Functional Characterization of Vacuolar and Plastidic sugar transporter genes within the “Major Facilitator Superfamily” of Arabidopsis thaliana

Funktionelle Charakterisierung von Genen für vakuoläre und plastidäre Zuckertransporter aus der “Major Facilitator Superfamily“ in Arabidopsis thaliana

vorgelegt von
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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>2PI</td>
<td>Protease Inhibitor</td>
</tr>
<tr>
<td>3-OMG</td>
<td>3-Ortho Methylglucose</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid(s)</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisicacid</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetate</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AtXYL</td>
<td><em>Arabidopsis thaliana</em> Xylose transporter</td>
</tr>
<tr>
<td>AtVGT</td>
<td><em>Arabidopsis thaliana</em> Vacuolar Glucose Transporter</td>
</tr>
<tr>
<td>BAR</td>
<td>Basta-Resistance</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>Col</td>
<td>Columbia</td>
</tr>
<tr>
<td>DAG</td>
<td>Days after germination</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy-Nucleotidetriphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylendiaminetetraaceticacid</td>
</tr>
<tr>
<td>Frc</td>
<td>Fructose</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>Glc6-P</td>
<td>Glucose 6-Phosphate</td>
</tr>
<tr>
<td>GPT</td>
<td>Glc6-P/Pi Translocator</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>T-DNA left boarder</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltosebinding Protein</td>
</tr>
<tr>
<td>mcs</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>µM</td>
<td>micro molar</td>
</tr>
<tr>
<td>NOS</td>
<td>Nopalin-Synthetase</td>
</tr>
<tr>
<td>oN</td>
<td>Overnight</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly ethylene glycol</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganicphosphate</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazin-N-N’-bis (2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>RB</td>
<td>T-DNA right boarder</td>
</tr>
<tr>
<td>Rib</td>
<td>Ribose</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Suc</td>
<td>Sucrose</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetra methyl ethylene diamine</td>
</tr>
<tr>
<td>TP</td>
<td>Triose Phosphate</td>
</tr>
<tr>
<td>TPT</td>
<td>Triose phosphate / Phosphate Translocator</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-(hydroxyl methyl)-1,3-propanediol</td>
</tr>
<tr>
<td>5’ UTR</td>
<td>5’ Un-translated region</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-3-D-galactoside</td>
</tr>
<tr>
<td>X-GLUC</td>
<td>5-bromo-4-chloro-3-indolyl-3-D-glucuronide</td>
</tr>
<tr>
<td>Xyl</td>
<td>Xylose</td>
</tr>
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MIPS-Numbers of *AtXYLs*:

- *AtVGT1*  
  At3g03090
- *AtVGT2*  
  At5g17010
- *AtXYL3*  
  At5g59250
1. Introduction

Sugar transport in plants

Higher plants represent a complex physiological mosaic of autotrophic and heterotrophic tissues. Carbon fixation occurs mainly in mesophyll cells of mature leaves, which are net exporters of sugars (source), while heterotrophic (sink) tissues depend on the import of carbohydrates.

Besides their role as carbon and energy source, sugars can act as regulatory signals that affect expression levels of several genes controlling key processes and hence plant development. Therefore appropriate partitioning of assimilates between source and sink is essential. Furthermore, this source/sink connection has to be highly regulated in order to allow adaptation of plant development to internal as well as external factors.

The transport of sugars across the membranes is fundamental to this allocation of assimilates and thus in living organisms sugar transporter gene families have evolved which are expressed at different developmental stages. In most plants, sucrose (is the main transport form of carbohydrates delivered by the phloem) and hexoses (which are obtained upon sucrose hydrolysis by extracellular invertases under certain conditions) are the main substrates for this carrier mediated transmembrane transport (Ward et al., 1998; Büttner and Sauer, 2000; Williams et al., 2000). To date, a great number of sugar transporters have been identified in plants (Sauer and Tanner, 1989, 1990; Bush, 1993) and many of them are characterized with respect to their transport properties, which are often studied by transgenic expression in yeast (Sauer and Tanner, 1993; Sauer and Stolz, 1994) and their expression patterns and functions in planta. Up to now, most known plant monosaccharide and disaccharide transporters actively translocate sugars across membranes driven by a proton electrochemical potential (Stadler et al., 1995). The tissue and cellular expression pattern of the respective genes indicate their specific and sometimes unique physiological tasks. The disaccharide symporter genes isolated were especially transcribed in mature leaves (Bauke Ylstra et al., 1998) whereas monosaccharide transporter genes were primarily transcribed in sink tissues (Sauer and Tanner, 1993). Some play a purely nutritional role and supply sugars to cells for growth and development, whereas, others involved in generating osmotic gradients required to drive mass flow or movement (Lorraine E. Williams et al., 2000).
On one hand, sugar transport occurs between different parts of the plant as long distance transport, and on the other hand for compartmentation within the cell. After the sucrose synthesis by photosynthetic CO$_2$ fixation in green leaves or glucose production by starch degradation, the soluble carbohydrates are transported to the various sinks via long distance transport. In source leaves, sucrose is loaded into the phloem cells symplastically and/or apoplastically (Van Bel, 1993). Symplastic transport requires symplastic connections called plasmodesmata, while the apoplastic phloem loading needs an energy-dependent active transport system, i.e. a sucrose transporter. In ‘sink’ tissues, either direct uptake of sucrose by a sucrose transporter occurs, or sucrose is hydrolyzed by apoplastic invertases and the resulting hexoses will be taken up by cells via hexose (monosaccharide) transporters. The subsequent intracellular compartmentation of sugars involves movement of solutes within a cell i.e between chloroplast, cytosol, vacuoles and mitochondria (Pollock and Kingston-Smith, 1997). *Arabidopsis thaliana* has more than 60 closely related genes encoding 53 ORFs encoding monosaccharide or cyclic and linear polyol transport proteins together included in large super family called MFS (Saier *et al*., 1999) and 9 ORFs encoding disaccharide transporter proteins (Lalonde *et al*., 1999). Carbohydrate transport across the membranes of different compartments via specific translocator genes is particularly discussed in this thesis.

**Sugar transport across the plasma membrane**

Any nutrient taken up by any cell must, at some stage, pass the plasma membrane (Sondergaard *et al*., 2004). Inside a plant tissue, a given solute can diffuse from cell to cell via plasmodesmata, which form a cellular continuum, the symplast. Plasmodesmal transport by diffusion is not very effective and for long-distance transport, the nutrient in question might have to leave the symplast. Specialized cells throughout the plant body serve as transport interfaces between symplast and apoplast, and intense transport occurs across the plasma membrane of these cells (Sondergaard *et al*., 2004).

The fluxes of carbohydrates across the plasma membrane of plant cells, is mainly catalyzed by hexose and sucrose proton symporters. The first plant sugar transporter genes were cloned from the green algae *Chlorella kessleri* by exploiting their rapid induction upon the addition of hexoses to the growth medium. The differential screening of cDNAs from autotrophic versus heterotrophic cells thus enabled the cloning of HUP1 (hexose uptake; Sauer and Tanner, 1989). Since then, numerous monosaccharide and disaccharide transporters in *Arabidopsis thaliana* and other species have been isolated, cloned and characterized. The
biochemical function and expression patterns of plasma membrane sugar transporters are described below.

### Disaccharide transporters of the plasma membrane

The first sucrose transporter in *Arabidopsis thaliana* has been identified by functional complementation of a yeast mutant, which cannot cleave sucrose extracellularly but internally (Riesmeier *et al.*, 1992). In total, 9 members of the sucrose transporter genes (named AtSUCs or AtSUTs) have been identified (Lalonde *et al.*, 1999) to date, of which *AtSUC6* and *AtSUC7* were found to be pseudogenes not encoding a functional protein (Sauer *et al.*, 2004). The sucrose transporting properties of the pollen specific *AtSUC1* (Ruth Stadler *et al.*, 1999) and companion cell specific *AtSUC2* were shown by Sauer and Stolz (1994) with $K_m$ values of about 450 $\mu$M and 530 $\mu$M, respectively. *AtSUC3* is a distinct member of the sucrose transporter family with extended N-terminus and middle loop, localized in sieve elements and upregulated in response to wounding (Mayer *et al.*, 2000, 2004). Schulze *et al.* (2000) reported a $K_m$ for sucrose of 11.7 mM, whereas Meyer *et al.* (2000) reported a considerably lower $K_m$ value of 1.9 mM. Interestingly, the endosperm specific sucrose transporter *AtSUC5* (Baud *et al.*, 2005) is also mediating the transport of Biotin (Vitamin H). Functional comparison of the *AtSUC5* transporter with previously characterized plant sucrose transporters revealed that biotin transport may be a general and specific property of all plant sucrose transporters (Ludwig *et al.*, 2000). The *AtSUC8* and *AtSUC9* are expressed in floral organs and the corresponding proteins transport sucrose with $K_m$ values of 0.15 mM and 0.5 mM, respectively. *AtSUC8* is the only sucrose transporter insensitive to p-(chloromercuri)-benzene sulfonic acid (PCMBs) (Sauer *et al.*, 2004). The transport properties of *AtSUC4* have been investigated in heterologous yeast expression system, demonstrating that *AtSUC4* is transporting sucrose with a $K_m$ value of 11.6 mM (Weise *et al.*, 2000), however subcellular localization of this protein is still ambiguous (see Discussion).

Several homologs of these sucrose transporters were also identified and characterized in other species like *Plantago major*, *Nicotiana tobaccum*, *Solanum tuberosum* and in other species. However, (except in *AtSUC2* mutant in which, dramatic reduction in plant size and a strongly decreased germination capacity of mutant seeds was observed (Gottwald *et al.*, 2000)) the analysis of single T-DNA insertion plants for these genes did not yield any significant phenotypes, revealing redundancy in their function.
Monosaccharide transporters of the plasma membrane

The ability to transport glucose across the plasma membrane is a feature common to nearly all cells from simple bacteria to highly specialized mammalian neurons. In the model plant Arabidopsis thaliana, 14 plasma membrane monosaccharide transporters (AtSTPs) were identified among the 53 members of the MST-like gene family (www.arabidopsis.org/browse/genefamily/Monos.jsp). AtSTP1, the first identified member of this family (Sauer et al., 1990), was characterized as a high affinity H⁺/symporter which is transporting several monosaccharides (Boorer et al., 1994, Stolz et al., 1994) and is expressed in leaves, stem, flowers and root (Sauer et al., 1990).

In contrast, AtSTP3 is a low affinity monosaccharide-H⁺ symporter with a $K_m$ for D-glucose of 2 mM (Büttner et al., 2000). Interestingly, AtSTP3 is expressed in all the green leaves such as cotyledons, rosette leaves, vasculine leaves and also in sepals, which is contrary to the sink-specific expression of all other AtSTPs. Furthermore, the promoter activity of AtSTP3 is upregulated during wounding. AtSTP3 is believed to function in the retrieval of monosaccharides that were released during cell damage and cell wall degradation (Büttner et al., 2000).

A surprisingly high number of AtSTP genes were expressed during pollen development. AtSTP2 is the first identified pollen specific MST in Arabidopsis, characterized as a high affinity, low specificity monosaccharide carrier. Immunolocalization studies revealed that AtSTP2 expression is confined to the male gametophyte especially during callose degradation (Sauer et al., 1999). AtSTP6 is also expressed during pollen maturation stage and in germinating pollen, probably functioning in supplying adequate sugars for germinating pollen and/or for growth of the pollen tube (Scholz-Starke et al., 2003). In contrast, AtSTP4, AtSTP9 and AtSTP11 are expressed in pollen grains and in pollen tubes. Even though the mRNAs of AtSTP4 and AtSTP9 are found during early pollen development, the protein could be detected only in mature pollen and pollen tube (Schneidereit et al., 2003). This preloading of mature pollen with specific mRNAs indicates an essential role for monosaccharide transport during pollen germination and pollen tube growth. In addition, AtSTP4 was found to be regulated by abiotic and biotic stresses (Truernit et al., 1996, Williams et al., 2003) and to be involved in increased glucose-uptake in response to powdery mildew infection, which creates an artificial sink in the plant (Williams et al., 2003). In contrast to other low specificity AtSTPs, AtSTP9 showed a high selectivity for glucose with a $K_m$ of 84 µM (Schneidereit et al., 2003). AtSTP11 is another pollen specific, high affinity
(K_m=25 µM) MST, expressed exclusively in pollen tubes (Schneidereit et al., 2005). Furthermore, microarray gene expression data (AtGenExpress Development) suggest a pollen specific expression also for AtSTP10 (Deborah A. Johnson et al., 2006). AtSTP13 is upregulated upon external stimuli like salt stress (Gong et al., 2001). Besides the transport properties of all the other STPs characterized so far, AtSTP14 is the first Arabidopsis MST which does not accept glucose as a substrate but instead, transports galactose and with lower rates xylose, both being cell wall components (Büttner M., unpublished). AtSTP7, AtSTP8, AtSTP10 and AtSTP12 were not fully characterized so far. No transport function could be identified for AtSTP5 when expressed in yeast (Barbara Hannich, Diploma thesis 2002). Interestingly, the AtSTP5 coding sequence has several divergent regions when compared in different ecotypes and thus was concluded to be a pseudogene. None of the so far identified AtSTP mutant plants show phenotypes, indicating a functional redundancy of these transporters.

Within the MST-like gene family, additional plasma membrane transporter genes were found, which code for transporters of linear and cyclic polyols. The corresponding cDNAs have been cloned and studies on function and transport properties of the encoded proteins have been initiated for most of the members (Klepek et al., 2005; Schneider et al., 2006).

**Sugar transport across the plastidic membranes**

Among the various types of plastids in plants, the chloroplast is the best characterized. Production and partitioning of photosynthetic carbon is one of the major determinants of the plant productivity and quality. Several phosphate translocators located across the chloroplast membranes, play a significant role in distributing the photosynthates throughout the plant.

In plastids, the family of phosphate translocators consists of at least three different members (Flügge, 1999) mediating transport of phosphorylated organic compounds such as TP or phosphoenol pyruvate (PEP) or glucose-6-Phosphate (Glc-6-P) in counter exchange with inorganic phosphate (Pi). These translocators are termed as Triose phosphate/phosphate translocator (TPT), Phosphoenol pyruvate/phosphate translocator (PPT) and glucose-6-Phosphate/Phosphate translocator (GPT) based on their substrate specificity. Analysis of the Arabidopsis genome revealed the complete set of 16 plastidic phosphate translocator (pPT) genes, coding for TPT, xylulose phosphate/phosphate translocator (XPT), PPT and GPT (Knappe et al., 2003). The carbon, fixed during the day from photosynthesis, exported from the chloroplasts into the cytosol in the form of TP as a precursor for sucrose biosynthesis in
the light is mediated by TPT (Fliege et al., 1978; Flügge et al., 1989) and an Arabidopsis mutants lacking TPT showed increased synthesis of starch compared to WT.

Although it is well known that some of the photosynthate is exported to the cytosol across the chloroplast envelope, a part of it is stored transiently upon conversion to assimilate starch when the demand for carbohydrates is reduced. Eventually, during dark phase the starch is degraded to maltose and glucose and will be exported to supply carbohydrates for sucrose synthesis or various metabolic reactions. An adenylate translocase system (ADP-glucose pyrophosphorylase) exists in the spinach chloroplast envelope membranes, utilizes glucose1-phosphate and photochemically generates ATP for the synthesis of ADP-glucose which serves as the immediate glycosyl donor for starch biosynthesis (Heldt, 1969).

Apart from the above mentioned phosphorylated sugars, unphosphorylated carbohydrates like glucose and maltose can be transported across the plastidic envelope (Schleucher et al., 1998). In C3 plants, a specific glucose transporter, pGlcT required for the export of glucose from the stroma as a product of amylolytic starch degradation was identified (Weber et al., 2000). A hexokinase anchored in the outer envelope membrane of chloroplasts converts glucose to glucose-6-Phosphate and thus establishes the concentration gradient of glucose required for export (Wiese et al., 1999). Maltose is the intermediary product during starch degradation and conversion to sucrose. A maltose transporter MEX1, identified and analyzed in Arabidopsis chloroplasts revealed that, maltose also is the predominant sugar being exported from the chloroplasts during night (Niittylä et al., 2004).

In contrast to the chloroplasts, starch biosynthesis in plastids of storage tissue (amyloplasts) proceeds continuously (Mohlmann et al., 1997). As most heterotrophic plastids do not possess fructose 1-6 bis phosphatase enzyme, intermediates to support the metabolic activities are imported from the cytosol (M. J. Emes and H. E. Neuhaus, 1997). Phosphate antiporters like hexose phosphate/phosphate translocator (HPT) which mediates the transport of Glc6-P or Glc1-P in exchange with Pi and an adenylate translocator that mediates ADP-glucose transport (Naeem et al., 1997; Wischmann et al., 1999) are involved in carbon transport to amyloplasts. The carbon transported into amyloplasts will be used for starch biosynthesis and the oxidative pentose phosphate pathway (Kammerer et al., 1998). It was already proved that glucose 6-phosphate (Glc6-P), glucose 1-phosphate (Glc1-P) and ATP or ADP Glucose (ADPGlc) will be imported to amyloplasts for starch biosynthesis during the day (M.J. Emes and H.E Neuhaus, 1997; Kammerer et al., 1998; Karsten Fischer et al., 2000). Genome analysis inferred existence of 16 such transporters in Arabidopsis thaliana. Analysis
of two plastidic GPTs (AtGPT1 and AtGPT2) revealed that disruption of AtGPT1 gene, leads to small and flattened pollen grains with reduced number of lipid bodies and vacuoles, and partial impairment of embryosac and seed development (Knappe et al., 2003 and Patrycja Niewiadomski et al., 2005).

Despite the plasma membrane sugar transporters, which did not show visible phenotypes, the so far identified and analyzed plastidic sugar translocators revealed significant/drastic phenotypes, indicates their unique role in carbohydrate allocation and starch turnover.

**Evidence for sugar transport across the tonoplast**

The central vacuole is the largest compartment of a mature plant cell which occupies more than 80% of the total cell volume and plays an essential role in maintaining cytoplasmic homeostasis of nutrients and ions. Vacuolar sap contains relatively high concentration of sugars such as glucose, fructose, sucrose and organic anions like malate, citrate and also certain amino acids in comparison to cytoplasm. Accumulation of these compounds in the vacuole on one hand serves for temporary or long-term storage of nutrients depending on the tissue type and, on the other hand for the production of large osmotic potential for maintenance of turgor.

The role of vacuole as storage organelle is greatly influenced by transport properties of the tonoplast (I.D. Milner et al., 1995). Since some agriculturally important plants like sugar beet (Doll et al., 1979; Getz, 1991; Getz and Klein, 1995) and sugar cane (Thom et al., 1982) store a considerable amount of sugars in the vacuoles of storage organs, there is a longstanding interest in this specific type of sugar partitioning. Experiments on isolated vacuoles provide biochemical evidence for the uptake of sucrose as well as hexoses into these organelles (Thom et al., 1982; Rausch, 1991; Keller, 1992). Interestingly, for the uptake of these sugars, both mechanisms passive diffusion as well as active transport were suggested (Thom and Komor, 1984; Martinoia et al., 1987; Martinoia et al., 2000). The mode of action of vacuolar sugar transporters might possibly depend on the tissue type and its function.

In leaves, carbohydrates accumulate in vacuole during the day, when phloem loading capacity is limited and are exported during the night whereas in storage tissue, carbohydrates accumulate in the vacuole during the vegetation period and will be used up as a source of energy for growth in subsequent periods (Martinoia et al., 2000). Experiments with protoplasts and rapid vacuole isolation have shown that products of the primary metabolism
are rapidly transferred into the vacuole (Kaiser et al., 1982). In a very careful compartmentation study Gerhardt et al., (1987) demonstrated that the diurnal changes of the malate content in spinach could be attributed to changes in the vacuolar malate content whereas negligible changes were observed in cytosol and stroma. Measurements of sugar concentrations using a non-aqueous fractionation technique in a variety of plants revealed that in leaves, the vast majority of glucose is found in the vacuole, while sucrose seems to be mainly in the cytoplasm (Wagner, 1979; Heineke et al., 1994; Pollock et al., 2000; Voitsekhovskaja et al., 2006). Even if the vacuole can account for more than 90% of the total volume in leaf mesophyll cells, the concentration of glucose in the vacuole is still higher than in the cytoplasm. Thus, an active import of glucose into the vacuole has to be postulated to allow this accumulation. The ATP dependent accumulation of glucose isomer of metabolically inactive 3-OMG into the vacuoles from pea leaf mesophyll cells (Guy et al., 1979) and maize coleoptile vesicles (Rausch et al. 1987) has been reported. A carrier-mediated transport system coupled with ATP was observed for glucose and fructose with corresponding $K_m$ values of 5 mM and 2.5 mM in protoplasts, isolated from immature apple fruit flesh (Yamaki and Asakura, 1987). Whereas in the isolated tomato fruit vacuoles, glucose and fructose uptake was not stimulated by ATP and showed saturation kinetics with $K_m$ values 122 mM and 120 mM respectively (Milner et al., 1995). Also in barley (Martinoia et al., 1987), celery (Daie and Wilusz, 1987) and pear fruit vacuoles (Shiratake et al., 1997), glucose transport by facilitated diffusion has been demonstrated. These results indicate that the vacuoles play an important role as an intermediate storage compartment for products of the primary metabolism, in order to maintain the cytosolic homeostasis necessary for metabolism.

