line, significant differences between genes are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$.

Subcellular localization of NtSUT1 in onion epidermal cells

To corroborate its function, the subcellular localization of NtSUT1-L was investigated, using the NtSUT1-L-GFP fusion protein transiently expressed in onion epidermal cells (Fig. 7). Consistent with the previous reports (Lemoine et al., 1996; Kühn et al., 1997; Schmitt et al., 2008), NtSUT1 seemed to be localized in the plasma membrane, since the fluorescence derived from NtSUT1::GFP was observed around the cells. In addition, GFP fluorescence was observed at the Hechtian strands in NtSUT1::GFP expressed onion cells, a part of the plasma membrane connected to the cell wall, in the plasmolyzed cells, but not in GFP expressed cells (Lang et al., 2004; Sasaki et al., 2010).

Discussion

In this study, two nearly identical *NtSUT1* genes (*NtSUT1-L*, and *NtSUT1-S*) were isolated from SL cells. In addition, using the specific primer sets, it was determined that the *NtSUT1-L* was dominantly expressed in SL cells. Subcellular localization of the *NtSUT1-L* was confirmed to be the plasma membrane.

Characterization of *NtSUT1* in cultured tobacco cells

Based on phylogenetic analysis (Fig. 5) and SUTs classification (Braun and Slewinski, 2009) (Fig. 1), NtSUT1-L and NtSUT1-S belong to Group 2 sucrose/H⁺ symporter from dicot plant species. In plants, its function is related to loading and unloading of phloem with photoassimilates (Fig. 3). The *NtSUT1* expressed in source leaf (photosynthetically active) and in sink tissue root suggested its dual roles of phloem loading in leaves and unloading in the root,

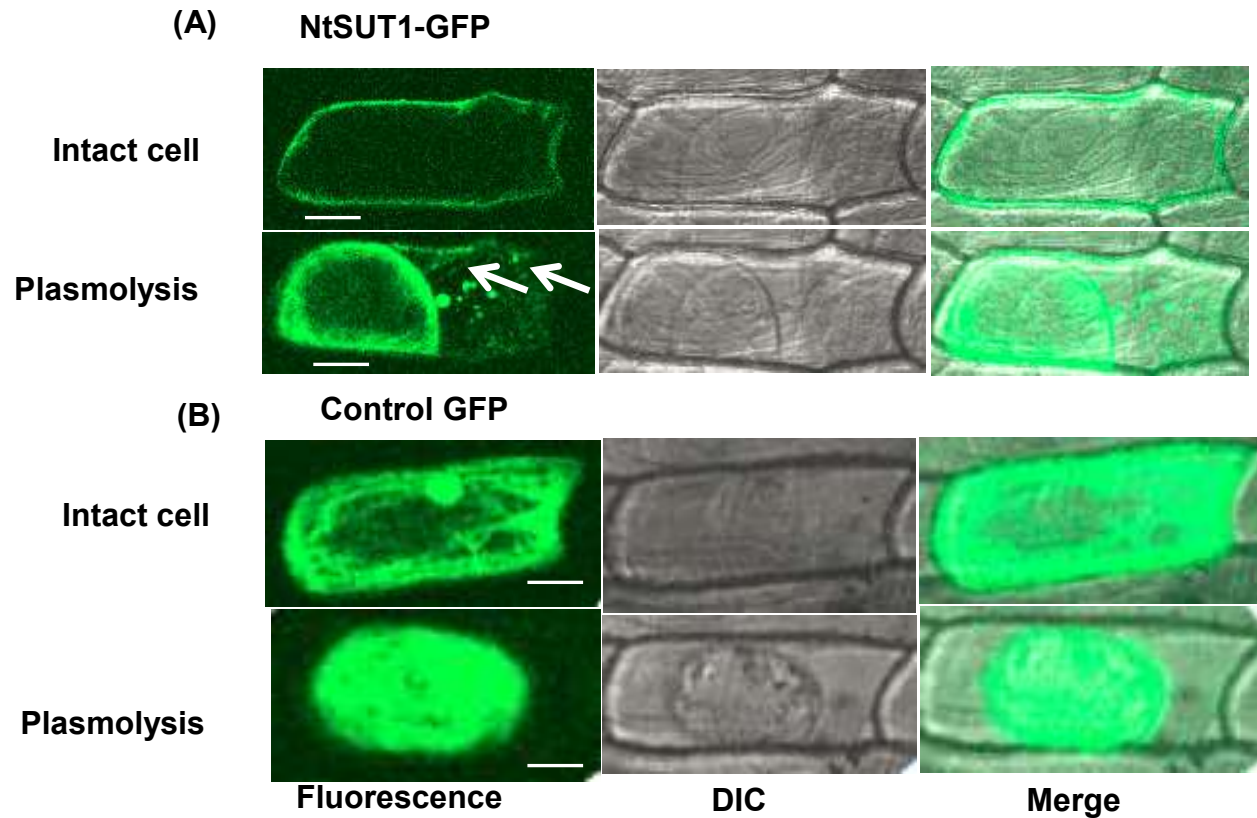


Figure 7. Subcellular localization of NtSUT1-GFP (A) and GFP (B) transiently expressed in onion epidermal cells. Onion cells were bombarded with the constructs for detection of subcellular localization of NtSUT1-GFP and GFP as described in Materials and Methods. Plasmolysis of NtSUT1-GFP expressing cells with 1 M mannitol shows the Hechtian strands indicated with arrows (attaching the plasma membrane to the cell wall) are labeled, indicating that the protein is localized at the plasma membrane. Left, GFP fluorescence images; Center, transmitted light images; Right, merged images, scale bars=50 μm .

even though expression was weak in other sink tissues such as sink leaf, floral parts and stem (Bürkle et al., 1998). NtSUT1 seems to be localized in the plasma membrane (Fig. 7) as reported previously (Schmitt et al., 2008). Therefore NtSUT1 localized in the plasma membrane seems to play a role for the uptake of sucrose from the medium in tobacco cultured cells, since tobacco cultured cells are heterotrophic and are totally dependent on nutrients from the medium. StSUT1 from potato is high affinity sucrose transporter and an ortholog of NtSUT1. However, the affinity to sucrose of NtSUT1 remains to be elucidated, even though it has been reported to be functional (Kühn, 2012). Tobacco genome has two orthologous SUT1 namely NtSUT1x and NtSUT1y (Schmitt et al., 2008), or NtSUT1-L and NtSUT1-S in this work. These orthologs have been arisen in allotetraploid tobacco (*Nicotiana tabacum*) due to cross pollination of two diploid tobacco plant species *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Kenton et al., 1993). However the functional characterization of these orthologs remains to be conducted. From higher expression of *NtSUT1-L* in SL cell line, it seems to be a major gene, which could be mainly responsible for sucrose uptake from the medium (Fig. 6). This finding is well consistent with the result of the ratio for the clone numbers of the *NtSUT1-L* and *-S* genes. The expression levels of *NtSUT1-L* and *NtSUT1-S* in BY-2 cell line seem to be at similar levels (Fig. 6), suggesting that these genes may contribute equally to sucrose uptake in BY-2.

By the analysis of subcellular localization, the plasma membrane-cell wall connections called as Hechtian strands were observed in an onion cell during plasmolysis (Mellersh and Heath, 2001; Lang et al., 2004), suggesting that the NtSUT1 is localized in the plasma membrane (Fig. 7). This finding is consistent with the previous finding of NtSUT1 (Schmitt et al. 2008).

Chapter II The role of sucrose transporter *NtSUT1* in growth capacity under normal growth condition

Introduction

Antisense plants of *NtSUT1* showed a variety of phenotypic characteristics in tobacco plants (Bürkle et al., 1998). Severe reduction of mRNA level in antisense *NtSUT1* plants showed a lack of usage of starch in the longer periods of darkness, accumulation of soluble sugars as a result of a decreased of photosynthesis rate in source leaves. Due to inability of sucrose export from leaves, necrosis and chlorosis phenotypes were observed in source leaves. Moreover, a short height of plants and a delayed flowering were also observed (Bürkle et al., 1998). Some similar observations were recorded in the antisense plants of *StSUT1* in potato, such as accumulation of carbohydrates which resulted in osmotic problems in leaves and retarded plant growth. These phenotypes also severely affected sink tissues such as reduced root development and low tuber yields (Riesmeier et al., 1994). T-DNA mutation in *AtSUC2* leads to abnormal phenotypes in *Arabidopsis* plants such as accumulation of high levels of soluble sugar, starch and anthocyanin, a delayed flowering and the inability to produce viable seeds (Gottwald et al., 2000). However, when these mutants were grown on MS agar medium supplemented with sucrose, the plants recovered from the stunted growth (Kircher and Schopfer, 2012). In tomato *LeSUT1* antisense plants were characterized by extreme alterations in plant development and leaf phenotype, and sink organs. Antisense *OsSUT1* rice plants showed similar typical phenotypes like other members of this group such as reduced grain filling, grain weight and low germination rate and retarded growth (Furbank et al., 2001; Ishimura et al., 2001). In maize mutants of *sut1*, the plants accumulated carbohydrates in source leaves, leaf chlorosis and premature senescence. Moreover, *sut1* mutants exhibited short stature, lower biomass, delayed flowering and stunted tassel

development which ultimately could affect yield in maize. Over all, physiological performance of the mutants showed the importance of *SUT1* in phloem loading of sucrose in maize leaves (Slewinski et al., 2009).

Therefore based on such important roles of sucrose transporters in the growth and development of plants, *NtSUT1* was evaluated in cell culture of tobacco BY-2 cell line.

Materials and methods

Tobacco cell lines and growth conditions

Aliquots of independently transformed calli of OX and RNAi cell lines (see below) were transferred into nutrient medium supplemented with 50 mg L⁻¹ kanamycin and 50 mg L⁻¹ carbenicillin, and grown until cell cultures reached to the stationary phase on a rotary shaker operated at 100 rpm at 25°C in the dark. Cells were maintained by subculturing 0.3 mL (BY-2 WT, OX) or 1.5 mL (BY-2 RNAi) cell suspension into 15-mL of fresh medium at about 7-d intervals as described previously (Yamamoto et al., 1994).

During the culture, fresh weight (FW) of the cells in 5-mL aliquots was determined every 24 h and was plotted on semi-log graph [FW vs. culture time (h)] to determine growth phases such as lag phase, logarithmic phase and stationary phase (Sakai et al., 2004). During a logarithmic phase, the mass doubling time was determined for each cell line as the time (h) necessary to double the FW of cells in 5-mL liquid culture aliquot. The mass doubling time was used for comparison of growth rates among the cell lines.

Construction of over-expressor (OX) and suppressor (RNAi) in BY-2 lines

Based on higher expression of the *NtSUT1-L* relative to *NtSUT1-S* (see Chapter I, Results), the *NtSUT1-L* was selected for the construction of over-expression (OX) and suppression (RNAi) transformants of BY-2 line (Fig. 8). For the OX construct, the full length open reading frame (ORF) of *NtSUT1-L* was cloned into Gateway entry vector pENTR 3C (Life Technologies, Carlsbad, CA, USA). For RNAi, the fragment started from 1 to 724 bp was cut with *Sac* I and blunt-end ligated to *Eco* RV site of the pENTR 3C. These entry plasmids were recombined to the destination vectors, pGWB2 driven by the CaMV 35 promoter for over-expression, kindly provided by Dr. Tsuyoshi Nakagawa (Nakagawa *et al.* 2007), or pBI-sense-antisense-GW for suppression (Inplanta Innovations, Kanagawa, Japan) by Gateway LR Clonase II enzyme mix (Life Technologies). These constructs were transformed into both SL and BY-2 cells by the use of *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986) and LBA4404 (Life Technologies). The transformed cells were selected on nutrient agar medium plate containing 200 mg L⁻¹ kanamycin and 500 mg L⁻¹ carbenicillin, and then cells were maintained in nutrient medium containing 50 mg L⁻¹ kanamycin and 50 mg L⁻¹ carbenicillin as described previously with minor modifications (Sasaki *et al.*, 2004). A higher number of transformants were obtained in BY-2 compared to SL cells. Therefore, BY-2 transformants were selected for further studies. All the plasmid constructs used in this paper were verified by standard sequencing method (ABI 3130xl or ABI 3100 sequencers, Applied Biosystems, Foster City, CA, USA).

