Functional Analysis of the *Arabidopsis thaliana* Monosaccharide Transporter AtSTP1

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Abstract

This thesis describes the analysis of AtSTP1 gene of *Arabidopsis thaliana* with regard to its physiological role. The AtSTP1 gene encodes a high affinity monosaccharide proton symporter which is located within the plasmamembrane. The gene is expressed in various tissues of *Arabidopsis* including stems, roots, flowers, sepals, ovaries and leaves where the AtSTP1 mRNA is most abundant. The AtSTP1 gene belongs to a gene family with at least 16 members in *Arabidopsis* The physiological function which has been proposed for individual members of this family includes the import of monosaccharides into symplastically isolated sink cells like pollen grains, embryos and guard cells or into cells with an increased carbohydrate demand due to wounding, pathogen infection or developmental processes. A role for monosaccharide transporters as part of one sugar sensing system which controls the regulation of the carbohydrate metabolism of the plant has been proposed as well. In order to elucidate the biological function of the AtSTP1 gene a STP1 knock-out line, a line which overexpresses the AtSTP1 gene under control of the 35S-CaMV promotor and wild type plants are analysed with regard to their ability of monosaccharide transport and phenotypic effects after feeding with monosaccharides. After feeding whole seedlings with μM concentrations of glucose the knock-out line shows 40% decrease in glucose uptake whereas the overexpressing line shows a two fold increase glucose uptake compared to wild type glucose uptake. When the seedlings are fed with 200 mM glucose there is no difference in the glucose concentration in the knock-out line and wild type line whereas the overexpressing line shows a clearly higher glucose contend. When the plants where grown in the absence of any added monosaccharides no phenotypic differences between the knock-out line, the overexpressing line and the wild type line can be observed. In the precence of 200 mM glucose seedlings of the overexpressing line show an increased growth rate and a higher antocyanin content compared to the wild type line and the knock-out line which have the same growth rate and antocyanin content. The monosaccharide galactose is toxic for *Arabidopsis* and causes a decreased growth rate and stunted roots. To concentrations of 1-100 mM galactose seedlings of the knock-out line show a hyposensitive phenotype whereas seedlings of the overexpressing line have a hypersensitive phenotype compared to the wild type line. The relevance of these results to the physiological role of the AtSTP1 gene will be discussed.

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Abbreviations

bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
CaMV	cauliflower mosaic virus
CCCP	carbonylcyanide M-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid
CF	5(6)-carboxyfluorescein
ds	double stranded
EDTA	ethenediaminetetraacetic acid
g	gramme
GFP	green fluorescent protein
h	hour
HPLC	high performance liquid chromatography
kb	kilobase
l	litre
m	meter
M	molar
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Murashige & Skoog medium
mRNA	messenger ribonucleic acid
PCMBS	p-chloromercuribenzene sulfunate
SDS	sodium dodecyl sulphate
sec	second
Tris	tris-(hydroxmethyl)-methylamine
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio

Table of content

1 Introduction

1.1 Aims of the introduction

AtSTP1 was the first monosaccharide proton symporter to be cloned from *Arabidopsis thaliana*. Although the biochemical transport characteristics of this protein have been well described by uptake studies performed in the heterologous systems of *Schizosaccharomyces pombe* and *Xenopus laevis* little is known about the physiological role of the protein in *Arabidopsis*. In this introduction the current understanding of hexose transport across the plasma membranes in plants, in particular in *Arabidopsis*, and the physiological context in which this transport occurs will be discussed. The current knowledge about the monosaccharide proton symporter AtSTP1 will be outlined.

1. 2.1 Long distance transport of carbohydrates in plants

Plants are photoautotrophic organisms that are able to synthesise carbohydrates from CO_2 and water using light energy. This process of photosynthesis occurs in all green plant tissue but not all plant cells are able to meet their carbohydrate demand by means of photosynthesis. Therefore carbohydrates have to be transported from cells that synthesise more carbohydrates than they need to fuel their own metabolism (source cells) to cells that are dependent on carbohydrate import (sink cells). Typical source cells are those of the leaf mesopyll. In roots and fruits no or very little photosynthesis with respect to their carbohydrate demand takes place.

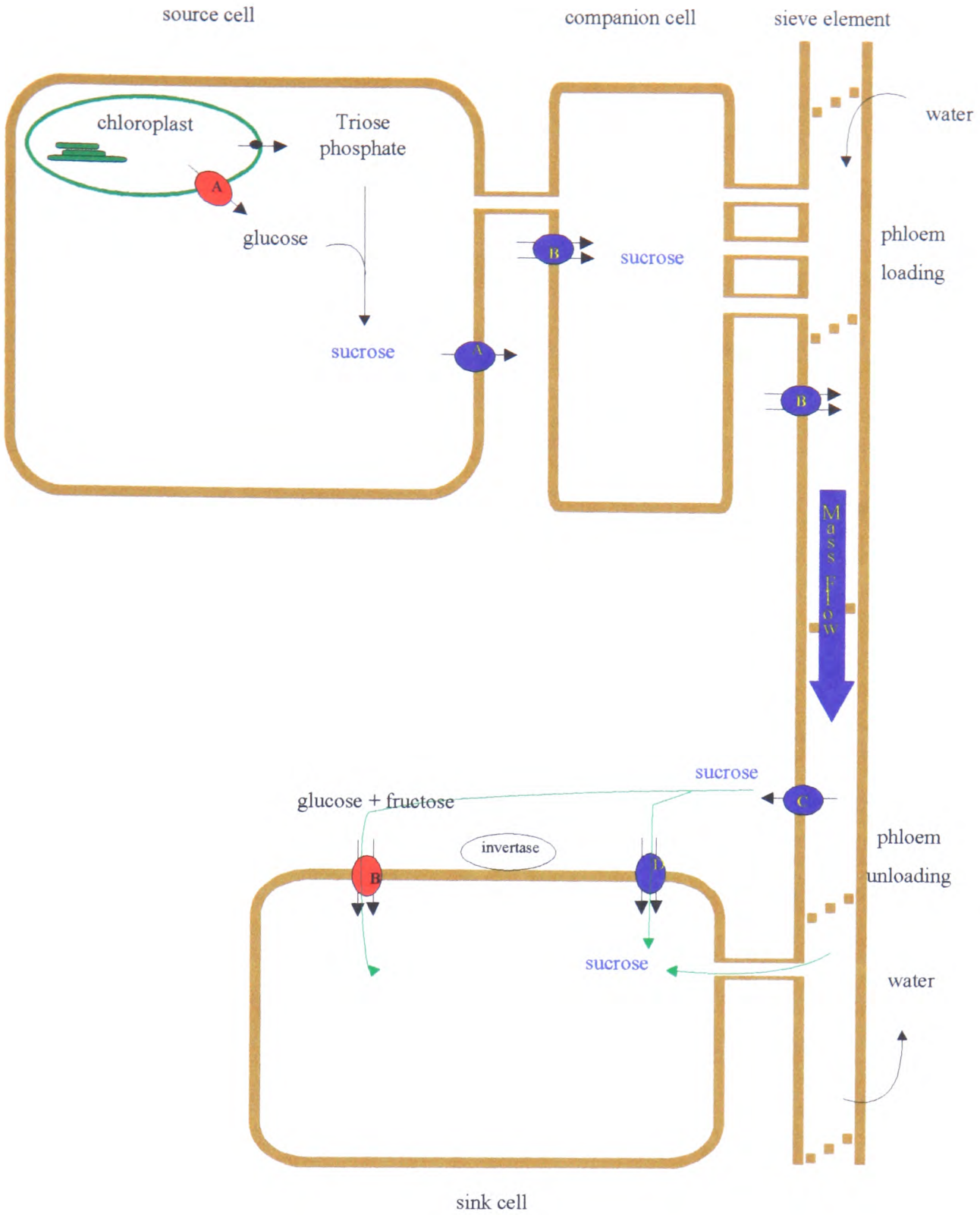


Figure 1: Schematic representation of sucrose partitioning. Blue circles represent sucrose transporters, red circles represent hexose transporters. Transporters with one arrow are faciliators. Transporters with two arrows represent proton coupled symporters. Green arrows indicate the various pathways of phloem unloading. The black circle represents a triose-phosphate translocator.

Therefore these tissues are considered as typical sink tissues. In most plant species including *Arabidopsis*, sucrose is the major carbohydrate that is translocated from source to sink tissues via the phloem. Some plant species use derivatives of sucrose such as raffinose, stachyose and verbascose or sugar alcohols such as mannitol sorbitol and dulcitol in addition to sucrose as the carbohydrate form that is transported over long distances. (Zimmermann and Ziegler, 1975).

The first step of long distance transport of sucrose is the efflux of sucrose from the source cells (sucrose transporter A in figure 1) Those transport activities have been described in sugar beet (Laloi et al.,1993). In the next step sucrose is actively loaded into the companion cells or into the sieve element in the area of phloem loading (sucrose transporters B in figure 1). In immunolocalisation experiments with a specific antibody against the *Arabidopsis* sucrose transporter SUC2 it was shown that this transporter is localised in the companion cells in the phloem (Stadler et al.,1996). SUC2 is a sucrose/proton symporter which allows sucrose to be imported into sieve element /companion cell complex against a sucrose concentration gradient (Stadler et al.,1995a; Stadler et al.,1996a). Phloem loading can also occur via passive cell to cell diffusion of sucrose through plasmodesmata connecting neighbouring mesopyll cells, bundle-sheath cells, phloem parenchyma and the companion cells. The extent to which either of these mechanisms is involved in phloem loading is correlated with transported sugar and the number of plasmodesmata connecting the sieve elements and companion cells to their surrounding photosynthetic cells. For plant species including *Arabidopsis* which possess few connections between the sieve element/companion cell complex (Bowman, 1994), and when sucrose is the major carbohydrate translocated between sink and source cells, apoplastic loading is

dominant. This applies to most temperate crops. On the other hand plant species which transport in addition to sucrose other oligosaccharides have abundant symplastic connections between the sieve element/companion cell complex and their surrounding photosynthetic cells, and symplastic loading is common (vanBel, 1996; vanBel, 1993). This situation is commonly found in tropical crops.

The mechanism of solute transport in the phloem between the sites of phloem loading and phloem unloading has been under debate since the 1930s. The model of phloem transport, which is currently favoured by most plant physiologists, was proposed in 1926 by E. Münch. (Münch, 1927). The pressure flow model proposed by Münch is based on an osmotically generated pressure gradient between sites of phloem loading and phloem unloading which drives the flow of solutes in the sieve elements. This gradient is established by the processes of phloem loading and phloem unloading. Phloem loading generates a low solute potential in the sieve elements and a difference in water potential between the phloem and the surrounding tissue. In response to this water potential gradient water enters the sieve elements and increases the turgor pressure (figure 1). Phloem unloading establishes a higher solute potential in the sieve elements of sink tissues. Due to the difference in water potential between the sieve elements and the surrounding tissue in the area of unloading water leaves the sieve elements, causing a decrease in the turgor pressure solutes.

Phloem unloading (reviewed in (Patrick, 1997)) can occur via two fundamentally different pathways. Sucrose can be unloaded from the sieve element / companion cell complex into the sink cells via a symplastic pathway or via apoplastic unloading (see figure 1). Symplastic unloading depends on the physical connection of the sink

cell with the phloem via plasmodesmata and plasmodesmal conductivity exerts the primary control over symplastic transport. The symplastic transport occurs by diffusion along the sucrose concentration gradient between the phloem and the sink cells. Sink cells maintain the sucrose concentration gradient by either metabolising sucrose or removing it from the cytoplasm into other compartments. In the case of apoplastic unloading sucrose efflux from the vascular tissue or from cells located along the symplastic pathway of post sieve element sucrose transport can occur by simple diffusion, by facilitated diffusion mediated by a proposed but so far unidentified sucrose transporter (figure 1 sucrose transporter C) (Dejong and Wolswinkel, 1995) or by active proton coupled export (Wang et al.,1995). The sucrose in the apoplast can be actively taken up into sink cells by a sucrose/proton symporter (figure 1 sucrose transporter D). The expression of such transporters in sink tissue has been demonstrated for various plant species including *Arabidopsis* ((Lalonde et al.,1999) and therein). Alternatively the sucrose in the apoplast can be hydrolysed by cell wall bound invertase into glucose and fructose and the hexoses subsequently can be taken up by sink cells via hexose/proton symporters (figure 1 hexose transporter B). The uptake of hexoses into sink cells via hexose/proton symporters will be discussed in the following section in more detail. For the individual contribution of the symplastic and apoplastic unloading pathways towards the total unloading process the following general statements can be made. The symplastic sieve element unloading route and post sieve element transport is the common and main unloading route. Due to the large transmembrane sucrose concentration differences, passive leakage of sucrose from the symplast into the apoplast is inevitable. This sucrose or glucose and fructose if apoplastic invertase

activity is present will have to be retrieved by apoplastic loading. Therefore it can be assumed that both unloading pathways do act in parallel. However in barley roots it was shown that the sugar retrieval from the apoplast did account for 20% of the photoassimilate import but that blocking of membrane transport did not alter photoassimilate import (Farrar, 1985). On the other hand it was demonstrated that nonvascular, symplastic diffusion of sucrose alone can not satisfy the carbon demands of the primary maize root tip (Bretharte and Silk, 1994) and involvement of apoplastic unloading was proposed. Under special circumstances when the sink cells are symplastically isolated from the phloem apoplastic unloading becomes the only option for carbohydrate supply for these cells. Examples for this situation are the embryo (Wang et al.,1995; Weber et al.,1995; Shanon, 1972; Weschke et al.,2000) the growing pollen tube (Ylstra et al.,1998), and developing pollen grain (Truernit et al.,1999) and guard cells (Palevitz and Hepler, 1985). Apoplastic unloading also is the major pathway of phloem unloading when sugars accumulate to high concentrations in the sink cells in the absence of an apoplastic barrier separating the phloem from the storage parenchyma cells. In this case the apoplastic step permits effective compartmentation of the stored sugars that would otherwise disturb the pressure differences that drive the unloading process. Examples for this are found in sugar cane where sucrose can accumulate to a concentration of up to 500 mM in cells in the stalk (Komor, 1994) and in the tap root of sugar beet (Fieuw and Willenbrink, 1990) and carrot (Tang and Sturm, 1999; Tang et al.,1999). In tomato fruit in the late stage of fruit development uptake via a hexose/proton symporter system accounts for 70 to 80% of photoassimilate uptake (Ruan and Patrick, 1995).

Recently a putative plastidic glucose transporter from spinach has been isolated and the homologues of this transporter from *Arabidopsis*, maize, potato, and tobacco have been identified (Weber et al.,2000). The authors suggest that this transporter (figure 1 hexose transporter A) could regulate the export of glucose from the chloroplast, which is produced by the breakdown of transitory starch by amylases. The amount of available glucose in the cytoplasm in turn will effect the amount of sucrose that is synthesised and available for transport into the phloem. Therefore the expression of the plastidic glucose transporter could have an impact on carbohydrate partitioning.

Little is known about the sugar transport across the tonoplast membrane, although the concentration of photoassimilates in the vacuole has major implications on the photoassimilate unloading process. Removal of sucrose from the cytoplasm into the vacuole of sink cells would maintain the symplastic concentration gradient of sucrose between the phloem and the sink cells. Up to now only one putative sugar transporter targeted to the tonoplast membrane has been identified (Chiou and Bush, 1996), but the characterisation of the transport mode and the identification of substrates for this transporter from *Beta vulgaris* are still missing. The relative acidity of the vacuolar compartment compared to the cytoplasm makes sugar/proton antiport or facilitated diffusion more likely modes of transport for transporters located in the tonoplast membrane. Transport studies with vacuoles isolated a from sugarcane suspension culture derived from storage parenchyma showed that the permeation of sucrose across the vacuolar membrane is a passive carrier mediated process since it was independent of membrane energization and could be inhibited by p-chloromercruibenzen sulfonate (PCMBS), a protein modifying agent (Preisser

and Komor, 1991). It was demonstrated that in these cells the sucrose concentration of the vacuole and the cytosol are the same (Preisser et al.,1992). Therefore it was suggested that sucrose-metabolising enzymes in the different compartments contribute to the regulation of the sucrose content in sugarcane storage parenchyma (Preisser et al.,1992). In tonoplast vesicles isolated from tomato fruit pericarp no evidence for a proton coupled transport of sucrose glucose or fructose could be found. The addition of p-chloromercruibenzen sulfonate (PCMBS) did inhibit the uptake of glucose (Milner et al.,1995). Therefore Milner *et al.* concluded that in tomato fruit pericarp the storage of hexoses in the vacuole is a passive carrier mediated process, rather than an active energy requiring one. In experiments where yeast derived invertase was targeted either to the cytosol, vacuole or apoplast in tobacco plants, the subsequent analysis the concentrations of sucrose, glucose and fructose in the cytoplasm stroma and vacuole indicated that an active uptake of hexoses into the vacuoles of tobacco leaves exists (Heineke et al.,1994). It is not surprising that the transport capabilities across the plasmalemma in sugarcane parenchyma, tomato fruit pericrap and tobacco leaf cells are different since these cells perform very different physiological functions. Sugarcane parenchyma and tomato fruit pericarp are specialised in storing photoassimilates. Both tissues import hexoses by apoplastic loading. In the sugarcane cells sucrose is the main storage carbohydrate (Komor, 1994), whereas tomato fruit pericarp accumulates glucose in high concentrations (Ruan and Patrick, 1995). Tobacco leaf represents a typical source tissue, which is involved in phloem loading.

The current knowledge about carbohydrate partitioning in plants suggests that hexose transport across the plasma membrane seems only to play an important role

in the process in apoplastic unloading when sucrose is hydrolysed by cell wall bound invertase.

1.2.2 Evidence for the involvement of hexose/proton symporter in apoplastic unloading

In tomato, unloading of photoassimilates into the storage parenchyma cells in the fruit switches from a symplastic to apoplastic mode at the onset of the stage of hexose accumulation during fruit ripening (Ruan and Patrick, 1995). This switch is associated with the activity of an extracellular invertase which hydrolyses the unloaded sucrose (Damon et al.,1988). The comparison of two tomato genotypes differing in fruit hexose content revealed that the fruit parenchyma of the genotype with the higher fruit hexose content had a higher influx of hexoses into the pericarp disks. Differences in the influx of hexoses between the genotypes were not due to differences in apoplastic supply of hexoses since the experiments were performed on pericarp disks incubated in identical solutions. This uptake was sensitive to treatment with PCMBS (Ruan et al.,1997). The authors conclude that hexose/proton symporters in the plasma membranes of the storage parenchyma cells mediate the subsequent retrieval of hexoses from the apoplast and that differences in hexose uptake do significantly contribute to differences in hexose accumulation seen in the two genotypes.

In tissue slices from sugarcane storage parenchyma active uptake of glucose and fructose could be demonstrated. The uptake of hexoses is coupled to proton uptake

and shows biphasic kinetics. There is a saturable phase with a K_m for glucose of 50 μM and a K_m for fructose of 550 μM, and a linear diffusion-like phase, which becomes obvious at substrate concentrations above 50 mM. No active uptake of sucrose could be demonstrated (Komor, 1994). These observations suggest that apoplastic unloading occurs in the sugarcane storage parenchyma which is dependent on the activity of a cell wall bound invertase and the accumulation of sucrose in the parenchyma cells is regulated by sucrose metabolising enzymes. Major differences in sucrose accumulation in sugarcane storage parenchyma between different genotypes of sugarcane can be attributed to the difference between activities of soluble acid invertase and sucrose phosphate synthase, provided that soluble acid invertase activity is below a critical threshold (Zhu et al.,1997). In the late stages of sucrose accumulation in the stalk apoplastic concentrations of sucrose have been measured in the order of 300 mM (Welbaum and Meinzer, 1990). It may be suggested that it is a regular natural situation during ripening, for sucrose to pile up in the apoplast. In this situation a diffusion-like system will tend to equilibrate sucrose concentrations between apoplast and cytoplasm and the active uptake of hexoses from the apoplast is only important during the early stages of ripening.

Sugar uptake studies with isolated protoplasts derived from sugar beet roots showed that transport of glucose is active and sensitive to carbonylcyanide M-chlorophenylhydrozone (CCCP), a protonophor. Fructose and sucrose were taken up by a lower rate than glucose. The uptake rate in conducting tissue exceeded that of storage tissue (Fieuw and Willenbrink, 1990). The authors hypothesise that in the tap root of sugar beet, sucrose gets unloaded from the sieve element/companion cell complex into the apoplast and then glucose, fructose and sucrose are taken up by the

conducting tissue via active transporter mediated processes. Sucrose is resynthesised and transported from the conducting tissue via symplastic connections into the storage cells.

In broad bean the developing embryo is symplastically isolated from the maternal tissue. During the storage phase of seed development sucrose is transported symplastically from the sieve element into the seed coat (Patrick et al.,1995), where it is unloaded by an apoplastic step into the endospermal space. In inhibitor studies and by selective removal of the inner cell layers of the seed coat, the thin-walled parenchyma was identified as the site of apoplastic unloading (Wang et al.,1995). From the endospermal space sucrose is taken up by the transfer cells into the embryo. Theses transfer cells develop where the embryo is in close proximity to the area of sucrose unloading from the seed coat into the endospermal space. Transfer cells are characterised by a high expression level of the sucrose proton symporter VfSUT1 (Weber et al.,1997). During the early stages of embryo development the cell wall bound invertase VfWINV1 is expressed specifically in the seed coat cell layer involved in assimilate unloading. This leads to an increased hexose/sucrose ratio in the endospermal space (Weber et al.,1995). The activity of the cell wall bound invertase coincides with the specific expression of the monosaccharide proton symporter VfSTP1 in the cotyledonary epidermis. Expression of VfSTP1 in *Schizosaccharomyces pombe* revealed that the transporter has a K_m of 30 µM for glucose and that yeast cells expressing the transporter had a 16 times higher transport rate of glucose compared to fructose (Weber et al.,1997). The parallel expression of the VfWINV and VfSTP1 not only ensures that the embryo is supplied with carbohydrates during the early stage of development, but also the hexose/sucrose

ratio in the embryo has been suggested to influence embryo development. A high hexose/sucrose ratio promotes cell proliferation in the embryo, whereas a low hexose/sucrose ratio favours cell expansion and accumulation of storage products (Weber et al.,1996; Borisjuk et al.,1998).

Antisense repression of the cell wall invertase in carrots alters the sucrose partitioning. Antisense plants have a bushy appearance due to the development of additional leaves. Leaves accumulated elevated levels of sucrose and starch. In addition the antisense plants develop a much smaller tap roots which contain decreased levels of carbohydrates. The dry weight leaf-to-root ratio is shifted from 1:3 in wild type plants to 17:1 in antisense plants (Tang et al.,1999). These results suggest that sucrose is not effectively translocated into the sink organs. The apoplastic hydrolysis of sucrose into glucose and fructose is essential for normal carbohydrate partitioning in carrots. Therefore it can be speculated that hexose transporters exist in the carrot tap root which transport the apoplastic glucose and fructose into the sink cells. In addition to the changes in sucrose partitioning somatic embryos of antisense plants show an altered phenotype when grown on sucrose as sole carbon source. The cotyledons of these embryos do not separate properly. The malformation of the embryos is alleviated when grown on a mixture of sucrose, glucose and fructose (1:2:2) as carbon source (Tang et al.,1999). By analogy to the observation in broad bean, these results suggest the hexose/sucrose ratio in the embryo influences embryo development.

The *Miniature1* seed (*mn1*) mutant of maize is characterised by an early withdrawal of the pedicel from the developing endosperm at 9 to 10 days after pollination. Consequently, the developing endosperm is starved for nutrients from the mother

plant, and its subsequent growth and development are reduced dramatically (Lowe and Nelson, 1946). In 1996 it was shown that the *Miniature1* seed locus encodes an endosperm specific cell wall bound invertase (Cheng et al.,1996). These findings imply that hexose uptake into the maize embryo occurs and is important for embryo development.

From grape berry the putative hexose transporter Vvht1 has been isolated. Vvht1 is expressed mainly in the berries, with a first peak of expression at anthesis, and a second peak about 5 weeks after veraison (Fillion et al.,1999). The expression of Vvht1 during ripening indicates that sucrose is at least partially cleaved before uptake into the berry.