The disaccharide sucrose uptake was also demonstrated in the vacuoles both by facilitated diffusion and active transport. Kaiser and Heber, (1984); Martinoia et al. (1987) showed that the uptake of sucrose into isolated leaf vacuoles is occurring by facilitated diffusion with an affinity of 20-30 mM, which is not inhibited by hexoses. Also in sugar cane cell cultures, sucrose transport by facilitated diffusion has been observed (Preissner and Komor, 1991). In contrast to the sucrose uptake by the leaf vacuoles, uptake into the vacuoles from root of red beet has been found to be stimulated by MgATP and occur via sucrose/H$^+$ antiport mechanism (Doll and Willenbrink, 1979). Sucrose accumulation in Stachys sieboldii tubers also is found to be stimulated by ATP. On the other hand, the affinity of these active sucrose carriers was similar (21 mM and 25 mM respectively) to that of passive transporter observed in barley leaf vacuoles.
Despite the largely characterized plasma membrane sugar transporters in *Arabidopsis thaliana*, no vacuolar glucose or sucrose transporters have been identified and analyzed in the last years. Recently, a sucrose transporter from barley, *HvSUT2*, was found to be localized to the vacuole in a proteomics approach and in transient expression studies using GFP fusion proteins (Endler *et al.*, 2006). However, results from expression studies of *HvSUT2* in yeast (Weschke *et al.*, 2000) as well as immunolocalization to the plasma membrane of its closest homologs in tomato (LeSUT4) and potato (StSUT4) are contrary to this finding (Weise *et al.*, 2000).

**Objectives**

The main objective of the present thesis is to functionally characterize the members of a newly identified monosaccharide transporter family of MFS. One of the approaches to elucidate the transport properties was isolation and functional expression of the cDNAs in heterologous system, baker’s yeast. Another major focus of this project is to investigate the detailed tissue and cell specific expression patterns of these transporters by generating transgenic promoter-reporter plants and by transient expression of their cDNA-GFP fusions in *Arabidopsis*. Further, this project is aimed to explore the physiological roles of these genes by isolation and analysis of the homozygous T-DNA insertion mutants.
2. Results

In silico analyses revealed a high degree of sequence homology to the known AtSTP gene family of monosaccharide transporters in Arabidopsis for three previously unknown open reading frames (ORFs), At3g03090 (AtVGT1), At5g17010 (AtVGT2) and At5g59250 (AtXYL3). The databases available for Arabidopsis, annotate these genes as homologues of bacterial H⁺/xylose symporters. Thus these three genes group as a distinct family previously named as xylose transporter family within the Major Facilitator Superfamily (MFS).

2.1 Functional characterization of AtVGT1

AtVGT1 is one of these genes with similarity to bacterial xylose permease genes. The AtVGT1 (MIPS code At3g03090) has a size of 1943 base pairs (bp) and is interrupted by 13 introns (Figure 2.1). The coding sequence has a length of 1512 bp which codes for a protein with 503 amino acids, calculated molecular mass of 53.543 kDa and an isoelectric point of 7.6.

![Figure 2.1: AtVGT1 gene in which 13 exons are represented with black blocks and 5’ UTR with a grey arrow.](image)

2.1.1 Isolation and cloning of the AtVGT1 cDNA

As AtVGT1 was strongly expressed in flowers, especially in pollen, flowers from Arabidopsis thaliana, ecotype Columbia, were used as raw material to isolate total RNA and to synthesize the transcript. The AtVGT1 cDNA was amplified using the specific primers AtXYL1c-15f and AtXYL1c+1522r, designed after the sequence obtained from the TAIR database. MfeI cloning sites were introduced at the very 5’ and 3’ ends of the primers, to allow cloning into MfeI or compatible EcoRI restriction sites.
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Figure 2.1.2: Schematic representation of *AtVGT1* gene with primer binding sites used to isolate its open reading frame along with their sequence.

The PCR product was ligated directly in sense as well as in antisense orientation into the unique EcoR1 cloning site of *E. coli*/*yeast shuttle vector NEV-E, yielding plasmids pSO114s and pSO114as. An error-free clone in each case was identified by sequencing and used for further cloning.

2.1.2 Heterologous expression of *AtVGT1* in *Saccharomyces cerevisiae*

To study whether the *AtVGT1* encodes a functional sugar transporter protein, the *AtVGT1* cDNA was expressed in bakers yeast. The plasmids pSO114s and pSO114as were transformed into the yeast strain EBY.VW-4000 (Boles *et al.*, 1999), in which all the endogenous hexose transporter genes were knocked out, yielding strains SAY114s and SAY114as respectively.

2.1.2.1 Substrate transport assay in transgenic yeast cells

Sugar uptake measurements were performed with intact cells of yeast strains SAY114s and SAY114as (§ 4.2.2.17). A range of radioactively labelled sugars including hexoses (glucose, fructose and galactose), pentoses (xylose and ribose), the disaccharide sucrose and the sugar alcohol sorbitol were tested to determine whether *AtVGT1* is a sugar transporter. The final sugar concentration was set to 100 µM in all cases. Transport measurements were carried out both with sense and antisense constructs at definite time intervals of 30 sec, 1 min, 2 min, 3 min, 5 min and 10 min. No significant transport activity was detected for any of the sugars tested (Fig. 2.1.3). The observed background activity is nearly identical in sense and antisense strains.
Figure 2.1.3: Radioactivity (Counts Per Min (CPM)) after 10 minutes incubation of transgenic yeast cells expressing *AtVGT1* in sense (dark grey coloured bars) and antisense (pale grey bars) orientation with different radiolabelled substrates. Mean values of two independent measurements are shown and the error bars represent standard deviation.

2.1.2.2 Growth complementation by *AtVGT1*

Result obtained from the substrate transport assays was further confirmed by growth complementation of hexose transport deficient yeast cells. All transformed EBY.VW-4000 strains have the ability to grow on maltose CAA plates. However, only the positive control (a yeast strain expressing monosaccharide transporter, *AtSTP8*, which is known to transport a wide range of monosaccharides including glucose and fructose, (Büttner M., unpublished)) was able to use glucose as sole carbon source but not the yeast clone expressing *AtVGT1* (Fig. 2.1.3). This shows that the AtVGT1 can not complement hexose transporter deficient yeast mutant.

Figure 2.1.4: Growth of EBY.VW-4000 strains expressing AtVGT1 on 2% maltose, 2% glucose and 0.2% glucose. After two days incubation at 29°C, only the positive control (SAY1) regained its growth on both concentrations of glucose.

2.1.3 Subcellular localization of *AtVGT1*

As no transport function was detected for AtVGT1 in yeast cells, it was assumed that the product of this gene might be localized in internal compartments of a cell. To investigate
this further, an *AtVGT1-GFP* fusion construct was generated and expressed in yeast as well as in *Arabidopsis* protoplasts.

### 2.1.3.1 Cloning of *AtVGT1* cDNA for GFP fusion

Flower specific cDNA (isolated from flowers of *Arabidopsis thaliana*, ecotype Columbia) was used as a template for PCR reaction with two specific primers AtXYL1c-20f and AtXYL1c+1526r which bind in *AtVGT1* gene on either side of the coding sequence. The stop codon of the original *AtVGT1* cDNA was replaced by an NcoI cloning site in the 3’ primer. The modified ORF of *AtVGT1* was ligated into pGEM-T easy (Promega) vector, yielding plasmid pSA115 which was transferred to *E.coli* strain DH5α.

### 2.1.3.2 Expression of an *AtVGT1* cDNA-GFP fusion construct in Yeast

To find out whether the AtVGT1 localized in the internal membranes of yeast cells, an *AtVGT1-GFP* fusion was expressed in yeast strain EBY.VW-4000. The modified ORF of *AtVGT1* from pSA115 was cloned into the yeast expression vector pEXTag_GFP2 (a modified pEXTag, with yeast plasma membrane ATPase promoter and *GFP* cassette), yielding plasmid pSA110 which was used to transform EBY.VW-4000. The resultant yeast strain SAY110 was examined for GFP fluorescence under the fluorescence microscope. The observed GFP fluorescence revealed that the AtVGT1-GFP fusion protein localized to internal compartments but not to the plasma membrane and GFP fluorescence in the membranes of isolated vacuoles proved its localization in the tonoplast (Fig. 2.1.5).

![Figure 2.1.5](image.png)

**Figure 2.1.5:** Analysis of *AtVGT1* cDNA-GFP fusion expression in yeast strain EBY.VW-4000. Yeast cells with GFP fluorescence resulted from AtVGT1-GFP fusion protein localized in internal compartments (A), GFP fluorescence in vacuoles isolated from yeast cells expressing AtVGT1-GFP fusion (B). (Scale bars: 8 µm in A and 12.09 µm in B).
2.1.3.3 Transient expression of AtVGT1-GFP fusion in Arabidopsis protoplasts

To check whether the vacuolar localization of the AtVGT1 GFP fusion protein in yeast vacuoles is true also in the case of plants, subcellular localization of the AtVGT1-GFP fusion was analyzed by transient expression in plant cells. To generate an AtVGT1-GFP fusion construct, the modified ORF from pSA115 was cloned into the unique NcoI restriction site of pSO35e which carries the strong CaMV35s promoter, the GFP ORF and the NOS (Nopaline synthase) terminator, yielding plasmid pSA120. The plasmid DNA of an error free clone was used to transiently transform Arabidopsis protoplasts and analyzed for GFP fluorescence by confocal laser scanning microscopy. As seen in Fig. 2.1.6, the GFP fluorescence of the AtVGT1-GFP fusion protein was confined to the vacuolar membrane (tonoplast) even after lysis of the protoplasts with mild osmotic shock.

Figure 2.1.6: CLSM analysis of PEG-transfected protoplasts expressing AtVGT1-GFP fusion construct. Intact protoplasts showing GFP fluorescence (A), chloroplast autofluorescence under GFP excitation light (B), the overlay picture of A and B, showing the chloroplasts, outside the fluorescence labelled tonoplast (C), Osmotically lysed protoplasts scanned under white light (D) and GFP excitation light (E), and the overlay picture showing the fluorescence clearly localized to tonoplast (F). (Scale bars: 10.9 µm in all).
2.1.4 AtVGT1 transport assay in isolated vacuoles of transgenic yeast

The AtVGT1 was proved to be localized to the vacuolar membrane in yeast as well as in plants. Thus, to determine the biochemical function of AtVGT1, transport measurements were performed with vacuoles isolated from yeast cells expressing AtVGT1.

2.1.4.1 Isolation and stabilization of yeast vacuoles

Vacuoles from yeast cells expressing AtVGT1 were isolated as described by Ohusumi and Anraku (1981), with few modifications. After isolation, protein content was determined by Bradford assay. One litre of culture with OD$_{600}$ of 1 yields approximately 700-1200 µg of vacuolar protein depending on the yeast strain. Isolated vacuoles were checked for viability, based on the biochemical activity of vacuolar ATPase, the major constituent of vacuolar proteins. The experiments were performed as described by Zhang et al., (2003). ATPase assay was based on regeneration of ATP, hydrolyzed by vacuolar ATPase This reaction is coupled to the oxidation of NADH (absorption maximum at 340 nm) to NAD$^+$ Following each cycle of ATP hydrolysis, the regeneration system, consisting of phosphoenol pyruvate (PEP) and pyruvate kinase (PK) converts one molecule of PEP to pyruvate, when the ADP was converted back to ATP. The pyruvate was subsequently converted to lactate by lactate dehydrogenase (LDH) resulting in the oxidation of one molecule of NADH. The assay measures rate of decrease in absorbance at 340 nm, which was proportional to steady state ATP hydrolysis which in turn was the measure of viability of vacuolar ATPase. Decrease in absorbance was observed after addition of 1µg vacuolar protein to the reaction mix, which is an indication of the viability of vacuolar ATPase and thus the vacuoles.

The stability of vacuoles after vacuum treatment during the uptake experiments was confirmed by using GFP fluorescing vacuoles isolated from SAY110. After suction onto nitrocellulose filters and washing with 2X buffer C, the SAY110 vacuoles were still intact, as was reconfirmed by fluorescence microscopy. Furthermore the quality of the isolated vacuolar protein was checked by measuring the uptake capacity of the vacuoles for amino acid lysine Ohusumi (1980). Uptake was measured at 100 mM initial outside concentration of $^{14}$C-Lysin (0.1 µCi) and determined to be 11 nmol lysine per mg protein and was comparable to the value of 14 nmol lysine per mg protein measured by Ohusumi et al., (1981).
2.1.4.2 Sugar transport assay with isolated yeast vacuoles

To use the vacuole isolation method of Ohusumi & Anraku (1981) for sugar uptake measurements, the protocol was modified as follows: the assay mixture (100 µl) consisted of 40-50 µg of vacuolar protein, 20 mM MES-Tris pH 7.9, 4 mM MgCl₂, 4 mM ATP. The above mixture was incubated at 29°C for 5 min and the reaction was started by adding the ¹⁴C-labelled substrate (100 mM, 0.1 µCi). After the desired reaction time, the mixture was diluted with 2 ml of cold buffer containing 20 mM MES-Tris pH 7.9, 5 mM MgCl₂ and 25 mM KCl to stop the reaction and vacuoles were quickly recovered on a nitrocellulose filter (0.2 µm pore size) and washed with 2 ml of above buffer. Vacuum was applied carefully to remove the excess buffer and unused radioactive substrate. The nitrocellulose filter was then added to 4 ml scintillation cocktail and the radioactivity was measured in a scintillation counter.

2.1.4.3 Sugar uptake into vacuoles of transgenic yeasts

Uptake experiments were performed with vacuoles isolated from transgenic yeast cells expressing *AtVGT1* in sense (SAY114s) and antisense (SAY114as) orientation. The assay was performed with different sugars in the presence and absence of ATP in the assay mixture. From the measured radioactivity, the amount of incorporated substrate was calculated per mg of vacuolar protein. As was shown in Fig. 2.1.7, *AtVGT1* is transporting glucose at a rate of 6.5 nmol/mg protein in the presence of ATP, while there was only background transport activity obtained with pSAY114s in the absence of ATP as well as with SAY114as in the presence of ATP.

To determine the substrate specificity of *AtVGT1*, transport assays were performed with different monosaccharides and the disaccharide sucrose (Fig. 2.1.8). The initial concentration of all the sugars tested was at 100 mM (0.1 µCi). In Fig. 2.1.8., the transport rate of glucose was set to 100% and compared to the relative transport rates of the other sugars. A lower but significant transport rate was observed for fructose (42%) and galactose (14%). However, uptake of the pentose xylose and the disaccharide sucrose was negligible. In all the cases, uptake of these sugars in the antisense strain was found to be negligible. This experiment indicates that the *AtVGT1* is basically a hexose transporter with glucose being the major substrate.
Figure 2.1.7: Transport of $^{14}$C labelled glucose, fructose, galactose and xylose in the presence or absence of ATP into vacuoles, isolated from yeast cells expressing *AtVGT1* in sense (SAY114s) and anti-sense (SAY114as) orientation.

Figure 2.1.8: Relative transport rates of radiolabelled D-glucose, fructose, galactose, xylose and sucrose in SAY114s vacuoles (black bars) and D-glucose in SAY114as vacuoles (grey bar) at initial outside sugar concentration of 100 mM. The transport rate observed for glucose was set to 100% and the relative transport rates for other tested sugars were calculated accordingly.
2.1.4.4 pH dependence of AtVGT1

The above substrate transport tests were performed at an outside pH of 7.9. To see whether the AtVGT1 transport activity depends on the pH of the reaction mixture, uptake experiments over a range of outside pH were performed. Vacuoles were resuspended in 2X Buffer C of pH 5.2, 5.8, 6.2, 6.9 and 7.9 and uptake experiments with $^{14}$C-glucose were performed as described. 2X Buffer C of respective pH was used to stop the reaction as well as to wash out the unused radiolabelled substrate. AtVGT1 driven sugar uptake was observed only in near neutral or slightly basic pH ranges but not at acidic pH. The transport activity observed at pH 5.2, 5.8 and 6.2 was similar to the background transport activity observed with vacuoles of pSAY114as. Glucose uptake at pH 6.9 was 3.99 nmole per mg of vacuolar protein whereas at pH 7.9, it was 6.5 nmole per mg protein (Fig. 2.1.9). This shows that the AtVGT1 activity increases with outside reaction conditions increasing from acidic to slightly basic pH, which strongly suggests a proton-antosport mechanism.

![pH dependence of AtVGT1](image)

**Figure 2.1.9:** Analysis of AtVGT1 transport ability in isolated vacuoles resuspended in 2X Buffer C from an acidic to basic pH range. The different symbols represent transport rate of Glucose (nmol per mg of vacuolar protein) at a particular pH (solid diamonds-pH 5.2, solid squares-pH 5.8, solid triangles- pH-6.2, asterisks-pH 6.9, solid circles-pH 7.9, open circles- in antisense constructs at pH 7.9).

2.1.5 Analysis of *AtVGT1*-expression by reporter plants

To study the expression patterns of *AtVGT1*, a GUS reporter plant was generated. To this end, a 1922 bp promoter fragment was amplified by PCR using primers AtXYL1g-1876f and AtXYL1g+66r. The primers introduced an N-terminal SphI site and a C-terminal NcoI site to the PCR fragment. The resulting 1400 bp fragment upon digested with SphI and NcoI restriction enzymes was cloned into pAF6 (a pUC19 derivative with GUS reporter gene) in
Results

front of the *GUS*-ORF and the NOS-terminator and the resulting plasmid pSA103 was sequenced. A 3.15 kb *AtVGT1* promoter-*GUS*-terminator trunk from an error free clone was transferred to the plant vector pGPTV-BAR (Becker *et al.*, 1992) over XmaI and EcoR1 restriction sites, yielding plasmid pSA104. This construct was then transferred to *Arabidopsis thaliana* (WT-Col) via *Agrobacterium tumefaciens* mediated transformation. First generation plants were selected for BASTA resistance and analyzed for GUS staining in different parts at various developmental stages of the plant. Of the 13 analyzed plants, 12 showed GUS staining after 4 hr incubation exclusively in anthers, predominantly in pollen (Fig. 2.1.10). The GUS expression was gradually increased to mature pollen. No other sites of GUS-expression could be detected, even after longer incubation (up to 24 hrs).

GUS stained flowers were embedded in Technovit (§ 4.2.2.21) and thin sections were made to obtain a more detailed picture of the staining pattern. The GUS staining observed in thin sections also revealed that the promoter activity was restricted to pollen and pollen sac.

![Figure 2.1.10](image)

**Figure 2.1.10:** Analysis of GUS activity under the control of *AtVGT1* promoter in representative transgenic *Arabidopsis thaliana* (WT-Col). GUS-Expression in anthers at different stages of flower development (A), Pollen sac with strong GUS-expression in mature pollen grains (B), Cross section of anthers of line #12 embedded in Technovit showing strong GUS staining in pollen grains (C). (Scale bars: 1 mm in A, 0.25 mm in B and 26 µm in C).

### 2.1.6 Isolation and analysis of T-DNA insertion mutants of *AtVGT1*

To investigate the physiological role of *AtVGT1* in plants, T-DNA insertion mutants were obtained and screened for homozygosity of the T-DNA insertion by genomic PCR.

#### 2.1.6.1 PCR analysis of *AtVGT1*-T-DNA insertion lines

The T-DNA insertion sites of two independent lines, SAIL_669-D03 (T-DNA insertion at position +2760, in the 9th intron) and SALK_000988 (T-DNA insertion at
position-1) (refer Table 4.1.2.2 for more information) were localized by PCR with genomic DNA

![Figure 2.1.11: A schematic diagram representing the insertion sites for SALK and SAIL T-DNAs in AtVGT1 gene.](image)

In the SAIL_669-D03, the T-DNA was detected by PCR using a T-DNA specific primer LB3 (5’-TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C-3’) and the AtVGT1 gene specific primer AtXYL1g+3052r (refer table 4.1.4.1 for primer sequences), yielding a PCR product of 269 bps (plus LB region of unknown size). Homozygosity of the T-DNA insertion was tested by genomic PCR using the two AtVGT1 gene specific primers AtXYL1g+2326f and AtXYL1g+3052r, which will span the T-DNA insertion site and yield a product of 726 bps in wild type and heterozygous insertion lines, but not in mutant lines homozygous for the T-DNA insertion.

Similarly, for the analysis of the SALK_000988 line, T-DNA specific primer Lba1 (5’-CGA TGG CCC ACT ACG TGA ACC AT-3’) and the AtVGT1 gene specific primer AtXYL1g+408r, yielding a PCR product of 408 bps (plus LB region of unknown size). Homozygosity of the T-DNA insertion was tested by genomic PCR using the two AtVGT1 gene specific primers AtXYL1g-20f and AtXYL1g+408r, producing a PCR fragment of 428 bps in wild type.

In WT plants, a 726 bps or a 428 bp PCR fragment can be amplified with the gene specific primer pairs AtXYL1g+2326f/AtXYL1g+3052r (Fig 2.1.12. A) or AtXYL1g-20f/AtXYL1g+408r (Fig 2.1.12. B), respectively. In the homozygous mutant lines, this PCR product was missing due to the large size of the inserted T-DNA. In heterozygous and homozygous SALK and SAIL lines, a PCR fragment with T-DNA specific primer and the corresponding gene specific primer can be amplified.
2.1.12 Analysis of the T-DNA insertion lines by genomic PCR.

Of the 7 SAIL_669_D03 lines, plants #3 and #6 (A), and of the 10 SALK_000988 lines, plants #3 and #4 (B) were homozygous for T-DNA.

The homozygous SAIL and SALK T-DNA insertion lines isolated, were analyzed for phenotypic differences to WT.