Real time RT-PCR

To determine the expression levels of *NtSUT1* gene, real time RT-PCR was performed as described previously (see Chapter I, Materials and Methods). For expression analysis of the *NtSUT1* in OX lines, *NtSUT1-L* gene specific primer set was used (see Table 1). For the

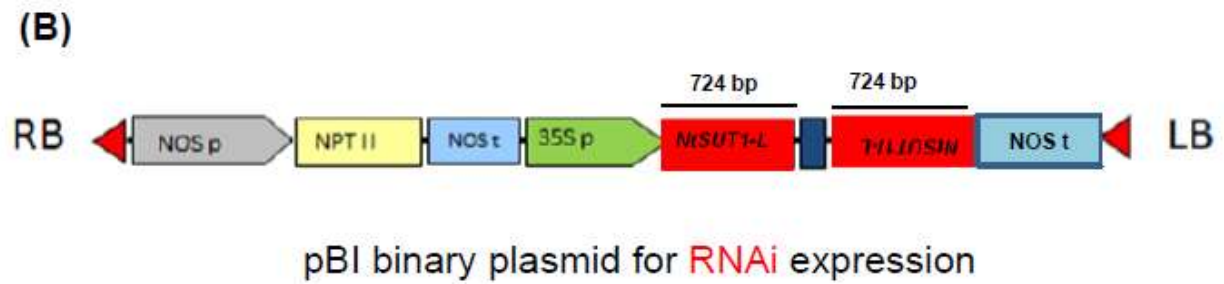
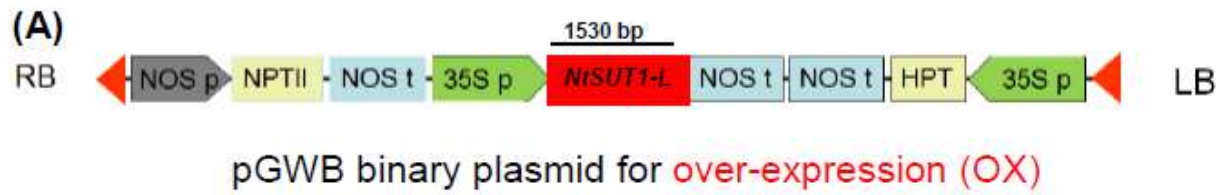


Figure 8. *NtSUT1* constructs for over-expression (A) and suppression (RNAi) (B) used to transform BY-2 cells.

expression analysis of *NtSUT1* in RNAi line, 3'UTR conserved region of *NtSUT1* was used for designing the primer set (Table 1) based on expressed sequence tag (EST) of the *NtSUT1* (accession no. EB441358). *Actin9* was used as an internal control (Table 1).

Preparation of crude membrane fractions and immunoblot analysis

Crude membrane fractions from tobacco cultured cells were prepared according to the method of Yamaguchi et al., (2005) with minor modifications. Briefly, cells were ground by mortar and pestle using an ice-cold grinding buffer containing 0.5 M sorbitol, 10 mM EGTA, 10 mM Na_3VO_4 , 10 mM NaF, 5% (v/v) polyvinylpyrrolidone, 25 mM Hepes-BTP (pH7.6) and five additional components [0.5% (w/v) bovine serum albumin (protease free, A-3294; Sigma, St. Louis, MO, USA), 1 mM dithiothreitol, 0.5 mM phenylmethyl sulfonyl fluoride, 5 $\mu\text{g mL}^{-1}$ leupeptin, and 0.5 $\mu\text{g mL}^{-1}$ pepstatin A] which were added just before use. The solution was further homogenized by Teflon homogenizer and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was centrifuged at 156,000 g for 30 min at 4°C and the pellet was resuspended in the suspension buffer containing 0.5 M sorbitol, 1 mM EGTA, 1 mM EDTA 1 mM Na_3VO_4 , 1 mM NaF, 5 mM MOPS-BTP (pH7.3) and three components (2 mM dithiothreitol, 0.5 $\mu\text{g mL}^{-1}$ leupeptin, and 0.5 $\mu\text{g mL}^{-1}$ pepstatin A) which were added just before use. The suspension was again centrifuged at 156,000 g for 30 min at 4°C, and the pellet was resuspended in the suspension buffer. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

The resultant crude membrane fractions were used for SDS-PAGE and immunoblot analyses performed as described previously (Sasaki et al., 2004, 2010). Briefly, denatured crude membrane fractions (15 μg for the detection of NtSUT1 and 7.5 μg for the plasma membrane

H⁺-ATPase) were loaded into 10% polyacrylamide gel for SDS-PAGE and electrophoresed to separate proteins (Laemmli, 1970), which were blotted to polyvinylidene difluoride membrane by immune blot analysis (Towbin et al., 1979), following standard protocols. The membrane was blocked with blocking buffer to reduce nonspecific bands. Primary antibody of anti-NtSUT1 antiserum was raised against peptides from 4 to 17 (GTKKLVSSSLAVEQ) and 493 to 506 (PSPPADAKPATTMG) of NtSUT1-L (Operon Biotechnologies, Tokyo, Japan). Anti-plasma membrane H⁺-ATPase antiserum was produced against the conserved amino acid peptide regions (PLFDPPRHDSAETIR) (accession number AY383599) among several plant species (tobacco, wheat, *Arabidopsis*) which was kindly provided from Dr. T. Sasaki. The blotted membrane was probed with the anti-polyclonal antibodies. The secondary antibody reaction was done with the ECL anti-rabbit IgG horseradish peroxidase linked F (ab')₂ fragment from donkey (GE Healthcare, Little Chalfont, BKM, UK) for 1 h, and ECL Prime Western Blotting Detection Reagents (GE Healthcare) were used for chemiluminescence for 5 minutes which was observed under detection system (LAS-1000, Fuji-film, Tokyo, Japan).

¹⁴C-Sucrose uptake

The rate of sucrose uptake in cells was determined as described previously with minor modifications (Abdel-Basset et al., 2010). Nutrient medium was used for assessment of sucrose uptake in cells at the logarithmic phase of growth. Cells were pre-cultured for 2 h at 25°C in modified MS medium at the respective cell densities of 50 mg FW per mL for normal growth. After each pre-culture, [U-¹⁴C] sucrose (16.095 GBq mmol⁻¹, 3.7 MBq mL⁻¹ from PerkinElmer, Boston, MA, USA) was added to each culture at 3.7 KBq mL⁻¹ and incubated. At designated times, two mL aliquots of each culture were harvested on a glass membrane filter by vacuum

filtration and washed four times with 5 mL of each medium. The cells on the filter were immediately inoculated into scintillation cocktail (Clear-sol I, Nacalai Tesque, Kyoto, Japan). After overnight incubation in the dark, the radioactivity was determined with a scintillation counter (PerkinElmer Life Sciences, Downers Grove, IL, USA).

Soluble sugar extraction and quantification

Soluble sugar was extracted from cells with 80% ethanol and was quantitated by the anthrone reaction as described previously (Abdel-Basset et al., 2010). In short, cells of 50 mg FW of cells at the logarithmic phase of growth were harvested soluble sugar was extracted and determined spectrophotometrically at 630 nm after the reaction with anthrone-sulphuric acid reagent, using glucose as a standard.

Statistical analysis

Statistical analyses were performed, using Microsoft Excel. Data showing significance was subjected to LSD test to show the significant differences among mean values at $P < 0.05$.

Results

Construction and expression analysis of over-expressor (OX) and suppressor (RNAi) lines of *NtSUT1* in BY-2 cells

For functional characterization of *NtSUT1*, the *NtSUT1-L* obtained from SL cells was used to construct two over-expressor lines (OX-1, OX-2) and one suppressor line (RNAi-1), respectively, in BY-2 cells (WT) (see Materials and Methods). In OX lines, the expression level

of *NtSUT1-L* was approximately 1.4 ~ 1.6 fold higher compared to WT (Fig. 9, Left). For the evaluation of transcriptional suppression of *NtSUT1* of both types [*L* (or *a*) and *S* (or *y*)] in RNAi line, the expression level of the 3'-UTR conserved region of the *NtSUT1* was examined. Compared to WT, the *NtSUT1* expression was strongly suppressed in RNAi line to about 19% of WT (Fig. 9, Right).

NtSUT1 protein levels in crude membrane fractions were also examined among three cell lines (WT, OX-2, RNAi-1) by immunoblot analysis, using the NtSUT1 specific antiserum (Fig. 10). Compared to WT, the protein level detected at 47 kDa was higher in OX (2 fold), while it was strongly suppressed in RNAi (0.2 fold), after image analysis and estimation by ImageJ software (Schneider et al., 2012). For comparison, the protein level of the plasma membrane H⁺-ATPase in the same crude membrane fractions was determined, using the ATPase specific antiserum. The ATPase levels were similar in these lines, OX (0.84) and RNAi (0.74), compared to WT, by ImageJ. These results suggest that in RNAi both *NtSUT1-L* (or *a*) and *NtSUT1-S* (or *y*) genes were efficiently suppressed.

Sucrose uptake rate in BY-2 cell lines (WT, OX, RNAi) during logarithmic phase of growth in a modified MS medium

The uptake rate of sucrose was determined among the lines maintained under normal growth conditions in a modified MS medium. The cells at the logarithmic phase of growth were labelled with ¹⁴C-sucrose for up to 90 min (Fig. 11). The initial uptake rate from 0 to 30 min (nmole mg FW⁻¹, n=2) were determined as follows: WT (2.23), OX-2 (3.71) and RNAi-1 (1.57), indicating that the uptake rate was enhanced in OX by 166% and suppressed in RNAi to 70% relative to

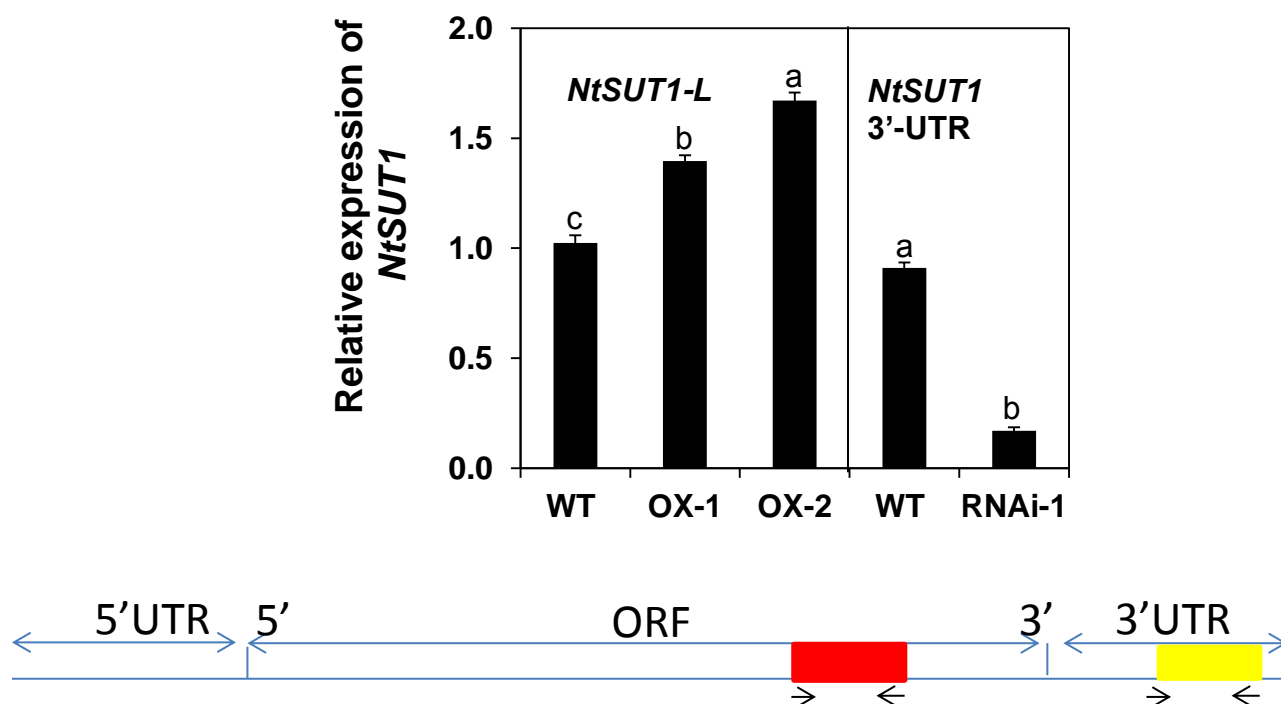


Figure 9. Characterization of OX and RNAi lines of *NtSUT1* in BY-2.

Expression analyses of *NtSUT1* were performed in BY-2 cell lines (WT, OX-1, OX-2, RNAi-1) during the logarithmic phase of growth in nutrient medium. *NtSUT1* expression levels were evaluated by real-time RT-PCR by using specific primer sets for the *NtSUT1-L* (WT, OXs) (shown as red bar) and the 3'-UTR conserved region of *NtSUT1* (WT, RNAi) (shown as yellow bar), respectively. Relative expression levels were normalized against the values of the *Actin9* transcripts. Each value represents the mean \pm SE of three samples from three independent experiments. For each analysis, significant differences among lines are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$.