1.2.3 The role of hexose/proton symporters in *Arabidopsis* in photoassimilate partitioning

In A*rabidopsis* the two main vegetative sink tissues are the roots and young leaves. In experiments where 5 (6)-carboxyfluorescein (CF), a fluorescent dye that follows very closely the pattern of movement of sucrose within the symplast, was injected into the phloem of *Arabidopsis* cotyledons, confocal laser scanning microscopy revealed that the dye accumulated in the two discrete phloem files of the root. Unloading of the dye from the phloem into the roots occurred only into the elongating zones of the main and the lateral roots. No dye was detected in the very tip of the roots and in cells in the zone of cell maturation. In addition no symplastic lateral unloading from the metaphloem was observed (Oparka et al.,1994; Oparka et al.,1995). In experiments where green fluorescent protein (GFP) was expressed under

control of the *ATSUC2* promotor, which regulates expression of the companion cell specific *AtSUC2* sucrose proton symporter gene (Truernit and Sauer, 1995; Stadler et al.,1996), the distribution of GFP within the root was similar to the distribution observed with CF (Imlau et al.,1999). The pattern of the CF and GFP distribution within the root tips correlates with the plasmodesmal frequency (Kragler et al.,1998). These observations suggest the symplastic unloading is the main pathway of photoassimilate supply to the actively growing parts of the root. However it is not clear via which pathway of phloem unloading cells in the maturation zone of the root are supplied with photoassimilates, since absence of CF and GFP does not necessarily prove that there are no symplastic connections from the cells in the maturation zone to the phloem. The expression of the sucrose/proton symporter AtSUC2 in the phloem of roots (Truernit and Sauer, 1995; Stadler et al.,1996) indicates that sucrose is lost from the phloem into the apoplast, since the proposed function of this transporter in roots is the retrieval of sucrose from the apoplast. Cells in the maturation zone of the root could meet their carbohydrate demand by using the sucrose leaked from the phloem. Whether this sucrose is taken up via sucrose transporters or the sucrose is hydrolysed by cell wall invertase and hexose transporters are involved in the uptake is not clear. Analysis of the expression of Atβfruct1 a cell wall bound invertase showed that the invertase is expressed in roots (TymowskaLalanne and Kreis, 1998), but the exact localisation of the expression of the invertase within the root is still missing. This observation supports the possibility of hexose transporters involved in carbon partitioning in the root. Up to now expression in roots for only two monosaccharide transporters from *Arabidopsis* has been demonstrated. AtSTP4 is expressed only in the elongation zones of primary and

lateral roots (Truernit et al.,1996). AtSTP1 is expressed in roots (Sherson et al., 2000, Sauer et al.,1990), but exact localisation of the expression is still not known. Analysis of *Arabidopsis* plants expressing GFP under control of the ATSUC2 promotor demonstrated that GFP fluorescence spread from the phloem of sink leaves (young leaves) into adjacent cells (Imlau et al.,1999). This indicates that phloem unloading and post-phloem transport in sink leaves is symplastic. The expression of the *Arabidopsis* monosaccharide transporters AtSTP1 (Sauer et al.,1990c), AtSTP3 (Buttner et al.,2000) and AtSTP4 (Truernit et al.,1996) has been demonstrated in leaves. AtSTP3 is induced by wounding (Buttner et al.,2000) and AtSTP4 is induced by wounding, pathogen infection and elicitors (Truernit et al.,1996). The induction of expression of AtSTP3 and AtSTP4 suggest that the increased carbohydrate demand of cells due to wounding or pathogen attack is met by an increased uptake of hexoses from the apoplast. This is in agreement with the observation that cell wall invertases in carrot are induced under various stress responses (Sturm and Chrispeels, 1990). The role of AtSTP1 in leaves is less clear. The expression of the various monosaccharide/proton symporters and of the cell wall invertase in leaves indicates that hexose transport does occur in leaves. The extent to which this transport of hexoses is involved in carbon partitioning is not clear.

The reproductive tissue of the flower represents a strong permanent sink and needs to be supplied with large amounts of assimilates. In the AtSUC2 promotor-GFP plants transport from GFP into petals anthers and ovules is observed (Imlau et al.,1999). This may reflect that symplastic unloading is the main mechanism of assimilate supply in these tissues. The developing male gametophyte is symplastically isolated, since prior to the meiotic divisions of the pollen mother cells

the plasmodesmata connecting the tapetum and the sporogenous cells disintegrate (Scott et al.,1991). Therefore the developing male gametophyte is dependent on apoplastic supply of carbohydrates. Expression of the monosaccharide/proton symporter AtSTP2 has been demonstrated to be confined to the male gametophyte and it has been suggested that this transporter is involved in the assimilate supply of the developing pollen grain with hexoses (Truernit et al.,1999). The developing embryo is also symplastically isolated from the maternal tissue (Mansfield and Briarty, 1991; Bowman, 1994) and therefore dependent on an apoplastic supply of carbohydrates. This is reflected in the fact that in the AtSUC2 promotor-GFP plants transport of GFP was confined to the seed coat and no GFP could be detected in the embryo. Up to now there is no evidence for the expression of a monosaccharide or sucrose transporter in the *Arabidopsis* embryo. Since in developing *Arabidopsis* seeds a similar change in the hexose/sucrose ratio (high during prestorage phase, low during storage phase) as in broad bean has been observed, (Footitt unpublished results). It can be hypothesised that by analogy to broad bean embryo specific monosaccharide and sucrose transporters may exist in *Arabidopsis*. Interestingly a flower specific invertase Atβfruc2 has been reported (TymowskaLalanne and Kreis, 1998), but it is not known when and where in the flower this invertase is expressed.

1.3.1 Cloning of *AtSTP1*

The first plant hexose proton/symporter *CkHup1* was cloned from the algae *Chlorella kessleri* in 1989 (Sauer and Tanner, 1989). *CkHup1* was isolated by differential screening of a *Chlorella* cDNA library exploiting the fact that the expression of hexose transporters in *Chlorella* is induced by incubation in glucose (Hilgarth et al.,1991). The cDNA clone of CkHup1 was subsequently used to screen an *Arabidopsis* genomic library to identify putative hexose transporters from *Arabidopsis*. In these experiments a 4447 bp lamda clone was isolated. This clone contained four putative exons, which give an open reading frame of 1566 bp encoding a protein of 522 amino acids with a predicted molecular mass of 57 581 daltons (Sauer et al.,1990). The predicted protein has a overall identity in the amino acid sequence of 47% compared to the CkHup1 and was given the name AtSTP1 (sugar transport protein 1). Isolation of a corresponding cDNA confirmed the predicted exon/intron structure of the gene. Northern blot analysis revealed that AtSTP1 is expressed in all tissues analysed including leaves, stems, roots, flowers, and siliques. The expression in leaves is most prominent (Sauer et al.,1990c). The cellular localisation of AtSTP1 is not yet definitely determined. There is strong evidence that the protein is located in the plasma membrane. The first line of evidence for the localisation of AtSTP1 in the plasma membrane is that highly homologous transporter CkHUP1 has been localised in the plasma membrane using anti-CkHUP1 antibodies on *Chlorella* thin sections (Stadler et al.,1995b). Although it has been demonstrated that homologous transporters can be targeted to different membranes. The human glucose uniporters GLUT2 and GLUT7 share 68% identity

in their amino acid sequence but GLUT2 is located in the plasma membrane whereas GLUT7 is located in microsomes (Wendell and Bisson, 1994). The second line of evidence comes from experiments where AtSTP1 was expressed in yeast and *Xenopus* oocytes. In both systems AtSTP1 was targeted to the plasma membrane. Again the fact that a protein is targeted to the plasma membrane in a heterologous expression system is strong evidence but no proof for the same localisation *in planta.* Overexpression of membrane proteins could interfere with the normal sorting process and the proteins are integrated by default rather than by intention into the plasma membrane.

1.3.2 Transport characteristics of AtSTP1

AtSTP1 was the first higher-plant sugar transporter to be expressed in yeast. By expression in *Schizosaccharomyces pombe* it was shown that AtSTP1 transports D-glucose, D-galactose whereas sucrose and D-fructose are not transported by AtSTP1. A K_m of 20 μM of AtSTP1 for D-glucose was measured in this system. The transport via AtSTP1 is energy dependent since it is sensitive to CCCP. Further energization of the cells by addition of ethanol resulted in an increase in transport activity (Sauer et al.,1990). Expression of AtSTP1 in *Xenopus* oocytes did lead to the accumulation of the non-metabolisable glucose analogue 3-O-methyl-D-glucose above the external 3-O-methyl-D-glucose concentration, which was accompanied by the depolarisation of the plasma membrane (Boorer et al.,1994a). These results indicate that AtSTP1 is a proton symporter. Uptake studies after the integration of purified AtSTP1 into

proteoliposomes containing cytochrome-c-oxidase, which establishes a proton gradient across the membrane in the presence of the electron donor ascorbate, did confirm that AtSTP1 is a monosaccharide/proton symporter (Stolz et al.,1994). In experiments with *Xenopus* oocytes it was demonstrated that Na^+ could not substitute the protons to drive the uptake via AtSTP1 and therefore clearly demonstrating that AtSTP1 is a proton and not a Na^+ symporter (Boorer et al.,1994b). Analysis of the substrate specificity of AtSTP1 in *Xenopus* oocytes revealed that D-mannose and 2-deoxglucose were preferred over D-galactose and 3-O-methy-D-glucose, over D-xylose, over D-glucose, over D-fucose, over D-fructose, over L-glucose, over L-arabinose, over D-arabinose as substrates. This large range of substrates may not indicate that all these substrates are the physiological substrates of AtSTP1. VfSTP1 shows similar substrate specificity but the physiological role of the transporter is believed to be the uptake of D-glucose and D-fructose from the apoplast into the epidermal embryo cells, although the transporter accepts D-mannose and D-galactose as substrates (Weber et al.,1997). The steady state kinetics of sugar uptake via AtSTP1 into *Xenopus* oocytes suggests a sequential mechanism for the binding of protons and sugar to AtSTP1 (Boorer et al.,1994b). In a sequential mechanism one of the substrates (proton or sugar) binds first to the transporter on the outside causing a conformational change. The loaded transporter then crosses the membrane, followed by the dissociation of the substrate releasing it into the cytoplasm. The transporter reorientates in the membrane to repeat the transport cycle with the second substrate.

1.3.3 AtSTP1 is a member of the major faciliator superfamily (TC # 2.1)

AtSTP1 is a member of the major faciliator superfamily which consists currently of 28 recognised families, each of which share common descent with the 27 other families of the superfamily (Saier, 2000; Buttner and Sauer, 2000; Walmsley et al.,1998; Pao et al.,1998; Bush et al.,1996; Sauer and Tanner, 1993; Gamo et al.,1994; Marger and Saier, 1993; Baldwin and Henderson, 1989). ATSTP1 belongs to the sugar porter family (TC # 2.A.1.1), which is the largest family with over 200 currently sequenced members. Members of the sugar porter family have been identified in bacteria, archaea and eukarya. Sugars transported by members of this family include glucose, fructose, mannose, galactose, arabinose, xylose, maltose, lactose, α-glucosides, quinate and myoinositol. The sugar porter family is not only diverse with respect to substrate specificity but also for the mode of transport which can be either uniport or cation symport (Saier, 2000). In addition, for two members of the sugar porter family SNF3p and RGT2p it has been demonstrated that they act as receptors, transmitting information from the cell surface into the cell in order to influence rates of gene expression (Ozcan et al.,1998). A similar receptor function has been reported in another faciliator family (Island and Kadner, 1993). This indicates that the sugar porter family is functionally diverse. The hydropathy profiles of the members of the sugar porter family are very similar and suggest 12 membrane spanning domains with the N- and C-terminus of the protein being intracellular (Saier, 2000; Buttner and Sauer, 2000). However the crystallisation of members of the sugar porter family has so far been unsuccessful and confirmation of the predicted topology is still missing, although experiments with fusion proteins,

peptide specific antibodies with several members of the family all support the predicted topology (Hresko et al.,1994; Davies et al.,1987; Calamia and Manoil, 1990). Considerable homology between the N- and C-terminal halves of the members of the family indicate that they may have evolved by gene duplication from a common ancestral gene with 6 membrane spanning regions. Table 1 shows the comparison of members of the sugar porter family.

Transporter	Percentage identity to AtSTP1	Percentage similarity to AtSTP1	Number of amino acids	Function	Organism
AtSTP1	-	-	522	H^+ symporter	*Arabidopsis thaliana*
AtSTP12	80.7	86.4	508	?	*Arabidopsis thaliana*
AtSTP2	50.2	72.2	499	H^+ symporter	*Arabidopsis thaliana*
AtERD6	30.7	40.6	496	?	*Arabidopsis thaliana*
NtMST1	79.5	88.6	523	H^+ symporter	*Nicotiana tabacum*
CkHUP1	47.7	68.0	533	H^+ symporter	*Chlorella kessleri*
Glut2	32.4	43.8	524	uniporter	*Homo sapiens*
Glut1	28.6	55.0	492	uniporter	*Homo sapiens*
HEX1p	30.7	42.8	570	uniporter	*Saccharomyces cerevisiae*
SNF3p	30.1	43.1	818	receptor	*Saccharomyces cerevisiae*
RGT2p	30.5	45.0	763	receptor	*Saccharomyces cerevisiae*
XylE	33.5	45.6	491	H^+ symporter	*Escherichia coli*
GalP	35.9	47.2	464	H^+ symporter	*Escherichia coli*

Table 1: Comparison of selected members of the sugar porter family. The percentage of identical and similar amino acid residues was calculated for the complete protein sequences. Calculations were performed using the program BESTFIT of the University of Wisconsin genetics computer group package (gap creation penalty=50, gap extension penalty=3).

Extensive phylogenetic analysis with 40 members of the sugar porter family, including 27 members from plants revealed that the plant members form a common cluster. Within this cluster the members from algae are separated from members from higher plants (Buttner et al.,2000). The plant transporters show strongest homology to bacterial proton symporters, yeast hexose uniporters and mammalian uniporters (see table 1). So far the biochemical transport characteristics of eleven members of the plant cluster have been analysed by expression in heterologous systems (Buttner and Sauer, 2000). All analysed transporters were found to be monosaccharide proton/symporters. Therefore is has been proposed all sugar porters within that plant cluster are monosaccharide/proton symporters. With the ongoing sequencing project of the *Arabidopsis* genome recently members of sugar porter family from *Arabidopsis* have been identified which show less homology to the other plant members of the sugar porter family and do not group in the common plant cluster in a phylogenetic analysis. Examples for those transporters are AtERD6, AAB6433.2 (accession number AC0023359) and AAD26954.1 (accession number AC007134). The deduced amino acid sequence of these genes have only 28 to 30% identity with the sequence of AtSTP1. Since the transport characteristics for none of these genes have been analysed so far, it is not clear whether these genes encode also monosaccharide / proton symporters.

1.3.4 Sugar porter family in *Arabidopsis thaliana*

Up to now four monosaccharide/proton symporters have been isolated and functionally characterised from *Arabidopsis thaliana* (Buttner et al.,2000; Truernit et

al.,1999; Truernit et al.,1996; Boorer et al.,1994; Sauer et al.,1990). Table 2 gives a summary of the data obtained for those four transporters. All four transporters have similar wide range of monosaccharides as substrates including D-glucose, D-galactose and D-mannose. The K_m for D-glucose for AtSTP1, 2 and 4 ranges from 15 µM to 50 mM, whereas AtSTP3 has a much higher Km of 2 mM. AtSTP1 is expressed in all tissues, which have been analysed and is most abundant in leaves. The expression of the *AtSTP1* gene is down regulated by the substrates of the transporter (this thesis). *AtSTP2* is only expressed in the male gametophyte during a small window of pollen development. Therefore the uptake of monosaccharides, which are derived from the degradation callose into the male gametophyte is the proposed physiological function of AtSTP2. The expression of *AtSTP3* and *AtSTP4* is up regulated by wounding. In addition to that the expression of *ATSTP4* is up regulated by exposure to elicitors and pathogens. The induction of *AtSTP3* in response to wounding is slow and continues over a long time in contrast to the response of *AtSTP4,* which is much faster but continues for a shorter time. It has been suggested that response to stress of AtSTP3 and AtSTP4 reflects the physiological necessity to support wounded tissue with additional carbohydrates for additional metabolic tasks.

	AtSTP1	AtSTP2	AtSTP3	AtSTP4
K_m D-glucose	20 µM	50 µM	2 mM	15 µm
leaves	+++		+++	++
root	+	male	-	+++
expression stem	++	gametophyte	+	+
flower	+	only	+	+++
silique	+		-	+
regulation of expression	↓ substrates	-	↑ wounding	↑ wounding ↑ elicitors ↑ pathogens

Table 2: Comparison of the 4 characterised monosaccharide/proton symporters from *Arabidopsis thaliana*. ↑↓ indicate up or down regulation of gene expression. Comparison of expression levels indicated by + and - is only valid for the expression in the various tissue for individual transporters. Indicated expression levels of individual transporters are not interrelated. There is no data available which compares the expression level of individual transporters. Data compiled from (Buttner et al.,2000; Truernit et al.,1999; Truernit et al.,1996; Boorer et al.,1994; Sauer et al.,1990).

In total 10 further putative monosaccharide transporters have been identified by database search and / or by PCR reactions with genomic DNA using degenerate

primers that hybridise to highly conserved domains in monosaccharide transporter genes (Buttner et al.,2000). These putative transporters show 50 - 80% identity to the amino acid sequence of AtSTP1. Due to the high degree of homology to the well characterised transporters it has been suggested that these genes also encode monosaccharide/proton symporters. (Buttner and Sauer, 2000; Buttner et al.,2000). It has been predicted that the whole *Arabidopsis* genome may encode up to 20 different monosaccharide transporters (Buttner and Sauer, 2000). Phylogenetic analysis revealed that the 14 *Arabidopsis* monosaccharide transporters do not form a clearly separated subgroup but rather are homogenously distributed within the cluster of monosaccharide transporters from higher plants (Buttner and Sauer, 2000). This observation indicates that the ancestors of higher plants already had several monosaccharide transporter genes.

In addition to that, several sequences in the *Arabidopsis* genome have been identified which encode members of the sugar porter family (1.3.3 and (Lalonde et al.,1999)). These sequences show considerably less homology to AtSTP1 (around 30% identity to the derived amino acid sequence) and therefore might not encode monosaccharide symporters. One of these sugar porters has been suggested to be a glucose uniporter that is located in the chloroplastic membrane (Weber et al.,2000), but direct evidence in this case is still missing.

The vast number of monosaccharide transporters in *Arabidopsis* suggests that individual members of this family might be expressed at distinct sites for a certain time during development or in response to a specific environmental signal. This is reflected in the fact that the so far characterised monosaccharide proton symporters have similar transport characteristics but differ in their expression pattern.

1.4 Sugar signalling in plants

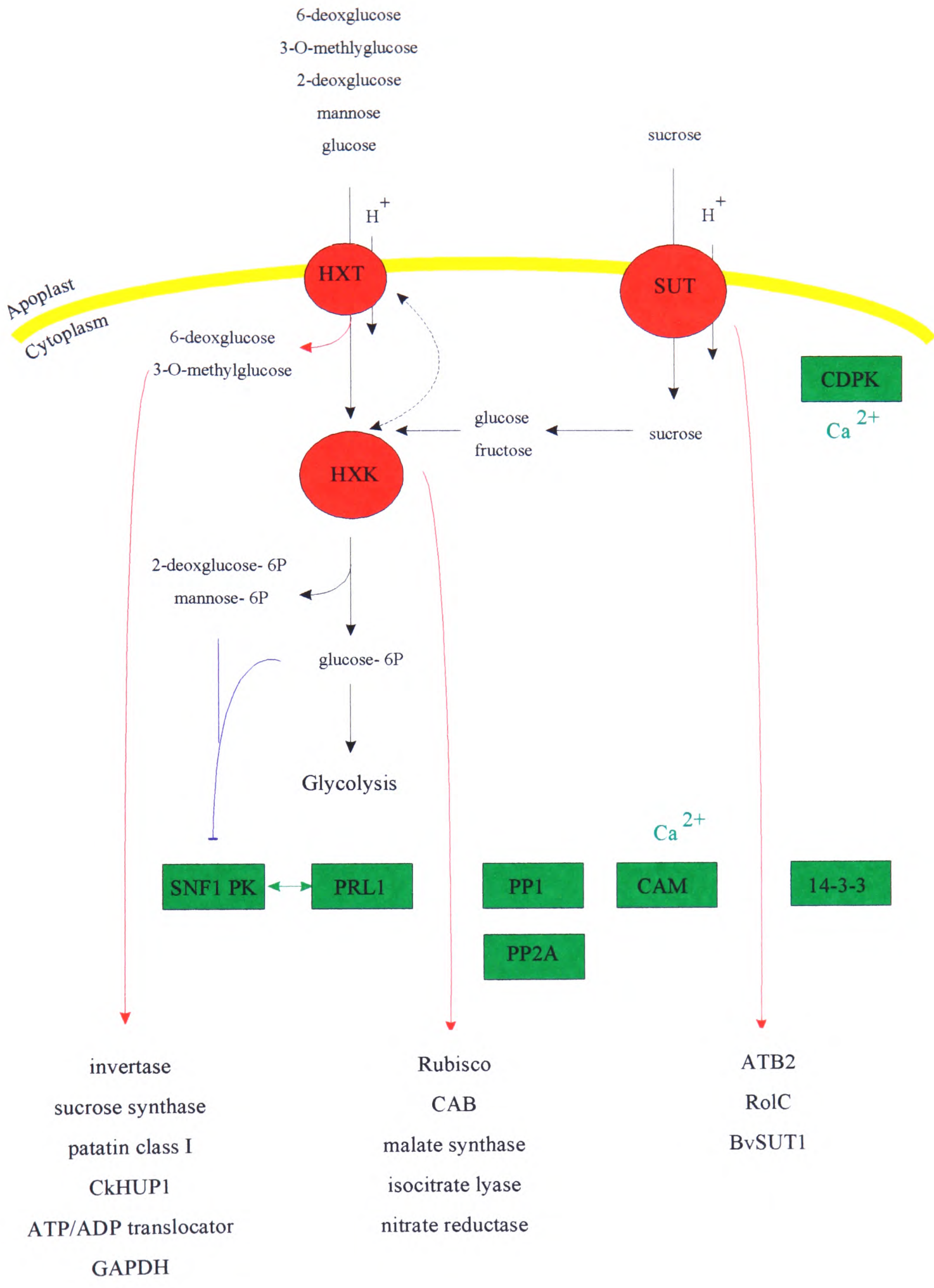
It is well known that sugars act as regulatory signals that effect gene expression and thereby influence such fundamental biological processes as growth and development. However in multicellular eukaryotes the mechanisms involved in the perception of sugar levels and the transduction of these signals are poorly understood.

Since plants are photoautotrophic organisms they have to co-ordinate the production of carbohydrates by means of photosynthesis with the demand for carbohydrates in sink tissues. Plants also have to co-ordinate the mobilisation of storage products such as starch and lipids, which are converted into carbohydrates in response to the demand for carbohydrates. It is known that the expression of several genes involved in these processes is regulated by sugars. Up to now over sixty genes involved in processes including photosynthesis, glycolysis, glyoxylate metabolism, nitrogen metabolism, sucrose and starch metabolism, carbohydrate transport, defence mechanisms and cell cycle control have been shown to be regulated by sugars (reviewed in (Graham, 1996; Koch, 1996)). In general high sugar levels repress the genes for sugar production and induce genes involved in utilisation of sugars and the synthesis of storage products. Low sugar levels exert opposite effects. Several lines of evidence suggest that the effects of sugars on gene expression involve specific signalling mechanisms and are not simply a result from their non-specific effects as substrates for plant growth. First, the effects of sugars on gene expression are selective; many genes are not affected. Second, sugars can repress as well as activate responsive genes. Third, in many cases slowly-metabolisable sugar analogues can

mimic the effects of sugars upon sugar responsive genes. Finally in some cases only a specific sugar causes an effect whereas other sugars do not affect the expression of those genes.

Currently the existence of at least three independent pathways of sugar sensing is discussed (reviewed in (Smeekens and Rook, 1997; Smeekens, 1998; Gibson and Graham, 1999; Lalonde et al.,1999): A hexose sensing mechanism, a hexokinase mediated sensing mechanism and a sucrose sensing mechanism (figure 2).

Figure 2: (next page) Schematic representation of proposed sugar sensing pathways in plants. Red circles represent proposed sugar sensors, red arrows link sensing mechanism with genes they regulate. Proteins and second messenger that are involved in signal transduction are indicated in green. Blue arrows link metabolites with proteins they inhibit. Abbreviations: HXT, hexose transporter; SUT, sucrose transporter; HXK, hexokinase; SNF1 PK, SNF1 protein kinase; PP1, protein Ser/Thr phosphatase 1; PP2A, protein Ser/Thr phosphatase 2A; CAM, calmodulin; CDPK, calmodulin dependent protein kinase; GAPDH, glyceraldehye-3-phosphate dehydrogenase(adapted from Lanonde *et al*.1999).