2.1.6.2 Analysis of homozygous AtVGT1 T-DNA insertion lines

The isolated homozygous T-DNA insertion lines were carefully analyzed for visible phenotypes in the subsequent generation with respect to growth and development. SALK_000988 mutants were denoted as Atvgt1-s and SAIL_669D03 mutants as Atvgt1-g. First, the seed germination rate on MS plates under standard long-day conditions (16 hrs light/8 hrs dark regime at 22°C and 60% relative humidity) was analyzed at different time intervals after 4 days vernalization. It was observed that the Atvgt1 seeds were germinating relatively slower than WT seeds. Eventually about 20% of the Atvgt1 seeds failed to germinate (Fig. 2.1.14), which was true for both the insertion lines.

![Germination rate graph](image)

Figure 2.1.13: Graphical representation of seed germination rate of Atvgt1-s (grey bars) and Atvgt1-g (white bars) in contrast to WT (black bars) under standard long day conditions followed by 4 days stratification.

Apart from seed germination, the Atvgt1 mutant lines were also checked for phenotypic differences in later stages of development. Initiation of flowering and primary
shoot development was monitored in 24 plants each of WT, Atvgt1-s and Atvgt1-g. As seen in Fig. 2.1.15-A, WT plants have started bolting in 21 days after germination (DAG) and 100% of the plants had produced a primary shoot in 28 DAG. In contrast, the Atvgt1 plants started bolting after 29 to 31 DAG and acquired 100% bolting only after 39 to 42 DAG.

Although bolting was delayed by 10 to 11 days in the mutant lines, these plants eventually, reached the height of WT (Fig. 2.1.16) in later stages of development. No significant differences to WT were observed in fertility, silique development and seed dormancy.

**Figure 2.1.14:** Analysis of bolting and flowering initiation time in Atvgt1 mutants. Graphical representation of relative bolting rates in Atvgt1-s, Atvgt1-g and WT. Solid squares representing rate of bolting in WT plants and open circles and solid triangles represent rate of bolting in Atvgt1-s and Atvgt1-g respectively (A), Atvgt1-s and Atvgt1-g plants in contrast to WT, 1 week after 100% initiation of bolting process in Atvgt1 plants (B). (Scale bar: 1.5 cm)

**Fig 2.1.15:** Overall morphology of 10 weeks old Atvgt1-s and Atvgt1-g plants in contrast to WT plants grown under similar conditions. Most of the siliques of WT plants were matured and start to dehisce where as the siliques of Atvgt1 plants were still green (Scale bar: 4.4 cm).
Taken together, the described transport function in isolated yeast vacuoles infers that the AtVGT1 is a vacuolar monosaccharide transporter with glucose being its major substrate. The observed pH dependency suggests H⁺ antiport mechanism for glucose transport. GUS reporter gene expression under the control of AtVGT1 promoter was detected only in pollen and pollesac, however microarray analysis suggesting a basal level expression in almost all the tissues. Analysis of T-DNA insertion lines indicated the important physiological role of AtVGT1 in seed germination and in flowering time determination.

2.2 Functional characterization of AtVGT2

A second gene of the here described new gene family is AtVGT2. The open reading frame (ORF) is 1512 bp long, which codes for 503 amino acids, with a calculated molecular mass of 53.54kDA. Similar to AtVGT1, AtVGT2 coding sequence is interrupted by 13 introns (Fig. 2.2.1) at conserved positions found in the gene family.

![Figure 2.2.1: Schematic representation of the intron-exon distribution in the AtVGT2 gene. The coding sequence consists of 14 exons (black blocks) and is interrupted by 13 introns (grey regions within the exons).]

2.2.1 Isolation and cloning of AtVGT2 cDNA

The AtVGT2 cDNA was synthesized by RT-PCR using total RNA isolated from Arabidopsis rosette leaves and the AtVGT2 specific primers AtXYL22g-41f and AtXYL2g+4482r, which introduced BbsI restriction sites at the very 5’ and 3’ ends (Fig. 2.2.2).

![Figure 2.2.2: Sequence and position of the primers used for PCR amplification of the AtVGT2 cDNA]
2.2.2 Expression of *AtVGT2* cDNA in yeast

The *AtVGT2* cDNA from pSA216 was ligated into the *E.coli*/*yeast* shuttle vector, NEV-E over EcoR1 restriction site in sense and antisense orientation, yielding plasmids pSA218s and pSA218as. Both the plasmids were transformed to yeast hexose transporter mutant (hxt) EBY.VW-4000 resulting in yeast strains SAY218s and SAY218as.

### 2.2.2.1 Growth complementation tests

To study the possible complementation of the yeast hexose transporter mutations (hxt) in EBY.VW-4000 by *AtVGT2*, the yeast strains SAY218s and SAY218as were grown on CAA plates containing 0.2% or 2% glucose and 2% maltose. The strain SAY1, carrying the known plasma membrane localized monosaccharide transporter *AtSTP8* was used as positive control. While the control strain SAY1 has regained its growth on glucose, the *AtVGT2*-expressing yeast clone SAY218s was not able to complement the yeast hxt mutations.

![Figure 2.2.3: Complementation of yeast hxt mutations by *AtVGT1* on D-glucose. After two days incubation at 29°C, both SAY1 and SAY218s regained growth on 2% maltose whereas only the positive control (SAY1) regained its growth on both concentrations of glucose.](image)

### 2.2.2.2 Substrate transport assay in transgenic yeast cells

Even though, the transgenic yeast cells expressing *AtVGT2* failed to complement yeast hxt-mutat, uptake experiments were performed, since some of the already characterized AtSTPs showed transport activity in yeast despite a missing growth complementation. Transport of $^{14}$C labelled monosaccharides and the disaccharide sucrose were tested with both sense and the antisense constructs. No transport activity could be detected for the SAY218 strains, since no significant difference in uptake was observed between the sense and antisense constructs and the counts for $^{14}$C labelled substrate measured after 10 minutes were very low (Fig. 2.2.4).
2.2.3 Subcellular localization of AtVGT2

One possible reason for the missing transport activity in the AtVGT2-expressing yeast strain SAY218 could be that the transporter does not localize to the plasma membrane. To verify the expression of AtVGT2 and to determine the subcellular localization of its gene product, a GFP fusion construct was expressed in yeast as well as in isolated Arabidopsis protoplasts.

2.2.3.1 Cloning of AtVGT2 cDNA for GFP fusion

The AtVGT2 coding sequence was amplified by RT-PCR from total RNA isolated from rosette leaves of Arabidopsis thaliana using AtVGT2 specific primers AtXYL2g-12f and AtXYL2g+4479r. The primers introduced an NcoI restriction site at the 5’end and a BbsI restriction site at the 3’ end which also replaced the stop codon of the original AtVGT2 cDNA to allow for transitional fusion to the GFP coding sequencing. The modified AtVGT2 ORF was ligated into the pGEM-T easy vector resulting in plasmid pSA217, the sequence of which was verified.

2.2.3.2 Expression of AtVGT2-GFP fusion in yeast

To determine the subcellular localization of AtVGT2 in yeast, an AtVGT2-GFP fusion construct was expressed in yeast strain EBY.VW-4000. The cDNA fragment from pSA217 was ligated to pEXtag-GFP2 (a modified pEX-tag vector with GFP cassette) over the NcoI cloning site yielding plasmid pSA219 which comprises the start ATG of the GFP-ORF. The
obtained plasmid pSA219 was transformed to yeast strain EBY.VW-4000 resulting in yeast strain SAY219. Analysis of SAY219 by fluorescence microscopy revealed that the GFP fluorescence clearly localized to internal compartments but not to the plasma membrane. GFP fluorescence in isolated vacuoles proved that the AtVGT2-GFP fusion protein was localized in the vacuolar membrane.

![Figure 2.2.5: Analysis of transgenic yeast cells expressing AtVGT2-GFP fusion construct. Localization of GFP fluorescence in the internal cell structures (A), GFP fluorescence in the vacuolar membrane isolated from the yeast strain SAY219 (B). (Scale bars: 9.4 µm in A and 13.14 µm in B).]

### 2.2.3.3 Transient expression of AtVGT2-GFP fusion in Arabidopsis protoplasts

To find out whether the vacuolar localization of AtVGT2 observed in yeast cells is also true for plants, an AtVGT2-GFP fusion construct was transiently expressed in isolated Arabidopsis protoplasts. To this end, the modified AtVGT2 ORF from pSA217 was ligated to pSO35e vector (a pUC19 derivative carrying the GFP-ORF behind the CaMV-35S promoter) over NcoI and BbsI (NcoI compatible) restriction sites, yielding plasmid pSA220. The resultant plasmid was used for transient expression in Arabidopsis protoplasts via PEG transfection. Confocal imaging of the GFP fluorescing protoplasts (Fig. 2.2.6) revealed that also in plants, the AtVGT2-GFP fusion protein was localized to the tonoplast.
2.2.4 Expression of *AtVGT2* gene in Planta

To explore the tissue specific expression patterns of *AtVGT2*, promoter-reporter (GUS) plants were generated. A 2.4 kb promoter fragment was used to drive the expression of GUS in transgenic plants. The promoter fragment was amplified by PCR with primers AtXYL2g-2356f (HindIII) and AtXYL2g+14r (NcoI), using genomic DNA isolated from rosette leaves of *Arabidopsis thaliana* as template. The PCR fragment was cloned into pAF6 (a derivative of pUC vector with GUS reporter gene) vector via HindIII/NcoI interfaces, yielding plasmid pSA221. The AtVGT2 promoter-GUS-terminator cassette form the resultant clone was ligated to the plant vector pGPTV-BAR over HindIII/SacI cloning sites, yielding plasmid pSA222, with which *Arabidopsis thaliana* plants were transformed via *Agrobacterium* mediated transfer. 12 transgenic plants were analyzed for GUS reporter gene expression in different developmental stages. As was shown in Fig. 2.2.7, the GUS staining was detected in hydathodes of cotyledons and in vasculature of 1 week old seedling root. Strong GUS staining after 4hrs incubation was observed in inflorescence and in rosette leaves. After overnight incubation at 37°C, GUS staining was observed also in the mid vein of siliques, in funiculi of the aborted seeds, and in the vascular bundles of the inflorescence stem. In young flowers, the GUS staining was restricted to the sepals, filament and ovary where as strong GUS staining was observed in petals of the mature flowers after 4hrs incubation. The GUS staining observed in pollen of the mature flowers (Fig. 2.2.7-D) might be because of diffusion of the GUS stain from the adjacent tissue.
Figure 2.2.7: GUS reporter gene expression under the control of AtVGT2 promoter. GUS staining in hypocotyl and in the hydathodes of cotyledons (A), in young rosette of AtVGT2-GUS plant, showing blue staining in leaf tip (B), GUS staining restricted to the leaf tip of a rosette leaf (C), in sepals, filament stigma and also in the inflorescence stem (D), in young flower in sepals filament and stigma (E), in older flower staining can be seen also in petals (F), GUS staining was restricted to proximal and distal ends in a silique and expressed strongly in stalk and funiculus of aborted seeds (inset: pointed by arrow) (G), GUS staining in cross section of an inflorescence stem (H), strong GUS staining in cross section of a filament (I), GUS staining in vasculature of roots (J), A cross section of the hypocotyl of an Arabidopsis seedling expressing GUS signal in vasculature and in the cortex (K). (Scale bars 2 mm in A, B, C and D, 1 mm in E, 1.5 mm in F, 3.4 mm in G, 50 µm in H and K).

2.2.5 Generation of Antibodies against AtVGT2

The extremely high homology between AtVGT1 and AtVGT2 made it impossible to raise specific antibodies against either protein. Thus, an antibody was generated against the N-terminus of AtVGT2, which was expected to also recognize AtVGT1.
2.2.5.1 Cloning for MBP-AtVGT2 fusion protein

For the production of anti *AtVGT2* antiserum, oligonucleotides encoding 15 N-terminus amino acids of AtVGT2 (REFGKSSGEISPERE, refer appendix for complete amino acid sequence of AtVGT1 and AtVGT2) were used. The annealed oligonucleotides (§ 4.2.2.9) were cloned into pMALc2 vector (New England Biolabs, Frankfurt am Main, Germany), yielding plasmid pSA205. The verified plasmid was transformed to the E.coli strains DH5α and Rosetta (Novagen, Madison, WI, USA). Expression of the MBP-AtVGT2 fusion was induced by Isopropyl thiogalactoside (IPTG). Soluble proteins from the transformed Rosetta strain were isolated as the protein induction was comparatively high in this strain. The MBP-AtVGT2 fusion protein was purified over an amylose resin column (New England Biolabs Frankfurt am Main, Germany) and lyophilized. Antiserum was generated by Pineda antikörper service (Berlin, Germany) by injecting 2 rabbits with purified MBP-AtVGT2 fusion protein.

To test the antisera, western blot analysis was performed with vacuolar vesicles isolated yeast cells expressing *AtVGT1* and *AtVGT2* cDNAs and their GFP fusions (i.e. from SA114s, SAY110; SAY218s and SAY219). A possible AtVGT-specific signal was detected with the different yeast extracts using the antisera obtained after 90 days.

![Western blot](image)

**Figure 2.2.8:** Western blot with vacuolar vesicles isolated from transgenic yeast strains SAY114s, SAY110, SAY218s and SAY219. The *AtVGT1* and 2 proteins run nearly at ~39 kDa and the GFP fusion proteins at ~66 kDa. This size shift corresponds to the molecular weight of GFP protein (~26 Kda).

2.2.6 Identification and analysis of *AtVGT2* T-DNA insertion mutants

To elucidate the physiological role of *AtVGT2*, T-DNA insertion lines were analyzed. Two independent insertion lines, SAIL_756_B12 and SALK-090827 were screened for homozygous insertions by genomic PCR method.
Figure 2.2.9: Schematic representation of the T-DNA insertion sites for SAIL_756_B12 and SALK-090827 lines. The T-DNA insertion for SAIL line was located in 1st intron, at 264 bps and the insertion line obtained from SALK harbouring T-DNA in 9th exon at position +3177 bp with respect to start ATG.

2.2.6.1 Isolation of homozygous T-DNA insertion lines for \textit{AtVGT2}

The SAIL_756_B12 insertion line has its T-DNA in the 1\textsuperscript{st} intron at 264 bp downstream of start ATG. DNA was isolated from progeny lines and analyzed by genomic PCR. The T-DNA allele was amplified with a T-DNA specific primer LB3 and gene specific primer AtXYL2g+595r, which yields a PCR fragment of approximately 530 bps, whereas two gene specific primers AtXYL2g+595r and AtXYL2g-41f which will span the T-DNA insertion site were used to amplify the genomic allele of 636 bp.

To screen the T-DNA insertion line SALK-090827, which harbours the T-DNA in 9\textsuperscript{th} exon at position +3,177 bp relative to the start codon, two gene specific primers AtXYL2g+2573f and AtXYL2g+3689r which will span the T-DNA insertion site were used to amplify the gene specific product. A T-DNA specific primer Lba1 and a gene specific primer AtXYL2g+2573f were used to amplify the T-DNA allele. In WT plants, a 1,130 bp PCR fragment could be amplified with gene specific primers, whereas this product was missing in homozygous mutant lines, due to the large size of the inserted T-DNA.

Figure 2.2.10: Screening of T-DNA insertion lines for \textit{AtVGT2} gene. Out of 6 plants screened for SAIL_756_B12, 3 (lines \# 1, \# 4 and \# 5) were homozygous and the remaining were WT, and 2 plants (#1 and #7) were homozygous out of 7 SALK-090827 plants analyzed.
2.2.6.3 Analysis of homozygous T-DNA insertion lines for \textit{AtVGT2}

The isolated independent hymozygous T-DNA insertion lines \textit{Atvgt2-g} and \textit{Atvgt2-s} were carefully analyzed for a phenotype in subsequent generations. The mutant plants were analyzed for phenotypic differences in seed dormancy and germination, bolting and flowering time, fertility in contrast to WT plants. No significant phenotype regarding any of the above mentioned features at any growth stage of the plant was observed (Fig. 2.2.11).

![Figure 2.2.11: Analysis of \textit{Atvgt2} T-DNA insertion lines. The \textit{Atvgt2} plants did not displayed any significant difference to WT plants at any of the developmental stages.]

2.3 Generation and analysis of \textit{Atvgt1/Atvgt2} double mutants

Studies on subcellular localization of \textit{AtVGT1} and \textit{AtVGT2} proved that both were localized to tonoplast in plants. The \textit{AtVGT1} was shown to be an active transporter of glucose into vacuoles and as such having an important physiological role in determining bolting time and stem elongation. However, the \textit{AtVGT2} mutant lines do not displayed any visible phenotype, and one possible reason could be that \textit{AtVGT1} may compensate the loss of \textit{AtVGT2}. Switching both the genes off could therefore reveal important hints towards the physiological role of these genes in plants. In order to examine this idea, double T-DNA insertion lines with respect to \textit{AtVGT1} and \textit{AtVGT2} were generated.
2.3.1 Generation of *Atvgt1/Atvgt2* double mutants

To generate double T-DNA insertion mutant plants, flowers of the *Atvgt1-s* (§ 2.1.5.2) plants were crossed with pollen from flowers of the *Atvgt2-g* (§ 2.2.5.1) lines so that, the offspring can be selected with two different resistance markers. The heterozygous T1 generation lines were analyzed by means of genomic PCR (Fig. 2.3.1). Primers used to analyze the individual T-DNA insertion lines of *Atvgt1-s* and *Atvgt2-g* were used to screen the *Atvgt1/Atvgt2* double mutants.

![Figure 2.3.1: Identification of *Atvgt1/Atvgt2* double mutants by genomic PCR analysis. Out of several plants screened in second generation, 7 plants found to be homozygous for both the genes. The lanes in each block indicates T-DNA allele for insertion in *AtVGT1* (T1), WT allele for *AtVGT1* (G1), T-DNA allele for insertion in *AtVGT2* (T2), WT Allele for *AtVGT2* (G2) respectively.](image)

Lines #1, #2, #3, #6, #7, #9 and #10 were homozygous for T-DNA insertions in both the genes and were used to analyze phenotypic differences.

2.3.2 Analysis of *Atvgt1/Atvgt2* double mutants

The homozygous double mutants with respect to *AtVGT1* and *AtVGT2* genes (indicated as *Atvgt1/Atvgt2*) were analyzed for phenotypic differences. The *Atvgt1/Atvgt2* seeds, when grown under standard long-day conditions, no further delay or impairment in seed germination to that of *Atvgt1* mutants was observed however, further development of seedlings was significantly delayed. Under complete darkness with or without sucrose, the *Atvgt1/Atvgt2* seeds were germinating much faster than WT seeds. As was seen in Fig. 2.3.2, this phenotype was more pronounced on sucrose containing medium (Fig. 2.3.2. left panel). WT seeds germinated but did not develope further when grown under dark, whereas in mutant seeds the hypocotyl was elongated. In contrast, on medium lacking sucrose, the hypocotyls of WT seedlings also developed slightly but still the hypocotyl elongation in *Atvgt1/Atvgt2* mutants was more pronounced (Fig. 2.3.2. right panel).
Figure 2.3.2: One week old etiolated seedlings of Atvgt1/Atvgt2 in contrast to WT grown on MS medium containing 2% sucrose or no sucrose.

In addition to hypocotyl development, etiolated seedlings grown for 1 week on sucrose lacking medium failed to develop chlorophyll even after exposed to light for 1 week (Fig. 2.3.3C), whereas seedlings of Atvgt1 and Atvgt2 single mutants showed photomorphogenesis similar to WT (Fig 2.3.3.A,B,D).

Figure 2.3.3: Analysis of seed germination on MS agar in the absence of sucrose under complete darkness. Seedlings of Avgt1-s (A), Atvgt2-g (B), Atvgt1/Atvgt2 (C), WT (D), grown for 1 week under complete darkness and exposed to light for 1 week.

As was seen in Fig. 2.3.4-A and B, also on soil the Atvgt1/Atvgt2 plants developing much slower than WT plants. Even in later stages of development, expansion of rosette, bolting process, stem elongation and branching were impaired. Bolting process observed in Atvgt1 mutants was further delayed for 3 to 5 days in Atvgt1/Atvgt2 double mutants. These plants developed very few branches compared to WT with comparatively weaker stems (Fig. 2.3.4-C and D).
Results

Figure 2.3.4: Phenotype of Atvgt1/Atvgt2 plants in contrast to WT. Delayed development in seedlings, 7 DAG (A), 14 days old double mutants with delayed rosette expansion (B), 6 weeks old double mutants in contrast to WT plants with delayed floral stem elongation and defective lateral flower stalk initiation (C), 10 weeks old Atvgt1/Atvgt2 double mutants which were not developed like WT plants, resulted in very low fresh weight of floral stem (D). (Scale bars: 1.7 cm in C and 3.8 cm in D).

Due to the impaired primary stem development and branching, the fresh weight of floral stem was reduced for about 60% in Atvgt1/Atvgt2 mutants (Fig. 2.3.5.A), and also possess a relatively low number of siliques. As shown in Fig. 2.3.5.B, the Atvgt1/Atvgt2 siliques harboured very few seeds and more empty positions. Although the bolting process, branching and silique development were delayed in Atvgt1/Atvgt2, these plants, ‘nearly’ reaching to the height of WT plants.