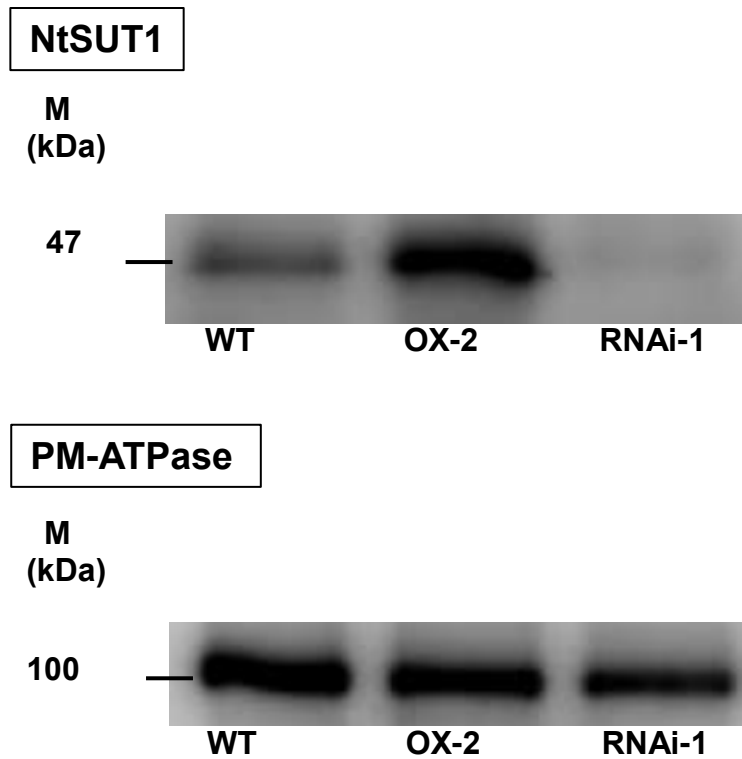


Figure 10. Immunoblot analysis of NtSUT1 and the plasma membrane H^{+} -ATPase in BY-2 cell lines (WT, OX and RNAi).

Proteins in crude membrane fractions were separated on SDS-PAGE for detection of NtSUT1 and the plasma membrane H^{+} -ATPase (PM-ATPase) by immunoblot analysis as described in Materials and Methods.

the WT level (100%). The RNAi line exhibited a strong suppression at both transcription and protein levels (~20% of WT), but it showed only a limited suppression of sucrose uptake rate, which could be due to the uptake of sucrose via other mechanisms different from NtSUT1 (see Discussion).

Relationship between soluble sugar content and mass doubling time among BY-2 cell lines (WT, OX, RNAi)

Using BY-2 cell lines (WT, OX-1, OX-2, RNAi-1), the physiological role of the *NtSUT1* in cell growth was evaluated using two criteria, soluble sugar content and mass doubling time, during the logarithmic phase of growth. The mass doubling time is defined as the time required to double the FW of 5-mL aliquot of each cell culture (see Materials and Methods). This value was used to estimate the growth rate at a mass level, not at a single cell level. Compared to WT, soluble sugar content was significantly higher in OX lines and lower in RNAi line (Fig. 12A).

Mass doubling time was shorter in OX lines and longer in RNAi line, although the differences among lines were not statistically significant (Fig. 12B). However, since the values of soluble sugar content were negatively correlated with the values of mass doubling time among these cell lines (Fig. 12C), it seems that *NtSUT1* contributes to enhance soluble sugar content, which leads to shorter mass doubling time, in other words, higher growth rate.

Discussion

Contribution of *NtSUT1* to growth capacity in nutrient medium

As major tools, using the *NtSUT1-L* cloned from SL cells, the OX and RNAi lines featuring the overexpression and suppression versions of *NtSUT1* gene, respectively, were constructed in

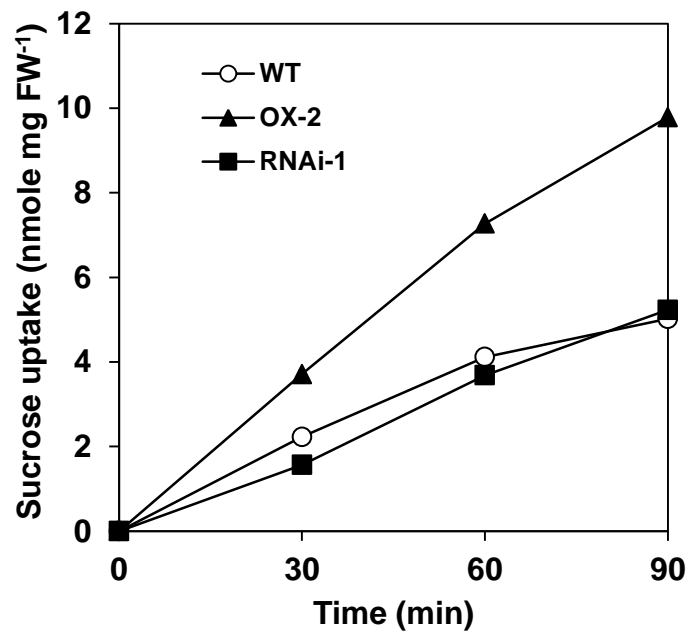


Figure 11. Sucrose uptake in logarithmic phase growth of BY-2 cells (WT,OX-2, RNAi-1).

Sucrose uptake as determined in a nutrient medium. Cells were suspended in a nutrient medium at 50 mg FW per mL and sucrose uptake was monitored at the times indicated after the addition of ¹⁴C-sucrose at 0 h, as described in Materials and Methods. The uptake values are shown per cells at one mg FW, based on the FW of the culture determined at 0 time. Each value represents the average value from two independent experiments.

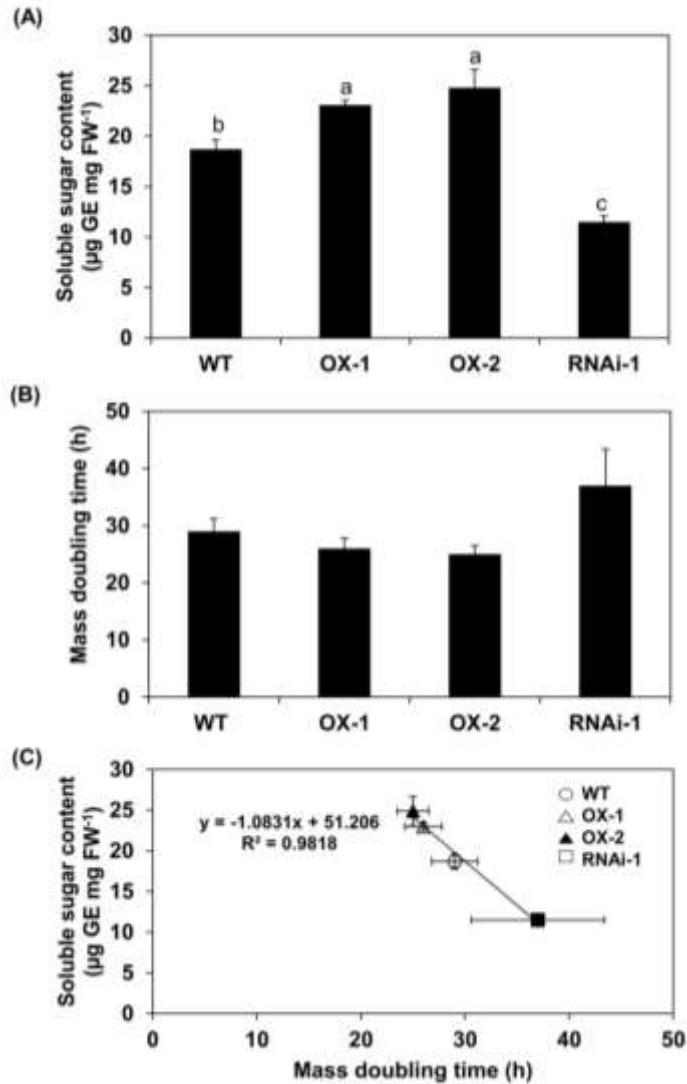


Figure 12. Soluble sugar content and mass doubling time during the logarithmic phase of growth in BY-2 cell lines (WT, OX-1, OX-2, RNAi-1). Soluble sugar content (A) and mass doubling time (B) were determined in each cell line as described in Materials and Methods. Each value represents the mean \pm SE of three independent experiments. In (A), significant differences among lines are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$. Data in (B) are not statistically different. In (C), soluble sugar content (Y) and mass doubling time (X) of each line is plotted. The relationship between these values can be shown as $Y = -1.0831X + 51.206$ ($R^2 = 0.9818$).

BY-2 cell line. By immunoblot analysis, NtSUT1-L was recognized as a 47-kD polypeptide (Fig. 10), as reported previously (Schmitt et al., 2008). OX line had 2-fold, while RNAi line contained only 20% of NtSUT1 protein, compared to WT (Fig. 10). Using these lines, it is shown that the over-expression of *NtSUT1* gene confers better sucrose uptake rates in BY-2 cells, which leads to higher soluble sugar content and higher growth rate during the logarithmic phase of growth in nutrient medium (Figs. 11, 12). The significant suppression of the *NtSUT1* expression in RNAi line gave negative effect on growth rate but this effect was only limited. Plant cells can uptake sucrose via three independent mechanisms: (a) H^+ -Sucrose symporter, (b) cleavage of sucrose by an apoplastic invertase and subsequent uptake by hexose transporters, (c) uptake by endocytosis (Sauer, 2007; Etxeberria et al., 2005). Thus, in the RNAi line, other mechanisms (like b, and c) seem to compensate for the deficiency of sucrose transporter.

Chapter III Effect of auxin (2,4-D) on the expression of *NtSUT1* and the role of *NtSUT1* in growth capacity under Al stress condition

Introduction

Although the inhibition of cell elongation by Al could be explained by several mechanisms, it has already been attributed, at least in part, to the acute (Abdel-Basset et al., 2010; Yang et al., 2012) or chronic (Rufyikiri et al., 2001) inhibition of water uptake. Soluble sugars, amino acids, organic and inorganic ions strongly affect osmotic properties of the cells, hence maintaining the steady state level of water uptake (Sharp et al., 1990). When Tabuchi et al., (2004) measured these components in wheat cultivars exhibiting differential Al sensitivity, only soluble sugar content was significantly reduced in the Al sensitive cultivar. In roots of peach seedling, Al caused severe reductions in reducing sugars, total soluble carbohydrates and total carbohydrates (Graham, 2002). Similar findings were also reported by Abdalla (2008) in barley regarding reducing sugars and total sugars under Al stress. Similarly, Abdel-Basset et al., (2010) did not find any remarkable changes in inorganic ions but found a clear decrease in soluble sugar content caused by Al that was related to decreased water content and osmolarity of cultured tobacco cells. In addition, it has been proposed that the inhibition of sucrose uptake by Al could be one of the mechanistic explanations for Al-imposed inhibition of water uptake and cell elongation (Abdel-Basset et al., 2010).

At cellular and physiological levels, auxin has great potential to promote cell elongation, cell division, and cell differentiation (Teale et al., 2006). Auxin induces transcription of several gene families by acting on the specific cis elements in their promoter regions (Abel and Theologis, 1996). Octopine synthase (OCS) elements are a good example of cis-acting auxin responsive elements. OCS elements are essential components of some promoters of

Agrobacterium genes expressed in the plant cells (Bouchez et al., 1989). Expression of at least two genes, nopaline synthase (nos) and mannopine synthase (mas), has been reported to be induced by auxin in transgenic plants (Langridge et al., 1989; An et al., 1990). OCS elements are also essential for the expression of plant viral promoters such as CaMV 35S which contains an OCS element and, correspondingly, responds to auxin application (Zhang and Singh, 1994). *In silico* analysis revealed that sucrose transporter genes (e.g. *OsSUT3*, *OsSUT4*, *AtSUC1*, *AtSUC5*, *AtSUC6* and *AtSUC9*) also contain regulatory elements for auxin response (auxin responsive elements) (Ibraheem et al., 2010).

In this chapter, the role of *NtSUT1* in cell growth under Al stress condition was investigated. It was found that *NtSUT1* gene expression strongly depends on the presence of 2,4-D in the culture medium, and that the over-expression of *NtSUT1* gene in the cells under Al stress enhances the growth capacity of the cells during post-Al treatment culture. The role of sucrose transporter under stress conditions is discussed in the context of exogenous 2,4-D application.