6-deoxglucose
3-O-methlyglucose
2-deoxglucose
mannose
glucose
sucrose
H+
H+
HXT
SUT
Apoplast
Cytoplasm
6-deoxglucose
3-O-methylglucose
CDPK
Ca 2+
glucose
fructose
sucrose
HXK
2-deoxglucose- 6P
mannose- 6P
glucose- 6P
Glycolysis
Ca 2+
SNF1 PK
PRL1
PP1
CAM
14-3-3
PP2A
invertase
sucrose synthase
patatin class I
CkHUP1
ATP/ADP translocator
GAPDH
Rubisco
CAB
malate synthase
isocitrate lyase
nitrate reductase
ATB2
RolC
BvSUT1

1.4.1. The hexose sensing mechanism

The suggestion that hexoses are generating a sugar signal is based on experiments with the sugar analogues 3-O-methylglucose and 6-deoxyglucose. These sugars are take up by plant cells with a similar efficiency as glucose and thought not to be metabolised at a significant rate in plant cells (figure 2). Therefore it can be argued that if these sugar analogues trigger a sugar response, this response is mediated by a sensing system which is activated by glucose and not by any metabolites of glucose. In a cell suspension culture of *Chenopodium rubrum* addition of glucose induces the expression of an extracellular invertase and sucrose synthase. This induction is mimicked by 6-deoxyglucose (Roitsch et al.,1995; Godt and Roitsch, 1997). In *Chlorella kessleri* the expression of the monosaccharide/proton symporter CkHUP1, an ATP/ADP translocator and of a glyceraldehyde-3-phosphate dehydrogenase is induced by the addition of glucose and 6-deoxyglucose (Hilgarth et al.,1991a). In transgenic *Arabidopsis* plants expressing the β-glucuronidase reporter gene under the control of a potato patatin class I promotor (B33) it is observed that the expression of the reporter gene is induced by glucose, 3-O-methylglucose and 6-deoxyglucose (Martin et al.,1997). In tobacco plants overexpression of a yeast invertase which was targeted either to the apoplast, the cytosol or vacuole lead to an increased glucose level in the compartment expressing the invertase. Only in plants that are overexpressing the invertase in the apoplast and the vacuole was the expression of the *CAB* gene repressed and the expression of the tobacco apoplastic invertase and defence related genes was induced. Increased glucose levels in the cytosol did not

alter the expression of those genes (Herbers and Sonnewald, 1998; Herbers et al.,1996). These observations can be explained in two ways: The glucose sensing is related with glucose transport via the plasma membrane. Therefore glucose that is produced in the cytoplasm is not sensed. Alternatively it can be argued that the sugar sensing system is located in the secretory systems, since only the invertase targeted to the vacuole and apoplast passes through those systems.

It has been speculated (Smeekens and Rook, 1997; Lalonde et al.,1999) the hexose sensing mechanism is associated with hexose transport and a dual function for hexose transporters has been proposed, although there is no direct evidence for such sensors in higher plants. In *Saccharomyces cerevisiae* the hexose transporter-like proteins SNF3and RGT2 act as such sugars sensors (Ozcan et al.,1998). The fact that SNF3 and RGT2 show about 30% identity at the amino acid sequence level to plant monosaccharide transporters lead to the speculation that proteins with similar function are present in plants. The C-terminal part of SNF3 and RGT2 which is involved in the signal transduction, and the proteins MST1 and STD1 which interact with the sensors, show no significant homology to any plant or other eukaryotic proteins (Schmidt et al.,1999). Therefore this type of sugar sensor may be restricted to yeast. The hexose sensing mechanism in plants may be independent from hexose transport and could be mediated by cytosolic hexose binding proteins.

1.4.2 The hexokinase mediated sensing mechanism (figure2)

Early indication that hexokinase is involved in sugar sensing came from experiments using the sugar analogue 2-deoxyglucose, which was thought to be phosphorylated by hexokinase but not to be further metabolised in plant cells. In maize protoplasts (Jang and Sheen, 1994) and in a cucumber cell culture system (Graham et al.,1994) 2-deoxyglucose could mimic the effects of glucose with regard to regulation of gene expression of several genes. However recently the use of 2-deoxyglucose as tool to study hexokinase mediated sugar sensing has been questioned (Klein and Stitt, 1998). In a detailed analysis of the metabolism of 2-deoxyglucose in a *Chenopodium rubrum* cell culture it was demonstrated that 2-deoxyglucose-6-phosphate is further metabolised at a significant rate and leads to complex changes in metabolism, and therefore makes the interpretation of the effect of 2-deoxyglucose very complex. It also has been demonstrated that mannose causes similar effects to 2-deoxyglucose and glucose on gene expression in maize protoplasts (Jang and Sheen, 1994) and cucumber cell culture (Graham et al.,1994). In plant cells mannose is phosphorylated by hexokinase. Mannose-6-phosphate is utilised slowly due to a deficiency of mannose-6-phasphate isomerase, which is necessary for its conversion to fructose-6-phosphate (Goldsworthy and Street, 1965). Therefore mannose is a more suitable sugar to test the role of hexokinase as a sugar sensor, although the accumulation of mannose-6-phosphate does lead to severe changes in phosphorylated intermediates (Klein and Stitt, 1998), and its utilisation requires an examination of those secondary changes potentially effecting gene expression. In addition to that it has been

demonstrated that the activity of a SNF1-related protein kinase from spinach is repressed by hexose-6-phosphates (Toroser et al.,2000). SNF1-related protein kinases have been suggested to function in sugar signal transduction (reviewed in (Halford and Hardie, 1998)). Therefore mannose-6-phosphate could influence gene expression via the inhibition of SNF1-related protein kinase (figure 2) rather than as a result of phosphorylation by hexokinase.

More direct evidence for hexokinase mediated sugar sensing was presented in experiments where two hexokinase genes from *Arabidopsis*, *AtHXK1* and *AtHXK2*, were expressed in sense and antisense direction in *Arabidopsis*. Overexpression of the hexokinases lead to a decreased amount of the transcript of the *RBCS* and *CAB* genes and the amount of transcript encoding nitrate reductase were increased. In addition to that, these plants were hypersensitive to the repression of hypocotyl elongation and greening of the cotyledons by high levels of glucose. AtHXK1 and AtHXK2 antisense plants showed a decreased sensitivity to high glucose levels with regard to hypocotyl elongation and greening of the cotyledons. In addition to that the plants overexpressing the yeast hexokinase 2 showed a reduced sensitivity to high glucose levels with regard to hypocotyl elongation and greening of the cotyledons. The interpretation was that by competing for the same substrate, glucose, the yeast enzyme bypassed the endogenous signalling hexokinases, resulting in reduced glucose sensitivity (Jang et al.,1997). This observation indicates that phenotypic effects seen in plants with altered expression levels of endogenous hexokinase activity are not only due to an altered metabolic system but rather an effect of sugar sensing via hexokinase.

In addition to the results in *Arabidopsis* the antisense repression of potato hexokinase1 in potato did lead to an overaccumulation of starch in leaves (Veramendi et al.,1999). Overexpression of *AtHXK1* in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence (Dai et al.,1999). In both systems the changes in hexokinase activity did lead to changes in metabolite levels. Again these results are no evidence that hexokinase is a sugar sensor since the effects observed could have been caused by the changes in metabolite levels. This highlights the problem to separate the effects of the catalytic function from the proposed signalling function of hexokinase in an experimental system.

1.4.3. The sucrose sensing mechanism (figure 2)

It has been demonstrated that the expression of a number of genes can be specifically regulated by sucrose, since a combination of glucose and fructose was less active in regulating the expression of those genes. Examples of sucrose regulated genes are the *Arabidopsis* transcription factor *ATB2* and the *Beta vulgaris* sucrose transporter *BvSUT1*. It has been demonstrated that in tobacco the transcription of genes under the control the *RolC* promotor from *Agrobacterium* is induced by sucrose (Yokoyama et al.,1994). Transcription of *ATB2* (Rook et al.,1998) and *BvSUT1* (Chiou and Bush, 1998) are specifically repressed by sucrose. The sucrose:glucose ratio in *Vicia faba* controls embryo development. In experiments where embryos were split in halves, and the halves cultured *in vitro* either on glucose or sucrose containing media it was demonstrated that the sucrose promotes cell differentiation and accumulation of storage products whereas glucose promotes cell division

(Weber et al.,1996; Borisjuk et al.,1998). This suggests that there are independent sensors for sucrose and glucose.

By analogy to the hexose sensing mechanism it has been speculated that sucrose transporter may have a dual function and also act as sensors (Lalonde et al.,1999; Smeekens and Rook, 1997). Direct evidence for this is lacking and a cytoplasmic sucrose binding protein could also fulfil the role as a sucrose sensor.

1.4.4 Transduction of sugar signals (figure 2)

In yeast the *SNF1*-encoded protein Ser/Thr kinase plays a major role in derepression of several glucose repressed genes. It has been demonstrated that several putative plant *SNF1* homologues can restore SNF1 function in yeast snf1 mutants ((Halford and Hardie, 1998) and references therein). Therefore a similar function in regulation of gene expression for SNF1 plant homologues has been proposed. In potato the inhibition of protein Ser/Thr phosphatase 1 (PP1) and 2A (PP2A), inhibits the sugar regulation of sporamin and β-amylase mRNA levels (Takeda et al.,1994). Inhibitors of calmodulin and Ca^{2+} also affected mRNA levels in this system, implying the involvement of calmodulin and Ca^{2+} in transduction of sugar signals. In experiments where cytoplasmic Ca^{2+} levels were monitored in tobacco leaves an increase in cytoplasmic Ca^{2+} levels was observed during the incubation with sugars. A membrane associated Ca^{2+} dependent Ser/Thr protein kinase was suggested to transmit these changes in Ca^{2+} (Ohto and Nakamura, 1995).

In response to exogenously supplied sucrose cell suspension cultures of *Vitis vinifera* accumulate anthocyanins. This response is inhibited by Ca^{2+} blockers and calmodulin

antagonists. Therefore it has been suggested that Ca^{2+} and calmodulin are involved in the sugar signal transmission in this system (Vitrac et al.,2000). The *Arabidopsis prl1* mutant shows an altered response to sugars and hormones. The mutants are hypersensitive to glucose and glucose regulated genes are derepressed. The *prl* locus was found to encode a nuclear WD protein (Nemeth et al.,1998). An interaction between SNF1-like protein kinases and the PRL1 protein was demonstrated (Bhalerao et al.,1999). This suggests that PRL1 is involved in the transmission of sugar signals. Recently it has been demonstrated that in *Arabidopsis* 14-3-3 proteins bind specific cytosolic proteins like sucrose phosphate synthase, glyceraldehyde-3-phosphate dehydrogenase and nitrate reductase. Under sugar starvation 14-3-3 proteins are no longer bound to their target proteins. These target proteins are degraded under sugar starvation. *In vitro* the binding of 14-3-3 can protect the target proteins from degradation by proteases. Therefore a role for 14-3-3 proteins in sugar sensing has been proposed (Cotelle et al.,2000).

2 Material and Methods

Note: All standard recombinant DNA techniques not mentioned here were performed after Maniatis *et al.* (1982).

2.1 Biological material

2.1.1 Plant material

The *stp1-1* mutant was kindly provided by Dr. Sarah Sherson (for details see (Sherson et al., 2000)in the appendix). This mutant was isolated from the INRA-Versailles T-DNA lines (Bechtold et al.,1993; Bouchez et al.,1993) and is a Wassilewskija ecotype. The wild type plants used were derived from the same seed pool from which the *stp1-1* mutant was isolated and are therefore as well Wassilewskija ecotype.

2.1.2 Bacterial Strains

Escherichia coli:

DH5α	*sup*E44 Δ*lac*U169 (Φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1
	Used as a host for recombinant manipulation (Hanahan, 1983)

Agrobacterium tumefaciens:

GV 3101 + PMP90RK	(Koncz and Schell, 1986; Van Larebeke et al.,1974) Used for plant transformation

2.1.3 Plasmids

Vector	**Source**	**Use**
pBluesrcipt II SK+	(Altingmees and Short, 1989)	subcloning, sequencing
pTF12108	N. Sauer unpublished results	35S::STP1 cDNA fusion
pTF405A	(Sauer et al.,1990)	*AtSTP1* cDNA
pGreens	(Hellens et al.,2000)	plant transformation

2.1.4 Oligonucleotides

reverse	5'CGGATAACAATTTCACACAGGA 3'
universal	5' CAACGTAAAACGACGGCCAGTCG 3'
stp1-out	5' GCAGCAACAACGCAAGTG 3'

2.2 Nucleic acid isolation, manipulation and analysis

2.2.1 Extraction of total RNA from plant tissue

All plant tissue was frozen in liquid nitrogen immediately after it was harvested and stored at -70 °C until the RNA was extracted. The tissue (up to 1 g) was ground using a prechilled pestle and mortar to a fine powder and transferred into a prechilled 15 ml Falcon centrifuge tube. 5 ml of RNA extraction buffer (100mM Tris-HCl pH 8.5, 6 % [w/v] para-aminosalicylic acid, 1 % [w/v] triisopropyl-naphthalene sulfonic acid sodium salt and 5ml Phenol/Chloroform (50:50 v/v)) were added and mixed with the sample. The organic and the aqueous phase were separated by centrifugation at 3500 g at 4 °C for 5 min and the aqueous phase was removed into a 30 ml corex centrifugation tube. The nucleic acids were precipitated out of the solution by adding 500 µl of 3 M sodium acetate pH 5.5 and 12.5 ml ethanol and incubation at -20 °C for 2 hours. After centrifugation at 12000 g at 4 °C for 10 min the supernatant was removed and the pellet redissolved in 2ml of ddH_2O. In order to separate the RNA from the DNA the RNA was precipitated out of the solution by adding 2ml of 5 M LiCl and an incubation for 1 hour at 4 °C. After centrifugation at 12000 g at 4 °C for 10 min the supernatant was removed and the pellet redissolved in 300 µl of ddH_2O. To further concentrate the RNA it was precipitated by adding 30 µl of 3 M sodium acetate pH 5.5 and 750 µl ethanol and incubation at -20 °C for 2 hours. After centrifugation at 12000 g at 4 °C for 10 min the supernatant was removed and the pellet redissolved in 100 µl of ddH_2O.

2.2.2 Quantification of RNA

RNA was quantified by measuring the A_{260} with a spectrophotometer. An A_{260} reading of 1 corresponds to approximately 40 $\mu g\ ml^{-1}$ of RNA.

2.2.3 Horizontal gel electrophoresis of RNA

RNA samples were size-fractionated by electrophoresis through 1.2 % (w/v) agarose gels in 1 X MOPS buffer (20mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate pH 7.0 , 1 mM EDTA pH 8.0) containing 6.6% (w/v) formaldehyde. RNA samples were prepared for electrophoresis by dessicating aliquots to complete dryness under vacuum and redissolving them in 5 µl of RNA gel loading buffer (1 X MOPS buffer, 50 % [v/v] formamide, 6.6 %[w/v] formaldehyde, 3% [w/v] Ficoll 400, 0.02 % [w/v] bromphenol blue, 50 $\mu g\ ml^{-1}$ ethidium bromide). Samples were heated at 70 °C for 5 min prior to loading. After electrophoresis in 1 X MOPS buffer RNA was visualised using a short wavelength transilluminator and photographed. The loading of approximately equal amounts of total RNA into each lane was checked by comparing the amount of the 18sRNA in each lane at this stage.

2.2.4 Northern Blotting

After fractionation, the RNA was transferred from the gel onto Hybond N filters (Amersham International plc.) by capillary action according to the method of Southern (Southern, 1975). The transfer was faciliated by imposing a salt gradient across the gel from a reservoir of 20 X SSPE (3.6 M NaCl, 0.2 M sodium

dihydrogen orthophosphate pH 7.7, 20 mM EDTA pH 8.0) to the filter. After transfer, the filters were rinsed in 2 X SSPE, and the RNA crosslinked to the filter by UV irradiation at 0.4 J cm^{-2} using a hybaid crosslinker.

2.2.5 Radiolabelling of ds DNA probes by random priming

Double-stranded DNA was labelled by the random priming method of Feinberg & Vogelstein (1983). The template for the *AtSTP1* probe for Northern blots was a PCR product using the reverse and universal primer with the vector pTF405A as template. For the hybridisation of the BAC library the 253 bp EcoRV / SacI fragment from the vector pTF405A was used. 50 ng template DNA was denatured by heating to 100 °C for 5 min. and subsequently chilled on ice. An appropriate volume of H_2O was added to the denatured DNA to give a final volume of 50 µl when the following were added:

10 µl	5 X oligonucleotide labelling buffer (OLB)
2 µl	BSA (10 mg ml^{-1})
3 µl = 30 µCi	(α-^{32}P) dCTP
1 µl = 1U	Klenow fragment DNA polymerase I

The radionucleotides were purchased from Amersham International plc. 5 X OLB: 250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM with respect to each of aATP, dGTP and dTTP,, 1 M HEPES (N-(2-hydroxyethyl)-N-(2-ethanesulfonic acid)), adjusted to pH 6.6 with sodium hydroxide, 1 mg ml^{-1} random

hexanucleotides. The labelling reaction was carried out at 37 °C for 45 min. Unicorporated nucleotides were removed by filtration through a G50 Nick Column. Before adding the radiolabelled DNA it was denatured by heating at 100 °C for 5 min.

2.2.6 Hybridisation of radiolabelled DNA probes to membrane-bound RNA

The membranes with the crosslinked RNA was prehybridised in 30 ml hybridisation buffer (4 X SSPE, 20 mM Tris-HCl pH 7.6, 1 % (w/v) SDS, 2 X Denhards solution , 10 % (w/v) dextrane sulpate) for at least 2 h at 65 °C. After prehybridisation the solution was replaced with 10 ml fresh hybridisation buffer and the denatured radiolabelled probe was added. The probe was hybridised for at least 16 h at 65 °C to the RNA. To remove all unspecifically bound radionucleotides the membranes were washed as follow:

Solution	**Conditions**
4 X SSPE, 1 % SDS	65 °C 30 min twice
2 X SSPE, 0,5 % SDS	65 °C 30 min

After washing the filters were wrapped in Saran wrap.

2.2.7 Autoradiography

DuPont Cronex 4 or Kodak Biomax X-ray Film was exposed to the filters in autoradiograghy cassettes with intensifier screens at -70 °C. The films were developed with a X-Omat developer.

2.2.8 Isolation of plasmid DNA from *E. coli*

2.2.8.1 Isolation of large plasmids representing BAC clones

The bacteria of 1.5 ml overnight culture were harvested by centrifugation and resuspended in 0.2 ml of lysis buffer containing:

50 mM	glucose
10 mM	EDTA
25 mM	Tris-HCl pH 8.0
5 mg ml^{-1}	lysozyme

After 5 min incubation on ice 0.4ml 0.2 N NaOH, 1% SDS was added and gently mixed. After a further 5 min incubation on ice 0.3 ml 3M potassium acetate pH 5.5 was added, mixed and incubated for 15 min at - 70° C. After centrifugation for 15 min at 13000g 0.75 ml of the supernatant was tranfered into a new reaction tube. To precipitate the DNA 0.45 ml of isopropanol was added and the solution incubated at -70° C for 15 min. After 5 min of centrifugation at 13000g the supernatant was removed and the DNA washed with 70% (v/v) ethanol. The DNA pellet was dried for 15 min at room temperature and redissolved in 40 µl TE Buffer pH 8.0 over night at 4° C .

2.2.8.2 Isolation of plasmid DNA after Holmes and Quigley(1981) (mod)

The bacteria of 1.5 ml overnight culture were harvested by centrifugation and resuspended in 150 µl of STEL buffer containing:

8 % (w/v)	sucrose
5% (v/v)	triton-X
50 mM	Tris-HCl pH 8.0
50 mM	EDTA
0,5 mg ml^{-1}	lysozym

The suspension was boiled for 30 sec in a water bath. After centrifugation for 20 min at 13000g the pellet was removed with a sterile toothpick. 180 µl of isopropanol were added and mixed with the supernatant. After 5 min centrifugation at 13000g the supernatant was removed and the pellet washed with 70 % (v/v) ethanol and the redissolved in 50 µl TE buffer.

2.2.9 Horizontal gel electrophoresis of DNA

DNA samples were analysed by electrophoresis through gels prepared from agarose at concentrations between 0.5 and 1.2% (w/v) according to the size of the DNA which was separated in TAE buffer (40mM Tris-HCl, 20 mM sodium acetate, 1mM EDTA, adjusted to pH 8.2 with glacial acetic acid). Samples were loaded in 0.2 volumes of loading buffer (5 X TAE, 15 % [w/v] Ficoll 400, 0.25 M EDTA, 0.04 %[w/v] bromophenol blue and 0.04 % [w/v] xylene cyanol FF). Ethidium bromide was either included into the gel matrix at a final concentration of 0.5 µg ml^{-1}, or the DNA was stained after electrophoresis by incubating the gel in a solution of ethidium bromide of that concentration. The ethidium bromide intercalated into the DNA was visualised using a short wavelength transilluminator. DNA size markers were

prapared from lamda phage DNA which was digested either with the restriction enzyme PstI, HindIII or StyI.

2.2.10 Southern Blotting

After fractionation, the DNA was transferred from the gel onto Hybond N filters (Amersham International plc.) by capillary action according to the method of Southern (Southern, 1975). The transfer was faciliated by imposing a salt gradient across the gel from a reservoir of 20 X SSPE (3.6 M NaCl, 0.2 M sodium dihydrogen orthophosphate pH 7.7, 20 mM EDTA pH 8.0) to the filter. After transfer, the filters were rinsed in 2 X SSPE, and the DNA crosslinked to the filter by UV irradiation at 0.4 J cm^{-2} using a hybaid crosslinker.

2.2.11 Hybridisation of radiolabelled DNA probes to membrane-bound DNA

The membranes with the crosslinked DNA was prehybridised in 30 ml hybridisation buffer (2 X SSPE, 1 % (w/v) SDS, 0.5 % [w/v] low fat milk powder) for at least 2 h at 65 °C. After prehybridisation the solution was replaced with 10 ml fresh hybridisation buffer and the denatured radiolabelled probe was added. The probe was hybridised for at least 16 h at 65 °C to the DNA. To remove all unspecifically bound radionucleotides the membranes were washed twice for 30 min in 2 X SSPE, 0,5 % SDS at 65 °C. After washing the filters were wrapped in Saran wrap.

2.2.12 DNA sequencing

DNA sequencing was performed with the D-rhodamine terminator cycle sequencing kit (Perkin Elmer) according to the manufacturers instruction. Template plasmid DNA was prepared with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturers instructions. Sequencing reactions were separated on a Perkin Elmer ABI Prism 377 DNA sequencer by the ICMB sequencing service.

2.3 Measurement of uptake of radiolabelled D-glucose into Arabidopsis seedlings

Seeds were germinated on ½ strength MS medium and the seedlings were grown for 7 days under constant light. 5 mg of seedlings (about 5 seedlings) were transferred into a 1.5 ml reaction tube and immersed in 0.2 ml ½ strength MS medium. The seedlings were vacuum infiltrated for 3 min and equilibrated for a further 30 min in that medium at room temperature. 0.1 μCi of [^{14}C]-D-glucose in 10 μl of H_2O, to give a final concentration of glucose of < 1.6 μM, was added. Uptake of the radioactive glucose was allowed to proceed for 30 min at room temperature. The incubation media was removed and the seedlings were washed 4 times in ice cold ½ strength MS medium containing 1mM unlabelled glucose. Radioactivity was extracted from the seedlings by incubation in 1 ml 80 % (v/v) ethanol at 56 °C for 30 min. The extraction was repeated once and the radioactivity in the combined extracts was determined by liquid scintillation counting.

2.4 Analysis of anthocyanins

2.4.1Quantification of anthocyanin after Rabino and Mancinelli (1986)

Seedlings were homogenised in methanol that contained 1 % HCl (v/v) at 4 °C and incubated at 4°C for 16 hours. After centrifugation the absorbance of the supernatant at 530 and 657 nm was measured and the formula A_{530} - 0.25 X A_{657} was used to calculate the amount of anthocyanin by compensating for the contribution of chlorophyll and it's degradation products.

2.4.2 Anthocyanin stain

Seedlings were incubated for 10 min at 70 °C in 70 % ethanol (v/v) to remove the chlorophyll. After removal of the chlorophyll the seedlings were placed into a methanol solution that contained 1% HCl (v/v) to decrease the pH.