Figure 2.3.5: Quantification of fresh weight gain by floral stem (A), and number of siliques per plant (B) in Atvgt1/Atvgt2 double mutants in comparison to WT.
The phenotype observed with this floral stem could be because of the altered development of cells in this region. The factors lead to weak floral stem and less frequent branching in Atvgt1/Atvgt2 plants were explored at cellular level. The floral stems of mature Atvgt1/Atvgt2 double mutants and WT plants were excised from 1 cm height to the rosette and stained with Propidium Iodide (PI). The PI stained stems were scanned through different longitudinal projections, with the aid of confocal laser scanning microscopy. From the fig. 2.3.6 shown below, it was very clear that the floral stems of Atvgt1/Atvgt2 mutants had very longer and less number of cortical cells compared to the floral stem of simultaneously grown WT plants.

![Image](image1)

**Figure 2.3.6:** Longitudinal sections of Propidium Iodide stained mature *Arabidopsis* floral stem. Cells in the cortical region of Atvgt1/Atvgt2 stem (A), in contrast to WT (B). The cortical cells of Atvgt1/Atvgt2 floral stems are significantly longer compared to WT. (Scale bars: 150 µm).

Length of 110 cortical cells each of Atvgt1/Atvgt2 and WT plants were measured which revealed that the cortical cell of Atvgt1/Atvgt2 floral stem was approximately 2.3 times longer than WT.

![Graph](image2)

**Figure 2.3.7:** Graphical representation of average cortical cell length in Atvgt1/Atvgt2 mutant floral stem in contrast to WT (N=110). The cortical cell of mutant is approximately 2.3 times longer than WT.
In addition, cross sections of *Atvgt1/Atvgt2* floral stems prepared by vibratome upon embedding the stems for oN in 5% Lower Melting Point (LMP) agar were analysed under light microscope (Fig. 2.3.7-A and B). The diameter of *Atvgt1/Atvgt2* primary floral stem was 3/4th to that of the diameter of WT. Furthermore, the cross sections were stained with FCA dye and examined by fluorescence microscopy. The stained cross sections revealed that formation of interfascicular fibres in floral stem of the *Atvgt1/Atvgt2* mutant was impaired (Fig. 2.3.7-A’ and B’). Also, the diameter of pith cells was much smaller in *Atvgt1/Atvgt2* plants as in WT.

**Figure 2.3.7:** Cross sections of mature *Arabidopsis* floral stems grown under standard long-day conditions. A 65 µm thick cross section of floral stem of an *Atvgt1/Atvgt2* double mutant (A), in contrast to the 40 µm thick cross section of a floral stem of WT (B). The FCA stained cross sections of the stem, revealed the defective interfascicular fibers in *Atvgt1/Atvgt2* mutants (A’) in comparison to WT (B’). (pc: pith cell; if: interfascicular fiber; ics: intercellular space). (Scale bars: 161.94 µm in A, 161.87 µm in B, 25 µm in A’ and B’).
Taken together, the Atvgt1/Atvgt2 seed germination rate was not delayed further than in Atvgt1 mutants. The cotyledons of etiolated seedlings grown on sucrose lacking medium failed to develop chlorophyll which was not observed with Atvgt1 or Atvgt2 single mutants. A clear difference in diameter of Atvgt1/Atvgt2 floral stem, cell size and lignification of secondary cell wall to WT plants was observed. The observed effects support a specific function for AtVGT2 during these processes.

2.4 Functional Characterization of AtXYL3

AtXYL3 is a member of xylose transporter gene family of Arabidopsis thaliana which is, highly homologous to D-xylose symporter from Lactobacillus brevis. The full length cDNA of AtXYL3 is 3084 bp long. The open reading frame is of 1677 bp long which corresponds to 559 amino acids and is interrupted by 13 introns (Fig. 2.4.1).

AtXYL3 is a third and distant member among the three genes of the above described monosaccharide transporter family. The amino acid sequence of AtXYL3 is 52% identical to AtVGT1 and 53% identical to AtVGT2 amino acid sequence. According to the protein targeting prediction tool of the ARAMEMNON database (http://aramemnon.botanik.uni-koeln.de), AtXYL3 has a plastidic translocation peptide (cTP) of 31 amino acids, starting from the very N-Terminal aminoacid. The cTP drives the proper localization plastidic proteins. Figure 2.4.1 represents the predicted cTP and intron-exon distribution in AtXYL3.

![Figure 2.4.1: Schematic representation of intron exon distribution and cTP in AtXYL3 gene. Grey arrow represents the database predicted cTP and dark blocks represent the AtXYL3 coding sequence.](image)

2.4.1 Subcellular localization of AtXYL3

To find out whether the database predictions regarding plastidic localization of AtXYL3 was true, a GFP fusion protein was expressed in isolated Arabidopsis protoplasts.
2.4.1.1 Isolation and cloning of AtXYL3 cDNA

To isolate the AtXYL3 cDNA, a transcript was synthesized from total RNA of Arabidopsis thaliana (WT-Col) as template and AtXYL3 specific primers AtXYL3c-11f and AtXYL3c+2894r, which introduced NcoI and BbsI cloning sites at their very 5’ and 3’ ends respectively. With this additional BbsI cloning site on 3’ primer, the stop codon of original AtXYL3 cDNA was altered. The PCR amplified, modified ORF of AtXYL3 was cloned into pGEM-T easy vector, yielding plasmid pSA319. An error free clone was identified by sequencing.

![Sequence and position of the primers in AtXYL3 which were used to amplify the modified open reading frame (ORF).](image)

2.4.1.2 Transient expression of XYL3-GFP fusion in Arabidopsis protoplasts

The vector pSO35e was used to express the XYL3-GFP fusion construct. The NcoI/BbsI fragment from pSA319 was ligated to pSO35e vector infront of the GFP ORF, yielding plasmid pSA320. Upon checking for the correct orientation of the inserted ORF, the plasmid was transformed to Arabidopsis protoplasts by PEG transfection method. The Confocal laser scanning microscopy analysis of the transfected protoplasts revealed that the AtXYL3-GFP fusion protein localized to chloroplasts (Figure 2.4.3.A and C).

![CLSM analysis of transfected Arabidopsis protoplasts. Fluorescence in chloroplasts under GFP excitation light (A), chloroplast auto-fluorescence (B), overlay projection revealed that the AtXYL3-GFP fluorescence was localized in chloroplasts (C). (Scale bar: 16.74µm).](image)
2.4.2 Generation of antibodies against *AtXYL3*

A 100 amino acid sequence at the N-terminus of AtXYL3 protein was fused to the *E.coli* Maltose binding protein (MBP) to generate an AtXYL3 specific antiserum.

2.4.2.1 Cloning for MBP-AtXYL3 fusion

Two primers AtXYL3c+9f (HindIII) and AtXYL3c+264r (EcoRI) were designed which have HindIII and EcoRI cloning sites at their very 5’ and 3’ ends respectively. The PCR product was cloned to pMALc2 vector over HindIII/EcoR1 cloning sites, yielding plasmid pSA305, which was transferred to *E.coli* strains DH5α and Rosetta. After sequence verification, Maltose binding protein fusion protein in both the clones was induced by isothio-propylgalactoside (IPTG). Soluble proteins were isolated from the clone SA305R as the induction was considerably higher than in the other construct SA305. The MBP-fusion protein from isolated soluble proteins was purified over an amylose resin column. Anti AtXYL3 antisera was generated by Pineda Antikörper service upon immunizing 2 rabbits with purified MBP-AtXYL3 fusion protein.

2.4.2.2 Western blot with isolated plastidic membrane proteins

The data obtained from transient expression of *AtXYL3-GFP* fusion construct in isolated Arabidopsis protoplasts only confirms its expression in chloroplasts. Antibodies raised against AtXYL3 protein were used to determine whether AtXYL3 was localized in plastidic membranes or in stroma. Membrane proteins were separated from stromal fraction as described in § 4.2.4.20 and western blot was performed with anti AtXYL3 antisera. A clear signal was identified with chloroplast membrane proteins (Fig. 2.4.5) which, corresponds to molecular weight of the predicted mature AtXYL3 protein.

![Figure 2.4.5](image-url)

*Figure 2.4.5:* Western blot performed with isolated plastidic envelope proteins. The lane A corresponds to stromal fraction shows no signal; a clear signal against membrane protein fraction in lane B corresponds to the size mature AtXYL3 protein.
2.4.2.5 Expression of AtXYL3-GFP fusion in yeast

Even though it was shown that AtXYL3 is a plastidic protein, AtXYL3-GFP fusion construct was expressed in yeast cells to check whether it is expressed in yeast cells and if so in which organelle. As yeast cells do not have chloroplasts, the protein if at all is made must be expressed in any of the organelles. The modified ORF of AtXYL3 (§ 2.4.2.2) was ligated into pEXtag-GFP2 (a modified pEXtag) vector over unique NcoI cloning site, yielding plasmid pSA321. The verified plasmid pSA321 was transformed to yeast strain EBY.VW-4000, yielding yeast strain SAY321. No GFP fluorescence detected in the resultant yeast strain, SAY321 indicates that AtXYL3 was not expressed in yeast cells.

2.4.3 Analysis of AtXYL3 expression by GUS reporter plants

To investigate the tissue specific expression patterns of AtXYL3, GUS reporter plant was generated. The transgenic plants were analyzed for reporter gene expression in subsequent generations.

2.4.3.1 Isolation and cloning of AtXYL3 promoter

A promoter fragment of 2097 bp was amplified by PCR, using primers AtXYL3g-2087f and AtXYL3g+10r. The primers introduced an N-terminal XbaI restriction site and a C-terminal NcoI restriction site to the PCR fragment. After digested with XbaI and NcoI restriction enzymes, the PCR fragment was cloned into pAF6 vector in front of the GUS ORF and the NOS-terminator, yielding plasmid pSA322. A 3.84 kb AtXYL3 promoter_GUS-terminator cassette from an error free clone was ligated to the plant vector pGPTV-BAR over SbfI/SphI cloning sites, yielding plasmid pSA323. The obtained construct was then transferred to Arabidopsis thaliana (WT-Col) by floral dip method via Agrobacterium tumifacience mediated transformation. The first generation plants were selected for BASTA resistance and analyzed for reporter gene expression.

2.4.3.2 Analysis of transgenic Arabidopsis plants for GUS expression

About 110 BASTA resistant transgenic Arabidopsis plants were analyzed for GUS staining (Fig. 2.4.4). Of the 110 analyzed plants, 79 plants showed extensive GUS staining in seedling cotyledons after 4 hrs incubation at 37°C. GUS staining was not observed in seedling hypocotyls at very early stage, however, strong GUS staining was observed in cortical region of 2 weeks old seedling root after 4 hrs incubation. Extensive GUS staining was observed in
pollen after 2 hrs incubation and upon longer incubation also developed in sepals. Significant staining was also observed in rosette and cauline leaves upon overnight incubation at 30°C. Staining was also observed in stalk of the silique where as no staining was detected in seeds or other parts of the silique.

2.4.4 Isolation and analysis of T-DNA insertion mutants of *AtVGT1*

To elucidate the physiological role of *AtXYL3* in plants, T-DNA insertion mutants were analyzed. Three different T-DNA tagged lines were screened by genomic PCR method to obtain homozygous T-DNA insertion mutants.
Results

2.4.4.1 PCR analysis of AtXYL3 T-DNA insertion lines

The SALK_N521796 insertion line was supposed to possess T-DNA insertion in the second intron at 447 bp down stream of start ATG (Fig. 2.4.7). The T-DNA was detected by genomic PCR using a T-DNA specific primer Lba1 and a gene specific primer AtXYL3g+1505r, yielding a PCR product of approximately 1250 bp. Homozygosity of the T-DNA insertion was tested by genomic PCR using two AtXYL3 gene specific primers AtXYL3g-11f and AtXYL3g+1505r, which will span the T-DNA insertion site and yield a product of 1516 bps in WT and heterozygous insertion lines. To amplify this 1516 bp product is not possible in homozygous mutant lines, because of the large size of the T-DNA.

![Schematic representation of the site of T-DNA insertion obtained from SALK institute in AtXYL3 gene.](image)

Figure 2.4.7: Schematic representation of the site of T-DNA insertion obtained from SALK institute in AtXYL3 gene.

![Identification of homozygous SALK_N521796 T-DNA insertion line by genomic PCR. 2 plants, line # 7 (homozygous) and line # 11 (heterozygous) were harbouring T-DNA insertion.](image)

Figure 2.4.8: Identification of homozygous SALK_N521796 T-DNA insertion line by genomic PCR. 2 plants, line # 7 (homozygous) and line # 11 (heterozygous) were harbouring T-DNA insertion.

Two independent lines SAIL_335_F05 and SAIL_1253_A02 were analyzed for T-DNA insertion. To screen the T.DNA insertion line SAIL_335_F05, which harbours the T-DNA at position -33, relative to the start ATG, two gene specific primers AtXYL3g-1244f and AtXYL3+294r which will span the T-DNA insertion site were used to amplify the gene specific product. A T-DNA specific primer LB3 and a gene specific primer AtXYL3+294r were used to amplify the T-DNA allele.

Similarly, to screen the T-DNA insertion line SAIL-1253_A02 which has its T-DNA insertion at 1,443 bp downstream of the start ATG, two gene specific primers AtXYL3g+1066f and AtXYL3g+1505r which will span the T-DNA insertion site were used to amplify the gene specific product. To amplify the T-DNA allele, a T-DNA specific primer LB3 and a gene specific primer AtXYL3g+1505r were used. Neither homozygous nor
heterozygous insertion plants were obtained for any of these SAIL lines, as only the gene specific product of 1538 bps and a 439 bps respectively could be amplified.

### 2.4.4.2 Analysis of homozygous \textit{AtXYL3} T-DNA insertion line

The homozygous SALK\_N521796 T-DNA insertion line alone was analyzed for phenotypic differences in different developmental stages. Under standard long day conditions, the \textit{Atxyl3} seedling displayed advanced development compared to WT. Also the initiation of bolting and flowering processes were slightly advanced (for 2 to 3 days) in \textit{Atxyl3} mutants. Despite of the initial advanced floral stem development, the mature \textit{Atxyl3} plants are similar to WT (fig.2.4.9).

![Analysis of \textit{Atxyl3} lines under standard long day (16hrs daylight / 8hrs darkness) growth conditions followed by 4days vernalization. 10days old \textit{Atxyl3} plants in contrast to WT showing advanced development (A), 20 days old \textit{Atxyl3} plants (B), 40 days old \textit{Atxyl3} plants with advanced floral stem development in contrast to WT (C), 60 days old \textit{Atxyl3} plants (Scale bars: 1.65 cm in A and B, 1.1cm in C and 4.2 cm in D).](image_url)

The silique and seed development was impaired in \textit{Atxyl3} plants, in contrast to the advanced development of vegetative plant. Number of seeds and empty positions per silique (N=110) and length of the silique (N=500) were quantified in contrast to WT. The individual siliques harboured very few no. of seeds in \textit{Atxyl3} lines even though the number of siliques per plant was comparable to WT (Fig. 2.4.10.A and B). As shown in fig. 2.4.10.D, the WT silique on an average had about 36 seeds and 2 empty positions whereas, in an average \textit{Atxyl3}
siliques. The only 7 positions were occupied with 24 empty positions. The Atxyl3 siliques were about 40% smaller, compared to the size of WT siliques.

Figure 2.4.10: Quantification of the siliques and seed development in Atxyl3 plants. A typical Atxyl3 siliques is significantly shorter in contrast to the WT siliques (chlorophyll was removed with 70% Ethanol) (A), an Atxyl3 siliques with very few seeds (above) and several empty positions indicated by arrows (below) (B), graphical representation of the average length of an Atxyl3 siliques in contrast to WT siliques (N=500 in each case) (C), number of seeds (black bars) versus empty position (white bars) in an average Atxyl3 and WT siliques (D). Atxyl3 siliques has very few number of seeds, whereas the WT siliques showed only 2 empty position on an average (N=100 in each case). (Scale bars: 0.25 cm in A and 0.20 cm in B).

As AtXYL3 is localized in chloroplasts, it was predicted that, knocking down this gene may yield varying phenotypes under different day light conditions. In order to investigate this prediction the Atxyl3 plants were grown under continuous light in contrast to the WT and observed for phenotypic differences.

2.4.4.3 Analysis of Atxyl3 mutants grown under continuous light

The phenotype of Atxyl3 with respect to the height of floral stem was more pronounced under continuous light. The floral stem of mature plant was much taller than WT
Results

(Fig. 2.4.11), in contrast to the phenotype under longday conditions. But this experiment shows only a preliminary set of data which need to be analyzed more intensively.

**Figure 2.4.11:** Analysis of *Atxyl3* T-DNA insertion mutants grown under continuous light. 5weeks old *Atxyl3* plants showing accelerated growth of floral stem compared to WT plants (A). Fully matured (senesced) *Atxyl3* plants, in which the floral stem was much taller than WT (B). (Scale bars: 4 cm in A and 4.8 cm in B).

The database predictions regarding the localization of AtXYL3, was proved by transient expression in *Arabidopsis* protoplasts in which the GFP fluorescence resulted from AtXYL3-GFP fusion protein was localized in chloroplasts. Strong signal observed by western blot against membrane fractions with AtXYL3 antibody further supports this localization. The AtXYL3 promoter was active in green as well as in non green tissue. Even though the transport activity was not determined for this gene, the observed phenotype indicates the important role of *AtXYL3* during plant development.
3. Discussion

In silico analyses revealed a new family of monosaccharide transporter like genes in *Arabidopsis thaliana*, consisting of three members (At3g03090, At5g17010, At5g59250) with strong homology to known bacterial H⁺/Xylose symporters. Besides the high sequence homology among these genes, the genomic gene structure is highly conserved with respect to the position and the number of introns. Interestingly in contrast to the average intron number of 5 found in *Arabidopsis* gene, the three new transporter homologs have an exceptionally high number of 13 introns. The aim of the presented work was to analyze these new transporter homologs with respect to their biochemical and physiological roles.

**Characterization of two new vacuolar sugar transporters in *Arabidopsis thaliana***

Among the three genes of this xylose transporter family, the amino acid sequence of the proteins encoded by At3g03090 and At5g17010 were closely related (79% identity; 82% similarity). Because of their homology to bacterial H⁺/Xylose symporters, these genes were previously named as *Arabidopsis thaliana* Xylose transporter 1 and 2 (AtXYL1 and 2). In fact, the homology between AtXYL1 and AtXYL2 and bacterial xylose transporters is not higher than the homology of the AtXYLs to other monosaccharide transporters in *Arabidopsis* and other species like *Vicia faba*. Based on the results presented here, the two highly homologous members At3g03090 and At5g17010 were renamed as Vacuolar Glucose Transporter 1 and 2 (AtVGT1 ans 2). The tissue specific and cell specific expression patterns and the biochemical and physiological roles of these genes were described in the following sections.

**AtVGT1, a vacuolar H⁺/glucose transporters**

To determine the transport properties, AtVGT1 was functionally expressed in baker’s yeast (*Saccharomyces cerevisiae*). As the uptake experiments in yeast cells yielded no results, the intracellular localization of AtVGT1 was determined. Expression of an *AtVGT1-GFP* fusion construct in *S.cerevisiae* (Fig. 2.1.5) revealed that the AtVGT1 gene product was localized in the vacuolar membrane.
Biochemical function of AtVGT1

As AtVGT1 is localized in the vacuolar membrane in yeast, vacuoles were isolated from transgenic yeast cells expressing AtVGT1 and uptake experiments were performed. Therefore a method described by Ohusumi and Anraku (1981) for the isolation and uptake experiments was slightly modified. Before performing the sugar uptake experiments, the isolated vacuoles were tested for viability by testing for amino acid uptake competence. The subsequent sugar uptake experiments with these vacuoles demonstrated that AtVGT1 is a monosaccharide transporter with glucose being its major substrate. Glucose was transported at a rate of 1.34 nmol·mg protein$^{-1}$·min$^{-1}$. Measurements recorded after 10 mins indicates that the transport process is saturated after 5 mins. AtVGT1 also transports fructose but only with 42% of the glucose transport rate and galactose (14%). Since several databases annotate AtVGT1 as a putative xylose transporter similar to bacterial xylose permease, a possible uptake of xylose (a pentose) was also tested as possible substrate. However, no transport activity was detected for xylose in vacuoles from At3g03090-expressing yeasts.

Several studies of the subcellular sugar distribution in a variety of plant species document significantly higher hexose concentrations in the vacuole in comparison to the cytosol (Wagner, 1979; Yamaki, 1984; Heineke et al., 1994; Moore et al., 1997; Voitsekhovskaja et al., 2006). Heineke et al. (1994) found up to 98% of the hexoses in the vacuole of tobacco leaves and proposed specific transporters for the active uptake of glucose and fructose against a high concentration gradient.

It is widely accepted that the vacuolar transporters function as H$^+$/sugar antiporters because of the low internal vacuolar pH. Plant vacuoles contain a proton-translocating ATPase which generates an inside acidic pH and a positive membrane potential (Thom and Komor, 1984). Uptake experiments with isolated yeast vacuoles, performed at different pH ranges indicated that AtVGT1 is an active transporter of glucose. No transport activity was detected for glucose from pH range 5.2 to 6.2. The transport rates observed at pH 7.9 were significantly higher than the transport rate at 6.9 (Fig. 2.1.9). The pH dependence of the newly identified glucose transporter AtVGT1 very well fit into the model that glucose-uptake was accompanied by a proton-efflux in a 1:1 stoichiometry, which clearly supports the model of a glucose/H$^+$ antiport.