Materials and Methods

Aluminum treatment conditions

Cells at the logarithmic phase of growth in nutrient medium were used for Al treatment without (control) or with 50 μM AlCl_3 as described previously with minor modifications (Abdel-Basset et al., 2010). Briefly, cells were washed with a medium containing 3 mM CaCl_2 and 3% sucrose (pH 5.0 adjusted with HCl) (washing medium). Washed cells were suspended in a medium containing 3 mM CaCl_2 , 3% sucrose and 20 mM MES at pH 5.0 adjusted with *bis-tris* propane

(BTP) (Al-treatment medium), either in the absence or presence of 1.5 μ M 2,4-D. Cell densities were adjusted at 10 mg FW per mL of culture, and the cells were cultivated for 18 h on a rotary shaker operated at 100 rpm at 25°C in the dark. Cells were harvested, washed and used for various assays as described below. For determination of growth capacity, cells contained in 10-mL culture aliquots were harvested, washed with washing medium, and cultured for 6 days in a nutrient medium (for the cells treated without 2,4-D) or for 4 days (for the cells treated with 2,4-D). The increase in FW over the designated period was termed as “growth capacity”, and expressed as a percentage of the control.

Expression analysis of *NtSUT1* in Al-treatment medium in the absence and presence of 2,4-D

After 18 h treatments without (control) or with Al (50 μ M) cells were harvested and total RNA was extracted as described (see Chapter 1, Materials and Methods). For expression analysis of the *NtSUT1* in OX and WT lines, *NtSUT1-L* gene specific primer set was used (Table 1).

¹⁴C-Sucrose uptake

The rate of sucrose uptake in cells was determined as described (see Chapter II, Materials and Methods) with minor modifications. Two types of media were used: (i) nutrient medium (see Chapter I, Materials and Methods) for assessment of sucrose uptake in cells during post-Al treatment culture; (ii) Al treatment medium (see above) with 1.5 μ M 2,4-D for assessment of Al effect on sucrose uptake in cells. For Al treatment, cells were pre-cultured for 2 h at 25°C in Al treatment media at 10 mg FW per mL. For post-Al treatment culture experiments, the cells at 10-mL aliquot of control and Al treatments (equivalent to 100 mg FW at a start of the treatment)

were harvested at 18 h of the treatment time, washed and resuspended with 10-mL nutrient medium and pre-cultured for 24 h. After each pre-culture, [U-¹⁴C] sucrose was added to each culture and incubated, and two mL aliquots of each culture were harvested at designated times and radioactivity was measured as described (see Chapter II, Materials and Methods).

Soluble sugar extraction and quantification

Soluble sugar was extracted from cells quantitated as described (see Chapter II, Materials and Methods). In short, cells of 50 mg FW of cells (for the cells at the logarithmic phase of growth) or 5-mL aliquot (for the cells after Al-treatment; equivalent to 50-mg FW at a start of the treatment) were washed with 3 mM CaCl₂, and soluble sugar was extracted.

Statistical analysis

Statistical analyses were performed, using Microsoft Excel. Data showing significance was subjected to LSD test to show the significant differences among mean values at $P < 0.05$.

Results

Expression analysis of *NtSUT1* in Al-treatment medium in the absence or presence of 2,4-D

For Al treatment of cultured tobacco cells, a simple treatment medium containing CaCl₂ (3 mM) and sucrose (3%) at pH 5.0 (adjusted with either HCl or 20 mM MES-BTP buffer) was developed (Ikegawa et al., 2000; Yamamoto et al., 2002; Abdel-Basset et al., 2010). Using this treatment medium, BY-2 cell lines at the logarithmic phase of growth were treated without

(control) or with AI for 18 h. Surprisingly, it was found that the *NtSUT1-L* expression was completely suppressed in both WT and OX-2 lines even after control treatment without AI (Fig. 13), without 2,4-D). Then, the differences of medium components between nutrient medium and the AI treatment medium were investigated, and finally found that 2,4-D was necessary for the *NtSUT1-L* expression. By the addition of 2,4-D at 1.5 μ M to the simple AI treatment medium, the *NtSUT1-L* expression was recovered in both WT and OX-2 lines (Fig. 13, with 2,4-D). These results indicate that the expression of *NtSUT1-L*, controlled by its own promoter (WT) or under the control of CaMV 35S promoter (OX-2), is strongly dependent on 2,4-D.

Effect of AI on sucrose uptake and soluble sugar content in BY-2 cell lines (WT, OX, RNAi) in the absence or presence of 2,4-D

In previous study in our laboratory, tobacco cell line SL was treated without or with AI under the same treatment conditions as described here, and the gains of FW and cell number in culture were evaluated (Abdel-Basset et al., 2010). Over the 18 h treatment, an increase in FW in control cells (58% over the initial value) and a reduction of the gain in FW by AI (12% over the initial) were observed in SL cells, which was similar to BY-2 cells in this study (see below). Interestingly, the gain of cell number was limited, but observed similarly in control and AI-treated cells (1.3~1.4 fold over the initial values), suggesting that AI inhibits cell elongation, but not cell division. Based on these findings, in this study, sucrose uptake rate, soluble sugar content and growth capacity were compared on the cells of culture basis, instead of FW basis, among the lines.

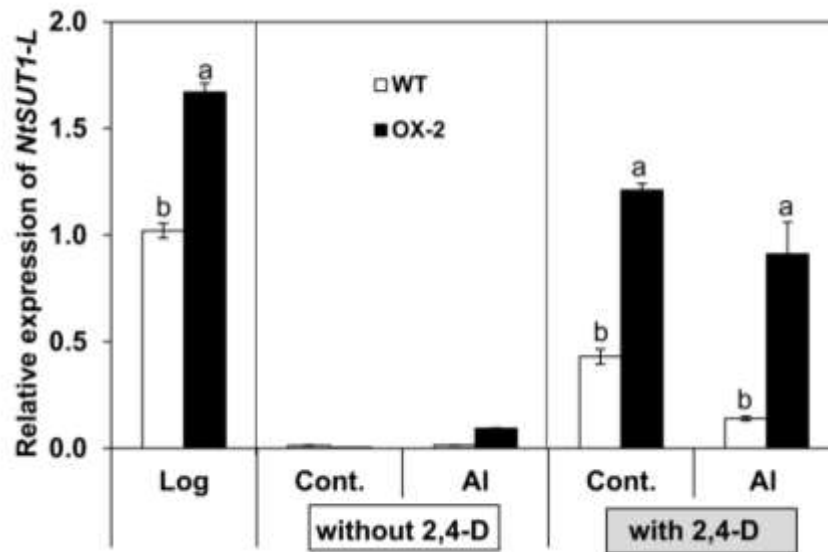


Figure 13. Effects of 2,4-D and Al on *NtSUT1-L* expression in BY-2 cell lines (WT, OX-2).

Cells at the logarithmic phase of growth were treated without (control) or with Al (50 μ M) in the absence or presence of 2,4-D (1.5 μ M) for 18 h. The *NtSUT1* expression levels of the cells were evaluated by real-time RT-PCR analysis using specific primers for *NtSUT1-L*. Relative expression levels were normalized against the values of the *Actin9* transcripts. Each value represents the mean \pm SE of three samples from three independent experiments. For each treatment condition, significant differences between lines are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$.

The uptake rate of ^{14}C -sucrose in the cells of one mL culture basis (containing 10 mg FW cells at a start) was investigated in the presence of 2,4-D in WT, OX-2 and RNAi-1 for 6 h after a start of treatment. Under control treatment, the sucrose uptake rate was slightly higher in OX-2 and slightly lower in RNAi-1 than in WT. During the first 3 h of treatment, the uptake rates in OX-2 and RNAi-1 lines were 124% and 84% of the WT (100%), respectively (Fig. 14A). The addition of Al suppressed the uptake rates in all lines to the level shown by RNAi-1, suggesting that Al blocks the sucrose uptake via NtSUT1, immediately and completely. However, a substantial rate of sucrose uptake was still maintained in the presence of Al (e.g. 66% of the control level in WT).

Soluble sugar contents were investigated at the start (initiation) and after 18 h treatment with or without Al in the presence of 2,4-D. After control treatment, in the cells of one mL culture basis, soluble sugar content increased in all lines (Fig. 14B). The net increases over the initial levels were similar in all lines. Such an increment was, however, completely blocked by Al in all used lines (Fig. 14B, with 2,4-D). To evaluate the findings described above, soluble sugar contents were investigated during treatments in the absence of 2,4-D, where the *NtSUT1* gene expression was suppressed both in WT and OX cell lines (Fig. 13, without 2,4-D). However, again a similar increment of soluble sugar content was observed in WT, OX and RNAi lines after 18-h control treatment, which was completely blocked by Al (Fig. 14B, without 2,4-D).

Taken together, in a simple treatment medium containing Ca, sucrose and MES (pH 5.0), it seems that NtSUT1 transporter only partly contributes to sucrose uptake, and that Al inhibits the sucrose uptake via NtSUT1, in immediate and complete manner. Similarly, an increase in soluble sugar content under control treatment was prevented by Al, and it seems that NtSUT1

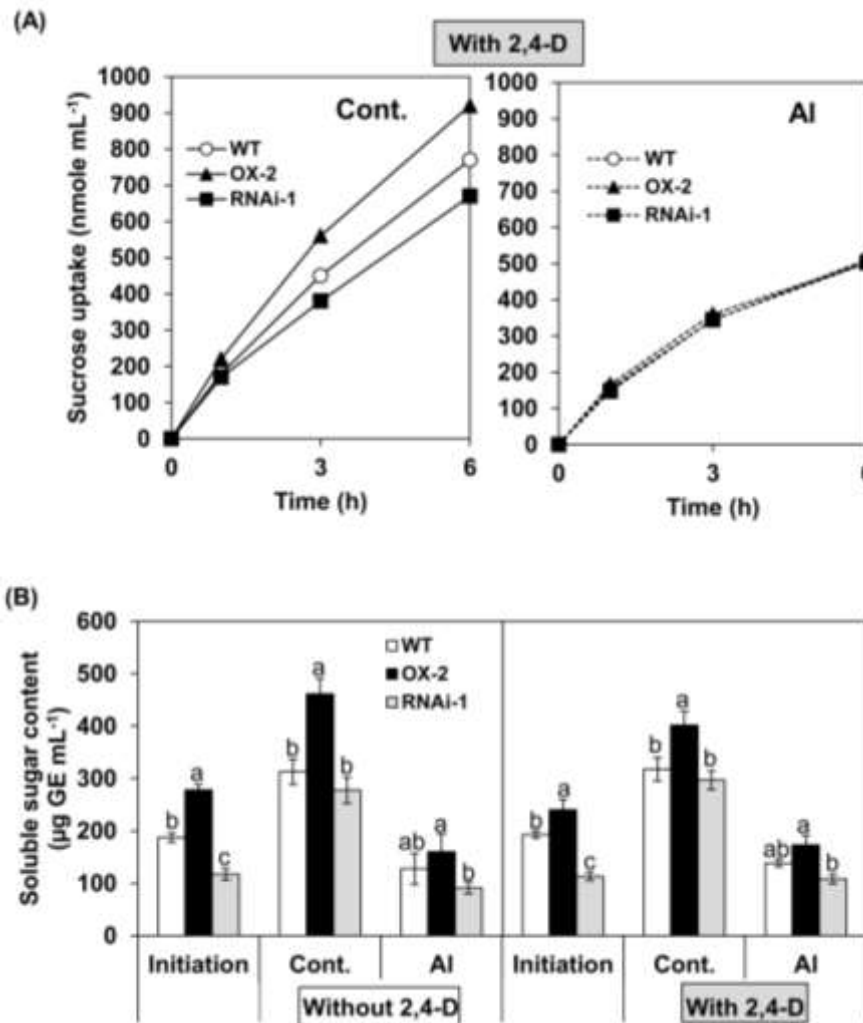


Figure 14. Effects of Al on sucrose uptake rate and soluble sugar content during Al treatment in BY-2 cell lines (WT, OX-2, RNAi-1). Cells at the logarithmic phase of growth were suspended in Al treatment medium at 10 mg FW mL⁻¹ (initiation) then incubated without (control) or with Al (50 μM) in the presence or absence of 2,4-D (1.5 μM). In (A), sucrose uptake was monitored only in the presence of 2,4-D for up to 6 h after the addition of ¹⁴C-sucrose at 0 h. The uptake value was shown per cells contained in one mL aliquot of the culture. Each value represents the average value from two independent experiments. In (B), soluble sugar

contents were determined after 18 h treatment in the absence or presence of 2,4-D as described in Materials and Methods and are shown per cells contained in one mL aliquot of the culture. Each value represents the mean \pm SE of three samples from three independent experiments. For each treatment, significant differences among lines are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$.

transporter is not involved in this process. It could be speculated that Al might inhibit sucrose uptake by other mechanisms (see Chapter II, Discussion) after 6 h during the 18-h treatment, or that Al might enhance the composition of soluble sugar during the treatment. These mechanisms remain to be elucidated.