2.5 Quantification of mono- and disaccharides by HPLC

Seedlings were grown on vertical agar plates containing ½ strength MS medium with 50 mM galactose. Seedlings were harvested and washed for 5 min in ddH_2O to remove any adhering galactose. 30 mg of seedlings were ground in 200 µL 15% (w/v) TCA in a 1.5 ml reaction tube for 30 seconds. After centrifugation at 13000g for 5 min the supernatant was removed and neutralised by the addition of 200 µl 2 M NaOH. Chlorophyll and anthocyanins were removed by mixing with ion exchange resin (Dowex 50WX8-200). The supernatant was filtered through a 0.2 µm syringe filter. 20µL of each sample was loaded onto a Dionex CarboPac-100 column

(250X4mm) attached to standard Dionex hardware. Sugars were eluted with 100 mM NaOH at room temperature and a flow rate of 1mL/min. Detection was by means of a Dionex pulsed amperometric detector fitted with a gold electrode.

2.6 Transformation

2.6.1 Plant Transformation

Agrobacterium tumefaciens mediated *in planta* transformation of *Arabidopsis* was performed according to Bechtold *et al.*(1993).

2.6.2 Transformation of *Agrobacterium tumefaciens*

An *Agrobacterium tumefaciens* onvernight culture was grown until it reached an OD_{680} of 0.5-1. The cells were harvested by centrifugation and resuspended in 1/50 of the volume in 20 mM ice cold $CaCl_2$. To 0.2 ml of the suspension 5 µg plasmid DNA in an volume of up to 10µl was added and mixed. The cells were frozen in liquid nitrogen for 30 seconds and then thawed by incubation for 5 min at 37°C. Before spreading the cells on LB agar plates with the appropriate antibiotics, 1 ml of LB medium was added and the cells were incubated at 28°C for 2-4 hours.

2.6.3 Transformation of *Escherichia coli*

E. coli was transformed by electro-transformation using a Bio RAD gene pulser and pulse controler according to the manufacturers instructions.

2.7 Growth of seedlings in sterile culture

Seeds were surface sterilised by incubation for one minute in isopropanol and five minutes in commercial thin bleach. After several washes with ddH_2O seeds were sown on ½ strength MS medium (Murashige and Skoog, 1962), 0.5% MES (w/v), 0.8% agar (w/v), pH 5.7. Carbohydrates were added as indicated. Plates were incubated in continuous light (30-60 $\mu mol\ m^{-2}\ s^{-1}$) at 22 °C.

3 Aim of the Project

Although the biochemical features of the *Arabidopsis thaliana* high affinity proton/monosaccharide symporter AtSTP1 have been well characterised by expression in heterologous systems *Xenopus* oocytes and *S. pombe* its physiological role within the plant still remains unclear. The physiological functions which have been proposed for plant monosaccharide transporters include the import of monosaccharides into symplastically isolated sink cells like pollen grains, embryos and guard cells or into cells with an increased carbohydrate demand due to wounding, pathogen infection or developmental processes as part of the carbon partitioning. Monosaccharide transporters may also be involved in the long distance transport of photoassimililates. Cell wall invertase can cleave sucrose into glucose and fructose at sites of apoplastic phloem unloading to generate the necessary sucrose gradient between sites of phloem loading and phloem unloading. Monosaccharide transporters import glucose and fructose from the apoplast into sink cells. A role for monosaccharide transporters as part of one sugar sensing system, which controls the regulation of the carbohydrate metabolism of the plant, has been proposed as well. It was the aim of this project to characterise the physiological role of the high affinity monosaccharide/proton symporter AtSTP1.

In order to obtain more information about the possible physiological role of AtSTP1 two strategies have been adopted to interfere with and therefore reveal the physiological function of AtSTP1. First a line with a T-DNA insertion into the AtSTP1 gene has been isolated by Dr. S. Sherson. This line does not express a functional AtSTP1 protein. The second strategy is based on the overexpression of the AtSPTP1 gene in *Arabidopsis* under the control of the CaMV 35S promoter.

4 Results

4.1 Expression analysis of *AtSTP1*

At the beginning of the project it was known that the *AtSTP1* gene is expressed in stems, roots, flowers, siliques and is most prominent in leaves. There has been no tissue found where *AtSTP1* is not expressed. A more detailed analysis revealed that the amount of *AtSTP1* mRNA is regulated by sugars. Wt seedlings were grown on ½ strength MS media without carbon source under constant light for 14 days. The seedlings were then placed into flasks containing water or increasing concentrations of glucose. After incubating for 3 hours in either light or darkness the seedlings were harvested and the total RNA extracted. Figure 3 shows a Northern blot experiment where the total RNA of the sugar treated seedlings was separated in an agarose gel, blotted onto a membrane and hybridised with an *AtSTP1* cDNA probe. Loading of equal amounts of total RNA into each lane was validated by ethidium bromide staining of the ribosomal RNA. Figure 3 shows that in the absence of light and glucose *AtSTP1* mRNA is most abundant. Treatment of the seedlings with 0.5 mM glucose in the dark leads to a decrease in *AtSTP1* mRNA compared to seedlings incubated in no glucose in the dark. When the seedlings were treated with 1 mM glucose in the dark a further decrease in *AtSTP1* mRNA is found. A further increase of the glucose concentration to 5 and 10 mM does not lead to a further decrease in AtSTP1 mRNA. When the seedlings were incubated in water in light, the amount of *AtSTP1* mRNA was similar to seedlings treated with 1 mM glucose in the dark and much lower than in seedlings incubated in water in the dark.

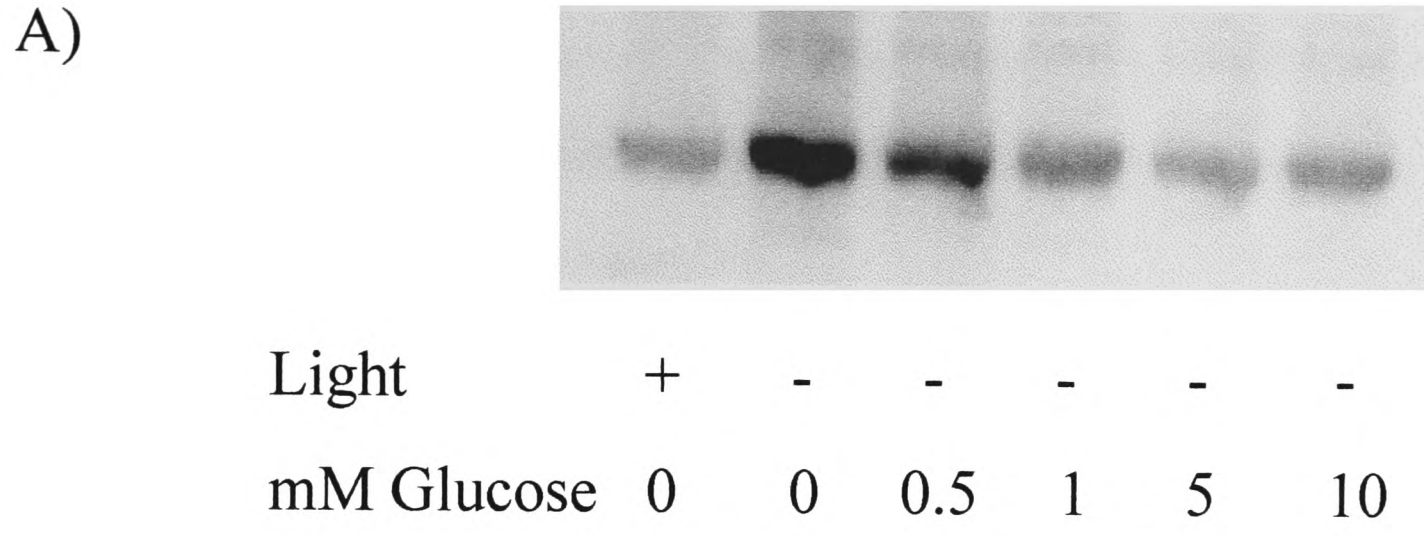


Figure 3: AtSTP1 mRNA abundance is regulated by D-glucose. RNA gel blot analysis of RNA isolated from 14 days old Arabidopsis seedlings grown on ½ MS medium. Prior to RNA isolation seedlings were incubated in the indicated concentration of D-glucose either in the light or the dark for 5 hours. A) Hybridisation with an *ATSTP1* specific probe. B) Ethidium bromide staining of the 18S rRNA.

In a similar experiment where detached leaves from 4 week-old-soil-grown plants were used as RNA source rather than seedlings, similar results were obtained (data not shown). Thus glucose regulates the *AtSTP1* mRNA levels. Concentrations of externally applied glucose of around 1 mM cause a decrease of *AtSTP1* mRNA amount. Higher glucose concentrations seem not to cause a further decrease in *AtSTP1* mRNA amount in these experiments.

In order to define the time frame in which glucose causes the decrease in *AtSTP1* mRNA as shown in Figure3 the following experiment was set up. Wt seedlings were grown on ½ strength MS medium without carbon source under constant light for 14 days. The seedlings were then placed into a flask containing 5mM glucose that was incubated in the dark. After 0, 1, 3 and 5 hours seedlings were harvested and the total RNA extracted. The autoradiograph of a Northern blot experiment using this RNA and *AtSTP1* DNA as a probe is shown is Figure 4. After one hour treatment with 5 mM glucose there is strong decrease in *AtSTP1* mRNA amount observed compared to levels at the beginning of the incubation (0 hours). After 3 hours the level of *AtSTP1* mRNA drops further compared to the level at 0 hours. There is only a small further decrease in the amount of *AtSTP1* mRNA after 5 hours incubation compared to the levels after 0, 1 and 3 hours (see Figure 4). Thus the decrease of *AtSTP1* mRNA amount caused by glucose is a relatively fast process which is most pronounced within 3 hours of the change in glucose levels.

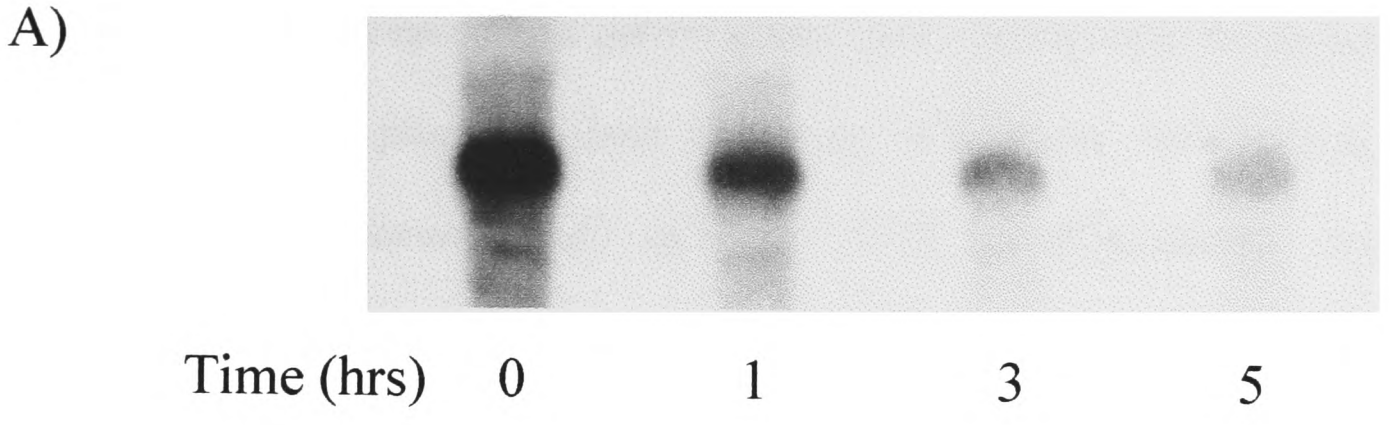


Figure 4: Time course of the regulation of the *AtSTP1* mRNA abundance by D-glucose. RNA gel blot analysis of RNA isolated from 14 day-old *Arabidopsis* seedlings grown on ½ strength MS medium under constant light. Prior to RNA isolation seedlings were incubated for 5 hours in the dark, then in 5 mM D-glucose in the dark for indicated time. A) Hybridisation with an *AtSTP1* specific probe. B) Ethidium bromide staining of the 18S rRNA.

To investigate whether other carbohydrates also have an effect on the *AtSTP1* mRNA levels the following experiment was performed. Wt seedlings were grown on ½ strength MS medium without carbon source under constant light for 10 days. The seedlings were then placed into flasks containing 5mM glucose, 5mM sucrose, 5mM fructose, 5mM mannose, 5mM galactose, 5mM 3-0-methylglucose, 5mM 2-deoxyglucose, 5mM mannitol or water. One sample of seedlings was harvested before the incubation (T0). All other samples were incubated in the different solutions for 5 hours in the dark. Subsequently the total RNA of all seedlings was extracted and used in a Northern blot experiment. Figure 5 shows the autoradiograph of the Northern blot experiment in which an *AtSTP1* DNA probe was used. In lane 1 we see the level of *AtSTP1* mRNA before incubation in solution in the dark (T0). A 5 hour incubation in water in the dark leads to an increase in the level of *AtSTP1* mRNA (Figure 5 lanes 1 and 2). The incubation in 5mM glucose, sucrose, fructose, mannose, and galactose leads to a decreased level of *AtSTP1* mRNA compared to levels before the incubation (Figure 5 lanes 1, 3-7). The response to the treatment with glucose, sucrose, fructose, mannose and galactose with regard to the level of *AtSTP1* mRNA is similar. Incubation in 3-0-methylglucose leads to a small increase in *AtSTP1* mRNA abundance (lanes1 +8). Whereas the treatment 2-deoxyglucose leads to a decrease in *AtSTP1* mRNA levels compared to the untreated sample, similar to the effect with glucose (lanes1, 2 + 9) Lane 10 shows the effect of mannitol which was used as an osmoticum. There is no difference in the *AtSTP1* mRNA level when the seedlings are treated with water or the osmoticum mannitol. *ATSTP1* mRNA level responds rapidly to a low concentration of metabolisable

sugars. Whether all these sugars trigger this response directly or only after being converted into another sugar is not clear from these experiments. The possibility that the osmotic effect of the sugars is causing the changes in *AtSTP1* mRNA levels can be ruled out since mannitol and polyethylen glycol (data not shown) fail to trigger that response.

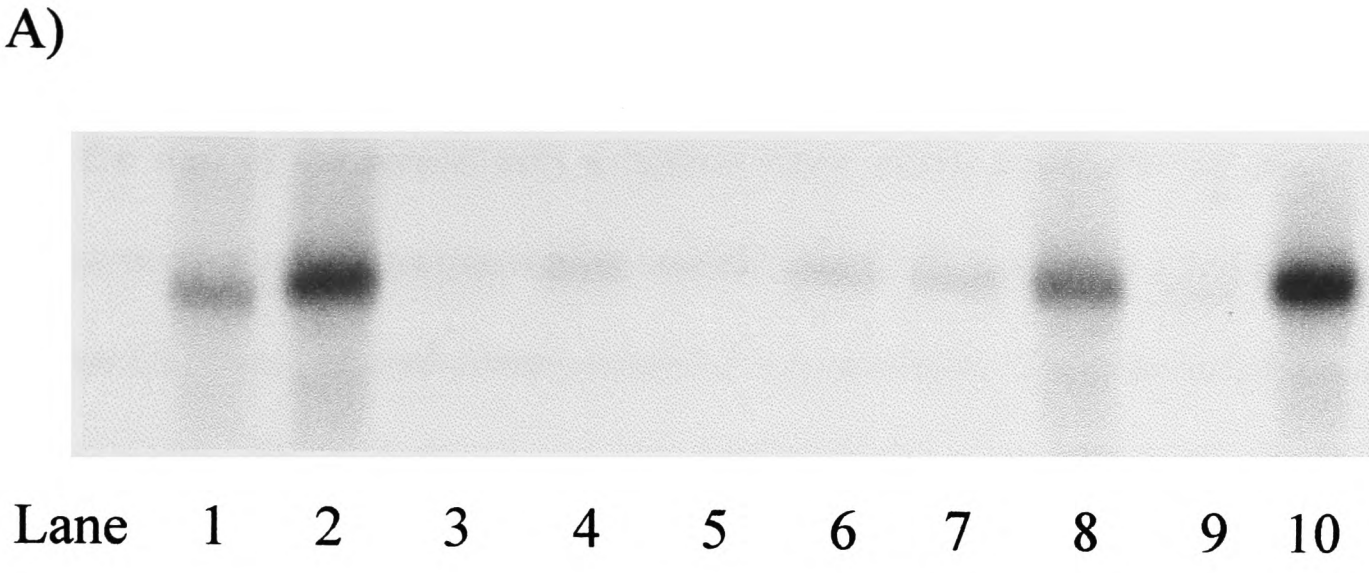


Figure 5: Regulation of the AtSTP1 mRNA abundance by carbohydrates. RNA gel blot analysis of RNA isolated from 10 days old Arabidopsis seedlings grown on ½ MS medium under constant light. Prior to RNA isolation seedlings were incubated for 5 hours in the dark in 5 mM D-glucose(lane3), 5 mM sucrose (lane4), 5 mM D-fructose (lane5), 5 mM D-galactose (lane6), 5 mM D-mannose (lane7), 5 mM 3-O-methyl-glucose (lane8), 5 mM 2-deoxy-glucose (lane9), ,5 mM manitol (lane 10).In lane 2 RNA from seedlings incubated in water was loaded and lane 1 contains RNA from seedlings before they were incubated in carbohydrates the dark. A) Hybridisation with an *ATSTP1* specific probe. B) Ethidium bromide staining of the 18S rRNA.

4.2 Isolation of a genomic *AtSTP1* clone and construction of the binary vector pGenSTP1

At the start of the project only a lambda clone which represents the whole coding sequence but not a functional promotor of the *AtSTP1* gene, and a full length cDNA clone (N.Sauer personal communication) were available. The position of the *AtSTP1* gene within the *Arabidopsis* genome was unknown. To isolate a complete *AtSTP1* gene which could be used to complement the AtSTP1 knock out plants the IGF BAC library (Monzo et al.,1998) was screened with a partial *AtSTP1* cDNA probe. Since almost all BAC clones of this library are physically mapped the identification of BAC clones containing the *AtSTP1* gene from this library had a very high chance to reveal the position of the *AtSTP1* gene within the *Arabidopsis* genome. The map position of *AtSTP1* could then be compared with the map positions of sugar signalling mutants to identify if one of those mutants is a AtSTP1 mutant.

Hybridisation of the IGF-BAC library with the partial *AtSTP1* cDNA identified 12 BAC clones (data not shown). Ten of these clones (F9I6, F17H19, F16J7, F11N4, F8G18, F21P12, F15E3, F4G18, F6C15 and F12I10) showed strong hybridisation and two clones (F9D19 and F9D15) showed a much weaker hybridisation with *AtSTP1* cDNA probe. The two clones with the weak hybridisation are overlapping clones, which map on chromosome IV contig3. They represent the part of the genome in which the gene for *AtSTP12* maps. *AtSTP12* shows the highest sequence similarity to *AtSTP1* of all so far identified *AtSTPs* in the *Arabidopsis* genome. Therefore it is quite likely that these two clones have been identified due to crosshybridisation

between the genomic sequence of *AtSTP12* and the partial *AtSTP1* cDNA. These two clones have not been further analysed. The ten clones, which gave a strong hybridisation signal, are overlapping clones. They map on chromosome I contig 1 between the marker mi443 and NCC1. This part of the genome had not been sequenced at the time the experiment was performed. To verify that these clones contain the gene for *AtSTP1* and to isolate a fragment which contains only the *AtSTP1* gene the BAC clone F4G18 was chosen for further analysis. The DNA of Clone F4G18 was subjected to Southern blot analysis. Digestion of the DNA of clone F4G18 with the restriction enzyme SpeI and hybridisation with a partial *AtSTP1* cDNA probe revealed a single hybridising band of approximately 10 kb (data not shown). This fragment was subcloned into the vector pBluescript II SK-. The subcloned fragment was analysed by restriction digest. The information gained from those experiments is shown in Figure 6.

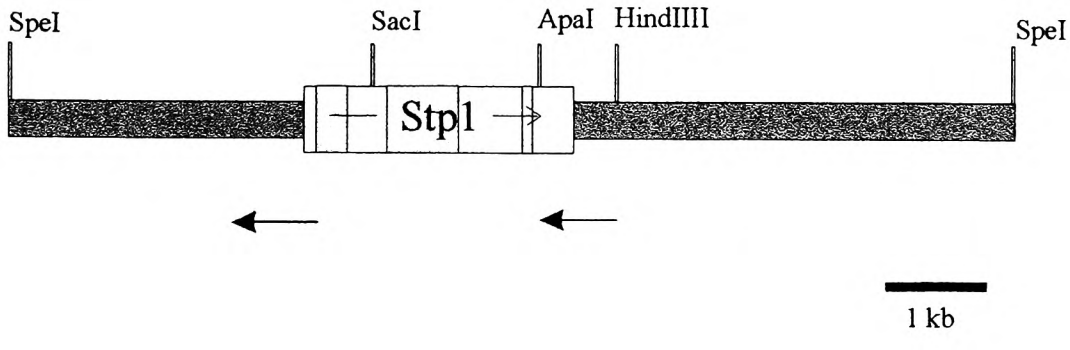


Figure 6: Schematic illustration of the genomic organisation of the AtSTP1 gene. The open rectangles represent the coding region of the gene and the light shaded rectangles illustrate the exon/intron structure of the gene. The dark shaded rectangles represent the 3′ and 5′ region of the gene which have been subcloned from the BAC F4G18. The arrows under the illustration indicate the part of the DNA which has been sequenced to confirm the identity of the subclone.

Based on the results of the restriction map of the subclone the SpeI / HindIII fragment which contains the *AtSTP1* coding region was subcloned into the SpeI and HindIII sites of the binary vector pGreen 0129 (Hellens et al.,2000) to give the vector pGenSTP1 as illustrated in Figure 7. To further confirm the identity of the clones the plasmid pGenSTP1 was partially sequenced. Using the primers STP1out and Universal, the parts of the clone as indicated in Figure 6 have been sequenced. This sequence is 100% identical to the published sequence of *AtSTP1* (data not shown).

On the 17th of August 1999 the complete sequence of the BAC T28P6 was published (Accession Number AC007259) as part of the *Arabidopsis* genome sequencing project. This BAC contains the complete SpeI fragment subcloned from F4G18. Therefore the complete sequencing of the so far unknown promotor region of the *AtSTP1* gene became obsolete. The SpeI / HindIII fragment of the vector pGenSTP1 contains 2936 bp upstream of the translation start site, the whole AtSTP1 coding region and 411 bp downstream of the stop codon. There are no other genes predicted to be in the subcloned SpeI / Hind III fragment. The predicted neighbouring genes of *AtSTP1* encode a syntaxin like protein which ends 7754 bp upstream of the start codon of the *AtSTP1* gene and a hypothetical protein with similarities to reverse transcriptases which ends 1002 bp downstream of the stop codon of the *AtSTP1* gene.