Despite the longstanding knowledge about sugar transport across the tonoplast in higher plants, to date no vacuolar sugar transport protein could be isolated and characterized. An immunological approach by Gietz et al. (1993) gave some indications that the sucrose
transport activity of the red beet tonoplast is associated with polypeptides in the range 55-60 kDa when reconstituted in proteoliposomes however, no further characterization was reported. Recently, a sucrose transporter from barley, HvSUT2, was found to be localized to the vacuole in a proteomics approach and in transient expression studies using GFP fusion proteins (Endler et al., 2006). However, results from expression studies of HvSUT2 in yeast (Weschke et al., 2000) as well as immunolocalization to the plasma membrane of its closest homologs in tomato (LeSUT4) and potato (StSUT4) (Weise et al., 2000) are contrary to this finding.

**AtVGT1 expression profile in planta**

According to the Genevestigator *Arabidopsis thaliana* Microarray database (https://www.genevestigator.ethz.ch/at/index.php), AtVGT1 is expressed at basal levels in almost all tissues in all developmental stages, with highest level of expression in mature pollen. However, analysis of GUS reporter plants showed AtVGT1 promoter activity only in anthers, mainly in pollen grains. Possibly, our reporter-construct is missing one or more regulatory elements, which could explain the observed discrepancy in expression patterns of microarray and reporter plants. Furthermore, the extremely high homology of AtVGT1 and AtVGT2 did not allowed to generate an antiserum specific for either protein, which also made it difficult to perform immunolocalization studies.

**Analysis of homozygous T-DNA insertion lines for AtVGT1**

To investigate the physiological role of *AtVGT1* in plants, two independent T-DNA insertion mutants were analyzed for phenotypic differences to WT during growth and development. Strikingly, about 20% of the seeds from both lines failed to germinate on agar plates. In addition, in both lines bolting, the first response after flower initiation is delayed by 9-14 days independent of the day length. Bolting is an important process in plants, which is regulated by several physiological and biochemical factors. It was also described that during bolting, the demand for carbohydrates, significantly increases and soluble sugars such as glucose and sucrose play a role in bolting time determination. According to the Genvestigator database, AtVGT1 displays slightly elevated expression in etiolated seeds and in the shoot apex above the otherwise basal levels. Floral induction was proposed to be a means of modifying the source/sink relationship within the plant so that the shoot apex receives a higher concentration of assimilates (Sachs and Hackett, 1969). Both of these effects, reduced germination as well as late flowering with retarded growth of the shoot, support a possible
function of AtVGT1 in vacuolar hexose accumulation and thus formation or maintenance of cell turgor, to drive cell expansion during floral transition. Further support for such a role of vacuolar hexoses comes from the functional analysis of vacuolar invertase knockouts defective in root elongation (Sergeeva et al., 2006) and from the finding that hexose accumulation accounts for a large proportion of the osmotic potential in the cell elongation zone of maize root tips (Sharp et al., 1990).

The highest level of *AtVGT1* expression found in pollen correlates with the expression of 5 AtSTPs (AtSTP2 (Truernit et al., 1999), AtSTP6 (Scholz-Starke et al., 2003), AtSTP9 (Schneidereit et al., 2003), AtSTP4, AtSTP11) in pollen, which are responsible for hexose import into this symplastically isolated strong sink (Büttner and Sauer, 2000). In addition, the sucrose transporters *AtSUC1*, *AtSUC3* and *AtSUC4* are also expressed in pollen and responsible for disaccharide import, possibly to allow synthesis of sufficient cell wall material during pollen germination and pollen tube growth. The rapidly elongating cell is not only controlled by cell wall expansion but also by internal turgor development (Kutschera and Köhler, 1994). It was well accepted that, the vacuolar sugars play an important role in building and/or maintaining cell turgor. In addition to the AtSTPs and AtSUCs, which allow sugar transport across the plasma membrane, the vacuolar sugar transporter *AtVGT1* might play an important role in building the necessary turgor for rapid elongation of pollen tube. However, impairment of this process could not be observed in the respective mutants, indicating that plants can compensate the loss of AtVGT1-mediated glucose transport into the vacuole by other transporters (e.g. AtVGT2) and/or alternative osmolytes.

**Characterization of AtVGT2**

Also for *AtVGT2* (MIPS code: At5g17010), the closest homolog of *AtVGT1* within the newly identified transporter family, vacuolar localization could be demonstrated in bakers yeast and in *Arabidopsis* protoplasts by expression of a GFP fusion.

**Expression profile of *AtVGT2* in Planta**

To obtain an estimate of the relative *AtVGT2* expression levels, the Genevestigator *Arabidopsis thaliana* Microarray Database ([https://www.genevestigator.ethz.ch/at/index.php](https://www.genevestigator.ethz.ch/at/index.php)) was queried. According to this database, *AtVGT2* is expressed nearly in all tissues and organs to significant levels (Refer to appendix fig: A27). Analysis of the transgenic *Arabidopsis* plants expressing a reporter gene (GUS) under the control of *AtVGT2* promoter reconfirmed these Microarray data and showed in more detail that *AtVGT2* was expressed in hydathodes of
early cotyledons, in rosette leaf, in flower parts except in pollen and pollen sac and in the
vasculature of 1 week old seedling root and in the inflorescence stem.

**Possible Biochemical function of AtVGT2**

The transport properties of AtVGT2 could not be determined in the present work. Despite the successful expression of an AtVGT2-GFP fusion in baker's yeast, uptake experiments performed with vacuoles isolated from yeast cells expressing AtVGT2 alone did not show accumulation of the tested sugars. However, due to the high degree of sequence homology to AtVGT1, a similar function as active monosaccharide transporter of the tonoplast can also be assumed for AtVGT2. Further experiments by expression of AtVGT2 in oocytes and performing sugar transport assays in plant vacuoles might reveal possible biochemical function of AtVGT2.

**Analysis of AtVGT2 T-DNA insertion lines**

Homozygous Atvgt2 T-DNA insertion lines were analyzed to uncover the physiological role of this putative transporter. Despite of its strong expression in most tissues, no impairment of growth or any of the developmental processes was observed in the mutant lines. This may be due to a possible compensation by its homolog AtVGT1. More sensitive approaches like HPLC analyses of the subcellular sugar distributions will be necessary to elucidate the physiological role of AtVGT2.

**Altered development of Atvgt1/Atvgt2 seedlings, rosette, bolting, silique, seed and interfascicular fibers**

As there is no significant phenotype observed for Atvgt2 mutant lines, an Atvgt1/Atvgt2 double mutant was generated and analyzed. Since in the Atvgt1 single mutants only 80% of the seeds germinated, this developmental step was also analyzed in the double mutant. It was observed that in Atvgt1/Atvgt2 mutants, the seed germination under standard long day growth conditions is delayed on soil. In contrast, when these seeds were grown on MS agar medium in the dark (in the absence or presence of 2% Sucrose), the double mutants were germinating much faster than WT seeds and also over Atvgt1 and Atvgt2 single mutants. The difference in advanced hypocotyl growth after germination is more prominent when grown on MS agar plates lacking sucrose. Furthermore, the greening of cotyledons in the Atvgt1/Atvgt2 double mutants was impaired even after exposed to light for 3 weeks, whereas the Atvgt1 and Atvgt2 showed normal photomorphogenesis when exposed to light. In a
number of sink tissues, it is known that the vacuoles play a significant role in accumulation of low molecular weight sugars (Pollock and Kingston-Smith 1997). Developing seeds are strong sinks and the carbon pools accumulated during seed development play an important role in distinct aspects of light-mediated de-etiolation in plants. It was shown by Elamrani et al. (1992 and 1994) that prolonged heterotrophy will lead to the depletion of internal carbon pools and is the major factor that determines loss of greening capacity in cotyledons. Furthermore studies on glucose starvation under controlled conditions in different tissues (Brouquisse et al, 1991; Tassi et al., 1992) revealed that, cells modify their metabolism under glucose starvation. The altered metabolism, though initially results in enhanced survival, finally leads to irreversible damage and cell death (Brouquisse et al., 1991). Taking in account, the observed germination phenotypes on soil as well as on synthetic growth medium in the presence and absence of light indicate the impaired glucose transport function of AtVGT1 and AtVGT2 mediated glucose transport leads to a reduced sugar loading capacity into the developing seeds which is required for the seedling survival in dark and development under light.

During further development, Atvgt1/Atvgt2 mutants showed impaired rosette expansion. In the source leaves, the assimilated sugars will be stored in the vacuole against a concentration gradient and thus create a high concentration gradient of sugars from the site of synthesis i.e from the chloroplasts to the cytosol which drives higher rates of carbon fixation during the light phase (Boller and Wiemken, 1986). High diurnal sugar concentrations in tobacco leaf mesophyll vacuoles measured by Heineke et al., (1994), supports this sort of phenomena. In this context the vacuolar sugar transporters AtVGT1 and AtVGT2 play an important role in compartmentation of sugars to the vacuole during light phase. Interruption of these genes might interfere with the above process and lead to low assimilate production during the light phase. This explains the delayed rosette expansion in Atvgt1/Atvgt2 double mutants. Strong promoter activity of AtVGT2 detected by GUS reporter gene expression in mature leaf tip further supports this argument. The bolting delay observed in Atvgt1 single mutants was further delayed for 3 to 5 days in Atvgt1/Atvgt2 double mutants. In double insertion mutants, in addition to the more pronounced bolting phenotype, the branching of inflorescence stem was defective. Compared to single knockouts and WT plants, Atvgt1/Atvgt2 double mutants showed a slender primary floral stem with very few branches, causing 60% low fresh weight of the floral stem. Analysis of the longitudinal sections showed that the Atvgt1/Atvgt2 plants have longer cells but less cell layers compared to WT. Due to the increased cell length, there might be less inter nodes which could lead to a decrease in
branching frequency observed in Atvgt1/Atvgt2 plants. Moreover a significant difference in the diameter. The differential staining of cross sections with FCA dye revealed that the primary floral stem of Atvgt1/Atvgt2 plant has less number of cell files with respect to sclerenchyma cells in interfascicular region and also the cells in the pith region have significantly smaller diameter compared to WT. These effects, together lead to the smaller diameter of Atvgt1/Atvgt2 inflorescence stem. A prominent anatomical feature in the inflorescence stem of Arabidopsis is the presence of fiber cells in the interfascicular region which provides the necessary support. Interfascicular fiber cells with thick secondary cell walls will be formed when the internodes of stems cease elongation (Zhong et al., 2001).

Several factors might lead to the impaired or altered cell structure of inflorescence stem. Vacuoles of a plant cell play an indispensable role in the maintenance of cell morphology by regulating numerous metabolic processes, especially during dormancy, sprouting, low photosynthetic activity, cell elongation and expansion (Kutschera and Köhler, 1994) by accumulating osmotically effective substances mainly hexoses and organic potassium salts. The role of vacuolar solutes in maintaining turgor and cell expansion was proved by analyzing the transgenic carrot lines in which cell expansion is reduced by antisense inhibition of subunit A of V-ATPase, which drives the solute uptake into vacuole along with H⁺-pyrophosphatase (Gogarten et al., 1992). One possible explanation for the here observed altered cell size in Atvgt1/Atvgt2 plants is that the osmotic homeostasis might be impaired, as sugar transport into vacuoles mediated by AtVGT1 and AtVGT2 was abolished. Another possible explanation might come from the interactive effects of sugar and hormonal signalling, which were known to modulate plant metabolic processes, like seed maturation, dormancy, cell differentiation and plant development by changing gene expression (Leon & Sheen, 2003; Moore and Sheen, 1999). Studies by Aloni R. (1976 and 1987) and Kirschner and Sachs (1972) have convincingly showed that auxin polar transport regulates fiber differentiation, and auxin together with gibberellin and cytokinin is required for normal development of fiber cells. Sugars were required for the biosynthesis of the fiber components cellulose, whereas auxin polar transport activity is responsible for cessation of internode elongation and initiation of secondary wall thickening (Zhong and Ye, 2001). This infers that the elongation of internodes and initiation of fiber cell differentiation in interfascicular region are tightly regulated. Although interactions between sugar and hormonal signalling pathways have been suggested, the mechanisms underlying the crosstalk between glucose and other signalling pathways remain obscure. Present analysis raises the question, whether the mutations in vacuolar sugar transporters themselves were responsible for this phenotype or
whether they somehow alter the expression of phytohormone responsive genes which in turn leads to this phenotype. Such an indirect effect is conceivable because sugars not only function as metabolites but also as osmolytes and as regulators of gene expression. In higher plants, glucose has been implicated to be the primary sugar signal through largely unknown mechanisms, that controls many aspects of plant development including germination, hypocotyl elongation, cotyledon greening and expansion, primary and lateral root growth, true leaf development, floral transition, and the onset of senescence. The fact, that the *Atvgt1* mutant phenotypes were even more pronounced in the *Atvgt1/Atvgt2* double mutants which, show additional differences to WT implicates an important role also for *AtVGT2*.

**Characterization of a putative plastidic sugar transporter-*AtXYL3***

Chloroplasts were organelles of endosymbiotic origin, which transferred most of their genetic information to the host nucleus during evolution (Jürgen Soll and Enrico Schleiff, 2004) and therefore have to import more than 95% of their proteins. The *AtXYL3* protein has an N-terminal extension predicted to be a chloroplast Transit peptide cTP. According to the protein targeting prediction tool of the ARAMEMNON database, *AtXYL3* has a plastidic translocation peptide of 31 amino acids, starting from the very N-terminal amino acid. In the present work, this was proven by transient expression of an *AtXYL3-GFP* fusion construct in *Arabidopsis* protoplasts. Consequently, this protein appeared in proteomic studies of the chloroplast envelope membrane proteins (Ferro et al., 2003), in which the *AtXYL3* protein was predicted as a member of the inner envelope membrane.

**AtXYL3 expression profile in planta**

According to the genevestigator *Arabidopsis thaliana* microarray database (https://www.genevestigator.ethz.ch/at/index.php), *AtXYL3* is expressed in green as well as in non green tissues, with highest percentage (46.3%) of total subfamily expression levels. The genevestigator data was reconfirmed by GUS reporter plants showing that the *AtXYL3* promoter can be found in cotyledons, in the shoot apex, in the rosette and cauline leaves, in sepals and pollen, in the cortical region of the seedling root and in the stalk of the siliqua.
Phenotype of *AtXYL3*-possible biochemical and physiological roles

In order to investigate the physiological role of *AtXYL3*, three T-DNA insertion lines were obtained. Unfortunately, only one line, SALK_N521796 was found to harbour a T-DNA insertion in the *AtXYL3* gene. Homozygous mutants were obtained and analyzed for phenotypic differences. In contrast to the phenotype observed with the *Atvgt1* single and *Atvgt1/Atvgt2* double mutants, the *Atxyl3* plants were developing faster than WT plants. The rosette expansion and the bolting processes were slightly advanced to that of WT. In contrast to this phenotypic difference of the vegetative plant, the silique and seed development in *Atxyl3* plants was impaired. The *Atxyl3* silique measures only 2/3rd to that of the WT silique and seed density is 5 times less. Despite the initial advanced development, the mature *Atxyl3* plants are similar to WT plants. On the other hand, when grown under continuous light, *Atxyl3* plants developed much faster than WT. In contrast to the phenotype under long-day, the *Atxyl3* plants grown under continuous light were much taller than wild type even by the time of senescence.

Possible biochemical function of *AtXYL3*

The plastidic localization of this putative sugar transporter made the expression and transport experiments in heterologous system difficult. Expression of a GFP fusion construct in *Saccharomyces cerevisiae* revealed that the *AtXYL3* was not expressed in yeast cells. Even though the transport properties of *AtXYL3* were not depicted in the present work, the phenotype of *Atxyl3* mutant under long day and continuous light indicates a possible function as plastidic glucose transporter. Carbon fixation, storage and transport inside the chloroplasts and transport to amyloplasts, is of major importance. Some of the carbon fixed during the day is stored in chloroplasts as transitory starch. Several researchers showed that sugar can be transported across the plastidic envelope membrane in phosphorylated or adenylated form. Besides the evidence for transport of glucose across the chloroplast envelope, no transporter has been proved until now assigning this function in *Arabidopsis*. Recently a plastidic glucose transporter pGlcT was identified by Weber *et al.* (2000) but the functional analysis of the corresponding protein failed. The expression patterns of *AtXYL3* in green and non green tissue could indicate that, the *AtXYL3* is a plastidic Glc transporter and might be involved in transitory starch metabolism.
Possible physiological role of *AtXYL3*

Carbon fixation in chloroplasts by photosynthesis is a highly controlled process with several regulatory levels. Assimilate concentration in plastids and sink strength is one of the key regulatory points that control photosynthesis. *Atxyl3* mutant plants were showing slightly advanced development compared to WT. This difference is more obvious, when these plants were grown under continuous light. Together, the *AtXYL3* expression pattern and the *Atxyl3* phenotype support the possible physiological role of *AtXYL3* in transitory starch utilization in chloroplasts and amyloplasts.

Finally, Affymetrix chip analysis of *Atvgt1/Atvgt2* and *Atxyl3* plants has already been initiated. Functional expression of *AtXYL3* in oocytes and in yeast with shortened N-terminus is also the forthcoming plan. With the aid of more sensitive approaches involving HPLC techniques, storage carbon content of *Atvgt1/Atvgt2* seeds and sugar contents in different organs of the mutants grown under different day light conditions should be analyzed. The possible interaction of Auxin responsive genes and the vacuolar glucose transporters must be elucidated.
4. Materials and Methods

4.1 Materials

The biological and chemical materials obtained or generated and used in the present work were mentioned in the following sections.

4.1.1 Micro-organisms

4.1.1.1 Non Transformed bacterial strains

<table>
<thead>
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<th>Organism</th>
<th>strain</th>
<th>Marker</th>
<th>Reference</th>
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</thead>
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<td><em>E. coli</em></td>
<td>DH5α</td>
<td>F-, endA1, hsdR17 (rk-, mk-), supE44, thi-1, recA1, gyrA96, relA1, Φ80d, lacZ[Δ]M15</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td>Novagen</td>
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<td>GV3101</td>
<td>GentR, RifR</td>
<td>Holsters <em>et al.</em>, 1980</td>
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</table>

Table 4.1: Non-transformed bacterial strains

4.1.1.2 Non transformed yeast strains

*Saccharomyces cerevisiae:*

<table>
<thead>
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<th>Strain</th>
<th>Marker</th>
<th>Reference</th>
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<td>EBY. VW-4000</td>
<td>Mata; leu2-3,112; ura3-52; trp1-289; his3-Δ1; MAL2-8c; SUC2; Δhxt1-17; Δgal2; Δstl1; Δagt1; Δmph2; Δmph3</td>
<td>Boles <em>et al.</em>, 1999</td>
</tr>
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</table>

Table 4.2: Non-transformed yeast strains used in the present work.

4.1.2 Plants

*Arabidopsis thaliana* (Ecotype: Columbia)

4.1.2.1 Transgenic *Arabidopsis* plants

<table>
<thead>
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<th>Construct</th>
<th>Selectionsmarker</th>
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<td>BASTA</td>
</tr>
<tr>
<td>pSA104A</td>
<td><em>AtVGT1</em> promoter-GUS-NOS terminator</td>
<td>BASTA</td>
</tr>
<tr>
<td>pSA222A</td>
<td><em>AtVGT2</em> promoter-GUS-NOS terminator</td>
<td>BASTA</td>
</tr>
<tr>
<td>pSA324A</td>
<td><em>AtVGT2</em> promoter-GFP-NOS terminator</td>
<td>BASTA</td>
</tr>
<tr>
<td>pSA323A</td>
<td><em>AtVGT3</em> promoter-GUS-NOS terminator</td>
<td>BASTA</td>
</tr>
<tr>
<td>pSA325A</td>
<td><em>AtXYL3</em> promoter-GFP-NOS terminator</td>
<td>BASTA</td>
</tr>
</tbody>
</table>

Table 4.3: *Arabidopsis thaliana* transformants generated in the present work.
Materials and Methods

4.1.2.2 Arabidopsis T-DNA insertion lines

<table>
<thead>
<tr>
<th>Name (Institute)</th>
<th>Site of Insertion (orientation)</th>
<th>Selection marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>669-D03 (SAIL)</td>
<td>+2760-I (VGT1::RB T-DNA LB::VGT1)</td>
<td>BASTA</td>
</tr>
<tr>
<td>013317 (SALK)</td>
<td>+2881-I (VGT1::LB T-DNA RB::VGT1)</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>000988 (SALK)</td>
<td>-1 (VGT1::RB T-DNA LB::VGT1)</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>756-B12 (SAIL)</td>
<td>+264-I (VGT2::RB T-DNA LB::VGT2)</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>090827 (SALK)</td>
<td>+3177-E (VGT2::LB T-DNA RB::VGT2)</td>
<td>BASTA</td>
</tr>
<tr>
<td>335-F05 (SAIL)</td>
<td>-33 (XYL3::RB T-DNA LB::XYL3)</td>
<td>BASTA</td>
</tr>
<tr>
<td>1253-A02 (SAIL)</td>
<td>+1443-E (XYL3::RB T-DNA LB::XYL3)</td>
<td>BASTA</td>
</tr>
<tr>
<td>021796 (SALK)</td>
<td>+447-E (XYL3::RB T-DNA LB::XYL3)</td>
<td>Kanamycin</td>
</tr>
</tbody>
</table>

Table 4.4: Arabidopsis thaliana T-DNA insertion lines, analyzed in the present work.