Sucrose uptake in BY-2 cell lines (WT, OX-2, RNAi) during post-Al treatment culture

To evaluate the contribution of NtSUT1 to Al tolerance mechanisms, the sucrose uptake rates and growth capacity during post-Al treatment cultivation of cells were investigated in control and Al-treated cultures of WT, OX-2 and RNAi-1 lines. From a start to 9 h of the post-treatment culture, no significant uptake of sucrose was detected in all lines (data not shown) but it was detected at 24 h. Therefore, ^{14}C -sucrose was added at 24 h of the post-treatment culture, and sucrose uptake by cells was monitored for up to 9 h (Fig. 15). The initial uptake rates during the first one h were compared between the lines. In control cells, compared to WT, OX-2 exhibited 3.3-fold higher rate, while RNAi-1 exhibited a half rate of ^{14}C -sucrose uptake. In Al-treated cells, OX-2 exhibited 2.5-fold higher rate than WT, while WT and RNAi-1 exhibited the same rates (Fig. 15).

Effect of Al on growth capacity in BY-2 cell lines (WT, OX, RNAi) in the absence or presence of 2,4-D

Growth capacity of the three lines was evaluated after 4 days of growth (recovery) in post-treatment culture. For control cells, OX-2 exhibited 1.6-fold higher growth capacity, while RNAi-1 exhibited lower capacity (48%) compared to WT. For Al treated cells, OX-2 exhibited 2.8-fold higher capacity than WT, while growth capacities of WT and RNAi-1 lines were similarly low (Fig. 16, with 2,4-D). Under these conditions, the relative growth capacity of Al-

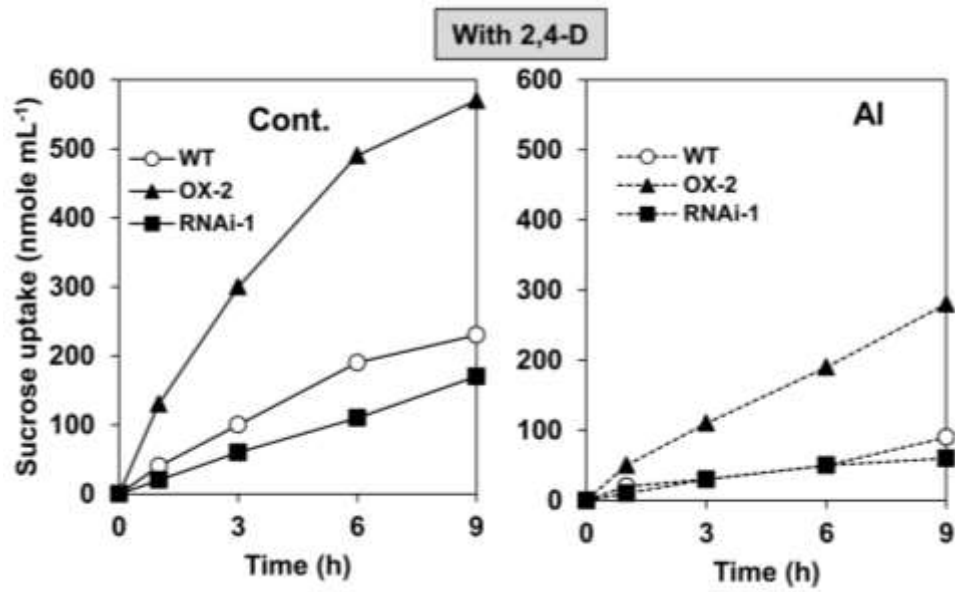


Figure 15. Effects of Al on sucrose uptake rate during post-Al treatment culture in BY-2 cell lines (WT, OX-2, RNAi-1). After Al treatment in presence of 2,4-D (1.5 μ M), cells were transferred into fresh nutrient medium and cultured under standard conditions. 14 C-sucrose was added at 24 h of the post-treatment culture and cellular radioactivity was determined at indicated times. The uptake value was shown per cells contained in one mL aliquot of the culture. Each value represents the average value from two independent experiments.

treated cells over the control cells in each line was 35% (WT), 56% (OX-2) and 36% (RNAi-1), respectively, indicating that OX-2 is more tolerant to Al than WT, while RNAi exhibits Al sensitivity similar to WT. Growth capacity during post-treatment culture was also evaluated in the lines treated with or without Al in the absence of 2,4-D. In the control cells, the highest capacity was observed in OX-2, while the lowest was, as expected, in RNAi-1 line. In Al treated cells, however, the growth capacity of OX-2 was repressed to the WT level (Fig. 16, without 2,4-D), although these Al-treated cells were grown in the standard nutrient medium containing 2,4-D during post-Al treatment culture.

Taken together, it seems that the over-expression of *NtSUT1* during Al treatment was able to confer Al tolerance in otherwise Al sensitive BY 2 cells.

Effect of Al on fresh weight in BY-2 cell lines (WT, OX-2, RNAi-1)

After the treatment in the presence of 2,4-D, FW of cells in culture basis increased in all three lines under control treatment, reaching 155% (WT), 176% (OX-2), 147% (RNAi) of the initial values (100%) respectively, while such increases were not observed in the presence of Al, reaching 113% (WT), 129% (OX-2), 104% (RNAi-1) respectively (Fig. 17). The FW values of three lines were not statistically different in both control and Al-treated cells indicating that *NtSUT1* transporter does not contribute to the FW increment in control cells, and that Al prevents the gain in FW almost completely.

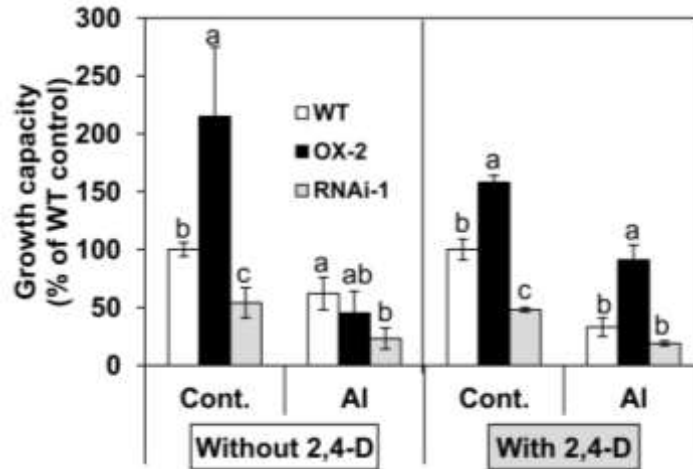


Figure 16. Effects of Al on growth capacity during post-Al treatment culture in BY-2 cell lines (WT, OX-2, RNAi-1).

Growth capacity was determined in the cells which had been treated without or with Al in the absence or presence of 2,4-D, and then cultured for 6 days (for the cells treated without 2,4-D) or 4 days (for the cells treated with 2,4-D), as described in Materials and Methods. Each value represents the mean \pm SE of three samples from three independent experiments. For each treatment, significant differences among lines are indicated with different lower case letters, which were determined by LSD test at $P < 0.05$.

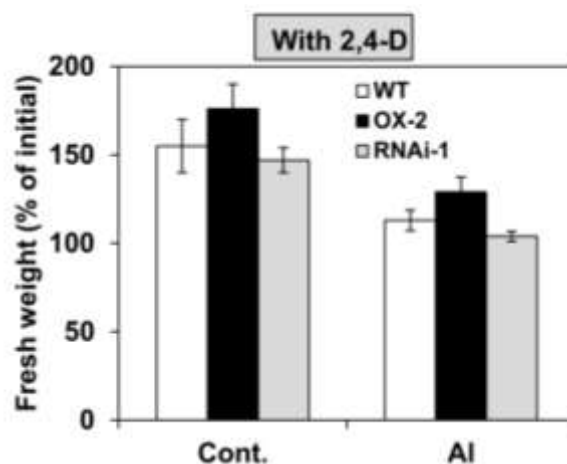


Figure 17. Effects of Al on FW during Al treatment in BY-2 cell lines (WT, OX-2, RNAi-1).

Cells were suspended in Al treatment medium at cell density of 10 mg FW per mL (Initiation) and cultured without (control) or with Al (50 μ M) in the presence of 2,4-D (1.5 μ M) for 18 h. FW of each cell culture was determined at a start (0 h) and 18 h, and the FW at 18 h was shown as % of the initial value. Each value represents the mean \pm SE from three independent experiments. Data among lines are not statistically different.

Discussion

In this chapter, it was found that the expression of the *NtSUT1* under its native promoter or under the CaMV 35S promoter is strongly depending on the presence of 2,4-D. Thereafter, cells were treated in the presence of 2,4-D. I found that Al strongly inhibits the sugar uptake via NtSUT1. Al also inhibits water uptake (evaluated by increment of FW), but it seems that NtSUT1 does not contribute to the water uptake. Most interestingly, I found that over-expression of *NtSUT1* during Al treatment confers higher growth capacity during post-Al treatment culture in BY-2 cells. These results suggest that over-expression of *NtSUT1* could increase Al tolerance in the actively growing cells. The actively growing cells at root apex could be involved.

Gene expression of the *NtSUT1* is auxin dependent

The application of synthetic auxin induced potato sucrose transporter *StSUT1* (84% similarity to *NtSUT1-L*) within a few hours at transcriptional (Harms et al., 1994) and translational (He et al., 2008) levels. The omission of 2,4-D from culture medium triggered several changes, such as starch accumulation, cessation of cell proliferation and cell elongation, while the application of auxin promoted cell elongation in BY-2 tobacco cells (Miyazawa et al., 1999). In this study, it was found that the expression of the *NtSUT1* is regulated by auxin (2,4-D) in WT and OX cell lines (Fig. 13). A CaCl₂ solution is typically used for Al treatments of plant roots. In our experiments, similar medium containing CaCl₂, sucrose, MES-BTP (pH 5.0) has been initially used for Al treatments of tobacco cells (Abdel-Basset et al., 2010). In fact, the responses to Al in tobacco cells in this Al treatment medium were comparable to those of pea roots in a CaCl₂ solution (Yamamoto et al., 2002). However, in this study, it was found that the expression of the *NtSUT1* under the native promoter in WT and the CaMV 35S promoter in OX line strongly

depends on the supply of 2,4-D (Fig. 13). In support of our findings, the presence of auxin responsive elements was already reported in the promoters of sucrose transporters in tomato, rice and *Arabidopsis* (Kühn, 2012). *In silico* analysis of *Arabidopsis* and rice sucrose transporters promoter regions revealed the presence of auxin response element (Ibraheem et al., 2010). One of OCS elements, CCACTGACGTAAGGGATGACGCACAATCC, contains auxin response element, TGACGTAA (Liu et al., 1994; Liu et al., 1997) which was also reported in the CaMV 35S promoter (Zhang and Singh 1994), possibly explaining the requirement of 2,4-D for the augmented expression of CaMV 35S::NtSUT1 construct in BY 2 cells.

In addition to auxin, the expression of sucrose transporter genes has been reported to be regulated by another class of phytohormones, cytokinins (Harms et al., 1994; He et al., 2008). Some variations in response to phytohormones have been reported among *SUT1s* of different plant origins. For example, rice *OsSUT1* was induced by gibberellic acid but not by abscisic acid (Chen et al., 2010), and apple *MdSUT1* was induced by gibberellic acid and abscisic acid but not by auxin (Peng et al., 2011). Abscisic acid responsive elements were also found to be important for the regulation of expression of the *AtSUC1* gene (Hoth et al., 2011). *In silico* analysis indicated the presence of ethylene responsive element in sucrose transporters (Ibraheem et al., 2010). Therefore, for complete evaluation of NtSUT1 and its role in the root system, the effects of multiple phytohormones and their cross talk on the *NtSUT1* gene expression should be considered.