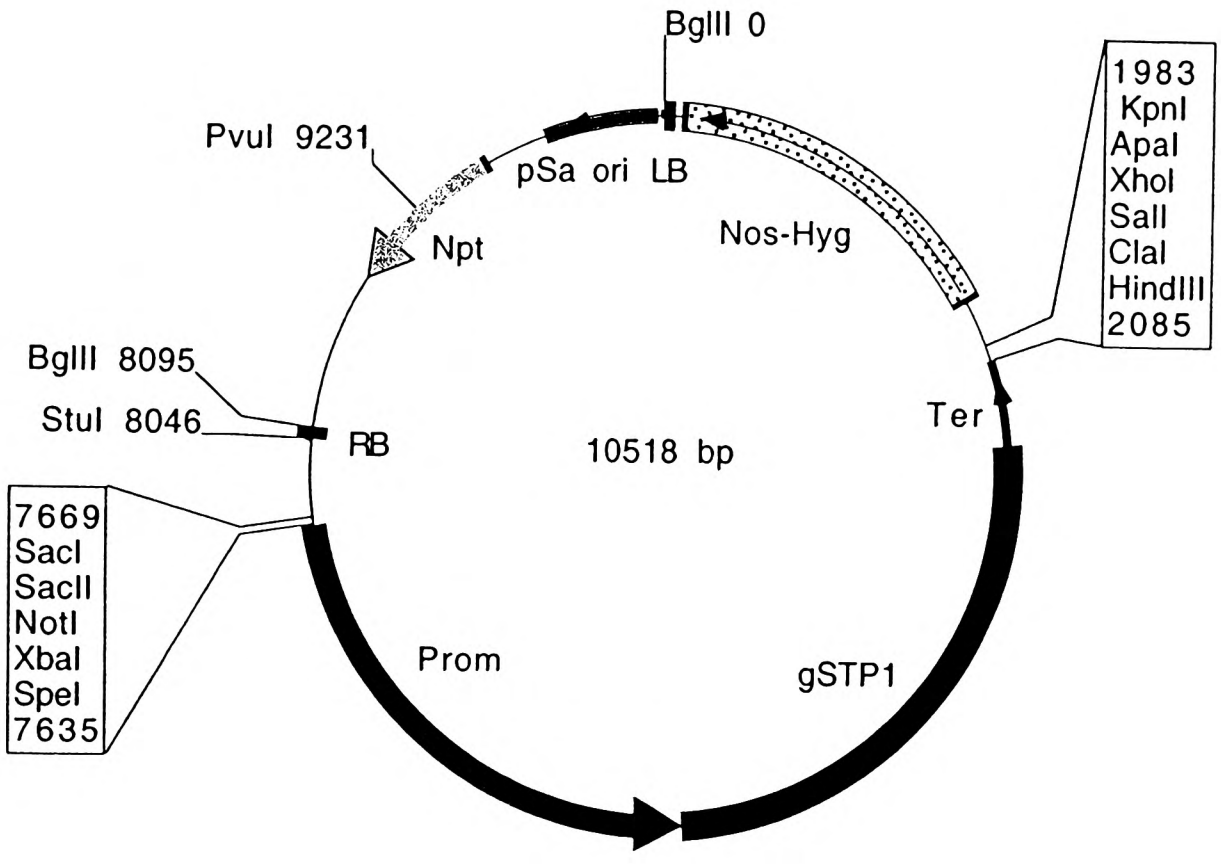


Figure 7: Schematic illustration of the binary vector pGenSTP1. The SpeI/HindIII fragment containing 2936 bp of the 5′ untranslated region, the complete coding region and 411 bp of the 3′ untranslated region of the AtSTP1 gene was subcloned into the SpeI/HindIII restiction sites of the vector pGreen0129. Selected restriction sites are indicated. Abbreviations used: Prom, 5′untranslated region; gStp1, coding region of the AtSTP1 gene; ter, 3′ untranslated region; Nos-Hyg, nopaline synthase promotor fused to the hygromycin gene; LB and RB, left and right T-DNA border sequences; Npt, NptII gene.

4.3 Construction of the binary vectors p35S-STP1bar and p35S-STP1hyg

In order to complement the AtSTP1 knock out line and to overexpress the AtSTP1 protein in wild type plants the binary vectors p35S-STP1bar and p35S-STP1hyg which contain the *AtSTP1* cDNA fused to the CaMV 35S Promoter and *AtSTP1* 3' untranslated region were constructed. Starting point was the vector pTF12108 (N. Sauer unpublished results) which contains the CaMV 35S promotor fused to the AtSTP1 cDNA fused to the 3' untranslated region of AtSTP1 gene. The complete fusion construct was excised by a partial Hind III/EcoRI restriction digest and cloned into the HindIII and EcoRI restriction sites of the binary vector pGreen 0229. This vector has been named p35S-STP1bar and is illustrated in Figure 8. In order to transform the AtSTP1 knock out line which already contains selectable marker genes NPTII and the gene for glufosinate resistance (BAR) the CaMV 35S::AtSTP1 fusion construct had to be cloned into a binary vector with a suitable marker gene. This was achieved by excising the XhoI / EcoRI fragment from the vector p35S-STP1bar and cloning it into the restriction sites XhoI and EcoRI of the binary vector pGreen 0129 which confers resistance to hygromycin. This vector has been named p35S-STP1hyg and is illustrated in Figure 9.

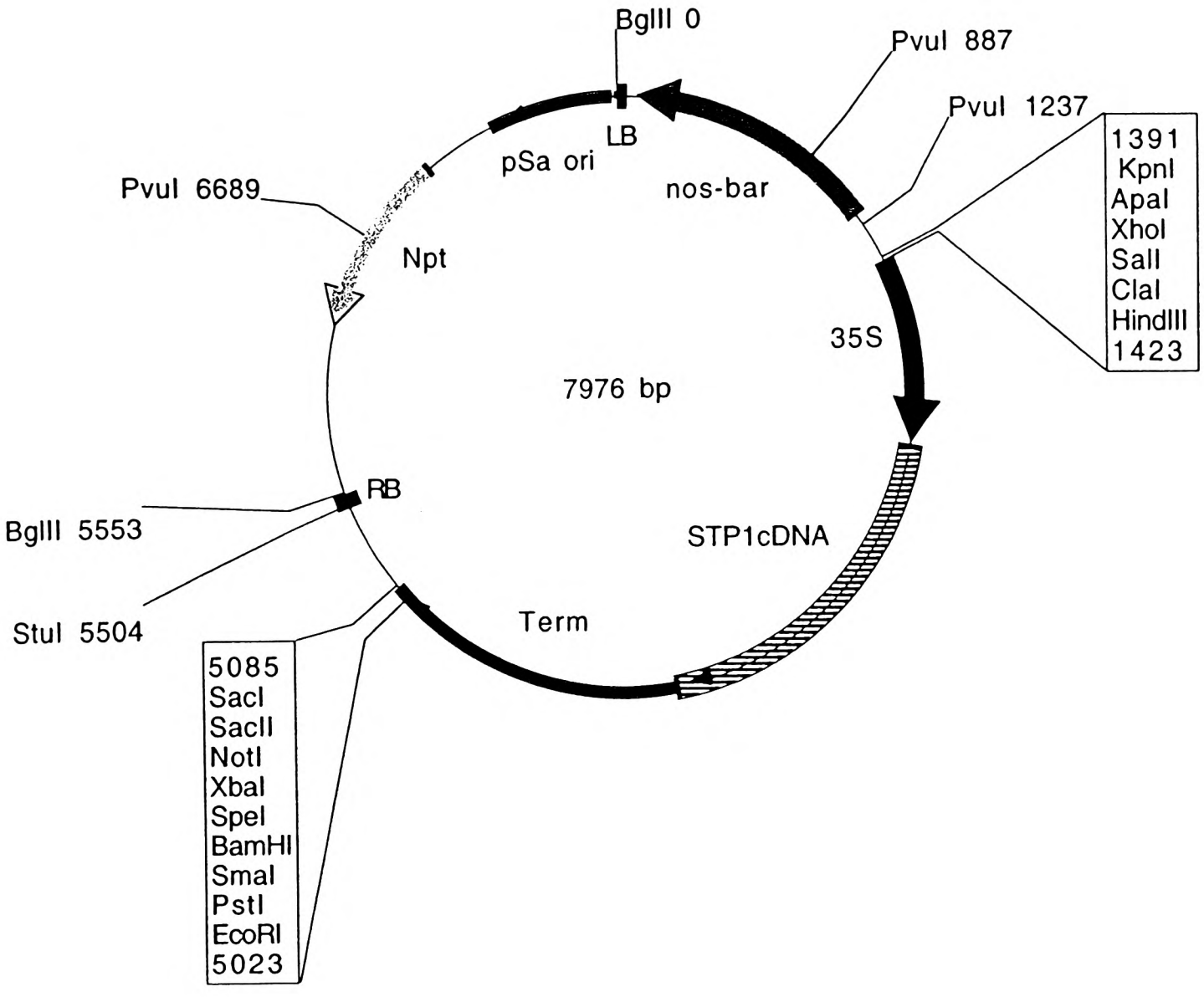


Figure 8: Schematic illustration of the binary vector p35S-STP1bar. The HindIII/EcoRI fragment containing 35SCaMV promotor fused to the AtSTP1 cDNA fused to the 3′ untranslated region of the AtSTP1 gene was excised from the vector pTF12108 (N.Sauer unpublished result) and subcloned into the HindIII/EcoRI restiction sites of the vector pGreen0229. Selected restriction sites are indicated. Abbreviations used: 35S, 35SCaMV promotor; Term, 3′ untranslated region; nos-bar, nopaline synthase promotor fused to the gene for glufosinate resistance (BAR); LB and RB, left and right T-DNA border sequences; Npt, NptII gene.

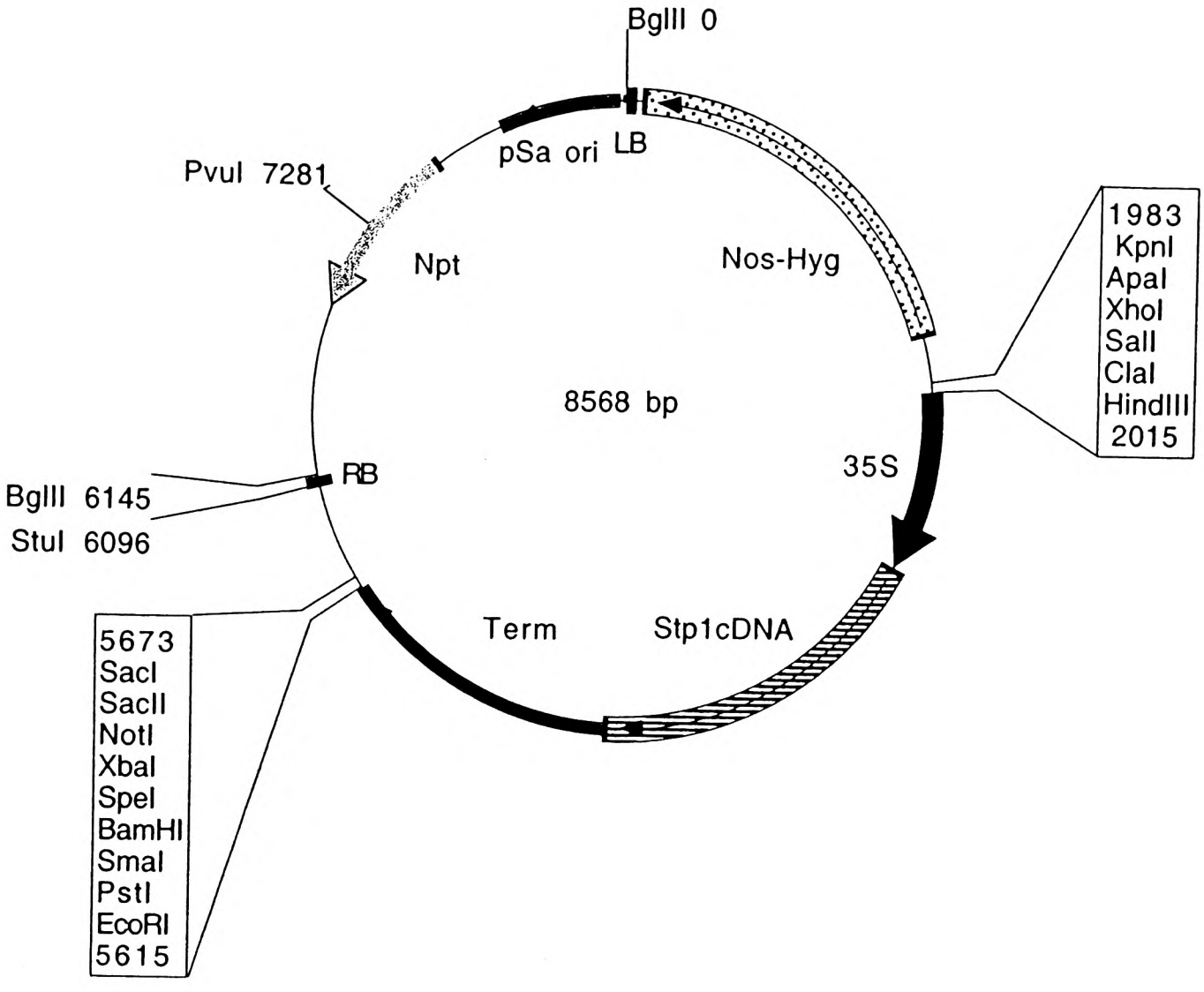


Figure 9: Schematic illustration of the binary vector p35S-STP1hyg. The XhoI/EcoRI fragment containing 35SCaMV promotor fused to the *AtSTP1* cDNA fused to the 3′ untranslated region of the AtSTP1 gene was excised from the vector p35S-STP1bar and subcloned into the XhoI/EcoRI restiction sites of the vector pGreen0129. Selected restriction sites are indicated. Abbreviations used: 35S, 35SCaMV promotor; Term, 3′ untranslated region; nos-bar, nopaline synthase promotor fused to the gene for hygromycin resistance; LB and RB, left and right T-DNA border sequences; Npt, NptII gene.

4.4 Transgenic plants

The binary vectors p35S-STP1bar, p35S-STP1hyg and pGenSTP1 (see previous sections) were used to transform *Arabidopsis* wild type and STP1 knock out plants. For the further analysis of the transgenic plants with respect to phenotypic effects and hexose transport characteristics it was important to have plants which were homozygous for the integrated construct. Therefore only plants with a single integration of the T-DNA were used for further analysis. To achieve this the primary transformants (T0) were allowed to self-pollinate to give the T1 generation of seeds. The T1 generation was then analysed for the presence of the cotransformed dominant marker gene. Only transformants, which showed a 3:1 ratio for the presence of the marker gene were allowed to self-pollinate to give seeds of the T2 generation. All transgenic plants which did not show a 3:1 segregation for the marker gene in the T1 generation were discarded at this stage. Individual plants of the T1 generation which produced seeds which showed 100% resistance to the selection for the marker gene used were considered to be homozygous for a single intergration of the T-DNA into their genome. To confirm this the T2 generation of these plants was backcrossed with wild type plants. When the progeny of this backcross showed a 1:1 segregation pattern for the presence of the marker gene, the T2 generation was considered to homozygous for a single integration of the T-DNA into the genome. These plants were then used for further experiments. To ensure that all individual lines are independent transformants all T0 transformants were isolated from

individual plants exposed to the *Agrobacterium* suspension (for details on the transformation process see material and methods).

Table 3 summarises the analysis of the transformation of wild type plants with the CaMV 35S Promotor-*AtSTP1* cDNA construct with regard to number of integrations of the construct for three lines. All three lines have P values >0,1 in the χ^2 Tests of the T1 generation and the backcross of the T2 to wild type plants. Therefore it has been concluded that the T2 generation of all three lines are homozygous for a single integration of the T-DNA. The T2 generations of these lines and progeny of them have been used in the further studies.

Line	Generation / Cross	Herbicide Selection		χ^2 Test
		Resistant	Sensitive	P
A	T1	298	110	0,36
	T2 X wt	131	0	1,00
B	T1	281	97	0,77
	T2 X wt	148	0	1,00
C	T1	314	123	0,13
	T2 X wt	97	0	1,00

Table 3: Characterisation of wild type plants transformed with the vector p35S-STP1bar with regard to the segregation of the glufosinate resistance (BAR) marker gene. The T1 generation and the backcross with wild type plants of the T2 generation of three independent transformants were grown on ½ stength MS medium for 1 week and then sprayed with a 150 mg /l basta solution. Resistance to the herbicide was scored after 6 days. For the T1 generation a 3:1 segregation was expected for a single locus integration. For the T2 generation backcrossed to wild type plants a 1:1 segregation was expected for a T2 generation which is homozygous for a single locus integration. P values for the χ^2 test based on the expected segregation ratios were calculated.

Table 4 summarises the analysis of the transformation of AtSTP1 knock out line *Atstp1-1* with the CaMV 35S Promotor *AtSTP1* cDNA construct with regard to number of integrations of the construct for two lines. Both lines have P values >0,1 in the χ^2 Tests of the T1 generation and the backcross of the T2 to wild type plants. Therefore it has been concluded that the T2 generation both lines are homozygous for a single integration of the T-DNA. The T2 generations of these two lines have been used in the further studies.

Line	Generation / Cross	Antibiotic Selection		χ^2 Test
		Resistant	Sensitive	P
A	T1	67	28	0,31
	T2 X wt	112	0	1,00
B	T1	96	32	1,00
	T2 X wt	132	0	1,00

Table 4: Characterisation of AtSTP1 knock out line *AtSTP1-1* transformed with the vector p35S-STP1hyg with regard to the segregation of the hygromycin resistance marker gene. The T1 generation and the backcross with wild type plants of the T2 generation of two independent transformants were germinated on ½ MS medium containing hygromycin B (40 $\mu g ml^{-1}$). Resistance to the antibiotic was scored after 10 days. For the T1 generation a 3:1 segregation was expected for a single locus integration. For the T2 generation backcrossed to wild type plants a 1:1 segregation was expected for a T2 generation which is homozygous for a single locus integration. P values for the χ^2 test based on the expected segregation ratios were calculated.

The transformation of the AtSTP1 knock out line *stp1-1* with the vector pGenSTP1, which includes the genomic sequence of *AtSTP1*, was carried out at a later stage.

Since at this point in time it became clear that the 35-S Promoter *AtSTP1* cDNA construct did complement the *Atstp1-1* phenotypes less attention was paid to the analysis of this construct in the *Atstp1-1* background. Table 5 shows that two independent lines have been isolated.

Line	Generation / Cross	Antibiotic Selection		χ^2 Test P
		Resistant	Sensitive	
A	T1	86	31	0,71
B	T1	96	27	0,43

Table 5: Characterisation of AtSTP1 knock out line *stp1-1* transformed with the vector pGenSTP1 with regard to the segregation of the hygromycin resistance marker gene. The T1 generation of two independent transformants were germinated on ½ strength MS medium containing hygromycin B (40 $\mu g ml^{-1}$). Resistance to the antibiotic was scored after 10 days. For the T1 generation a 3:1 segregation was expected for a single locus integration. The P values for the χ^2 test based on the expected segregation ratio were calculated.

4.5 Uptake of glucose into wild type, AtSTP1 knock out, and AtSTP1 overexpressing seedlings at low substrate concentration

Uptake of glucose into 7 days old seedlings was determined by incubation of the seedlings in liquid ½ strength MS medium containing radioactive D-glucose for 30 min. After washing the seedling sugars from the seedlings were extracted with 80%

ethanol. The amount of radioactivity in the extract was quantified by liquid scintillation counting.

As illustrated in Figure 10 the uptake of glucose at low glucose concentrations into seedlings of the AtSTP1 knock out line *stp1-1* is decreased by 45% compared to uptake into wild type seedlings. Overexpression of the AtSTP1 gene under control of the CaMV 35S promoter in wild type seedlings leads to an increase of glucose uptake in these seedlings. The increased rate of glucose uptake in the different transgenic lines varies. Line A shows, with a 4 fold increase compared to wild type uptake the biggest increase. Whereas line C with the smallest increase in the rate of glucose uptake accumulates still more than twice as much radioactivity than wild type. Since Line A shows the biggest increase in glucose uptake this line has been used in the subsequent analysis.

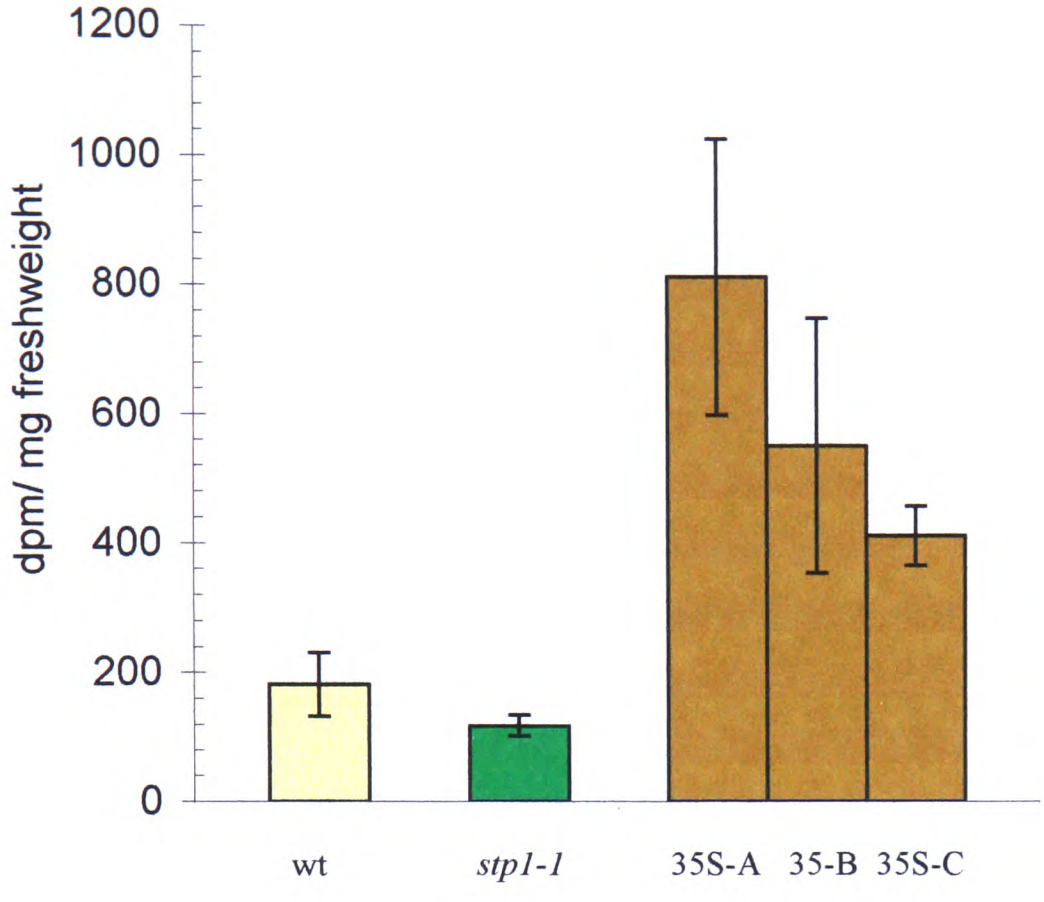


Figure 10: Uptake of [^{14}C]-D- glucose into wild type, AtSTP1 knock out, and AtSTP1 overexpressing seedlings. Seven-day old wild type (□ wt), *Atstp1-1* (■*stp1-1*) seedlings and three lines of wild type seedlings transformed with the 35S::STP1 cDNA construct (■35S A,B,C) were each incubated in 156 µM D-glucose containing 0.1 µCi [^{14} C]-D-glucose for 30 min, then washed and the uptake of radioactivity determined. Results shown are the mean of four replicates with standard deviations.

4.6 Comparison of the AtSTP1 mRNA levels in wild type plants and transgenic plants with the 35S::*ATSTP1* construct

As demonstrated in the previous sections the level of *AtSTP1* mRNA is regulated by exogenous applied sugars. It is not clear whether this regulation occurs on a transcriptional level, on the level of mRNA stability or by a combination of both. In order to demonstrate that 35S::STP1 plants have an increased *AtSTP1* mRNA level at mM glucose concentration compared to wild type plants the following experiment was performed: Plants were grown in the presence of 5 mM D-glucose and RNA subsequently isolated from those plants. Figure 11 shows the autoradiograms of a Northern blot experiment in which total RNA of seedling from wild type plants (lane 1) and seedlings expressing the 35S:AtSTP1 construct in wild type background (lane2) was separated and hybridised with an *AtSTP1* specific probe. For this experiment RNA from seedlings of the transgenic line A, which shows the highest glucose uptake under low substrate concentration of the transgenic lines (See figure 10) was used. After 6 hours of exposure (Figure 11 A) *AtSTP1* mRNA is only detected in the 35S::AtSTP1 line. Exposure for 6 days reveals that there is *AtSTP1* mRNA present in the wild type seedlings (Figure 11 B). The *AtSTP1* mRNA level in the 35S::AtSTP1 seedlings is much higher than in wild type seedlings under these conditions. However from these results no valid conclusions about the mode of regulation of the mRNA level can be drawn.