4.1.3 Vectors

4.1.3.1 Empty vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Selections marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBS (pBluescript II SK)</td>
<td>Amp&lt;sup&gt;K&lt;/sup&gt;</td>
<td>Stratagene, LaJolla</td>
</tr>
<tr>
<td>NEV-E</td>
<td>Amp&lt;sup&gt;K&lt;/sup&gt;, URA3</td>
<td>Sauer und Stolz, 1994</td>
</tr>
<tr>
<td>pEXTag-GFP2</td>
<td>Amp&lt;sup&gt;K&lt;/sup&gt;, URA3</td>
<td>Sabine raab (Besenbeck, 1998)</td>
</tr>
<tr>
<td>pGEM®-T Easy</td>
<td>Amp&lt;sup&gt;K&lt;/sup&gt;</td>
<td>Promega, Madison</td>
</tr>
<tr>
<td>MAL c2</td>
<td>Amp&lt;sup&gt;K&lt;/sup&gt;</td>
<td>New England Biolabs, Schwalbach</td>
</tr>
</tbody>
</table>

Table 4.5: Empty vectors

4.1.3.2 Vectors with inserts

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Destination vector</th>
<th>Insert</th>
<th>Microbial strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSA101</td>
<td>pAF1</td>
<td>\textit{AtVGT1} promoter</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA102</td>
<td>pGPTV-Bar</td>
<td>\textit{AtVGT1} promoter-GFP</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA102A</td>
<td>pGPTV-Bar</td>
<td>\textit{AtVGT1} promoter-GFP-NosT</td>
<td>GV3101</td>
</tr>
<tr>
<td>pSA103</td>
<td>pAF6</td>
<td>\textit{AtVGT1} promoter</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA104</td>
<td>pGPTV-Bar</td>
<td>\textit{AtVGT1} promoter-GUS</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA104A</td>
<td>pGPTV-Bar</td>
<td>\textit{AtVGT1} promoter-GUS-NosT</td>
<td>GV3101</td>
</tr>
<tr>
<td>pSO114s</td>
<td>NEV-E</td>
<td>\textit{AtVGT1} cDNA in sense</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSO114as</td>
<td>NEV-E</td>
<td>\textit{AtVGT1} cDNA in anti sense</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSAY114s</td>
<td>NEV-E</td>
<td>\textit{AtVGT1} cDNA in sense</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSAY114as</td>
<td>NEV-E</td>
<td>\textit{AtVGT1} cDNA in anti sense</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA110</td>
<td>pEXtag-GFP2</td>
<td>\textit{AtVGT1} cDNA (*no stop)</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA110</td>
<td>pEXtag-GFP2</td>
<td>\textit{AtVGT1} cDNA (*no stop)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA115</td>
<td>pGEM</td>
<td>\textit{AtVGT1} cDNA (*no stop)</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA116</td>
<td>pGEM</td>
<td>\textit{AtVGT1} cDNA</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA120</td>
<td>pSO35e</td>
<td>\textit{AtVGT1} cDNA (*no stop)</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA205</td>
<td>pMAL c2</td>
<td>\textit{AtVGT2} (N-Terminus)</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA205R</td>
<td>pMAL c2</td>
<td>\textit{AtVGT2} (N-Terminus)</td>
<td>Rosetta</td>
</tr>
<tr>
<td>pSA216</td>
<td>pGEM</td>
<td>\textit{AtVGT2} cDNA</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSA217</td>
<td>pGEM</td>
<td>\textit{AtVGT2} cDNA (*no stop)</td>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Constructs Used</td>
<td>Expression Pattern</td>
<td>Host</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>--------------------</td>
<td>------</td>
</tr>
<tr>
<td>pSA218s</td>
<td>NEV-E</td>
<td><em>AtVGT2</em> cDNA (in sense)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA218as</td>
<td>NEV-E</td>
<td><em>AtVGT2</em> cDNA (in antisense)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSAY218s</td>
<td>NEV-E</td>
<td><em>AtVGT2</em> cDNA (in sense)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSAY218as</td>
<td>NEV-E</td>
<td><em>AtVGT2</em> cDNA (in antisense)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA219</td>
<td>pEXtag-GFP2</td>
<td><em>AtVGT2</em> cDNA (*no stop)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA219</td>
<td>pEXtag-GFP2</td>
<td><em>AtVGT2</em> cDNA (*no stop)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA220</td>
<td>pSO35e</td>
<td><em>AtVGT2</em> cDNA (*no stop)</td>
<td>DH5α</td>
</tr>
<tr>
<td>SA221</td>
<td>pAF6</td>
<td><em>AtVGT2</em> promoter</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA222</td>
<td>pGPTV-Bar</td>
<td><em>AtVGT2</em> promoter-<em>GUS</em></td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA222A</td>
<td>pGPTV-Bar</td>
<td><em>AtVGT2</em> promoter-<em>GUS</em>-NosT</td>
<td>GV3101</td>
</tr>
<tr>
<td>pSA224</td>
<td>pGPTV-Bar</td>
<td><em>AtVGT2</em> promoter-<em>GFP</em></td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA224A</td>
<td>pGPTV-Bar</td>
<td><em>AtVGT2</em> promoter-<em>GFP</em>-NosT</td>
<td>GV3101</td>
</tr>
<tr>
<td>pSA305</td>
<td>pMAL c2</td>
<td><em>AtXYL3</em> (N-Terminus)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA305R</td>
<td>pMAL c2</td>
<td><em>AtXYL3</em> (N-Terminus)</td>
<td>Rosetta</td>
</tr>
<tr>
<td>pSA308</td>
<td>pGEM</td>
<td><em>AtXYL3</em> cDNA</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA308s</td>
<td>NEV-E</td>
<td><em>AtXYL3</em> cDNA (in sense)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA308as</td>
<td>NEV-E</td>
<td><em>AtXYL3</em> cDNA (in antisense)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSAY309s</td>
<td>NEV-E</td>
<td><em>AtXYL3</em> cDNA (in sense)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSAY309as</td>
<td>NEV-E</td>
<td><em>AtXYL3</em> cDNA (in antisense)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA319</td>
<td>pGEM</td>
<td><em>AtXYL3</em> cDNA (*no stop)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA320</td>
<td>pSO35e</td>
<td><em>AtXYL3</em> cDNA (*no stop)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA321</td>
<td>pEXtag-GFP2</td>
<td><em>AtXYL3</em> cDNA (*no stop)</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSAY321</td>
<td>pEXtag-GFP2</td>
<td><em>AtXYL3</em> cDNA (*no stop)</td>
<td>EBY.VW-4000</td>
</tr>
<tr>
<td>pSA322</td>
<td>pAF6</td>
<td><em>AtXYL3</em> promoter</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA323</td>
<td>pGPTV-Bar</td>
<td><em>AtXYL3</em> promoter-<em>GUS</em></td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA323A</td>
<td>pGPTV-Bar</td>
<td><em>AtXYL3</em> promoter-<em>GUS</em>-NosT</td>
<td>GV3101</td>
</tr>
<tr>
<td>pSA324</td>
<td>pAF1</td>
<td><em>AtXYL3</em> promoter</td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA325</td>
<td>pGPTV-Bar</td>
<td><em>AtXYL3</em> promoter-<em>GFP</em></td>
<td>DH5α</td>
</tr>
<tr>
<td>pSA325A</td>
<td>pGPTV-Bar</td>
<td><em>AtXYL3</em> promoter-<em>GFP</em>-NosT</td>
<td>GV3101</td>
</tr>
</tbody>
</table>

**Table 4.6:** Plasmids generated in the present work
4.1.4 Oligonucleotides

Oligonucleotides used in the present work were obtained from biomers.

4.1.4.1 Oligonucleotides used for cloning and sequencing of \textit{AtVGT1}

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtXYL1c-15f(MfeI)</td>
<td>CAAGCTTCAATTGCCATGGGGTTTGAT</td>
</tr>
<tr>
<td>AtXYL1C+1526r(NcoI)</td>
<td>AGATGAGTAACCATGGAGACACATTTG</td>
</tr>
<tr>
<td>AtXYL1C+1522r(MfeI)</td>
<td>GAGTCAATTGTTAGAGACATTTGGCTTCAATTTC</td>
</tr>
<tr>
<td>AtXYL1G-1876f(SphI)</td>
<td>GATGTTGGAAGCATGCATATATGG</td>
</tr>
<tr>
<td>AtXYL1g+66r</td>
<td>CGATAATGAGAAAGCGAACC</td>
</tr>
<tr>
<td>AtXYL1c-20f</td>
<td>CATACCAAGCTTCCGTAGCC</td>
</tr>
<tr>
<td>AtXYL1g-382r</td>
<td>TGTTAATCAAATTTCCAGTTTCG</td>
</tr>
<tr>
<td>AtXYL1g+3052r</td>
<td>CGCTAACACACAAAGAGTAAG</td>
</tr>
<tr>
<td>AtXYL1g+1965f</td>
<td>CTCAATGTACATTGCAGAGC</td>
</tr>
<tr>
<td>AtXYL1g+1382f</td>
<td>CATAGAAACAAGCAACTCTTTG</td>
</tr>
<tr>
<td>AtXYL1g+408r</td>
<td>CTTTTCACGACTAATCCACC</td>
</tr>
<tr>
<td>AtXYL1g+2326f</td>
<td>GGGACAAGGAAATGGGAGGAGATC</td>
</tr>
<tr>
<td>AtXYL1g+3029r</td>
<td>GTTCCTTTCAACTCTTCG</td>
</tr>
<tr>
<td>AtXYL1g+2207f</td>
<td>CGGTTATTTCTGGTGCGCCA</td>
</tr>
</tbody>
</table>

\textbf{Table 4.7:} Oligonucleotides used for cloning and sequencing of \textit{AtVGT1}. 
### 4.1.4.2 Oligonucleotides used for cloning and sequencing of *AtVGT2*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtXYL2g+613r</td>
<td>CGGGAGAATGGCGCGAAGCAGCG</td>
</tr>
<tr>
<td>AtXYL2c+1020r</td>
<td>TGCAGGCTGAAAGGAGCGCG</td>
</tr>
<tr>
<td>AtXYL2g-423r</td>
<td>CATTTATGCAAAATAGGAGATGGAAG</td>
</tr>
<tr>
<td>AtXYL2g-1907f</td>
<td>ACCACATATACCTAAATGTTTCCCAATTTTC</td>
</tr>
<tr>
<td>AtXYL2g-1407f</td>
<td>TTGAGGTGATGTTGTTGTTGAGG</td>
</tr>
<tr>
<td>AtXYL2g+14r(NcoI)</td>
<td>GGATCAAGCGGCATGCGGTGTTGAGG</td>
</tr>
<tr>
<td>AtXYL2g-12f(NcoI)</td>
<td>CAAATACAGGCGGCATGCGGTGTTGAGG</td>
</tr>
<tr>
<td>AtXYL2g+4479r(BbsI)</td>
<td>GAAGAAGTTCATGAGCATTTGGTCCTCAATTTC</td>
</tr>
<tr>
<td>AtXYL2g-41f(BbsI)</td>
<td>GAAGACTAATTAGACATTAGTGCTGTCGTC</td>
</tr>
<tr>
<td>AtXYL2g+4482r(BbsI)</td>
<td>GAAGACTAATTAGACATTAGTGCTGTCGTC</td>
</tr>
<tr>
<td>AtXYL2g-2356f</td>
<td>GAGAGAGAGAGTTCTAGAAGGAGGAAGGC</td>
</tr>
<tr>
<td>AtXYL2g-361f</td>
<td>CGACATATACAAACCAGTGACC</td>
</tr>
<tr>
<td>AtXYL2g-1801f</td>
<td>CGCGCAATAGATTTAGTGACAT</td>
</tr>
<tr>
<td>AtXYL2g-1347f</td>
<td>GGTGTTGCGAGTTATGAGAAGGAG</td>
</tr>
<tr>
<td>AtXYL2g+2573f</td>
<td>CAGAGAGTTATGAGAAGGAG</td>
</tr>
<tr>
<td>AtXYL2g+3689r</td>
<td>CAGGCGCAATAAAGCAGCG</td>
</tr>
<tr>
<td>AtXYL2c+46f(MBP)</td>
<td>CTACAGAGTTTGGTAAGTCATCTGGTGAGTACGCG</td>
</tr>
<tr>
<td>AtXYL3g+10r(NcoI)</td>
<td>CGAAAGCCATGGTGTTTGCAG</td>
</tr>
<tr>
<td>AtXYL3c-11f(NcoI)</td>
<td>CCGGATCCGACCATGCGGTGAGTGC</td>
</tr>
<tr>
<td>AtXYL3c+413f</td>
<td>ACTTTCACCTGTGTTGCTGAGTCC</td>
</tr>
<tr>
<td>AtXYL3g+10r(NcoI)</td>
<td>CGGAAGACCATGCGGTGAGTGC</td>
</tr>
<tr>
<td>AtXYL3c-11f(NcoI)</td>
<td>CCGGATCCGACCATGCGGTGAGTGC</td>
</tr>
<tr>
<td>AtXYL3g+2894r(BbsI)</td>
<td>GAAGACCTCATGCACTTCAAGATTTGGATTCATTTTC</td>
</tr>
<tr>
<td>AtXYL3c+1f</td>
<td>ATGGCTTTGCTGCTGTCG</td>
</tr>
<tr>
<td>AtXYL3g+2897r</td>
<td>TCACCTGCAAGATTTTGTGAGTCAATTTTC</td>
</tr>
<tr>
<td>AtXYL3g-2087f(Xmal)</td>
<td>CTTCAGACCAGCGGTGVAAATTCTCCCTTC</td>
</tr>
<tr>
<td>AtXYL3c+43f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3c+94f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3g+635r</td>
<td>CCGAGGCCATGCCATGCTGAC</td>
</tr>
<tr>
<td>AtXYL3c+28f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3g+1f(EcoRI)(MBP)</td>
<td>CGCGGAATTTCGCTGCTGCTGCGGTAGTACGAC</td>
</tr>
<tr>
<td>AtXYL3g+294r(STOP-HindIII)</td>
<td>GCGCAAGCTTCAATCAGCGCGCGAAGGAGTACGAC</td>
</tr>
</tbody>
</table>

Table 4.8: Oligonucleotides used for cloning and sequencing of *AtVGT2*.

### 4.1.4.3 Oligonucleotides used for cloning and sequencing of *AtXYL3*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtXYL3g+106f</td>
<td>GCTGCTGTCTCTATCTCTCCTC</td>
</tr>
<tr>
<td>AtXYL3g+1524r</td>
<td>CATCAGCAGGACAAACCGGAGG</td>
</tr>
<tr>
<td>AtXYL3g+1266r</td>
<td>GAGATACATAACCTGTGCAAC</td>
</tr>
<tr>
<td>AtXYL3g-1244f</td>
<td>GATCAGCTCTCTAAATGTCGA</td>
</tr>
<tr>
<td>AtXYL3g-381r</td>
<td>GTGGTGATTTGAGTACGC</td>
</tr>
<tr>
<td>AtXYL3c+413f</td>
<td>ACTTTCACCTGTGTTGCTGAGTCC</td>
</tr>
<tr>
<td>AtXYL3g+10r(NcoI)</td>
<td>CGAAAGCCATGGTGTTTGCAG</td>
</tr>
<tr>
<td>AtXYL3c-11f(NcoI)</td>
<td>CCGGATCCGACCATGCGGTGAGTGC</td>
</tr>
<tr>
<td>AtXYL3g+2894r(BbsI)</td>
<td>GAAGACCTCATGCACTTCAAGATTTGGATTCATTTTC</td>
</tr>
<tr>
<td>AtXYL3c+1f</td>
<td>ATGGCTTTGCTGCTGTCG</td>
</tr>
<tr>
<td>AtXYL3g+2897r</td>
<td>TCACCTGCAAGATTTTGTGAGTCAATTTTC</td>
</tr>
<tr>
<td>AtXYL3g-2087f(Xmal)</td>
<td>CTTCAGACCAGCGGTGVAAATTCTCCCTTC</td>
</tr>
<tr>
<td>AtXYL3c+43f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3c+94f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3g+635r</td>
<td>CCGAGGCCATGCCATGCTGAC</td>
</tr>
<tr>
<td>AtXYL3c+28f(NcoI)</td>
<td>CCATGGGCGTTAAAAACGAGGCC</td>
</tr>
<tr>
<td>AtXYL3g+1f(EcoRI)(MBP)</td>
<td>CGCGGAATTTCGCTGCTGCTGCGGTAGTACGAC</td>
</tr>
<tr>
<td>AtXYL3g+294r(STOP-HindIII)</td>
<td>GCGCAAGCTTCAATCAGCGCGCGAAGGAGTACGAC</td>
</tr>
</tbody>
</table>

Table 4.9: Oligonucleotides used for cloning and sequencing of *AtXYL3*
4.1.5 Culture media

4.1.5.1 Bacterial culture media

<table>
<thead>
<tr>
<th>LB-Medium (Luria Broth)</th>
<th>0.5% Bacto® Yeastextract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% Bacto® Tryptone</td>
</tr>
<tr>
<td></td>
<td>1% NaCl</td>
</tr>
<tr>
<td>SOB-Medium</td>
<td>0.5% Bacto® Yeastextract</td>
</tr>
<tr>
<td></td>
<td>2% Bacto® Tryptone</td>
</tr>
<tr>
<td></td>
<td>10mM NaCl</td>
</tr>
<tr>
<td></td>
<td>2.5mM KCl</td>
</tr>
</tbody>
</table>

Appropriate amounts of antibiotics were added to the medium after autoclaving.

Ampicillin 50µg/ml
Kanamycin 50µg/ml

4.1.5.2 Yeast culture media

<table>
<thead>
<tr>
<th>CAA-Medium</th>
<th>0.67% YNB (Yeast Nitrogen Base) without amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% CAS amino acids</td>
</tr>
<tr>
<td></td>
<td>2% Maltose</td>
</tr>
<tr>
<td>YPD-Medium</td>
<td>1% Bacto® Yeast extract</td>
</tr>
<tr>
<td></td>
<td>2% Bacto® Peptone</td>
</tr>
<tr>
<td></td>
<td>2% Glucose</td>
</tr>
</tbody>
</table>

Solid media contain 2% Agar in addition.

Yeast strain EBY.VW-4000 (Boles et al., 1999) used in this work will grow on maltose as sole carbon source. Tryptophan (19.2 µg/ml end concentration) was added to the medium before use.

4.1.5.3 Soil composition and media used to grow plants

<table>
<thead>
<tr>
<th>Soil composition</th>
<th>65% compost soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25% Sand</td>
</tr>
<tr>
<td></td>
<td>10% Granulate</td>
</tr>
<tr>
<td>MS medium</td>
<td>0.44% (w/v) Salt-Vitamin Mixture</td>
</tr>
<tr>
<td></td>
<td>0.05% (w/v) MES</td>
</tr>
<tr>
<td></td>
<td>2% (w/v) Sucrose pH adjusted to 5.7 with 1N KOH</td>
</tr>
<tr>
<td></td>
<td>0.8% Phytagar</td>
</tr>
</tbody>
</table>
4.1.6 Solutions

2PI (Protease inhibitor 100x)  
100 mM PMSF  
250 mM p-Amino-Benzamidin  
dissolved in DMSO

Acryl amide Stock solution  
30% Acryl amide  
0.8% Bisacryl amide

Betain Buffer  
400mM Betaine  
30mM KCl  
20mM HEPES, adjusted to pH 7.2 with KOH  
added 0.1% BSA and 0.1M DTT before use

Blocking buffer  
50 mM Tris/HCl  
150 mM NaCl  
1% Dry milk powder  
0.1% Triton X-100

Bradford-solution (5x)  
23.5% Ethanol  
42.5% Phosphoric acid  
0.05% Coomassie Brilliant Blue R-250

Buffer A (for yeast vacuole isolation)  
10mM MES-Tris pH 6.9  
0.1mM MgCl₂  
12% Ficoll  
1/100 vol. 2Pi

Buffer C (for yeast vacuole isolation)  
10mM MES-Tris pH 6.9, 7.9  
5mM MgCl₂  
12.5mM KCl  
1/100 vol. 2Pi

Buffer PII  
50 mM Potassium phosphate buffer pH 6.3  
20% Glycerine  
1 mM EDTA

Carrier-DNA  
Heringssperma-DNA (10 mg/ml). Denatured by heating for 5mins at 95°C before use.