Al inhibits the sucrose uptake mediated by NtSUT1

In the Al treatment medium, the presence of 2,4-D strongly supported the *NtSUT1* expression in WT and OX cells. Under these conditions, Al prevented the uptake of ^{14}C -sucrose via NtSUT1 in complete and immediate manner (Fig. 14A), suggesting that NtSUT1 is one of the likely target

proteins of Al ion in the plasma membrane. The molecular mechanism of Al during inhibition of NtSUT1 has not been elucidated yet. Recently, some functionally important amino acids were proposed for the transport mechanism of rice OsSUT1. These were charged amino acids such as aspartate, glutamate and arginine located at specific sites within membrane spans, and the mutations of these sites (D177N, R188K, D331N, E336Q) led to a complete loss or substantial decrease of transport activity (Sun et al., 2012). These specific sites and amino acids are also conserved in NtSUT1. Since Al ion has a preference for binding the carboxyl groups in aspartate and glutamate, it is likely that Al ion might interact with these critical amino acids to prevent the sucrose transport activity.

In addition to Al affecting NtSUT1 protein levels as described above, the inhibitory effect of Al on the *NtSUT1* expression, especially under its native promoter (in WT) was observed in the present study (Fig. 13). As Al-responsive transcripts, several sugar transporter genes (including hexose transporter) were reported to be increased or decreased in *Arabidopsis* roots, following exposure to Al (Kumari et al., 2008). It remains to be elucidated how Al ion could affect the expression of the *NtSUT1* and other sugar transporter genes.

Chapter IV Expression analysis of sucrose transporters *NtSUT3* and *NtSUT4* in BY-2 cells

Introduction

Sucrose transporter *NtSUT3* belongs to Group 2 of SUTs from dicot plant species and is expressed in late stages of development of flower (Lemoine et al., 1999). No expression of *NtSUT3* was observed in other parts of flower except pollen or anther, therefore it was proposed as late pollen gene as *pmt1* (Scott, 1994). *NtSUT3* seems to supply sucrose or other nutrients during pollen tube growth, because the amount of mRNA level in pollen tube was stable up to 6 h (Lemoine et al., 1999).

NtSUT4 belong to Group 4 SUTs majorly localized in tonoplast and small portion in the plasma membrane and was involved in cell shape in mini protoplast culture (Okubo-Kurihara et al., 2011). The ortholog of *NtSUT4*; *StSUT4* in potato, has diverse role during plant growth and development. Early flowering and tuber production in the *SUT4*-suppressed potato plants exhibited higher sucrose export from leaves and increased sucrose and starch accumulation in sink organs like tubers. The *SUT4* suppression affected the expression of enzymes related to biosynthesis of gibberellin and ethylene. Furthermore, the *SUT4*-suppressed plants could not follow diurnal rhythm for ethylene production (Chincinska et al., 2008; Chincinska et al., 2013). The suppression of *SUT4* affected the expression of circadian rhythm genes such as *StFT*, *StCO* and *STSOC1* which could be involved in a photoperiod- dependent tuberization. Overall, *SUT4* expression seems to be linked to photoreceptor-perceived information, light quality, length of the

day with ethylene biosynthesis and the expression of diurnal regulated genes (Chincinska et al., 2013).

In this chapter, the expression levels of NtSUT3 and NtSUT4 were investigated in BY-2 cells, focusing on the effect of 2,4-D and AI on the expression levels.

Materials and Methods

Expression analysis of the *NtSUT3* and NtSUT4 in AI-treatment medium in the absence or presence of 2,4-D

Cells at the logarithmic phase of growth or after 18 h treatments without (control) or with AI (50 μ M) were harvested, and RNA was extracted as described (see Chapters II and III, Materials and Methods).

For the semi quantitative RT-PCR analysis, the expression of *NtSUT3* and *NtSUT4* was determined by using the primer sets (Table 1), while *Actin9* was used as internal control. For semi RT-PCR, thermal cycling conditions were: 94°C for 2 min; 94°C for 30 sec, 60°C for 30 sec and 72°C for one min using various numbers of cycle (as mentioned in Fig. 18); 72°C for 5 min for final extension cycle, using a thermal cycler (Eppendorf, Hamburg, Germany). PCR products were separated by agarose gel electrophoresis, and visualized by staining with ethidium bromide.

AI treatment conditions

AI treatment conditions were performed as described (see Chapter III, Materials and Methods).

Results and Discussion

Effects of 2,4-D and AI on *NtSUT3* and *NtSUT4* expression

In BY-2 WT cells, the expressions of the *NtSUT3* and *NtSUT4* were detected during the logarithmic phase of growth. Therefore, the effects of 2,4-D and AI on the expression levels were investigated in BY-2 cells after treatment without (control) or with AI for 18 h. In contrast to the *NtSUT1*, expression levels of the *NtSUT3* (Fig. 18A) and *NtSUT4* (Fig. 18B) were not affected by 2,4-D or by AI. To support our observation, expression of the *StSUT4* which is ortholog of *NtSUT4* could not be induced by the application of 2,4-D (He et al., 2008).

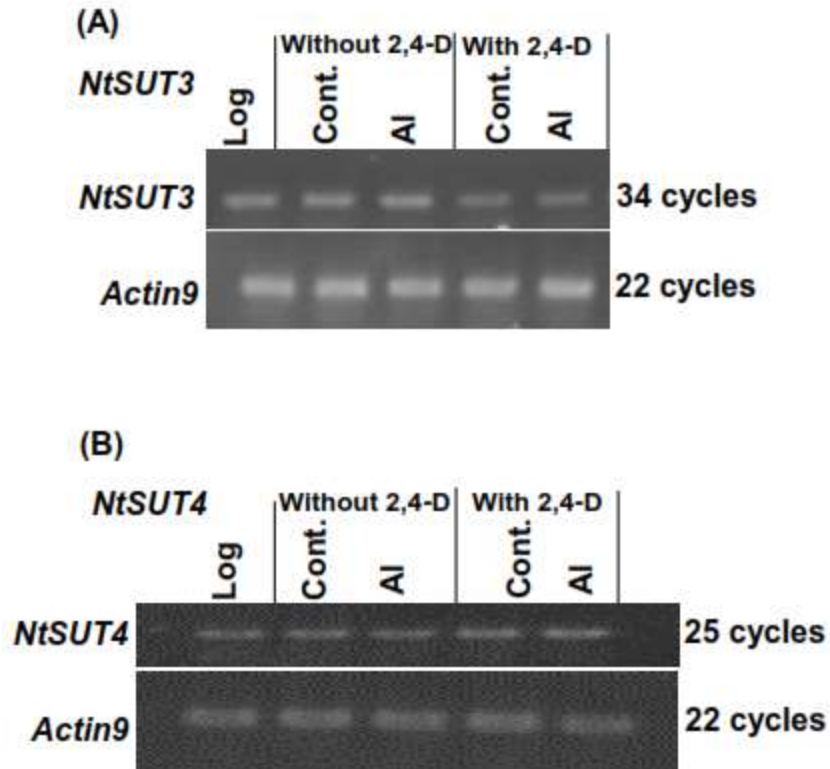


Figure 18. Effects of 2,4-D and AI treatment on expression levels of the *NtSUT3* (A) and *NtSUT4* (B) in BY-2 cell line (WT). Cells were harvested at the logarithmic phase of growth or after 18-h treatments without or with AI in the presence or absence of 2,4-D as described in Materials and Methods. Total RNA was extracted and cDNA was synthesized. Semi-quantitative RT-PCR was performed as described in Materials and Methods.

Chapter V Construction of transgenic tobacco plants of *NtSUT1*

Introduction

The role of sucrose transporter SUT1 has been investigated for plant growth development in various plant species. Therefore, based on the important roles of sucrose and the SUT1 in plant growth and development, it is interesting to investigate the role of NtSUT1 in plant roots under Al stress. For this aim, over-expression and suppression lines of the NtSUT1 transformants were constructed in tobacco plant and was performed preliminary characterization of these lines.

Materials and Methods

Construction of over-expressor (OX) and suppressor (RNAi, antisense) lines of the *NtSUT1-L* in tobacco plant (cv Samsun)

The *NtSUT1-L* cloned from cell line SL was used for the construction of OX (Fig. 19A) and RNAi (Fig. 19C) lines as described (see Chapter II, Materials and Methods). For the construction of antisense tobacco line, the inverted ORF of *NTSUT1* was inserted to the expression vector (Fig. 19B). Plasmid constructs were used for the transformation of leaf explants of young tobacco seedlings grown hydroponically for 4-6 weeks by use of *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) and LBA4404 (Life Technologies). The transformed explants were selected on nutrient agar medium and grown (Wang et al., 2004).

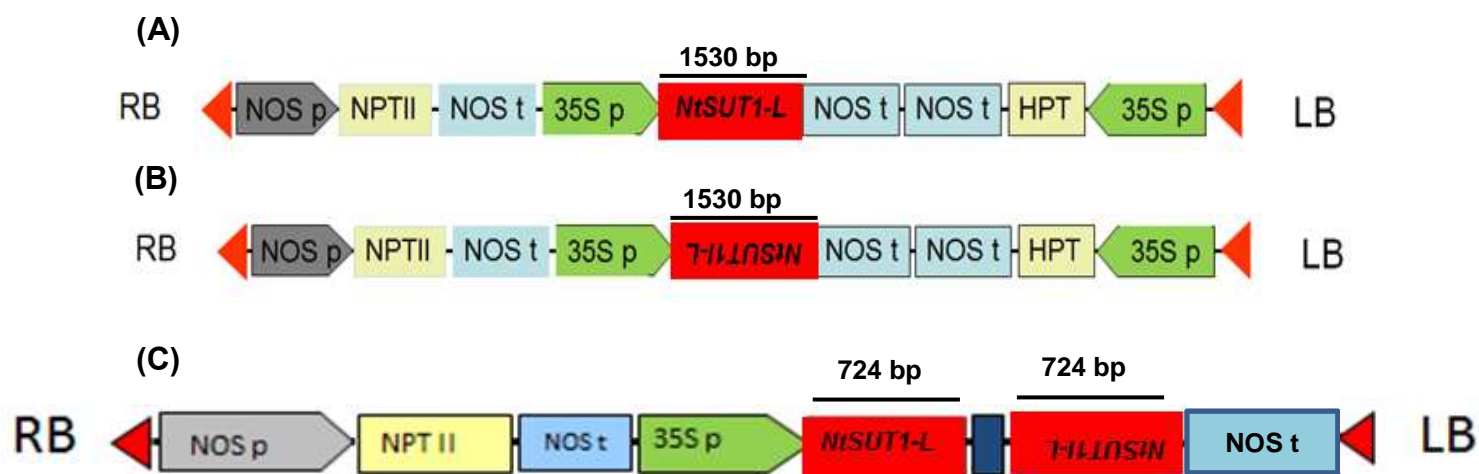


Figure 19. *NtSUT1* constructs for over-expression (A) and suppression lines (antisense and RNAi) (B and C) used to transform tobacco plants.

Growth conditions of T₁ tobacco seedlings

Seeds of the *NtSUT1* transgenic tobacco lines (T₁) were surface sterilized with 0.2% NaOCl and grown on MS agar containing 150 mg L⁻¹ kanamycin (Km) and 100 mg L⁻¹ carbenicillin for the selection of transgenic lines. Hundred seeds of each line were grown. Transgenic lines showing 3:1 ratio of Km^T vs Km^S on MS agar plates were selected in order to obtain transgenic lines carrying probably one copy of *NtSUT1* gene in the genome. The second or 3rd leaf of each transgenic line was used for PCR confirmation of transgene. After PCR confirmation, these T₁ transgenic tobacco lines were transferred from MS plates to the pots containing peat soil and acclimatized for two weeks in growth chamber. The pots were covered with transparent polythene sheet in order to maintain high humidity. Then, polythene sheets were gradually punctured and then removed. Then plants were grown in the green house of P1P level.

T₂ seeds of T₁ transgenic lines were harvested 4-5 months after sowing. Some RNAi and antisense lines exhibited poor growth and started to bloom after 8 months and then T₂ seeds were harvested.

Evaluation of homozygosity of the transgene in T₂ seeds

Transgenic T₂ seeds were surface sterilized with 0.3% NaOCl and were grown for 15-20 days on MS agar plates containing 150 mg L⁻¹ kanamycin (Km) and 100 mg L⁻¹ carbenicillin to determine the homozygosity of the transgene.

AI treatment conditions

Surface sterilized seeds of WT, OX 1-2-12 and RNAi 4-2-2 were placed on mesh and grown by floating the mesh on Ruakura medium which includes low phosphorus (5 µM) (Snowden and

Gardner, 1993) at pH 5.8. After 7 days, the root length of each seedling was measured with ruler and exposed to control and Al treatments in Ruakura medium at pH 4.5 adjusted with HCl. Sucrose was added to the medium at 30 mM. The treatment was performed for 24 h, and then again root length was measured.