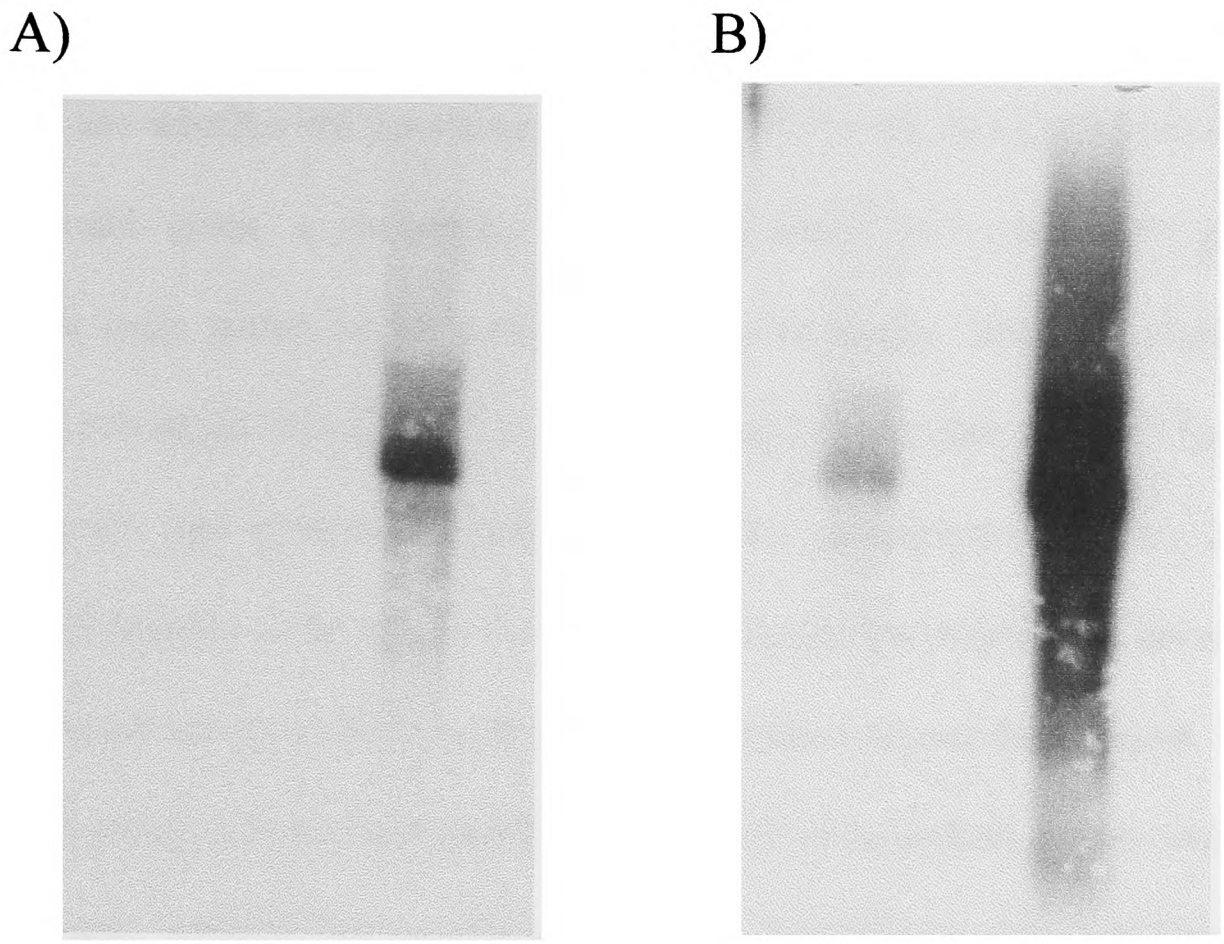


Figure 11: Comparison of AtSTP1 mRNA abundance in wild type seedlings and seedlings overexpressing AtSTP1 after growth on 5 mM D-glucose. RNA gel blot analysis of RNA isolated from 14 day-old Arabidopsis seedlings grown on ½ strength MS medium containing 5 mM D-glucose. Lane 1 was loaded with total RNA from wild type seedlings and lane 2 with total RNA from seedlings overexpressing AtSTP1 in wild type background (Line A see figure 10). A) Hybridisation with an *AtSTP1* specific probe and exposure of the blot for 6 hours. B) 6 days exposure of the blot used in A. C) Ethidium bromide staining of the 18S rRNA.

4.7 AtSTP1 knock out plants and plants overexpressing AtSTP1 display no phenotypic difference compared to wild type plants when grown on soil

In order to see whether the knocking-out of AtSTP1 or the ectopic expression of AtSTP1 would cause a phenotypic effect, the plants with the three genetic backgrounds were grown on soil under identical conditions. Since the change of expression level of *AtSTP1* did lead to a clear change of in hexose transport function in these plants (see previous section and Wallace unpublished results) it was hypothesised that this could lead to a change in carbohydrate distribution throughout the plants which could result in a phenotypic difference among these plants. It has been demonstrated that nutrient supply and removal of reproductive sink tissue do affect seed size, -viability and -germination rate in *Arabidopsis* (Sills and Nienhuis, 1995). Therefore a detailed analysis of the seed was performed. Figure 12 shows a photograph of a wild type plant, an AtSTP1 knock out plant and a plant overexpressing AtSTP1 under control of the 35S promotor in wild type background, grown on soil in a 16 h light / 8 h dark cycle. Comparison of 40 plants of each genetic background under these conditions revealed that there is no difference in flowering time and number of rosette leaves among these plants (data not shown).

Figure 12:(next page) Photograph of a wild type (wt), AtSTP1 knock out (*stp1-1*) and an AtSTP1 overexpressing plant (35S::STP1) which have been grown on soil at 22° C in a 16 hours light /8 hours dark cycle.

WT

stp1-1

35S::STP1

Table 6 summarises the data of the comparison of seed weight and number of seeds per silique. No statistically significant difference with regard to seed weight and number of seeds in siliques from the main stem could be found when wild type plants where compared to AtSTP1 knock out plants or AtSTP1 overexpressing plants.

	Wild type		stp1-1			35S::AtSTP1		
Seed weight / 300 seeds	4,90	+/- 0.07	4,88	+/- 0,1	p 0,83	4,84	+/- 0,08	p 0,82
Seeds per silique	56.3	+/- 1.9	56.4	+/- 1.8	p 0,94	56,7	+/- 2,0	p 0,72

Table 6: Comparison of seed weight and number of seeds per silique of wild type plants, AtSTP1 knock out plants and plants overexpressing AtSTP1. 40 plants of each genetic background where grown in a 16 hours light 8 hours dark cycle at 22° C. The mean (n=10) seed weight of batches of 300 seeds in mg and the standard deviation are given. For the number of seeds per silique siliques were collected at random from the middle of the main stem from individual plants. The mean (n=15) and the standard deviation are given. P -values were calculated using a T-Test by comparing the results for AtSTP1 knock out plants (stp1-1) to wild type and the results for the AtSTP1 overexpressing line (35S::STP1)(line A2) to wild type.

The germination rate and frequency of seed germination from the three genetic backgrounds was analysed by germinating the seed on ½ strength MS medium without added carbohydrates. As shown in figure 13 no difference in germination rate and frequency could be found for the seeds from the three genetic backgrounds.

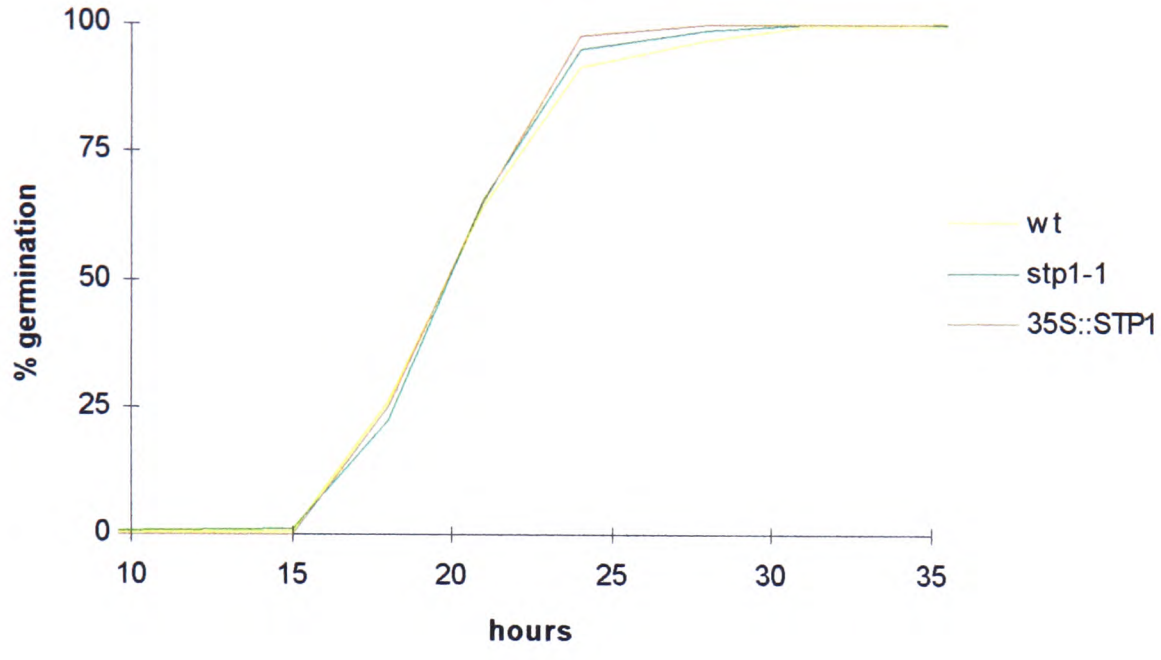


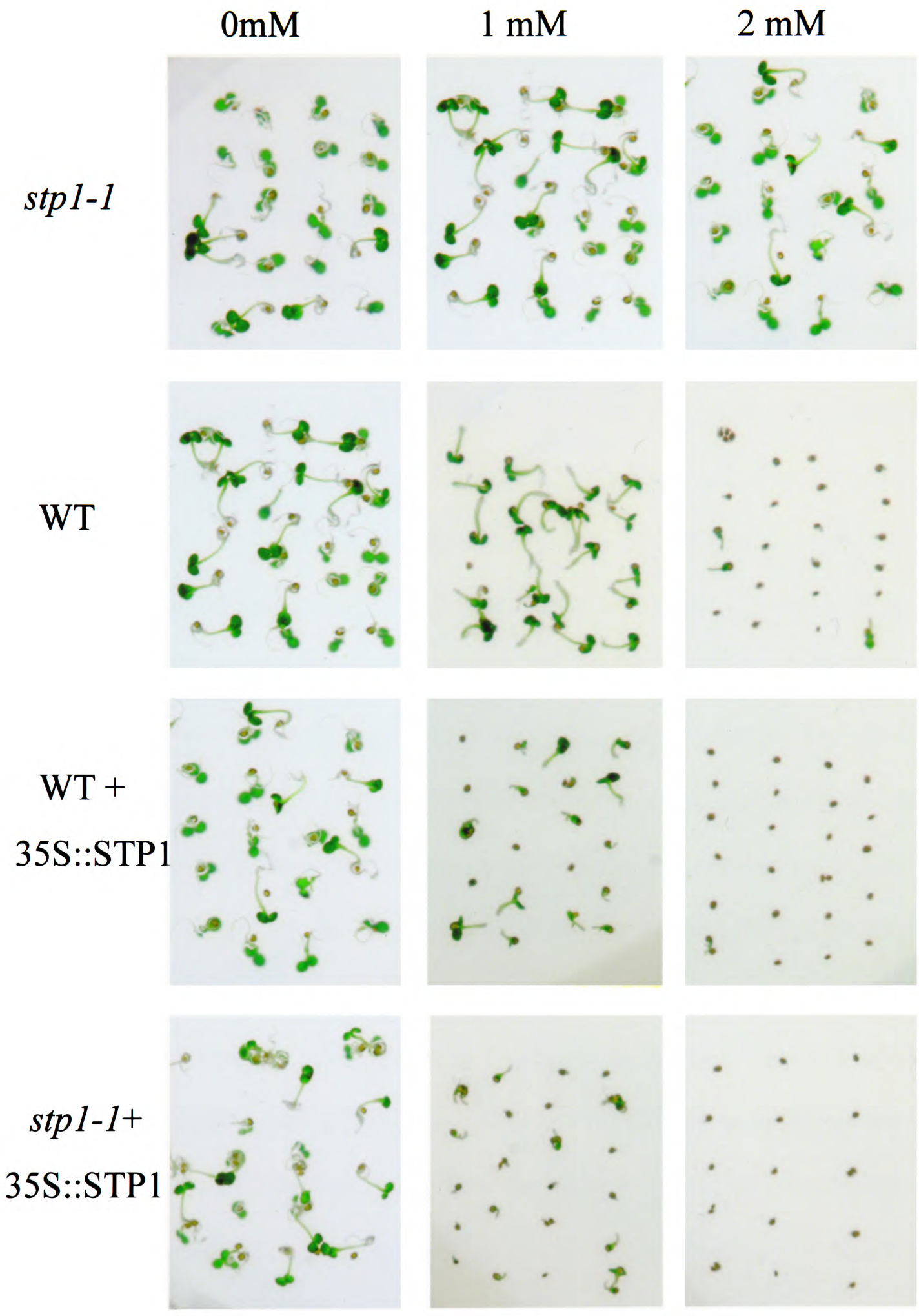
Figure 13: Germination rate of wild type, AtSTP1 knock out and AtSTP1 overexpressing seed. Seed were vernalised for 48 hours at 4° C in the dark on ½ strength MS medium before the experiment. Seed were germinated at 22° C under constant light. Germination was defined as emergence of the radicel from the seed coat. 255 wild type seed (yellow line), 240 AtSTP1 knock out seed (green line) and 258 AtSTP1 overexpressing seed (brown line) were scored after 0, 15, 18, 21, 24, 28, 31, 34 and 40 hours.

4.8 Effect of D-mannose on the germination of seed from wild type plants, ATSTP1 knock out plants and plants overexpressing AtSTP1

Low concentrations of exogenous applied D-mannose inhibits the germination of *Arabidopsis* seeds (Pego et al.,1999). This observation has been used to characterise and isolate potential sugar sensing *Arabidopsis* mutants. Since D-mannose is a substrate for AtSTP1 it was hypothesised that AtSTP1 could be involved in the manifestation of this phenotypic effect. In order to test if AtSTP1 plays a role in the inhibition of germination by D-mannose, seeds from wild type plants, AtSTP1 knock out plants and seeds from plants overexpressing AtSTP1 in the two genetic backgrounds were tested for their ability to germinate in the presence of various concentrations of D-mannose. Figure 14 shows photographs of seedlings from a germination experiment where seeds have been incubated for one week in the presence of 0, 1 and 2 mM D-mannose. On medium without D-mannose, seeds from all four different genetic backgrounds do germinate at a frequency of almost 100%. Wild type seeds do germinate in the presence of 1 mM D-mannose at a slightly lower frequency than in the absence of D-mannose, whereas 2mM D-mannose causes a large decrease in the germination frequency of the wild type seeds. The AtSTP1 knock out seeds are hyposensitive to the effect on germination caused by D-mannose. The germination frequency of AtSTP1 knock out seeds is hardly affected by 1 and 2mM D-mannose. On 7.5 mM mannose these seeds still display a germination frequency of 35%, when germination is defined as the emergence of a radicle from the seed coat which is at least 1mm long. (data not shown).

Overexpression of AtSTP1 in wild type plants leads to a hypersensitivity of their seed to D-mannose with respect to germination. These seeds display a much lower germination frequency on 1 and 2mM D-mannose compared to wild type seeds. Overexpression of AtSTP1 in the AtSTP1 knock out line reverts the hyposensitivity of the seeds to D-mannose with respect to germination into hypersensitivity of the seeds. These seeds show a germination frequency, which is similar to the germination frequency of the seeds from wild type plants overexpressing AtSTP1.

Figure 14: (next page) Effect of D-mannose on the germination of seed. Seeds from wild type plants, *Atstp1-1* plants, wild type plants overexpressing AtSTP1 and seeds from *Atstp1-1* plants overexpressing AtSTP1 were vernalised for 48 hours at 4° C in the dark. Then the seeds were incubated for 1 week at 22°C and constant light on ½ strength MS medium with the indicated concentration of D-mannose, and photographed.

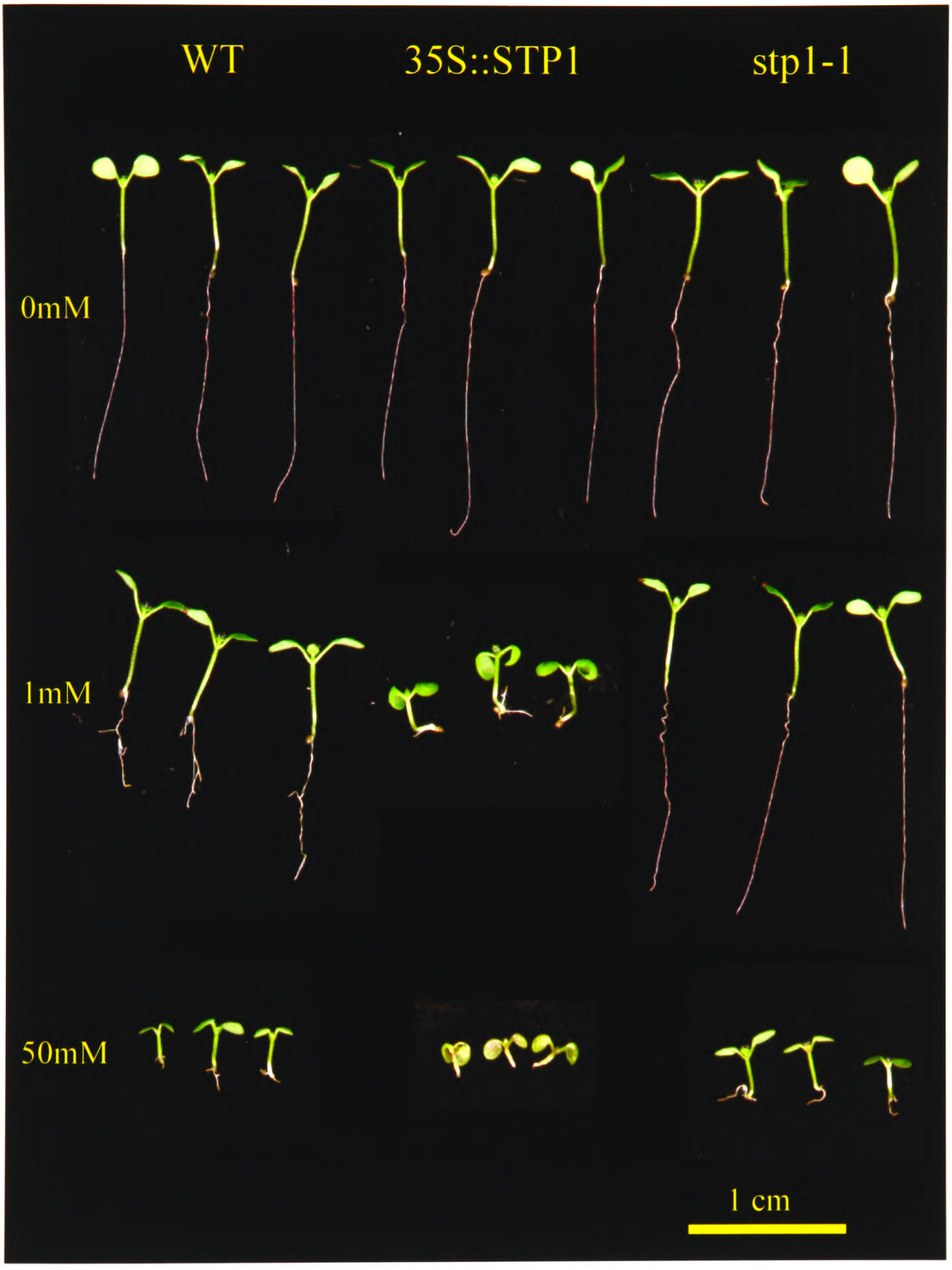

0mM
1 mM
2 mM
stp1-1
WT
WT +
35S::STP1
stp1-1+
35S::STP1

4.9Effect of D-galactose on the growth of wild type, ATSTP1 knock out and plants overexpressing AtSTP1

D-galactose is highly toxic to many plants including *Arabidopsis* when supplied exogenously in concentrations as low as 1 mM. Since D-galactose is a substrate for AtSTP1 it was investigated whether different expression levels of AtSTP1 in the different genetic backgrounds would influence the level of toxicity to exogenously supplied D-galactose. Figure 15 shows the toxic effect of D-galactose on *Arabidopsis* seedlings. Seeds from wild type plants, AtSTP1 knock out plants and seeds from wild type plants overexpressing AtSTP1 were germinated, grown for one week on ½ strength MS medium containing either no, 1 or 50 mM D-galactose. In the absence of D-galactose the seedlings with the three genetic backgrounds with different expression levels of AtSTP1 display the same phenotype. The seedlings have long main roots. There is no induction of lateral roots visible. The shoots start to initiate true leaves and the cotyledons are expanded. Wild type seedlings which are grown on 1mM D-galactose show a decreased length of the main root, and formation of lateral roots. The shoots of wild type plants display no phenotypic effect on 1 mM D-galactose. Application of 50 mM D-galactose to wild type seedlings leads to a drastic decrease in main root length and the size of the shoot is decreased. The cotyledons are thicker compared to wild type seedlings grown in the absence of D-galactose. Seedlings of AtSTP1 knock out plants are hyposensitive to the effects caused by growth on D-galactose. There is no phenotypic difference between seedlings grown in the absence or in the presence of 1 mM D-galactose. When grown on 50 mM D-

galactose AtSTP1 knock out seedlings display a phenotype similar to wild type seedlings under these conditions. Overexpression of AtSTP1 in wild type plants makes their seedlings hypersensitive to the toxic effects of D-galactose. In the presence of 1 mM D-galactose the seedlings show a phenotype similar to that of wild seedlings on 50 mM D-galactose. In addition to the short roots and the thick cotyledons the hypocotyl is bent. On 50 mM D-galactose there is no root detectable without optical aid. The accumulation of anthocyanins in the cotyledons becomes apparent.

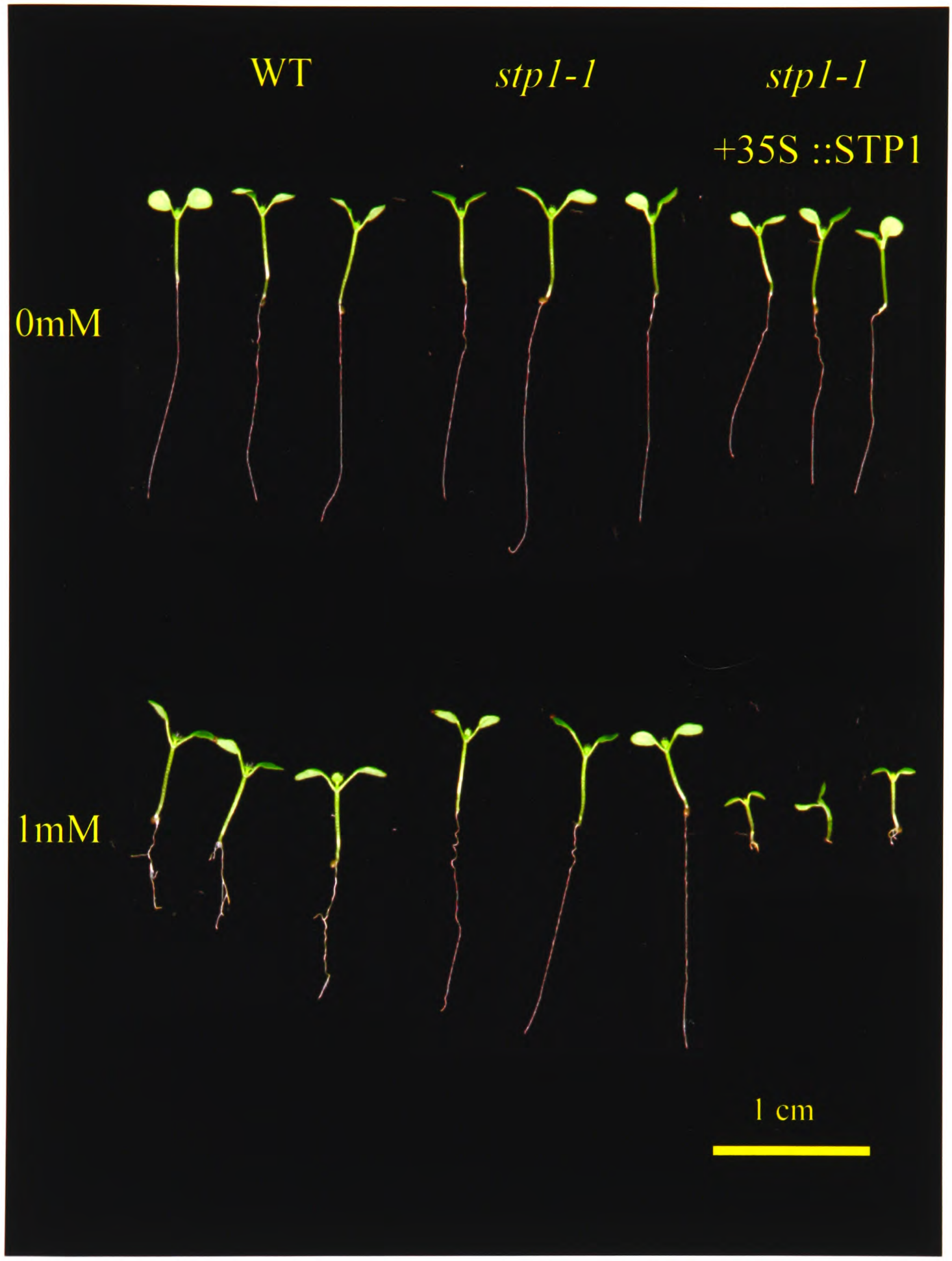
Figure 15:(next page) Effect of D-galactose on the growth of wild type seedlings, ATSTP1 knock out seedlings and wild type seedlings overexpressing AtSTP1. Seeds of wild type plants (WT), seeds of wild type plants overexpressing AtSTP1(35S::STP1) and seeds of AtSTP1 knock out plants (*stp1-1*) were germinated and grown for one week on ½ strength MS medium with the indicated concentration of D-galactose. Three seedlings for each genotype and concentration were photographed.