Coomassie-dye  
0.05% Coomassie Brilliant Blue R-250  
25% Isopropanol  
10% Acetic acid
<table>
<thead>
<tr>
<th>Material (RNase-free)</th>
<th>Composition</th>
</tr>
</thead>
</table>
| Digestion Buffer     | 0.03% Pectolyase Y23  
                     | 0.75% Cellulase YC, dissolved in MCP Buffer |
| EB-Buffer (RNase-free) for RNA-Extraction | 100 mM NaCl  
                                 | 10 mM Tris/HCl pH 7.5  
                                 | 1 mM EDTA  
                                 | 1% SDS |
| All the solutions (except Tris/HCl) were treated with 0.1% DEPC, to inactivate RNases. Tris/HCl solution is prepared with DEPC treated water. |
| Enzyme solution for protoplast isolation | 1% Cellulase  
                                 | 0.2% Macerozyme (dissolved in Solution A) |
| Ethidium bromide-Stock solution | 10 mg/ml dissolved in H2O |
| Glycerin buffer      | 50 mM Potassium Phosphate Buffer pH 6.3  
                     | 20% Glycerin  
                     | 1 mM EDTA |
| Glycine buffer pH 2.2 | 5 mM Glycine pH 2.2  
                        | 0.5 M NaCl |
| K3 Medium            | 400mM Sucrose  
                     | 0.44% MS salt-vitamin mixture  
                     | 16.5mM xylose  
                     | CaCl$_2$ |
| Ligation buffer (10x) | 0.5 M Tris/HCl pH 7.5  
                        | 100 mM MgCl$_2$  
                        | 100 mM DTT  
                        | 10 mM ATP |
| Loading Dye (10x)    | 100 mM EDTA pH 8  
                     | 60% Glycerin  
                     | 0.25% Bromphenol blue  
                     | 0.25% Xylene Cyanol |
| LP-Mix               | 40% Polyethylene glycol 400  
                        | 0.1 M Lithium acetate  
                        | 10 mM Tris/HCl pH 7.5  
                        | 1 mM EDTA |
**Materials and Methods**

Lysis Buffer
- 200mM Sorbitol
- 10% Ficoll
- 20mM EDTA
- 10mM HEPES, adjusted to pH 8.0 with KOH

MaMg Buffer
- 400mM Sorbitol
- 15mM MgCl₂
- 5mM MES, adjusted to pH 5.6 with KOH

MCP
- 0.5M Sorbitol
- 1mM CaCl₂
- 10mM MES, adjusted to pH 5.6-6.0 with KOH

PEG-CMS Buffer
- 400mM Sorbitol
- 100mM Ca(NO₃)₂
- 40% PEG 4000 adjusted to pH 8.0 with KOH and stabilized for 2 to 3hrs

Percoll pH 6.0
- 0.5M Sorbitol
- 1mM CaCl₂
- 20mM MES, adjusted to pH 6.0

Percoll pH 7.2
- 0.5mM Sorbitol
- 20mM HEPES

pH Jump Buffer
- 10 mM Tris/HCl pH 7.5
- 1 mM EDTA
- 1 mM MgCl₂
- 1/100 Vol. 2Pi

Resolving gel buffer (3x)
- 1.126 M Tris/HCl pH 8.8
- 0.3% SDS (store at 4°C)

SDS-Running buffer
- 25 mM Tris
- 0.1% SDS
- 192 mM Glycin

SDS-sample buffer (4x)
- 250 mM Tris/HCl pH 6.8
- 20% Glycerin
- 20% β-Mercaptoethanol
- 8% SDS
- 0.4% Bromphenol blue

Seed wash solution
- 70% ethanol
- 0.01% Triton X-100
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>0.5M Sorbitol 1mM CaCl&lt;sub&gt;2&lt;/sub&gt; 5mM MES adjusted to pH 5.5 with KOH</td>
</tr>
<tr>
<td>Sorbitol for Barley</td>
<td>400mM Sorbitol 30mM KCl 20mM HEPES, adjusted to pH 7.2 with KOH</td>
</tr>
<tr>
<td>Spheroplasting buffer</td>
<td>1M Sorbitol 2.5U/ml Zymolyase (dissolved in Zymolyase buffer)</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>139 mM Tris/HCl pH 6.8 0.11% SDS (store at 4°C)</td>
</tr>
<tr>
<td>STET-Buffer</td>
<td>50 mM Tris/HCl pH 8.0 50 mM EDTA 8% Sucrose 5% Triton X-100</td>
</tr>
<tr>
<td>TB-Buffer</td>
<td>10 mM Pipes (Na-Salt) 55 mM MnCl&lt;sub&gt;2&lt;/sub&gt; 15 mM CaCl&lt;sub&gt;2&lt;/sub&gt; 250 mM KCl</td>
</tr>
<tr>
<td>pH was adjusted to 6.7 before adding MnCl&lt;sub&gt;2&lt;/sub&gt; and sterile filtered after adding MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>TBE-Buffer (5x)</td>
<td>445 mM Tris 445 mM Boric acid 5 mM EDTA</td>
</tr>
<tr>
<td>TBS-Buffer</td>
<td>50 mM Tris/HCl pH 7.5 50 mM NaCl</td>
</tr>
<tr>
<td>TBST-Buffer</td>
<td>50 mM Tris/HCl pH 7.5 150 mM NaCl 0.1 % Triton X-100</td>
</tr>
<tr>
<td>TE-Buffer</td>
<td>10 mM Tris/HCl pH 7.5 1 mM EDTA</td>
</tr>
<tr>
<td>TE/RNase</td>
<td>10 mM Tris/HCl pH 7.5 1 mM EDTA RNase A (~100µg/ml, store at 4°C)</td>
</tr>
</tbody>
</table>
Materials and Methods

W5 Solution
154mM NaCl
125mM CaCl₂
5mM KCl
5mM Glucose
1.5mM MES, adjusted to pH 5.6 with KOH

Western-Transfer-Buffer
20 mM Tris
150 mM Glycin
20% Methanol
0.02% SDS

Yeast cell lysis buffer
50 mM Tris/HCl pH 7.5
5 mM EDTA
1/100 Vol. 2Pi (Proteaseinhibitor 100x)

Zymolyase Buffer
50mM Tris-HCl pH 7.5
1mM EDTA
50% Glycerol

4.1.7 Other chemicals and enzymes

Agar-Agar (Difco Laboratories, Detroit)
Agarose ultra pure (GIBCO BRL, Life Technologies, New York)
Amino acids (SIGMA-ALDRICH, Deisenhofen)
Ampicillin (Roth, Karlsruhe)
Bio-Dry milk powder (Lasana)
Bio-Dry mikl powder (Uelzena)
BSA (Albumin Fraction V), (BioLabs, Schwalbach)
Chemicals to prepare Media (Difco Laboratories, Detroit)
Coomassie Brilliant Blue R-250 (Serva, Heidelberg)
DEPC (Diethylpyrocarbonat), (SIGMA-ALDRICH, Deisenhofen)
Desoxynucleotide (ROCHE, Mannheim)
DMSO (Dimethylsulfoxide) (SIGMA-ALDRICH, Deisenhofen)
DNase I, RNase-free (ROCHE, Mannheim)
Glycerin (Roth, Karlsruhe)
IPTG dioxane free (Isopropyl-β-D-thiogalactoside) (Roth, Karlsruhe)
Lumi-Light Western Blotting Substrate (ROCHE, Mannheim)
Lysozyme (ROCHE, Mannheim)
MES (SIGMA-ALDRICH, Deisenhofen)
N,N-Dimethylformamide (SIGMA-ALDRICH, Deisenhofen)
NEB-Buffersystem for Restrictiondigest (BioLabs, Schwalbach)
Oligo dT-Primer, pd(T)12-18 (Amersham Pharmacia Biotech, Freiburg)
Oligonucleotide (Interactiva, Ulm)
PEG 4000 (Polyethyleneglycol), (Fluka, Buchs/Schweiz)
Phosphatase, alkaline (ROCHE, Mannheim)
Ponceau S (SIGMA-ALDRICH, Deisenhofen)
Materials and Methods

Proteinstandard Roti®-Mark Standard (Roth, Karlsruhe)
Restriction enzymes (Bio Labs, Schwalbach)
Reverse Transcriptase, M-MuLV rev. Transcriptase (MBI Fermentas, St. Leon-Rot)
Ribonuclease-Inhibitor (MBI Fermentas, St. Leon-Rot)
RNase A (Roth, Karlsruhe)
Roti-Phenol (Roth, Karlsruhe)
Sucrose (14C- marked) for Yeast uptake experiments (SIGMA-ALDRICH, Deisenhofen)
SDS (Serva, Heidelberg)
Sequence-Mix „Big Dye Terminator RP Mix“ (Perkin Elmer, Überlingen)
Taq-DNA-Polymerase (Q-Biogene, Heidelberg)
Technovit
TEMED (Roth, Karlsruhe)
Triton X-100 (Serva, Heidelberg)
Zymolyase 20T (Shikagaku, Tokyo, Japan)

Chemicals, those are not mentioned here were obtained from Roth, Karlsruhe.

4.1.8 Secondary antibody
Anti-Rabbit-IgG/Peroxidase-Konjugate (SIGMA-ALDRICH, Deisenhofen)

4.1.9 Materials used
Microscopy slides Marienfeld HistoBond® (Linaris, Wertheim-Bettingen)
Amylose resin “Column material” (New England Bio labs, Schwalbach)
KODAK Developer D-19 (Integra Biosciences, Fernwald)
Folded filter (Ges. f. Laborbedarf mbH, Würzburg)
Filter-Tips (Roth, Karlsruhe)
KODAK Fixer Unifix (Integra Biosciences, Fernwald)
Glasbeads 0,5 mm (Braun, Melsungen)
Macrotiterplates (Greiner, Nürtingen)
Microconcentrator Centric C-30 (Amicon, Bedford/USA)
Nitrocellulose-Membrane (Sartorius, Göttingen)
NUCLEOBOND®-Columns AX 100 –Kit (Macherey-Nagel, Düren)
pGEM®-T Easy Vector Kit (Promega, Madison)
E.Z.N.A.® Gel Extraction Kit (PEQLAB, Erlangen)
QIAquick Gel Extraction Kit (Qiagen, Hilden)
QIAquick PCR Purification Kit (Qiagen, Hilden)
QuantiTect™ SYBR® Green PCR Kit (Qiagen, Hilden)
RNasy Plant RNA-Extraction Kit (Qiagen, Hilden)
X-ray film KODAK X-OMAT AR (Integra Biosciences, Fernwald)
Syringe filter-sterile (Roth, Karlsruhe)
Scintillation cocktail LumisafeTM Plus (Lumac, Groningen)
Whatmannfilter (GLW, Würzburg)
Materials and Methods

4.1.10 Machines

Cell-Lysis-Machine (Edmund Bühler, Johanna Otto GmbH, Bodelshausen)
Confocal laser scanning microscope (Leica, Bensheim)
Dounce homogeniser (Roth, Karlsruhe)
Fluorescence Microscope, Axioscope (Zeiss, Göttingen)
General Glassware (Roth, Karlsruhe)
HPLC analysis system (Dionex corporation, Sunnyvale, USA)
Liquid Scintillation counter TRI-CARB 2100 TR (Packard®, Meriden)
Microscope und Stereomicroscope with camera attachment (Zeiss, Göttingen)
PCR-Machine T-Gradient (Biometra, Göttingen)
Real-Time PCR-Machine Rotor Gene 2000 (Corbett Research, Mortlake, Australia)
Refrigerated centrifuges (Avanti Beckman, USA)
SDS-PAGE Apparatus (Biorad, München)
Sequencer ABI PRISM™ 310, Genetic analyser (Perkin Elmer, Überlingen)
Spectro photometer, Uvikon 922 (Kontron Instruments)
Stereomicroscope Stemi SV 11 (Zeiss, Göttingen)
Sterile bench (Hölzl, Wörth)
Thermocycler GeneAmp PCR System 2400 (Perkin Elmer, Überlingen)
Ultramicrotome ULTRACUT R (Leica, Bensheim)
Ultracentrifuge L 8-60 M (Beckman, München)
Videosystem for Microscope und Stereolupe (Zeiss, Göttingen)
Western-Blotting Apparatus (Biorad, München)

4.2 Methods

4.2.1. Culturing the organisms used

4.2.1.1. Microbial cultures (Bacteria and Yeast)

_E coli_ strains used usually grow at 37°C, whereas the yeast strains grow better at 29°C.
Liquid cultures were always grown while shaking at respective temperatures. Selective components were added to the medium according the plasmid requirements.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>X-Gal</td>
<td>40 µg/ml</td>
</tr>
<tr>
<td>IPTG</td>
<td>23 µg/ml</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.4 µg/ml</td>
</tr>
<tr>
<td>Maltose</td>
<td>2%</td>
</tr>
<tr>
<td>Glucose</td>
<td>2%</td>
</tr>
</tbody>
</table>
4.2.1.2 Growing *Arabidopsis* plants

The seeds were stored at room temperature and always sown on more dampened soil and transferred to the short-day phyto chamber (8h lighting, approx. 22°C, and 60% air humidity) after vernalization for 3 to 4 days. The pots containing the seeds were kept covered by foil until the seeds develop to four-leaf stage. Three weeks after sowing, the plants were changed to individual pots and transferred to the long-day phyto chamber after 5 weeks (under 16h light/8h dark and approx. 22°C, 60% humidity).

4.2.2 General molecular biology methods

Standard molecular biological methods were performed for plasmid preparations, restriction digests, agarose gel electrophoresis, cloning etc., as described in the Handbook “Molecular Cloning, A Laboratory Manual” (Sambrook *et al.*, 1989). Methods, which are not mentioned in the manual, were described below.

4.2.2.1 Stock cultures

By inoculating a single colony, the cells were cultured O/N in liquid medium. To 1ml of culture in a sterile Eppendorf (in case of yeast 1ml of culture was centrifuged, discarding the supernatant and adding one more ml of yeast culture in order to concentrate the cells), added 80% Glycerine up to a final Glycerine concentration of 15%. The cells were immediately shock frozen in liquid nitrogen and stored at -80°C.

4.2.2.2 Isolation and purification of DNA from *E.coli*

A modified version of Holmes and Quigley (1981) method was used to isolate plasmid DNA from *E.coli*. Larger amounts of plasmid DNA was isolated using NUCLEOBOND column AX100. In addition to the examination on gel, the amount of DNA after column purification was measured using fluorometer.

4.2.2.3 Isolation of DNA from *Arabidopsis thaliana*

Larger amounts of *Arabidopsis* genomic DNA was isolated based on modified CTAB method (Aitchit *et al.*, 1993). For isolation of genomic DNA in smaller amounts, CTAB method was modified in an easy way. In brief, 10 mg of plant tissue in 400 µl of DEB and 4 µl of 10% DTT was homogenized with drilling machine or with cell lyser (Quiagen), and incubated for 30 min at 65°C. Equal volume of Chloroform was added, vortexed at least for 1
min and centrifuged for 15 min at 14000 rpm in tabletop centrifuge at RT. Supernatant was collected to a new Eppendorf tube and equal volume of Isopropanol was added. Mixed gently by inverting the tubes for 4-5 times and centrifuged for 15 mins at 14000 rpm. Pellet was washed with 500 µl of 70% Ethanol and centrifuged for 5 mins at 14000 rpm, air dried and dissolved in 30 µl of sterile distilled water or in TE buffer.

4.2.2.4 Isolation of mRNA

Phenol ice cream method was used to isolate mRNA from plant cells. In brief, 200 mg of plant material was harvested and immediately frozen in liquid nitrogen. 150 µl of Phenol and 500 µl of EB were added to the plant material and grinded in mortar and pestle while adding liquid nitrogen in between. 300 µl of phenol was added in addition after grinding and vortexed to form a homogenous suspension and centrifuged for 3 mins at 14000g. To the supernatant in a new eppi, equal volume of Chloroform was added and centrifuged like in previous step after vortexing. Equal volume of 4M LiCl was added to supernatant and incubated for 3-15 hr at 4°C. Precipitation was terminated by centrifuging the above solution for 10 mins at 14000 rpm. To the pellet, 300 µl of DEPC treated water, 33 µl of 3M NaOAc (pH 5.2), 830 µl 100% Ethanol were added and incubated for 2 hr at -20°C. After centrifugation for 10 mins at 14000g, the pellet was washed with 70% Ethanol and air dried and resuspended in appropriate volume of DEPC treated water.

4.2.2.5 RNA preparation for gene chip analysis

Total RNA was isolated using RNeasy plant mini kit (Quiagen, Hilden). The purity of RNA isolated, was determined by photometric analysis and also by RNA gel.

4.2.2.6 Determination of DNA and/or mRNA concentration

The DNA- and/or RNA solution was diluted 1:80 or 1:1000 and the absorption between 220 and 320 nm was determined. The concentration can be calculated by means of following relation

\[
\text{DNA: } 1 \text{ OD}_{260} \text{ corresponds to } 50 \mu g \text{ DNA/ml} \\
\text{RNA: } 1 \text{ OD}_{260} \text{ corresponds to } 40 \mu g \text{ RNA/ml}
\]
4.2.2.7 DNA purification and precipitation

After PCR, the DNA was purified by means of QUIAGEN columns to get rid of the primer dimmers or precipitated by adding 1/10 volumes of 3M NaOAC pH 4.8-5.2 and 2.5 volumes of 100% Ethanol and centrifuged for 15 mins at 14000 rpm in micro-centrifuge. The pellet was washed with 70% ethanol, centrifuged for 5 mins at 14000 rpm, air dried and resuspended in appropriate volume of water (HPLC water for sequencing) or buffer.

4.2.2.8 Analysis of DNA sequence

DNA sequence was analysed based on Sanger’s chain termination method (Sanger et al., 1977) using fluorescence labelled dideoxy nucleotides (Perkin Elmer Big Dye Terminator RP Mix) and a Thermocycler (Perkin Elmer). The DNA sequence was determined by capillary electrophoresis using the generalised programme in the laser machine ABI PRISM 310 (PERKIN ELMER).

For the sequence PCR, 5 pmoles of specific primer and 2 µl of sequence mix were added to approximately 200 ng of DNA and the volume was made up to 10 µl. Obtained PCR product was precipitated as stated in § 4.2.2.7. The pellet was resuspended in 20 µl of HPLC water and was again diluted to 1:5 with HPLC water before sequencing.

4.2.2.9 Annealing and 5’ phosphorylation of oligonucleotides

Primers AtXYL2c+46f (MBP) and AtXYL2c+90r mentioned in Table 4.8 used for antibody generation against AtXYL2 protein were annealed in the following way before use:

\[
\begin{align*}
1 \mu l & \quad \text{Oligo1} \\
1 \mu l & \quad \text{Oligo2} \\
10 \mu l & \quad 5x \text{T4 PNK-Buffer} \\
\text{upto } 50 \mu l & \quad \text{H}_2\text{O}
\end{align*}
\]

The Primers were annealed by incubating the above mix at 85°C for 2 mins and 65°C for 15 mins and finally at 37°C for 15 mins. Annealing reaction was stopped by transferring the mix unto ice and then phosphorylation reaction was carried out as follows:

\[
\begin{align*}
3,5 \mu l & \quad \text{double stranded oligonucleotide} \\
2 \mu l & \quad 5x \text{T4 PNK-Buffer} \\
0,5 \mu l & \quad \text{T4 Polynucleotide-Kinase} \\
0,5 \mu l & \quad 10 \text{ mM ATP} \\
\text{upto } 10 \mu l & \quad \text{H}_2\text{O}
\end{align*}
\]

The above reagents were pipetted together and incubated for 1 hr at 37°C. The double stranded oligonucleotide was subsequently ligated to the vector pMALC2 via EcoRI.
4.2.2.10 Sample preparation for HPLC analysis

0.5 to 1ml of 80% ethanol was added to 100mg of plant material and incubated for 1hr at 80°C. The samples were cooled down for 15 mins by switching off the incubator and then centrifuged for 5 mins at 14000 rpm and at 4°C. The supernatant was transferred to a new Eppendorf tube and evaporated in speed-vac. Pellet was resuspended in 250 µl of HPLC grade water while mixing for 10 min and was used for measurement along with standard mix with a defined concentration of sugars.

4.2.2.11 Isolation of protoplasts from Arabidopsis thaliana

Rosette leaves of Arabidopsis thaliana were used to isolate protoplasts. All the centrifugation steps were performed at minimum acceleration and decelerations. The lower surface of rosette leaves was rubbed with a sand paper to disrupt the epidermis and put them in a large Petri dish containing solution A. After the required number of leaves placed in Petri dish, solution A was removed by aspiration (or with a pipette) and 10 ml of enzyme solution was added. The leaves were incubated for 3h at 29°C-37°C, while shaking in between. Protoplasts in the enzyme solution were filtered through 120 µm nylon cloth and the material left in Petri plate was washed again with 10-15 ml of solution A. The suspension was centrifuged at 100g for 5 min in a swing out rotor. Protoplasts were resuspended in solution A and centrifuged for 5 min at 40g.

4.2.2.12 PEG transfection

Protoplasts, isolated in the above method were resuspended in MaMg Buffer at 5x10^6 cells/ml and 300 µl of this suspension was used for one sample. To 300 µl of protoplast suspension in a plastic tube, 50 µg of DNA and 300 µl of PEG-CMS buffer were added quickly and mixed by shaking gently and incubated at RT for 30 min. W5 buffer was then added at 5 min intervals in volumes of 600 µl, 1 ml, 2 ml and 4 ml. Mixed gently after each step and centrifuged for 5mins at 60g. Protoplasts were resuspended in 2 ml of 400 mM sorbitol and 500 µl W5 buffer. The suspension was centrifuged finally for 5 min at 60g and resuspended in 3 ml of K3 medium. The suspension transferred to small Petri dishes and incubated oN at 18°C and examined for GFP fluorescence in the next morning.
4.2.2.13 Isolation of vacuoles from *Arabidopsis thaliana*

Well expanded young rosette leaves were used to isolate vacuoles. As in the case of protoplast isolation all the centrifugation steps were carried out at RT and at minimum acceleration and deceleration. The lower surface of leaves was rubbed with sand paper to remove cuticle and placed the leaves in a Petri dish (φ 20cm) as wet surface facing the dilute MCP (1:2 with water) containing 1 mg/ml BSA. MCP buffer was removed by aspiration when the petri plate was filled with leaves. Enzyme solution was then added and incubated for 2hrs at 29°C. Protoplasts were released by shaking the petri dish and the content was added to a 50 ml Falcon tube. Contents in the Petri dish were washed with 10-15 ml of MCP buffer and added to the previous suspension. A cushion was formed by adding 2 ml of 100% Percoll pH 6.0 to the bottom of the falcon tube and centrifuged at 1500 rpm for 8 min at RT. Protoplasts were recovered from the top of Percoll layer and the suspension was set to have final Percoll concentration of 35-40%. 7.5 ml of 25% Percoll followed by 5 ml of Sorbitol for barley were added to protoplast suspension to form a gradient and centrifuged for 8 min at 1200 rpm. Purified protoplasts layered between sorbitol for barley and 25% Percoll were recovered to a 50 ml falcon tube and were warmed just by keeping the falcon tube in hand for few mines. Same volume of lysis buffer (42°C) was added to protoplasts suspension and the incubated at RT for 10 mins. Precedence of lysis process observed for every 2 min and the suspension was transferred to ice immediately after completion of lysis process. A second gradient was now formed to obtain purified vacuoles which contains from bottom to top 7 ml of lysate, 5 ml of lysis buffer : betaine buffer (1:1) and 1 ml of betaine buffer. The gradient was centrifuged for 8 mins at 1200 rpm. Vacuoles were recovered from higher interface and stored on ice until use.