Results and Discussion

After 3-4 weeks, seedlings growing on the selective MS agar plates containing kanamycin could be distinguished clearly if they were transgenic or non-transgenic, by the color of green (transgenic) and yellow (non-transgenic) respectively. Only the lines which exhibited 3:1 ratio (green to yellow) were selected as single gene insertion lines for the over-expression line, except for the 1-2 OX line which showed 22:1 ratio (Table 2, Figs. 20). They were grown to obtain T₂ seeds. The seedlings and mature plants of the line 1-2 OX exhibited expanded large leaf size than WT, 1-1 OX line, antisense and RNAi lines (Figs. 20, 21).

In the control treatment without Al, OX line exhibited higher root elongation over 24 h periods than WT and RNAi. After Al treatment, root elongation was inhibited severely in all lines (Fig. 22). Interestingly, the root elongation of RNAi lines was much more inhibited by Al than that in WT and OX lines.

Among 3 OX lines obtained, only one OX line, the NtSUT1 OX line (OX 1-2), exhibited larger leaf size and height than other OX lines and the repression lines (antisense and RNAi). The OX 1-2 line seemed to be inserted probably with multicopies of the NtSUT1 (Table 2). Therefore, the increase in biomass could be due to poly insertion of *NtSUT1* in this line. Repression lines (antisense and RNAi) were delayed in bud formation and flowering and exhibited stunted growth (data not shown), as reported previously (Bürkle et al; 1998).

Table 2. Evaluation of the single gene insertion of the transgene in the *NtSUT1*-transgenic tobacco seedlings (T₁ generation) by antibiotic selection

Line name	No. of total seeds	Germination (%)	Green (Live)	Yellow (Dead)	Ratio
1-1 OX	100	100	70	30	3:1
1-2 OX	100	94	90	4	22:1
1-3 OX	91	34	16	18	1:1
2-1 antisense	100	100	92	8	9:1
2-2 antisense	100	100	74	26	3:1
2-3-2 antisense	100	100	77	23	3:1
4-2 RNAi	100	85	65	20	3:1
4-3 RNAi	100	100	69	31	3:1
5-1 RNAi	100	100	77	23	3:1
5-2 RNAi	100	92	70	22	3:1
5-3-1 RNAi	100	91	69	22	3:1
5-4-1 RNAi	100	100	99	1	99:1

OX lines of tobacco plant (T_1)

Antisense and RNAi lines of tobacco plant (T_1)

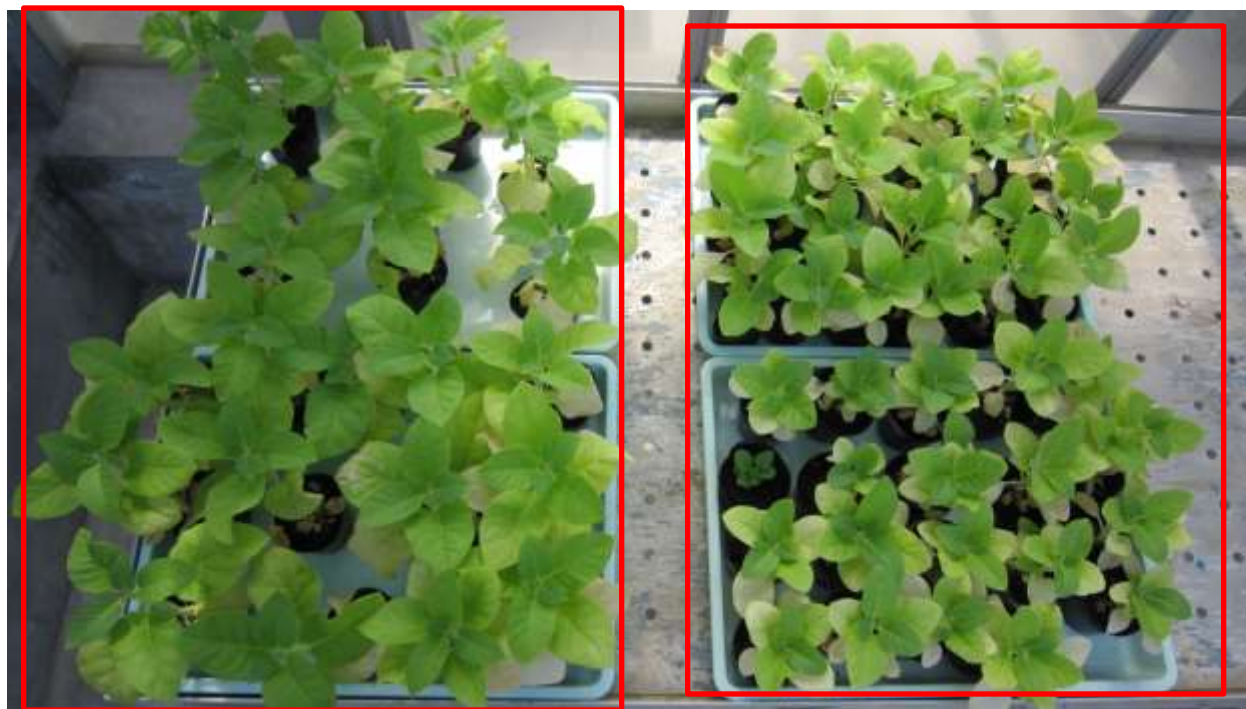


Figure 20. Young seedlings at T_1 generation

Table 3. Evaluation of homozygosity of the transgene in T₂ seeds of OX and RNAi lines

Line#	No. of seeds germinated	Green (Live)	Yellow (Dead)	Comments
Samsun WT	30	0	30	Non transgenic
OX tobacco plant lines				
1-1-1 OX T ₂	63	38	25	2:1
1-1-2 OX T ₂	87	80	7	11:1
1-1-3- OX T ₂	73	56	17	3:1
1-1-4- OX T ₂	60	0	60	Non transgenic
1-1-7- OX T ₂	46	40	6	6:1
1-1-8- OX T ₂	73	73	0	Homozygous
1-1-9 OX T ₂	110	95	15	6:1
1-1-10 OX T ₂	80	67	13	5:1
1-1-11-OX	91	90	1	90:1
1-2-5 OX T ₂	30	30	0	Homozygous
1-2-3 OX T ₂	30	30	0	Homozygous
1-2-10 OX T ₂	30	30	0	Homozygous
1-2-12- OX T ₂	30	30	0	Homozygous
RNAi tobacco Lines				
4-2-2 RNAi T ₂	30	30	0	Homozygous
5-1-1 RNAi T ₂	30	23	10	Heterozygous
4-2-10 RNAi T ₂	30	30	0	Homozygous

T₂ seeds were evaluated for homozygosity on MS agar containing antibiotics (Table 3). Then T₂ seeds of WT Samsun, OX 1-1-8, OX 1-2-12 and RNAi 4-2-2 were grown on MS agar without antibiotics for one month and were transferred to peat soil after one month (Fig. 21).



Figure 21. One-month old tobacco seedlings grown on MS agar were transferred to peat soil and were taken photos immediately.

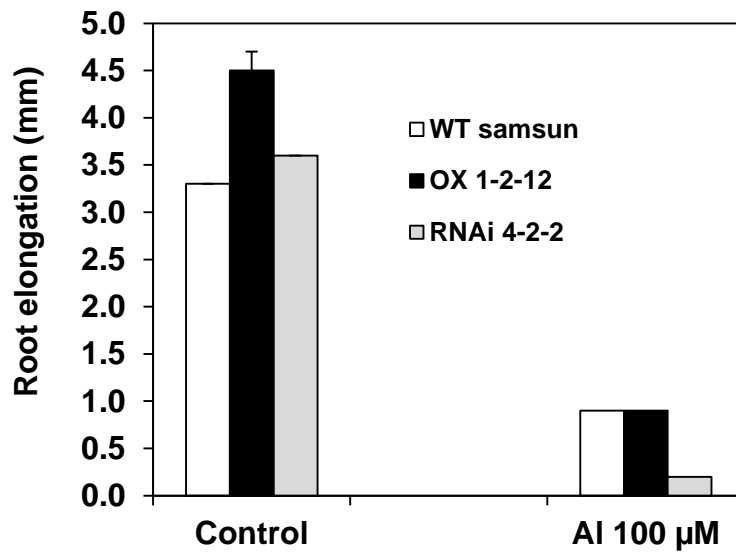


Figure 22. Effect of Al on the root length increment in tobacco seedlings. Seven days old seedlings were treated in Ruakura medium at pH 4.5 with 30 mM sucrose without (control) or with Al (100 µM) for 24 h. Each value represents the mean \pm SE from two independent experiments, using 8-9 seedlings of each tobacco line for each treatment.

The role of *NtSUT1* under Al stress was evaluated, using WT, OX (1-2-12) and RNAi (4-2-2) in T₂ generation and the preliminary result suggests a contribution of NtSUT1 to root growth under Al stress. The root growth under Al stress should be elucidated in the presence of 2,4-D since it was found that the expression of the *NtSUT1* under native and CaMV 35S promoter requires 2,4-D in BY-2 cell system.

General discussion

Sucrose transporters have essential roles in plant growth and development. Therefore, the role of sucrose transporter NtSUT1 was studied in BY-2 cells during normal growth and under Al stress. NtSUT1 sucrose transporter belongs to Group 2 SUTs from dicot plant species (Figs. 1, 5). Due to amphidiploid nature, tobacco (*Nicotiana tabacum*) has two orthologous of *NtSUT1*. From tobacco cultured SL cell line, *NtSUT1-L* and *NtSUT1-S* were cloned and real time RT-PCR expression analysis revealed that *NtSUT1-L* is majorly expressed gene (Fig. 6). The plasma membrane localization of NtSUT1 was confirmed (Fig. 7) as previously reported (Schmitt et al., 2008).

NtSUT1 transformed tobacco cell lines (OX, RNAi) were used to investigate the role of *NtSUT1* in normal growth. In the cells at the logarithmic phase of growth, overexpressed cell lines (OX-1, OX-2) showed higher levels of soluble sugar content and higher growth rates (mass doubling time) than WT and RNAi line showed lower soluble sugar content and lower growth rate (Figs. 10, 11 and 12). During the actively growing phase, however, the sucrose uptake rate was not remarkably lower in RNAi than WT, suggesting possible involvement of other mechanisms of sucrose uptake such as endocytosis or cell wall invertase.

The role of NtSUT1 in growth under Al stress was evaluated. Several reports suggest the effects of Al on sugar uptake or sugar metabolisms at physiological level (Graham, 2002; Abdalla, 2008; Abdel-Basset et al., 2010) as well as transcriptomic level (Kumari et al., 2008).

In this study, BY-2 cell line and its transformants of the *NtSUT1-L* (OX-2, RNAi-1) were treated without or with Al in simple Ca-sucrose medium at pH 5.0 adjusted with MES-BTP. Surprisingly, it was found that the expression under both native and CaMV 35S promoters

depends on 2,4-D. Subsequently, 2,4-D was added in Al treatment medium. It was found that Al ion strongly inhibits sucrose uptake via NtSUT1 protein, suggesting that NtSUT1 could be one of the target proteins. However, interestingly, it was found that the over-expression of *NtSUT1* in OX line during Al treatment confers both higher rate of sucrose uptake and higher growth rate during post-Al treatment culture than WT. Thus, the overexpression of *NtSUT1* confers Al tolerance in BY-2 cells. It is speculated that functionally important amino acids, aspartic acid and glutamic acid in rice (Sun et al., 2012) also conserved in NtSUT1 (Fig. 23), due to carboxylic group could be target sites of Al^{3+} ion hence sucrose uptake is immediately and completely blocked (Sameeullah et al., 2013). It remains to elucidate the molecular details to explain how Al inhibits sucrose uptake via the *NtSUT1* and how overexpression of the *NtSUT1* during Al treatment confers higher growth capacity. Here is shown my working hypothesis to explain how the overexpression of *NtSUT1* confers Al tolerance (Fig. 24). It seems Al ion to be a strong and irreversible inhibitor of the *NtSUT1* function. Therefore, when cells were transferred into Al-free medium the higher accumulation level of the *NtSUT1* transcript in OX line than that WT under Al stress (Fig. 13) could lead to higher uptake level of sucrose during post Al treatment culture.

It was found that both *NtSUT3* and *NtSUT4* are also expressed in BY-2 cells. Contrast to the *NtSUT1*, the expression of the *NtSUT3* and *NtSUT4* did not depend on 2,4-D and was not affected by Al. It is interesting to elucidate possible functions of *NtSUT3* and *NtSUT4* under Al stress.

Preliminary results of NtSUT1 in transgenic tobacco suggest positive roles in root growth under normal and Al stress conditions. Further study should be performed using these transgenic lines under hydroponic culture in the presence of 2,4-D or IAA.