WT
35S::STP1
stp1-1
0mM
1mM
50mM
1 cm

4.10 Hyposensitivity of AtSTP1 knock out seedlings to the effect D-galactose is reversed by overexpression of AtSTP1

Since the AtSTP1 knock out line contains a second unidentified T-DNA integration in the genome (Sherson unpublished results) it is not clear whether the integration into the AtSTP1 gene or the second unidentified T-DNA integration is responsible for phenotypic effect caused by the exogenous application of D-galactose. Therefore AtSTP1 was expressed in the knock out line in order to see if the phenotypic effects could be reversed. Figure 16 shows that the overexpression of AtSTP1 in the AtSTP1 knock out line leads to hypersensitivity of the seedlings to the effect of exogenous application of 1 mM D-galactose. These seedlings display a similar phenotype to the wild type seedlings overexpressing AtSTP1 (see Figure15).

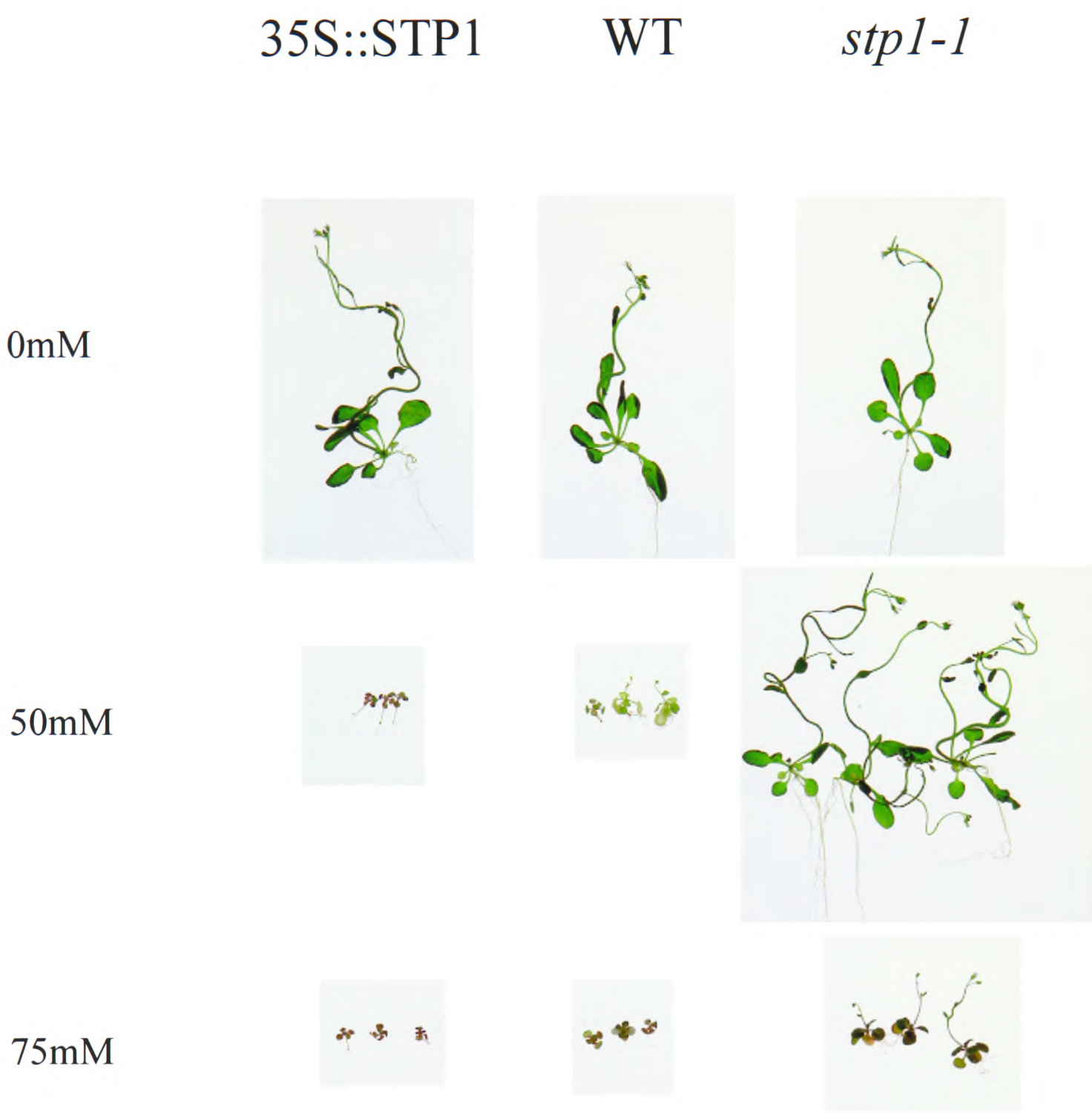
Figure 16: (next page) Effect of D-galactose on the growth of wild type seedlings, ATSTP1 knock out seedlings and ATSTP1 knock out seedlings overexpressing AtSTP1. Seeds of wild type plants, AtSTP1 knock out plants and AtSTP1 knock out plants overexpressing AtSTP1 were germinated and grown for one week on ½ strength MS medium with no D-galactose or 1 mM D-galactose added to the medium as indicated. Three seedlings for each genotype and each concentration were photographed.


WT
stp1-1
stp1-1
+35S ::STP1
0mM
1mM
1 cm

4 .11 Three weeks old AtSTP1 knock out seedlings are insensitive to up to 50 mM D-galactose

Seeds were germinated and grown on ½ strength MS medium to the stage where the seedlings have four true leaves (approx. 3 weeks). Then the seedlings were transferred onto ½ strength MS medium containing increasing concentrations of D-galactose. As illustrated in Figure 17 the growth of AtSTP1 knock out seedlings is unaffected by 50 mM D-galactose since these seedlings display the same phenotype as control seedlings which were grown in the absence of D-galactose. When the external D-galactose concentration is increased above 50 mM AtSTP1 knock out seedlings display the phenotypical effects of arrest in development and accumulation of anthocyanins caused by external application of D-galactose. Wild type seedlings and AtSTP1 overexpressing seedlings display those phenotypic effects already on 50 mM D-galactose. In the AtSTP1 overexpressing seedlings this phenotype is more pronounced than in wild type seedlings.

Figure 17: (next page) Effect of D-galactose on three week old wild type, ATSTP1 knock out and AtSTP1 overexpressing seedlings. Seedlings were grown for 3 weeks on ½ strength MS medium under constant light (four leaf stage) and then transferred onto ½ MS medium supplemented with the indicated concentration of D-galactose. The seedlings were grown on this medium until the control plants (0 mM D-galactose) started to flower. For each D-galactose concentration three plants for each genetic background are shown. For the control (0 mM D-galactose) only one plant per genetic background is shown. All plants are reproduced at the same scale.


35S::STP1
WT
stp1-1
0mM
50mM
75mM
100mM
200mM

4.12 Effect of D-glucose on the growth and anthocyanin accumulation of wild type, AtSTP1 knock out and AtSTP1 overexpression plants

Exogenously applied D-glucose can promote the growth of *Arabidopsis* seedlings and induces at high concentrations anthocyanin synthesis (Vitrac et al.,2000). To test if the different expression levels of AtSTP1 in wild type, AtSTP1 knock out and AtSTP1 overexpressing plants have an effect on growth and anthocyanin levels, seedlings were grown on different concentrations of D-glucose. Figure 18 shows that the accumulation of dry weight in seedlings is D-glucose dependent. Wild type and AtSTP1 seedlings show a similar response to the D-glucose concentrations. On 50 mM D-glucose, seedlings overexpressing AtSTP1 have after two weeks more than 2,5 times higher dry weight compared to wild type and AtSTP1 knock out seedlings. On 200 mM D-glucose this difference is less pronounced. In the absence of D-glucose the seedlings of all three genetic backgrounds have a similar dry weight.

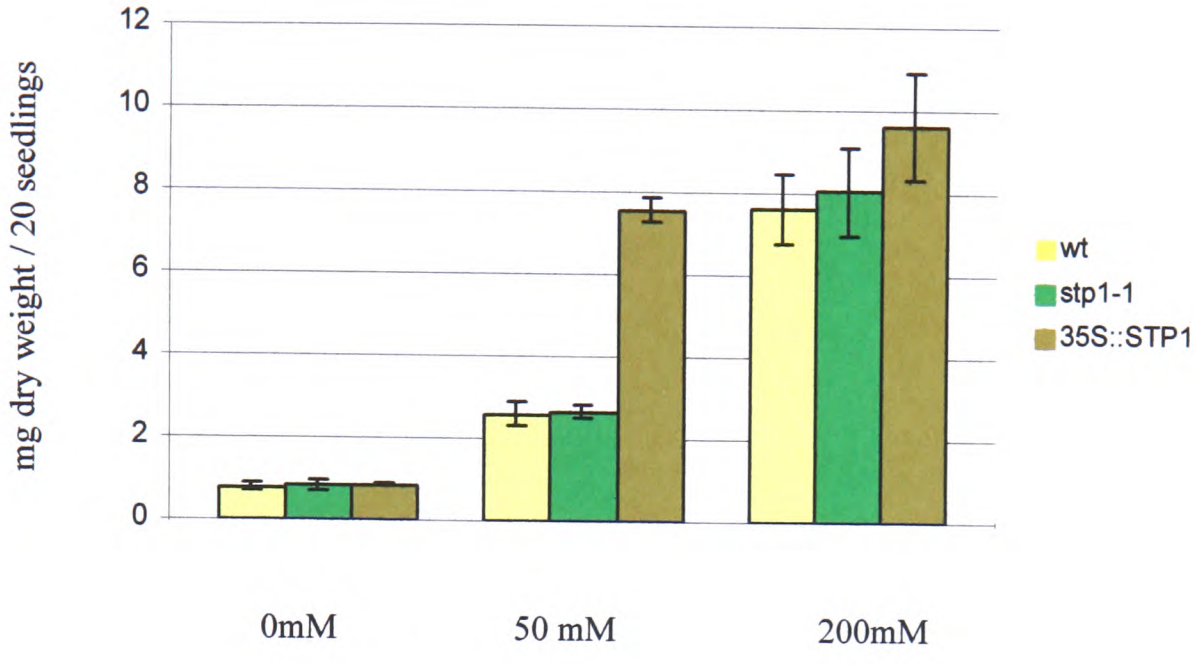
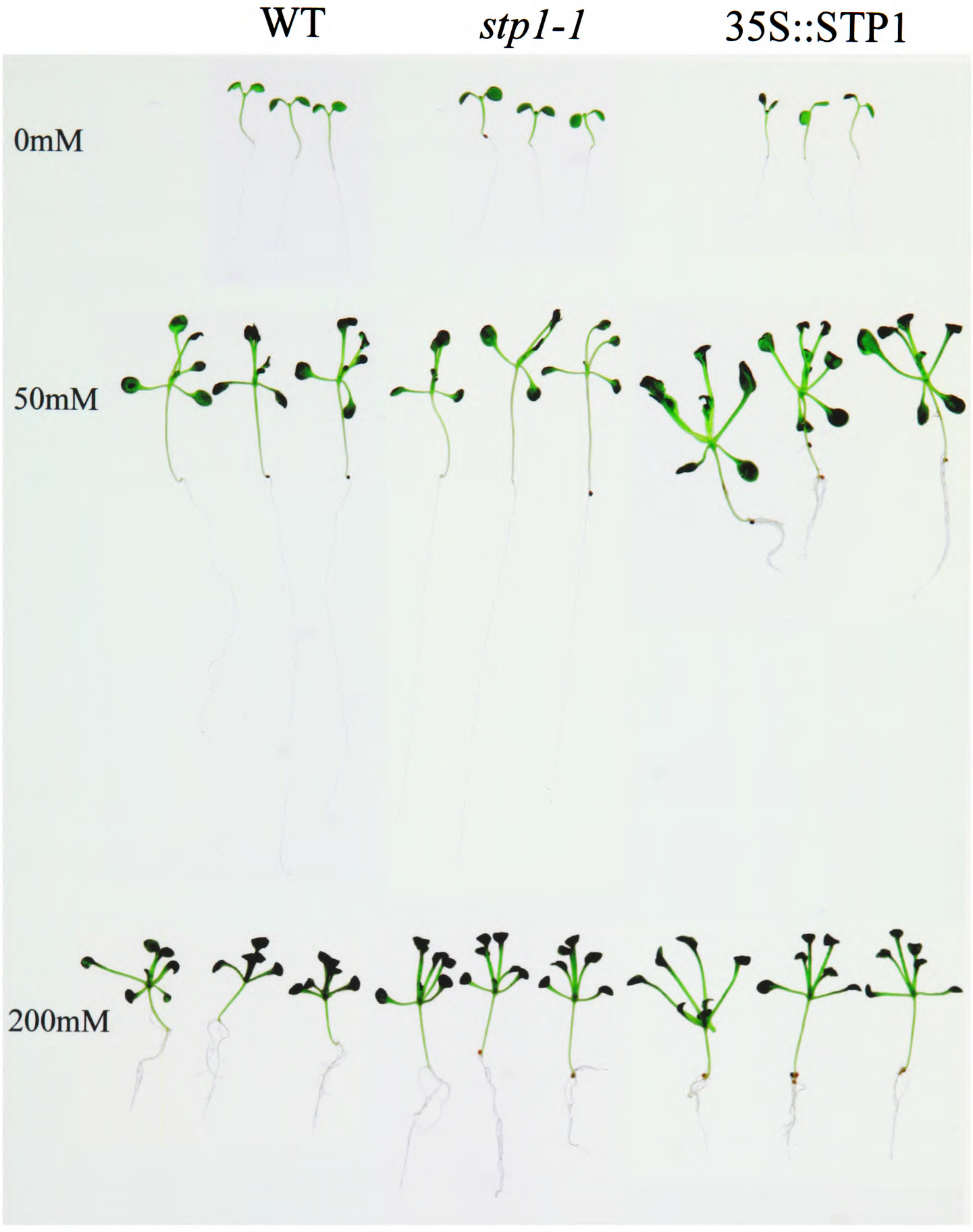


Figure 18: Accumulation of dry weight on different D-glucose concentrations in wild type (wt) AtSTP1 knock out (*stp1-1*) and AtSTP1 overexpressing (35::STP1) seedlings. Seedlings were germinated and grown for tow weeks on ½ MS medium with the indicated D-glucose concentration. Each value represents the mean of three independent experiments. Error bars indicate standard deviations.

Figure 19 Shows photographs of seedlings germinated and grown for two weeks either in the absence or on 50mM and 200mM D-glucose. As evident from figure 18 D-glucose promotes the growth of the seedlings. On 50 mM D-glucose seedlings overexpressing AtSTP1 have more leaves and have formed lateral roots in comparison to wild type and AtSTP1 knock-out seedlings, which have under these condition only a long main root and less leaves. On 200 mM D-glucose all three genetic backgrounds have lateral roots.

Figure 19. (next page) Phenotypic effects caused by different concentrations of D-glucose on wild type (WT), AtSTP1 knock out (*stp1-1*) and AtSTP1 overexpressing (35S::STP1)seedlings. Seedlings were germinated and grown for two week on ½ srength MS medium supplemented with the indicated D-glucose concentration.


WT
stp1-1
35S::STP1
0mM
50mM
200mM

As shown in figure 20 anthocyanin levels increase when seedlings are transferred to D-glucose. Wild type and AtSTP1 knock out seedling have a similar response to different D-glucose concentrations with regard to anthocyanin levels. Seedlings overexpressing AtSTP1 accumulate more anthocyanins in response to glucose over the tested range on concentrations, than wild type and AtSTP1 knock out seedlings. In the absence of exogenous D-glucose the anthocyanin levels in all three genetic backgrounds are similar.

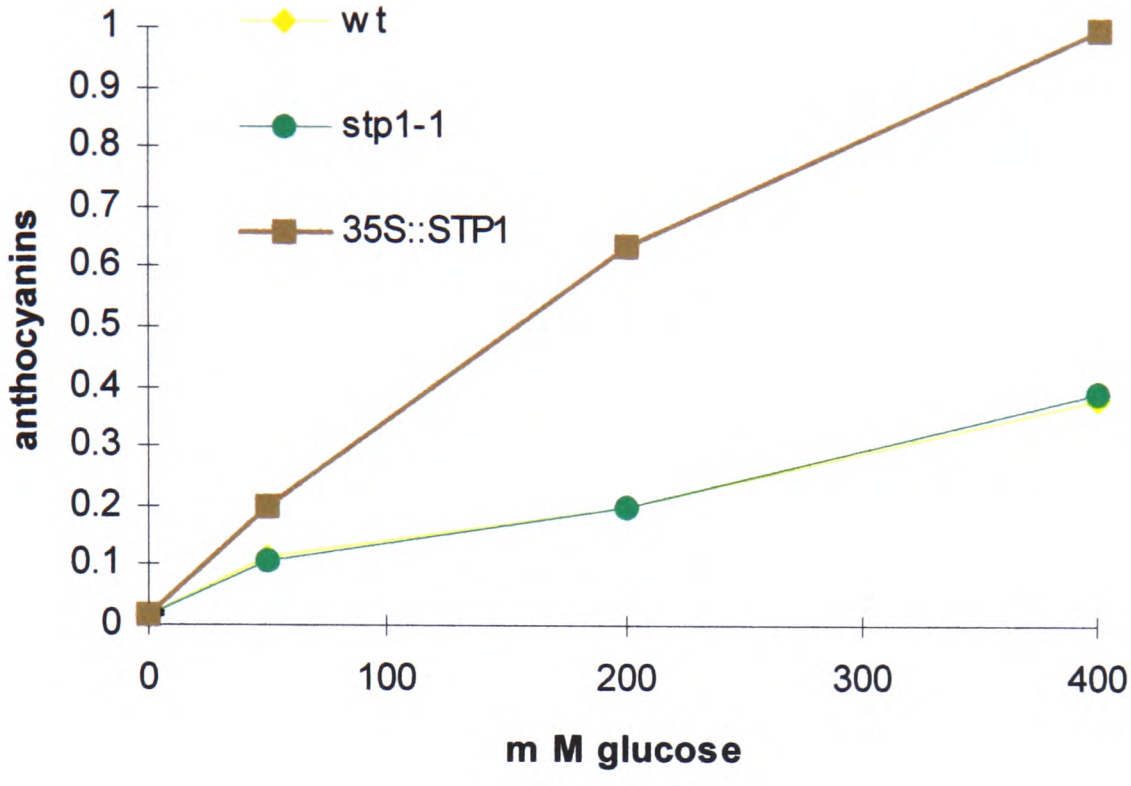


Figure 20: Anthocyanin levels in wild type (wt), AtSTP1 knock out (stp1-1) and AtSTP1 overexpressing plants (35S::STP1) after growth on different D-glucose concentrations. Seedlings were grown for 3 weeks on ½ MS medium without carbon source and then transfered onto ½ MS medium containing 0, 50, 200, or 400 mM D-glucose. After one week seedlings were harvested and anthocyanin content determined. Each data point represents the mean of three independent measurements. Anthocyanin content is expressed as relative anthocyanin content per mg fresh weight in relation to the anthocyanin content in AtSTP1 overexpressing seedlings grown on 400 mM D-glucose. Standard deviation were less than 10% for each data point.

Figure 21 shows seedling that have been grown on 200 mM D-Glucose and chlorophyll has been removed to reveal the presence of anthocyanins. In wild type and AtSTP1 knock out plants anthocyanins accumulate in the petioles, whereas in the leaves there is little anthocyanin. In AtSTP1 overexpressing seedlings high amounts of anthocyanins accumulate in the petioles and leaves.

Figure 21: (next page) Localisation of anthocyanin accumulation in wild type (WT), AtSTP1 knock out and AtSTP1 overexpressing seedlings (35S::STPP1). Seedlings were grown for three weeks on ½ strength MS medium without carbohydrates. The seedlings were then transferred for one week onto medium containing 200 mM D-glucose. Chlorophyll was removed before the seedlings were photographed.

35S::STP1

WT

stp1-1

4.13 Analysis of galactose and glucose concentrations in seedlings grown on 50 mM galactose

The fact that AtSTP1 knock out seedlings are resistant to the toxic effects of 50 mM galactose whereas wild type seedlings and seedlings overexpressing AtSTP1 are clearly sensitive to this galactose concentration (see 4.11) is quite surprising. The observation that at high glucose concentrations (50mM and 200mM) knock out and wild type seedlings show no difference in dry matter and anthocyanin accumulation (see 4.12) suggests that the contribution to glucose uptake by AtSTP1 is minimal under such high substrate concentrations. This is in agreement with the down regulation of *AtSTP1* mRNA levels by sugars (see 4.1). Therefore is seems to be not that trivial to explain the differences observed in wild type and knock out seedlings in resistance to the toxic effects 50 mM galactose simply by a difference in galactose uptake. To address this question the accumulation of galactose and glucose in seedlings grown on 50 mM galactose was analysed. Seedlings were germinated and grown for 2 weeks on ½ strength MS medium and then transferred onto ½ strength MS medium supplemented with 50 mM galactose. The seedlings were exposed up to 72 hours to the galactose. After exposure to galactose the sugars were extracted form the seedlings and quantified by HPLC. Unfortunately this kind of experiment does not give direct information on galactose uptake since galactose is metabolised by the seedlings, but the amount of galactose and glucose in the seedlings are related to galactose uptake. Uptake assays with radioactive sugars which would avoid these problems are very difficult to perform at high substrate concentration due to limitations of specific activity of the radioactive sugar and amount of the plant

material (G. Wallace and S. Smith personal communication). Therefore it was not attempted to set up a radioactive uptake assay for high substrate concentrations. Figure 22 shows the changes of galactose and glucose concentrations in wild type seedlings (a), in AtSTP1 knock out seedlings (b) and in AtSTP1 overexpressing seedlings (c) over time. In all three different genetic backgrounds the amount of galactose increases with incubation time on galactose. At time point 0 no galactose was detected in the seedlings (data not shown). In wild type and AtSTP1 knock out seedlings the galactose concentration peaks at 48 hours after transfer onto galactose and levels then off. In contrast thereto the galactose concentration in AtSTP1 overexpressing seedlings peaks much earlier at around 16 hours and levels off from there. As shown in figure 23 a) the relative amount of galactose in the three different genetic backgrounds is different. In AtSTP1 knock out seedlings the galactose concentration is lower than in wild type seedlings and much lower than in AtSTP1 overexpressing seedlings. After 72 hours incubation on galactose AtSTP1 knock-out seedlings accumulate with 0.61 µmol galactose per gram fresh weight 3.7 times less galactose than wild type seedlings and 5.8 less galactose than AtSTP1 overexpressing seedlings. Apart from galactose, the concentration of glucose, sucrose and fructose did increase in the seedlings after incubation on galactose. The accumulation of glucose was most prominent and occurred earlier than the accumulation of sucrose and fructose (data no shown). The accumulation of these sugars is likely to be a result of the metabolism of galactose, which is the sole externally supplied carbon source. As shown in figure 22 the amount of glucose in AtSTP1 overexpressing seedlings increases constantly over time. In contrast hereto the ATSTP1 knock out seedlings show strong increase in glucose concentration after

16 hours of exposure to galactose. From 16 hours to 36 hours after exposure to galactose the glucose concentration in these seedlings drops to show a strong increase thereafter until the final time point of 72 hours. The changes in the glucose concentration in wild type seedlings is similar to those observed for AtSTP1 knock-out seedlings, although the early peak of glucose accumulation is less pronounced and delayed. As shown in figure 23 b) after 72 hours of exposure to galactose all three genetic backgrounds have s similar concentration of glucose. The glucose concentration after 72 hours is much higher than the galactose concentration in all three genetic backgrounds (figure 22 a) b) c)). Since this data was collected in a single experiment a repetition of this experiment is necessary to confirm the observed trends in changes in glucose and galactose levels.