4.2.2.14 Yeast transformation

*Saccharomyces cerevisiae* was transformed according to the Lithium acetate/single-stranded carrier DNA/Polyethylene glycol (LiAc/ssDNA/PEG) method (Agatep et al., 1998; Gietz and Woods, 2002). Briefly, yeast (2-5 ml culture) was grown under permissive or selective conditions overnight, shaking at 29°C. Following determination of cell titre, the overnight culture was diluted to a final concentration of 5x10⁶ cells/ml in CAA medium and grown while shaking at 29°C until the titre reaches at least 2 x 10⁷ cells/ml (3-5 hours). Cells were harvested via centrifugation, washed once in ½ volume ddH₂O, and resuspended in 1-10 ml of ddH₂O, depending upon the volume of the starting culture. This homogenate was
further divided into aliquots of 50 μl – 3 ml and subjected to another round of centrifugation. The following ingredients were then added to the resulting pellet (3 ml aliquot used as an example) in the order indicated, with gentle mixing after each addition:

- 2.4 ml 50% PEG
- 360 μl 1 M LiAc
- 500 μl ssDNA (from salmon testis, 2 mg/ml)
- x μl plasmid DNA (1-5 μg)
- 340-x μl ddH₂O

The homogenate was vortexed to ensure complete suspension and incubated for 30 minutes at 29°C. The yeast was then subjected to a heat shock for 15-30 min at 37°C. The cells subsequently harvested by centrifugation, were resuspended in ddH₂O and spread onto selective media.

4.2.2.15 Isolation of soluble proteins from *S. cerevisiae*

Isolated colonies were incubated overnight at 29°C in 2 ml of CAA medium supplemented with appropriate amino acids. Yeast cells were collected by centrifugation (5,000g for 5 minutes at 0°C), the supernatant discarded and the pellet resuspended in 5 volumes (usually 250 μl) yeast lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 0.5% SDS). Acid-washed glass beads (0.45-0.50 mm) were added to the suspension to the level of the meniscus and the entire solution vortexed for 5 times of 20 sec each (the cell suspension was cooled on ice for 1 min between each cycle of vortexing). The cell extract was recovered via centrifugation (3,000g for 2 minutes at 4°C) into a fresh tube, centrifuged again at 12,000g for 5 mins at 4°C for clarification, protease inhibitor (2PI) was added and the extract was stored at -20°C until further use. In some cases, the starting cultures, as well as the subsequent steps were scaled up to 100X.

4.2.2.16 Western blot analysis

Following SDS PAGE, proteins were transferred to Nitrocellulose (Amersham Bioscience, Germany) in transfer buffer (25 mM Tris, 192 mM Glycin, 20% methanol). Transfer was performed at a constant voltage of 400V for 20 mins. After completion of transfer, the membrane was washed briefly in water and stained for 1 min in Ponceau S. The membrane was then incubated in Blocking buffer (5% skim milk powder TBST Buffer) for 30 mins at RT. Primary antibody was diluted in blocking buffer and added to the membrane; incubated at RT for one hour or o/N at 4°C. The membrane was then washed for two times with blocking buffer, for a minimum of 15 min. The secondary antibody (Anti-Rabbit IgG-
Peroxidase conjugate) was added to the membrane after diluted by a factor of 4000 with
blocking buffer and incubated for 1 hr at RT. The membrane was washed for two times with
blocking buffer and incubated for 15 min in the same. Detection was performed using
Lumilight western blotting substrates.

**4.2.2.17 Transport assay with yeast cells**

To characterize the transport function, putative AtXYL genes were heterologously
expressed in yeast strain EBY VW 4000 and uptake experiments were carried out with
radioactively labelled sugars. In brief, Yeast cells harbouring the cDNA in sense as well as in
antisense orientation were grown on in CAA medium at 29°C until an OD$_{600}$ of 1.0-1.5. Cells
were harvested by centrifuging for 5 min at 3500 rpm and washed for the first time with water
and then with 25 mM Sodium Phosphate buffer pH 5.0. Cells were resuspended at the end in
25 mM Sodium Phosphate buffer to obtain an OD$_{600}$ of 20 and stored on ice until use.

To measure the uptake, 1 ml of cells were taken in 25 ml Erlenmeyer flask and
incubated for 1 min at 29°C while shaking. 10mM sugar of 0.02 µCi was added to cells and
100 µl aliquots pipetted on to Nitrocellulose membrane of pore size 0.8 µm at definite time
intervals (15 sec, 1, 2, 5 and 10 mins). Vacuum was applied to suck the buffer and the
membrane was placed in 4 ml Scintillation cocktail after washing for 2 times with sodium
phosphate buffer. Radioactivity was measured in Scintillation counter.

**4.2.2.18 Isolation of vacuoles from Saccharomyces cerevisiae**

Yeast cells were grown to OD$_{600}$ of 1 or below. The cells were sedimented and washed
with distilled water for two times by centrifuging the culture at 4500g for 5 min. Each pellet
resuspended in 30 ml of spheroplasting buffer and incubated at 29°C for 1 hr. Spheroplasts
were harvested and washed twice with 1M Sorbitol by centrifugation at 2200g for 5min.
Pellet was resuspended in 12% Ficoll buffer of pH 6.9. Spheroplasts were lysed osmotically
in addition to mechanical stress by using the dounce homogeniser. Cell debris was removed
by centrifuging the homogenate at 2200g for 10 min. Supernatant was transferred to an
ultracentrifuge tube, over-layered with fresh 12% Ficoll and centrifuged for 1hr at 60,000g
using sw28 rotor of ultracentrifuge. Vacuoles were harvested from the surface of 12% Ficoll,
just by scooping with a spatula and resuspended in appropriate volume of 2X Buffer C.
4.2.2.19 Uptake experiments with vacuoles

Uptake measurements with isolated vacuoles were carried out basically as in the case of whole cells after few modifications. Nitrocellulose membrane of 0.2 µm was used instead of 0.8 µm. Initial sugar concentration was set to 100 µM with 0.1 µCi. 50 µg of vacuolar protein (diluted to 100 µl with 2X bufferC) was used as uptake mix for each time point along with 4 mM ATP and 4 mM MgSO\(_4\). Radioactive substrate was added after 5 min preincubation of uptake mix at 29°C. 100 µl aliquots were pipetted onto nitrocellulose membrane at each time point and vacuoles were washed for 2 times with 2X Buffer C of pH 7.9. Vacuum was applied very slowly to remove the excess buffer and unused radioactive substrate. Nitrocellulose membrane was added to scintillation cocktail and the radioactivity was measured.

4.2.2.20 Isolation of plastidic membrane fraction

Leaf tissue was added to isolation buffer at a ratio of 1:4 (w/v) and homogenized in dounce homogenizer for 2 min. The homogenate was filtered through 4 layers of nylon mesh (50 µm). The filtrate was layered over isolation buffer containing 40% Percoll (v/v). The intact chloroplasts were pelleted after centrifugation at 4000g for 5 min. The chloroplasts were lysed osmotically by incubating in hypotonic lysis buffer for 15 min. The suspension was centrifuged at 105,000g for 1 hr, and the resultant pellet was used as membrane protein fraction and the supernatant as stromal fraction.

4.2.2.21 Embedding the plant material in Technovit

The GUS stained plant material of interest was dehydrated by incubating in 90% ethanol for 30 min and 2 times in 100% ethanol, incubated for 1 hr each time. The plant material was then infiltrated by incubating in 100% ethanol: preparing solution (v/v) for 2 hr at RT. To embed, the plant material was taken in a 0.2 ml Eppendorf cup and embedding solution was added and incubated for 4 hr to let the embedding solution polymerize.
5. Summary

A new subfamily of the monosaccharide transporter genes, consisting of three members At3g03090 (AtVGT1), At5g17010 (AtVGT2) and At5g59250 (AtXYL3), was identified within the Major Facilitator Superfamily. The cDNAs of all three genes were cloned and used for further analysis. For the highly homologous members AtVGT1 and AtVGT2, vacuolar localization was demonstrated by expression of the GFP fusions in yeast as well as in Arabidopsis protoplasts. The functional expression of AtVGT1 in yeast and substrate transport assays with vacuoles revealed that AtVGT1 is a vacuolar H⁺/glucose antiporter. This is the first identified and functionally analyzed plant vacuolar sugar transporter. The analysis of GUS reporter plants showed AtVGT1 promoter activity only in pollen, while results from our RT-PCRs as well as the Genevestigator microarray database indicate, that AtVGT1 is expressed in most tissues at a low basic level. Analysis of AtVGT1 T-DNA insertion mutants revealed the important role of this gene in seed germination and determination of flowering time, since 20% of the mutant seeds failed to germinate and the bolting process was delayed by 9 to 14 days. An important osmotic function of AtVGT1-mediated glucose-transport was proposed.

AtVGT2, which also localized to the vacuole, is expressed in most of the tissues, throughout the plant development. However, Atvgt2 T-DNA mutants did not show visible phenotypes. Atvgt1/Atvgt2 double-mutant plants were analyzed to investigate a possible functional compensation of the loss of AtVGT2 by AtVGT1. In addition to the seed germination and bolting phenotypes observed in Atvgt1 mutants, in Atvgt1/Atvgt2 plants lignification of the cell wall was impaired, and increased cell elongation with impaired cessation of the internode elongation was observed. Together, these effects resulted in a weak floral stem and fewer branches, thus leading to lower fresh weight. In addition, these plants also showed delayed rosette development and impaired silique development.

The third and more distant member of this family, AtXYL3 has an extended N-terminal sequence, predicted to be cTP. The plastidic localization of this protein was determined by transient expression of a GFP fusion in Arabidopsis protoplasts. Since AtXYL3 is not expressed in yeast, a functional analysis of this transporter homolog remains to be elucidated using a different system. Tissue specific expression analyzed by GUS-reporter plants revealed that AtXYL3 is expressed in most tissues. Analysis of Atxyl3 mutants showed that disruption of this gene leads to advanced vegetative plant development, whereas silique
and seed development is defective. Under continuous light the enhanced vegetative plant development was even more pronounced. This suggests an important role of *AtXYL3* in diating glucose fluxes across the plastidic envelope, and in transient starch metabolism.
6. Zusammenfassung

Innerhalb der „Major Facilitator Superfamily“ wurde eine neue Unterfamilie von Monosaccharid-Transportern, bestehend aus den drei Mitgliedern At3g03090 (AtVGT1), At5g17010 (AtVGT2) und At5g59250 (AtXYL3), identifiziert. Von allen drei Genen wurde die cDNA gekloniert und zu weiteren Analysen herangezogen. Für die hoch homologen Mitglieder AtVGT1 und AtVGT2 konnte durch Expression eines GFP Fusionsproteins sowohl in Hefe, als auch in Arabidopsis Protoplasten eine vakuoläre Lokalisation beobachtet werden. Die funktionelle Expression von AtVGT1 in Hefe und folgende Aufnahmemessungen mit isolierten Vakuolen zeigten, dass es sich bei AtVGT1 um einen vakuolären H⁺/Glucose Antiporter handelt. Hiermit konnte zum ersten Mal ein pflanzlicher vakuolärer Zucker-Transporter identifiziert und funktionell analysiert werden. Bei der Analyse von GUS Reporterpflanzen wurde AtVGT1 Promotoraktivität ausschließlich im Pollen beobachtet, wohingegen RT-PCR Experimente und Microarray Daten aus der Genevestigator Datenbank auf eine niedrige, aber gleichmäßige Expression in allen Geweben hindeuten. Die Untersuchung von AtVGT1 T-DNA Insertionslinien weist auf eine wichtige Rolle von AtVGT1 bei der Samenkeimung und Blühinduktion hin, da 20% der Samen der KO-Mutante nicht keimten und sich der Beginn der Blütenprososphbildung um 9-14 Tage verzögerte. Diese Ergebnisse deuten daraufhin, dass der AtVGT1-vermittelte Glukose-Transport eine wichtige Funktion bei der Osmoregulation einnimmt.

Für AtVGT2 konnte ebenfalls eine vakuoläre Lokalisation gezeigt werden. Obwohl AtVGT2 während der gesamten Entwicklung in fast allen Geweben exprimiert wird, zeigen Atvg1 T-DNA Mutanten keinen sichtbaren Phänotyp. Aus diesem Grund wurden Atvg1/Atvg2 Doppelmutanten analysiert, um festzustellen, ob der Verlust von AtVGT2 möglicherweise durch AtVGT1 kompensiert werden kann. Zusätzlich zu dem in der Atvg1 Mutante beobachteten Phänotyp bezüglich Samenkeimung und Sprossbildung zeigten sich in der Doppelmutante weitere Auswirkungen. So war zum einen die Lignifizierung der Zellwand beeinträchtigt, zum anderen wurde eine verstärkte Zell-Elongation verbunden mit verlängerten Internodien im Stengel beobachtet. Insgesamt führten diese Effekte zu einer verminderten Stabilität des Stengels und weniger Seitentrieben, was ein geringeres Gesamtgewicht zur Folge hatte. Zusätzlich zeigten diese Pflanzen auch eine verzögerte Entwicklung der Blattrosette und Störungen bei der Schotenbildung.
7. References


References


8. Appendix

The plasmid maps that are generated in the present thesis were displayed under this section which also included the amino acid sequence of the three transporters of newly identified monosaccharide family and the tissue specific expression patterns (Genevestogator microarray database).

**Figure A1:** Plasmid map of pSO114. PCR fragment of the *AtVGT1* cDNA was ligated into *E.coli*/yeast shuttle vector NEV-E, as described in § 2.1.1 and used for transport measurements in yeast and for complementation of yeast hxt mutant.

**Figure A2:** Plasmid map of pSA115. The modified ORF of *AtVGT1* cDNA PCR fragment was ligated into mcs of pGEM-T easy vector as described in § 2.1.3.1 and used for further cloning into pEX tag GFP2 and pSO35e vectors.
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Figure A3: Plasmid pSA110. *AtVGT1* cDNA was ligated into pEX tag-GFP2 vector in between plasma membrane ATPase promoter and GFP ORF via NcoI cloning site and used to determine the subcellular localization of AtVGT1 in yeast as described in §2.1.3.2.

Figure A4: Plasmid map of pSA120. *AtVGT1* cDNA ligated into NcoI cloning site of pSO35e vector, inbetween CaMV 35s promoter and NOS terminator as described in § 2.1.3.3 and used for transient expression in *Arabidopsis* protoplasts.
Figure A5: Plasmid map of pSA103. *AtVGT1* promoter was cloned in front of the GUS reporter gene over HindIII/NcoI cloning sites as described in § 2.1.5 and AtVGT1 promoter-GUS reporter gene_NOS Terminator cassette was transferred to plant vector pGPTV-BAR.

Figure A6: Plasmid map of pSA104. The *AtVGT1* promoter-GUS reporter_NOS terminator cassette from pSA103 was ligated to pGPTV-BAR vector over XmaI/EcoRI cloning sites and used to generate transgenic *Arabidopsis* plants via *Agrobacterium* mediated transfer.
Figure A7: pSA101 plasmid map. PCR fragment of the *AtVGT1* promoter was cloned into pAF1 vector infront of the GFP ORF over the SphI/NcoI cloning sites.

Figure A8: Plasmid map of pSA102. The *AtVGT1* promoter-GFP cassette from the pSA101 plasmid was cloned into pGPTV-BAR vector infront of the NOS-Terminator over XmaI/SacI cloning sites.
**Figure A9:** pSA218 plasmid map. *AtVGT2* cDNA (BbsI (EcoRI compatible)) was ligated into NEV vector over EcoRI cloning site and used for the functional expression *AtVGT2* in yeast.

**Figure A10:** Plasmid map of pSA217. PCR fragment of the modified ORF of *AtVGT2* cDNA (NcoI/BbsI) was ligated into pGEM-T easy vector as described in § 2.2.3.1 and after sequence verification was used for further cloning into pEX-Tag-GFP2 and pSO35e vectors.
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Figure A11: Plasmid pSA219. The modified ORF of *AtVGT2* (along with 5’ NcoI cloning site, the 3’ primer of *AtVGT2* cDNA has BbsI (NcoI compatible) restriction site) ligated into pEXtag-GFP2 vector over NcoI cloning site, in between pMA1 promoter and GFP ORF and was used for expression of *AtVGT2*-GFP fusion in yeast as described in § 2.2.3.2.

Figure A12: Plasmid map of pSA220. *AtVGT2* cDNA (*no* stop) was cloned over NcoI/BbsI (NcoI compatible) into pSO35e vector, inbetween CaMV35s promoter and NOS terminator as described in § 2.2.3.3 and was used for transient expression of *AtVGT2*-GFP fusion in *Arabidopsis* protoplasts.
The AtVGT2 amino acid sequence in comparison to its homolog, AtVGT1. The grey regions indicate completely identical amino acids, the black regions indicate strongly similar positions and the white regions are with no or weak identity. The amino acids from 15 to 30 represented in block letters were used to generate the antibodies against AtVGT2 which can also be directed against AtVGT1 protein.

The AtVGT2 amino acid sequence in comparison to its homolog, AtVGT1. The grey regions indicate completely identical amino acids, the black regions indicate strongly similar positions and the white regions are with no or weak identity. The amino acids from 15 to 30 represented in block letters were used to generate the antibodies against AtVGT2 which can also be directed against AtVGT1 protein.

**Figure A13:** Plasmid map of pSA205. A 45 bp sequence of N-terminus of AtVGT2 cDNA (annealed oligos) was ligated into pMALc2 vector over BamH1/HindIII cloning sites.
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Figure A14: Plasmid pSA222. *AtVG2* promoter ligated into pAF6 vector infront of the GUS reporter gene over HindIII/NcoI cloning sites and the *AtVG2* promoter-GUS-Terminator cassette from this plasmid was transferred to plant vector pGPTV-BAR.

Figure A15: Plasmid map of pSA222. *AtVG2* promoter-GUS-terminator cassette was cloned into pGPTV-Bar vector and was used to generate transgenic *Arabidopsis* plants expressing GUS reporter gene under the control of *AtVG2* promoter.
Figure A16: Plasmid map of pSA223. The PCR fragment of *AtVGT1* promoter was ligated to pAF1 vector infront of the GFP ORF.

Figure A17: Plasmid map of pSA224. The *AtVGT2* promoter-GFP cassette from pSA223 plasmid was cloned into pGPTV-BAR vector infront of the NOS Terminator.
Figure A18: Plasmid map of pSA308. PCR fragment of *AtXYL3* cDNA was cloned into mc of pGEM T-easy vector.

Figure A19: Plasmid map of pSA320. The modified ORF of *AtXYL3* (NcoI/BbsI (NcoI compatible)) was ligated into pEXtag-GFP2 vector over NcoI cloning site and used for expression *AtXYL3-GFP* fusion in yeast.
Figure A20: Plasmid map of pSA321. The modified ORF of AtXYL3 was ligated into pEXtag GFP2 vector over NeoI cloning site and was used for expression of AtXYL3-GFP fusion in yeast.
Amino acid sequence of AtXYL3 in comparison to the other members of the newly identified monosaccharide transporter family: Amino acid sequence, represented in block was the predicted cTP and those represented in block letters were used to raise anti AtXYL3 antibodies. The identical regions were represented against pale grey background. Completely diverse regions were represented against dark grey background.

Figure A21: pSA305 plasmid map. A 285 bp N-terminal cDNA sequence of AtXYL3 was cloned into mcs of pMALc2 vector and the resultant plasmid pSA305 was used to generate MBP-AtVGT2 fusion protein.
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Figure A22: Plasmid map of pSA322. AtXYL3 promoter was cloned into pAF6 vector over XbaI/NcoI cloning sites inform of the GUS reporter gene.

Figure A23: pSA323 plasmid map. AtXYL3 promoter-GUS cassette from pSA322 was cloned into pGPTV-BAR vector and was used to generate transgenic *Arabidopsis* plants expressing GUS reporter gene under the control of AtXYL3 promoter.
Figure A24: Plasmid map of pSA324. PCR fragment of the AtXYL3 promoter was ligated into pAF1 vector infront of the GFP ORF over XbaI/NcoI cloning sites.

Figure A25: Plasmid map of pSA325. AtXYL3 promoter-GFP cassette from pSA324 plasmid was cloned into pGPTV-BAR vector infront of the NOS-Terminator over XbaI/Sacl cloning sites.
Figure A26: Genevestigator microarray analysis for *AtVGT1* gene showing highest level of expression in stamen and basal level expression in all the other tissues except in root hair and root tip.
Figure A27: Genevestigator microarray analysis for *AtVGT2* gene which, significantly expressed in most of the developmental stages.
Figure A28: Genevestigator microarray analysis of *AtXYL3* gene significantly expression in most of the plant tissues with highest level of expression in cotyledons.
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