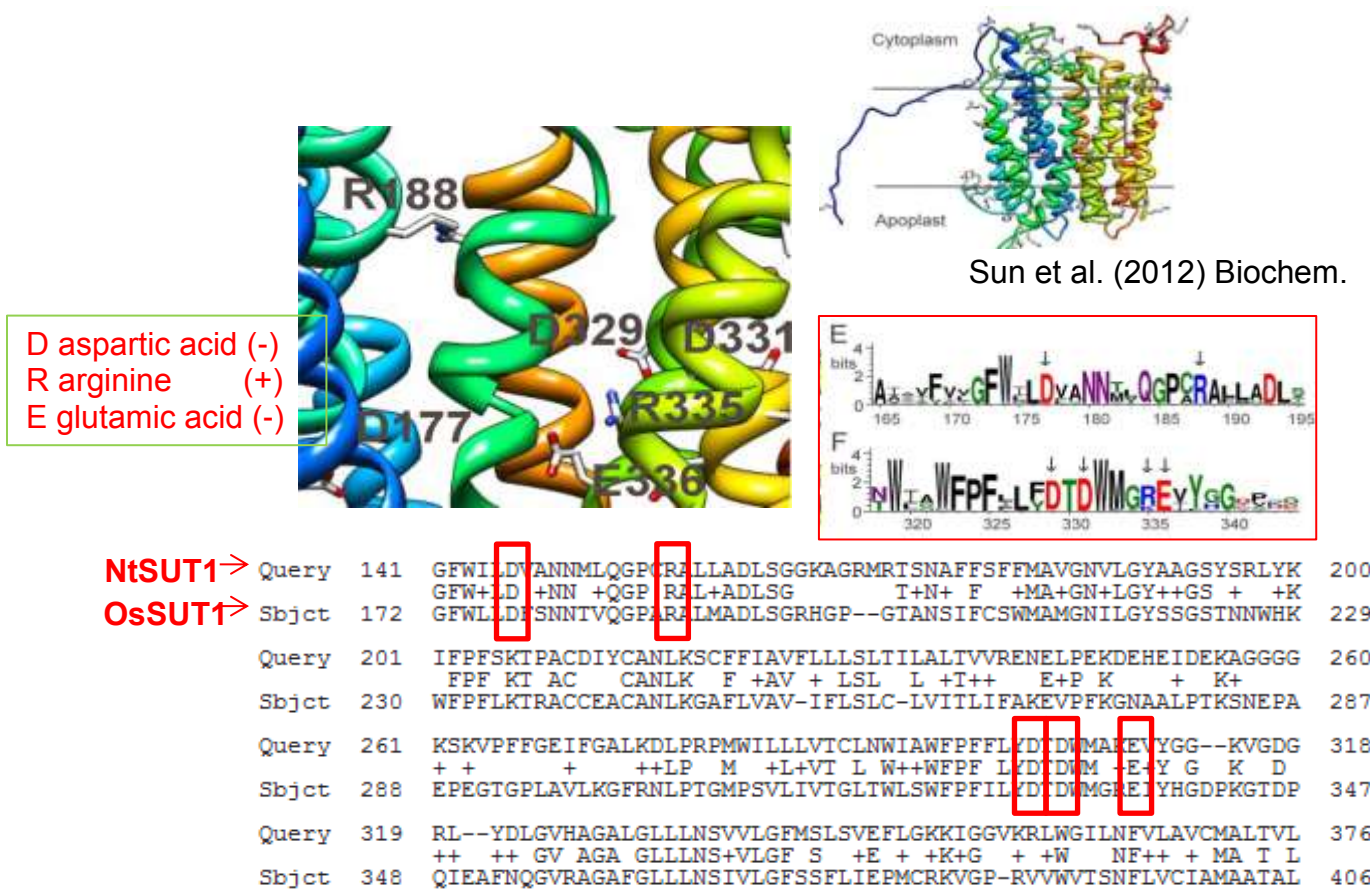


Figure 23. Putative functionally important amino acids for sucrose uptake in NtSUT1 based on OsSUT1 as reported by Sun et al. (2012).

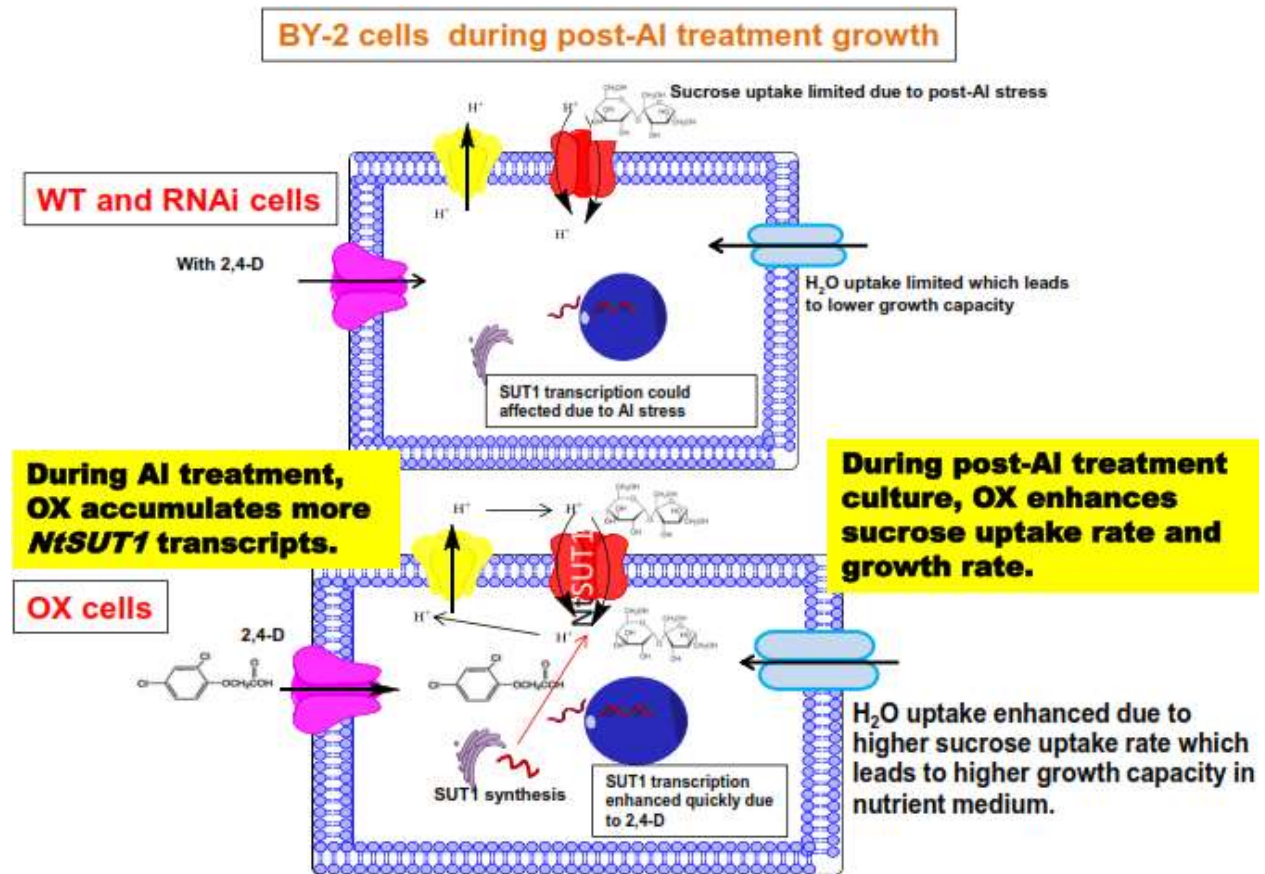


Figure 24 (B) BY-2 cells during post-Al treatment culture in nutrient medium.

A working hypothesis illustrating possible interactions of Al ion with NtSUT1 and other proteins in the plasma membrane. A model illustrates about the sucrose uptake via NtSUT1 in the presence of 2,4-D under Al stress. During Al treatment even in the presence of 2,4-D, sucrose uptake mediated by NtSUT1 is inhibited by Al ion (Fig. 24 A). However, during the post-Al treatment culture in nutrient medium, NtSUT1 works functionally to uptake sucrose and hence enhances an increase in fresh weight (Fig. 24 B). Transcription of *NtSUT1* could be activated by 2,4-D which is incorporated into cells via PINs (auxin transporters) or ABP1 (high affinity auxin binding protein 22 kD). Under the conditions, NtSUT1 transcripts might be accumulated and be ready to be translated to NtSUT1 protein, when cells were transferred into Al-free nutrient

medium. The replacement of functional NtSUT1 protein from the Al-inactivated NtSUT1 should be quicker in OX than in WT, leading to a supply of carbon source to repair the damage caused by Al and to start regrowth, more efficiently in OX than that in WT.

Summary

The role of plasma membrane-localized sucrose transporter (NtSUT1) was investigated using cultured tobacco cell line BY-2. Since tobacco (*Nicotiana tabacum*) is an amphidiploid, it has two *SUT1* orthologs which were cloned from tobacco cell line SL and were named as *NtSUT1-L* and *NtSUT1-S*. The expression analysis of *NtSUT1* in actively growing SL cells at the logarithmic phase of growth revealed that *NtSUT1-L* was majorly expressed. The localization of NtSUT1-L in the plasma membrane was confirmed, using heterologous expression system of onion epidermal cells. For further functional analysis under normal and aluminum (Al) stress conditions, the *NtSUT1-L* was used to construct over-expressor (OX) and suppressor (RNAi) lines in BY-2 wild type (WT) cells.

BY-2 cell lines (WT, OX, RNAi) were investigated during normal growth condition in a modified Murashige-Skoog (MS) medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D). Expression analysis of *NtSUT1* at transcriptional and translational levels indicated higher expression of *NtSUT1* in OX and negligible expression in RNAi compared to WT. The sucrose uptake rate during logarithmic phase was determined by use of radio-tracer; OX line exhibited higher sucrose uptake rate than WT and RNAi line. However, the difference in sucrose uptake between WT and RNAi line was not remarkable, suggesting the sucrose uptake in RNAi line via other mechanisms different from NtSUT1, such as endocytosis or by the cleavage of sucrose by cell wall invertase and subsequent uptake by hexose transporters. The OX and RNAi lines exhibited significantly higher and lower soluble sugar contents than WT, respectively, and soluble sugar contents were negatively correlated to the time necessary to double fresh weight in culture in these lines. These results indicated that NtSUT1 enhances growth rate by increasing soluble sugar content under normal growth condition in nutrient medium.

BY-2 lines (WT, OX, RNAi) were used for investigation of the role of NtSUT1 under Al stress condition. For Al treatment, cells were incubated without (control) or with Al (50 μ M) in a simple medium containing 3 mM CaCl₂ and 88 mM sucrose at pH 5.0 adjusted with 20 mM MES-BTP. Using this medium, it was revealed that *NtSUT1* expression under its native promoter or under the control of strong constitutive cauliflower mosaic virus (CaMV) 35S promoter was strongly dependent on the presence of 2,4-D. Thereafter, cells were treated in the presence of 2,4-D, under the treatment conditions. Then, it was found that Al ion inhibits expression of *NtSUT1* more strongly in WT cells than that in OX cells. During 6 h after a start of the control treatment, sucrose uptake rates were slightly higher in OX and lower in RNAi than that in WT, respectively. The addition of Al reduced the sucrose uptake rates immediately in all lines to the level of RNAi line indicating that Al inhibits sucrose uptake via NtSUT1. Taken together, these results suggest that Al ion inhibits NtSUT1 at transcriptional level (*NtSUT1* expression) and also at functional level (sucrose uptake).

During the post-Al treatment culture of control and Al-treated cells in Al-free nutrient medium, sucrose uptake rates were much higher in OX compared to WT and RNAi lines, which were closely and positively correlated with the growth capacity of the cells. Judging from the growth capacity of Al-treated cells relative to that of control cells, OX cells were more tolerant to Al than WT and RNAi.

Taken together, we conclude that over-expression of *NtSUT1* confers higher growth capacity in actively growing cells under normal growth condition as well as under Al stress.

The OX and RNAi lines of the *NtSUT1* were constructed in a whole plant system using cv. Samsun. The OX line at T₂ generation indicated larger leaf size and longer root length than WT, indicating positive role of NtSUT1 in normal growth. After Al treatment in Ruakura

medium, RNAi line exhibited shorter root length than OX and WT. These preliminary results suggest a positive role of NtSUT1 in root growth capacity under both normal and Al stress conditions, which should be elucidated in the future.

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