Figure 22: (next page) Changes of the galactose and glucose **concentration over time in two week-old seedlings of different genetic backgrounds grown on 50 mM galactose.** Seeds of wild type plants (a wt), AtSTP1 knock out plants (b *stp1-1*) and of AtSP1 overexpressing plants (c 35::STP1)were germinated and grown for two weeks on ½ strength MS medium without carbohydrates. At time point 0 the seedlings were transferred onto ½ strength MS medium containing 50 mM galactose. Seedlings were harvested at the indicated time , washed and the sugars extracted and quantified by HPLC. Each time point represents the mean of three samples (30 mg fresh weight each). Error bars indicate the standard deviation. Were no error bars are shown the standard deviation was smaller than the symbol of the data point.

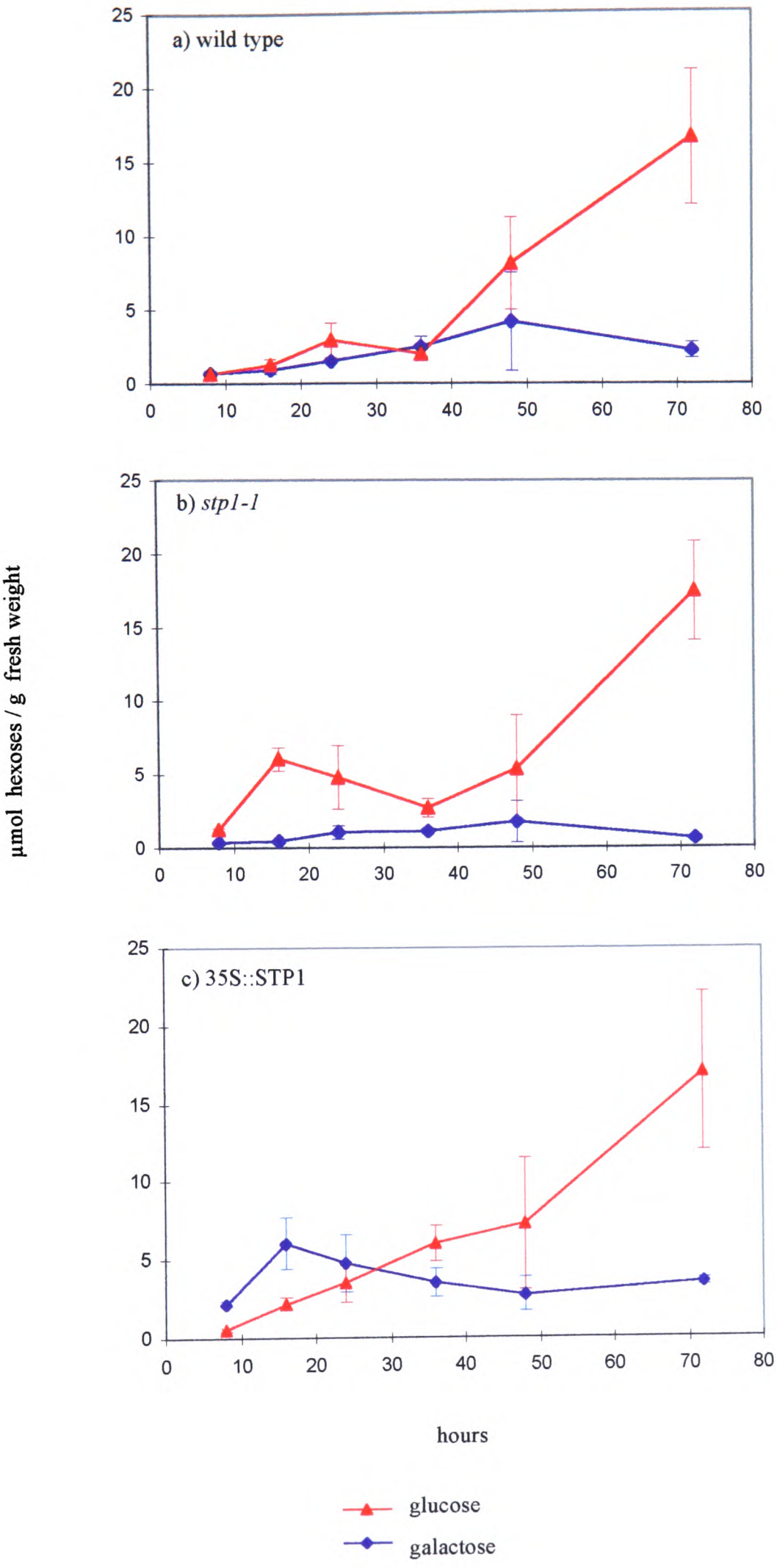
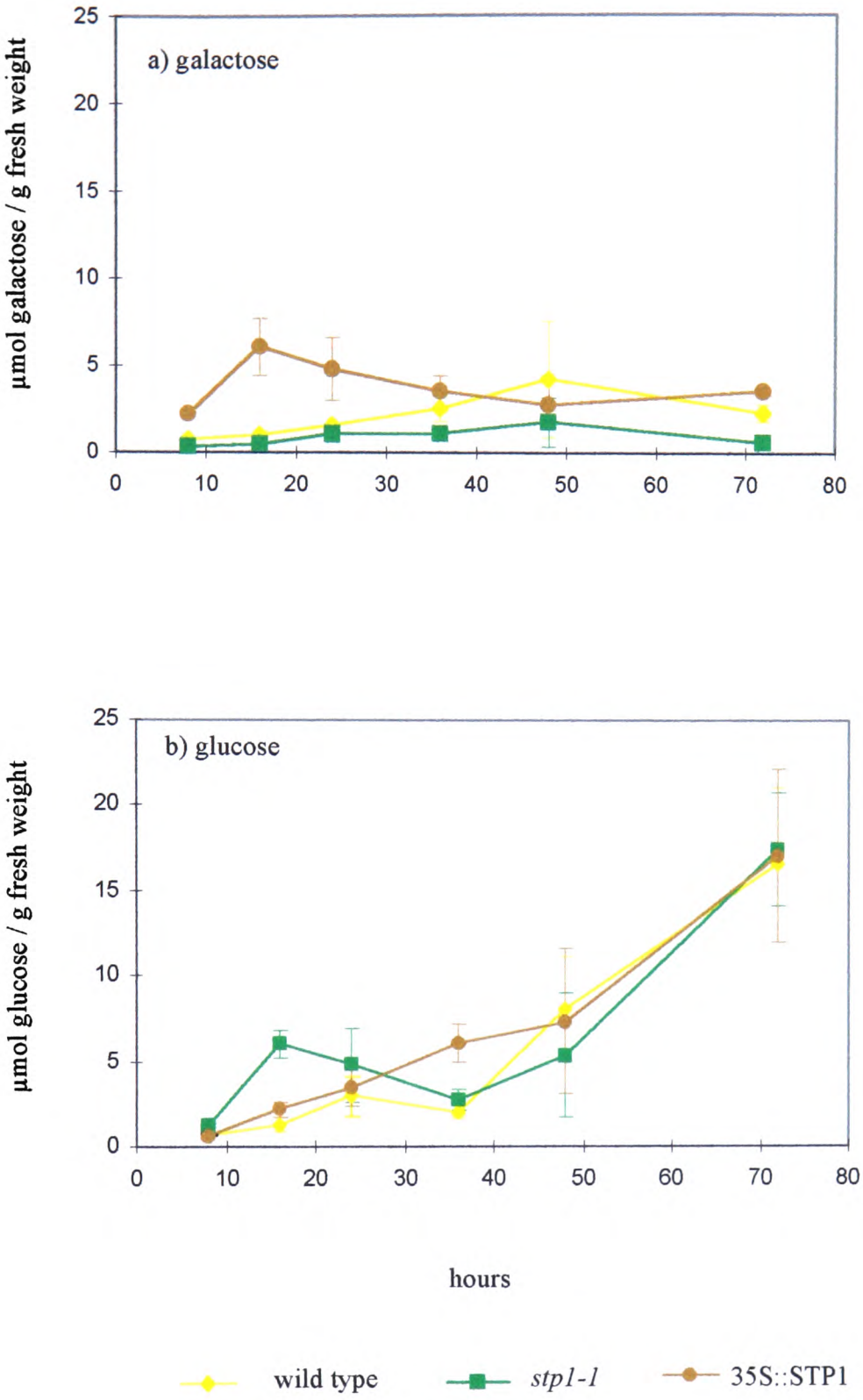

a) wild type
b) stp1-1
c) 35S::STP1
µmol hexoses / g fresh weight
hours
glucose
galactose

Figure 23: (next page) Comparison of the changes of the galactose and glucose concentration over time in two week-old seedlings of different genetic backgrounds grown on 50 mM galactose. The data presented in this figure is identical to the data of figure 22. For an easier comparison between the changes of galactose and glucose concentration in wild type seedling , AtSTP1 knock out seedlings (*stp1-1*) and seedlings overexpressing AtSTP1 (35S::STP1) the data has been plotted in one graph. Part a) illustrates the changes in the galactose concentration and part b) the changes in glucose concentration.


a) galactose
µmol galactose / g fresh weight
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
b) glucose
µmol glucose / g fresh weight
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
hours
wild type
stp1-1
35S::STP1

5 Discussion

5.1 Regulation of *AtSTP1* mRNA levels

The data presented in section 4.1 demonstrates that the level of *AtSTP1* mRNA is regulated by sugars. In response to sugars the *AtSTP1* mRNA level decreases. This response is triggered by exogenous glucose concentrations as low as 0.5-5 mM (figure 3). The change in mRNA levels in response to glucose is rapid. The major change in *AtSTP1* mRNA levels is observed within 3 hours after supply with glucose (figure 4). This suggests that turnover of the AtSTP1 protein might be very rapid as well. This would not be uncommon since the rapid protein turnover for a plant sucrose transporter has been reported (Kuhn et al.,1997). All tested monosaccharides that are metabolised and sucrose triggered the response (figure 5). As demonstrated for galactose these monosaccharides can be interconverted into each other within the plant (see section 4.13). Therefore it is not possible to conclude from the experiments presented in this thesis whether only one specific monosaccharide or all individual sugars tested are directly involved in the regulation of the *AtSTP1* mRNA level. That the changes in *AtSTP*1 mRNA level are not due osmotic stress caused by the exogenous supply of sugars is indicated by the fact that supply of the osmoticum mannitol (figure 5) and polyethylene glycol (Money, 1989)(data not shown) did not cause a change in *AtSTP1* mRNA level. The observation that incubation in 2-deoxyglucose leads to a decrease in *AtSTP1* mRNA level whereas 3-O-methylglucose has little effect does suggest that hexokinase is involved in the regulation of *AtSTP1* mRNA. A sugar sensing function for plant hexokinases has been proposed by several authors (reviewed in (Smeekens and Rook, 1997; Lalonde et al.,1999)) Although these results must be interpreted with caution since 2-

deoxglucose is metabolised at a slow rate and its secondary metabolites can interfere with gene expression (Klein and Stitt, 1998).

Since *AtSTP1* belongs to a gene family which shares high homology there is always the possibility that the *AtSTP1* specific probe cross-hybridises to other members of the gene family in a Northern blot experiment (see 4.2). Therefore the interpretation of such Northern blot experiments can be difficult. This possibility can be ruled out for the conditions and *AtSTP1* specific probe used in the experiments presented in this thesis, since under the hybridisation conditions used the AtSTP1 specific probe does not hybridise to any mRNA extracted from the AtSTP1 knock out plants (Sherson et al. 2000). Since these plants are only lacking *AtSTP1* mRNA any cross-hybridisation with other members of the family is possible but not detected.

From the results presented in this thesis it is not possible to conclude whether the changes in *AtSTP1* mRNA levels caused by sugars are due to changes in the rate of transcription of the AtSTP1 gene or due to a change in *AtSTP1* mRNA stability. A combination of both mechanisms could be possible as well.

5.2 Subcloning of the *AtSTP1* gene

A genomic fragment containing the complete coding region of the *AtSTP1* gene and 2936 bp upstream of the translation start site and 411 bp downstream of the stop codon has been subcloned from a genomic BAC library (Monzo et al.,1998). The identity of this subclone has been confirmed by partial sequencing. The whole fragment has been used to transform AtSTP1 knock out plants. Two independent

transformants with a single integration of the T-DNA have been obtained. This was done to see whether the genomic *ATSTP1* fragment does complement the phenotypic effects seen in the AtSTP1 knock out line. This would then prove that the phenotypic effects observed in the AtSTP1 knock-out line are due to the insertion in the *ATSTP1* gene and not due to a second unidentified T-DNA insertion in this plant. Due to the lack of time the phenotypic analysis of those transformants is still missing and are currently performed by Dr. Sarah Sherson. It was demonstrated that the overexpression of the *AtSTP1* cDNA under control of the 35S-CaMV promotor does revert the phenotypic effects seen in the AtSTP1 knock out line (section 4.8 and 4.9). This is already strong evidence that the phenotypic effects observed in the AtSTP1 knock out line are due to the T-DNA insertion into the *AtSTP1* gene and not caused by the second unidentified T-DNA insertion in these plants.

In collaboration with the PhD student H. Alford the subclone of *AtSTP1* genomic fragment has been used to create an *AtSTP1* promotor-luciferase-*AtSTP1* terminator transcriptional fusion construct. *Arabidopsis* plants are currently transformed with this reporter construct. Although the use of GFP as a reporter gene would be more suitable to localise expression of AtSTP1 the luciferase reporter construct will hopefully give more information about the cellular localisation of the AtSTP1 expression. Knowledge of the cellular localisation of the AtSTP1 expression could help to define a physiological function for that gene. Attempts to localise the expression of AtSTP1 with promotor-reporter gene constructs using a much shorter *AtSTP1* promotor fragment have been unsuccessful (N. Sauer personal communication).

If the reporter construct shows a similar response to exogenous applied sugars as to the *AtSTP1* gene, plants with the reporter construct will be used to isolate sugar sensing mutants. The isolation of such mutants can be based on the ethyl methanesulfonate (EMS) mutagenisis of seeds, which carry the sugar responsive reporter construct. Subsequently out of the mutagenised population individual seedlings with altered expression of the reporter gene in response to sugars are identified. Using a similar strategy several *Arabidopsis* sugar sensing mutants have been isolated. So far promotors from the plastocyanin gene (vanOosten et al.,1997; Dijkwel et al.,1996), the β-amylase gene (Mita et al.,1997b; Mita et al.,1997b) and from a patatin class I gene (Martin et al.,1997) have been used to isolate such mutants. The drawback of the promotors used so far is that unphysiologically high sugar concentrations have to be used (< 88 mM) to trigger the change in expression of the reporter genes. The use of the *AtSTP1* promotor in such a strategy promises to be able to perform the screening at much lower sugar concentrations.

5.3 Monosaccharide transport via AtSTP1

As shown in figure 10 the uptake of radioactive glucose under low substrate concentration into AtSTP1 knock out seedlings is decreased by 45% compared to uptake into wild type seedlings. Overexpression of AtSTP1 leads to a two to four fold increase of glucose uptake in different transgenic lines. For other substrates of AtSTP1 an up to 60% decrease in uptake into AtSTP1 knock out seedling compared to wildtype seedlings was observed (Sherson et al., 2000). This observation implies

that AtSTP1 is the major monosaccharide transporter in *Arabidopsis* seedlings, although pleiotropic effects caused by the knock out of AtSTP1 can not be ruled out to be the cause of this change in monosaccharide uptake. It could be possible that the absence of ATSTP1 leads to decreased transport activity and / or expression of other monosaccharide transporters. This seems rather unlikely and the more logical consequence of a loss of transport activity due to the knock out of AtSTP1 would be an increased expression and /or activity of other monosaccharide transporters to compensate for the decreased uptake. There is no evidence for either of such pleiotropic effects.

The observation that the knock out of only one monosaccharide transporter out of a large family with an estimated number of 20 members (Buttner et al.,2000) causes such a pronounced change in monosaccharide uptake underlines the importance of AtSTP1 for monosaccharide uptake into seedlings under these conditions.

In contrast to the observation that AtSTP1 plays a major role in monosaccharide uptake into seedlings at low substrate concentration (< 1 mM) at high glucose concentration (> 50 mM) AtSTP1 seems to contribute little to the total glucose uptake into seedlings. It is very difficult to perform radioactive sugar uptake assays into *Arabidopsis* seedlings at high substrate concentrations. Therefore only experiments, which monitor the glucose uptake into seedlings indirectly, have been performed. As shown in figure 18 and 19 addition of glucose to the growth medium stimulates development and accumulation of dry matter of wild type seedlings in a concentration dependent manner over the range of 0 to 200 mM. It can be hypothesised that the differences in glucose uptake into the seedling should be reflected in seedling development and dry matter accumulation. As shown in figure

18 and 19 there is no difference in development and dry matter accumulation between wild type and AtSTP1 knock out seedlings. In contrast seedlings overexpressing AtSTP1 show a faster development and a higher dry matter accumulation with increasing glucose concentration compared to wild type seedlings. The second line of indirect evidence that AtSTP1 does not contribute significantly to the uptake of glucose at high substrate concentrations comes from the analysis of anthocyanin accumulation in seedlings. It has been demonstrated that the accumulation of anthocyanin in plant cells is related to glucose concentration in the culture media (Vitrac et al.,2000). Differences in glucose uptake therefore should be reflected as differences in anthocyanin content. As shown in figure 20 the amount of anthocyanin in wild type *Arabidopsis* seedlings is related to the glucose concentration in the growth medium. The anthocyanin content of AtSTP1 knock out seedlings is not different to the content of wild type seedlings. Seedlings overexpressing AtSTP1 have an increased anthocyanin content on increasing glucose concentrations compared to wild type seedlings (figure 20, 21).

The observation that AtSTP1 may not significantly contribute to the glucose uptake at high substrate concentration into wild type seedlings is not surprising. In uptake studies with corn protoplasts (Lin et al.,1984), sugarcane suspension cells (Komor et al.,1981; Komor, 1994) and tomato fruit pericarp (Ruan and Patrick, 1995) a biphasic uptake of hexoses was observed. There is a saturable phase exhibiting a relativly low K_m value (below 1mM) and a linear diffusion-like phase which becomes obvious only at high concentration (> 20-50 mM). These observations suggested that at high substrate concentrations active uptake via transporters becomes saturated and a diffusion-like unsaturable uptake pathway is the main

pathway of substrate uptake. In this context it is interesting that the overexpression of AtSTP1 did lead to a difference in development, dry matter and anthocyanin accumulation in seedlings grown on high glucose concentration compared to wild type seedlings. These observations suggest that AtSTP1 is capable to contribute significantly to glucose uptake under high substrate concentrations when expressed under the control of the CaMV 35S promotor. This suggest that the fact that no differences between wild type and knock out seedlings were observed when grown on high concentrations of glucose was due to the fact that expression of AtSTP1 in wild type plants is repressed at high glucose concentrations (see 4.1 and figure 11 for comparison of mRNA levels between overexpressing and wild type plants) and not due to the possibility that the AtSTP1 protein does not significantly contribute to glucose uptake under these conditions when it is expressed.

The observation that two week-old seedlings overexpressing AtSTP1 do form visible lateral roots whereas wild type and AtSTP1 knock out seedlings have no lateral roots when grown on 50 mM glucose (figure 19) is interesting. There are two possible explanations for this: One possibility is that the overexpressing seedlings take up more glucose and therefore develop faster than the wild type and knock out seedlings. This is reflected in the higher number of leaves and the higher dry weight of AtSTP1 overexpressing seedlings. The presence of lateral roots in these seedlings could simply reflect a different stage of development. Alternatively the presence of lateral roots in AtSTP1 seedlings grown for two weeks on 50 mM glucose could reflect a different carbon:nitrogen ratio in the seedlings compared to wild type and knock out seedlings. It has been suggested that the activation of the meristem of lateral roots in *Arabidopsis* is regulated by the carbon:nitrogen ratio (Zhang and

Forde, 2000; Zhang et al.,1999). A low carbon:nitrogen ratio inhibits activation of the meristem of lateral roots. In the case of the AtSTP1 overexpressing seedlings an increased glucose uptake could lead to an increased carbohydrate level and a shifted carbon:nitrogen ratio. This would cause an activation of lateral root meristems. The observation that the formation of lateral roots can be induced at very early stages of seedling development (two to four leaf stage) under a suitable carbon:nitrogen ratio (Zhang et al.,1999) does favour the suggestion that the lateral roots observed in AtSTP1 overexpressing plants are due to an increased carbohydrate level in these seedlings and not a simple developmental process.

5.4 AtSTP1 knock out seed have a decreased sensitivity to germination on D-mannose

D-mannose inhibits the germination of *Arabidopsis* seeds at mM concentrations. D-mannose is taken up by the seeds and phosphorylated by hexokinase. Mannose-6-phosphate does not enter glycolysis at a significant rate and accumulates (Goldsworthy and Street, 1965). Neither ATP depletion nor phosphate depletion seem to be the cause of the inhibition of germination by mannose (Pego et al.,1999). It has been proposed that mannose inhibits germination via a hexokinase mediated step. This claim is supported by the fact that the germination of seeds from the sugar sensing mutant *sun6* (vanOosten et al.,1997) show a decreased sensitivity to mannose (Pego et al.,1999). It has been proposed that *sun6* mutation affects a

process involved in the hexokinase-mediated signal transduction pathway (vanOosten et al.,1997).

The observation that AtSTP1 knock out seeds have a decreased sensitivity to germination on D-mannose (figure14) could be explained by decreased uptake of D-mannose into the seeds. This would be in agreement with the observation of a decreased D-mannose uptake into knock out seedlings at low substrate concentrations (Sherson et al., 2000). Because of the technical difficulty to measure D-mannose uptake into ungerminated *Arabidopsis* seeds a detailed analysis of the observed phenotype is missing. However the possibility that AtSTP1 is involved in a hexokinase mediated signal transduction process can not be ruled out. One could imagine that only a specific isoform of hexokinase is capable of mediating a signal. This isoform could be localised at the plasma membrane in close proximity to AtSTP1. The absence of AtSTP1 would lead to a situation in which less mannose is phosphorylated by the signalling competent isoform of hexokinase. This is pure speculation. There is no experimental evidence to support this suggestion.

5.5AtSTP1 knock out seedlings have a decreased sensitivity to the toxic effects of D-galactose

D-galactose is highly toxic when supplied exogenously to plants in concentrations of 1-10 mM (Maretzki and Thom, 1977). D-galactose was found to prevent growth in different plant tissues. Yamamoto *et al.*(1988) could show that incubation in the presence of D-galactose caused an increase of galactose-1-phosphate and UDP-

galactose in oat coleoptiles and azuki bean epicotyls. For *Arabidopsis* seedlings grown in the presence of 55 mM galactose a two fold increase in UDP-galactose and an eight fold increase in starch was observed (Dormann and Benning, 1998). Overexpression of UDP-glucose epimerase did lead to a decrease in the sensitivity of *Arabidopsis* seedlings to the toxic effects of exogenously supplied D-galactose. *Arabidopsis* seedlings overexpressing UDP-glucose epimerase did not accumulate UDP-galactose and starch to such a high extent when exposed to 55 mM D-galactose (Dormann and Benning, 1998). Dörmann und Benning (1998) conclude from these observations that the UDP-glucose epimerase is the rate-limiting enzyme in the conversion of D-galactose to UDP-glucose. Figure 24 illustrates the proposed pathway for the conversion of D-galactose into UDP-glucose (Frey, 1996a; Frey, 1996a) in *Arabidopsis*. In the first step galactose is phosphorylated by galactokinase (E.C.2.7.1.6). The conversion of galactose-1-phosphate into UDP-galactose is either catalysed by a transferase (E.C.2.7.7.12) or a pyrophosphorylase (E.C.2.7.7.10). Which of those enzymes or if both are present in *Arabidopsis* is not clear. The last step is catalysed by the epimerase (E.C.5.1.3.2). The mechanism that causes the toxicity of D-galactose in *Arabidopsis* is not understood. It has been proposed that accumulation of UDP-galactose and galactose-1-phosphate lead to sequesteration of inorganic phosphate and an energy depletion, which could be responsible for the toxic effect of galactose (Prosselkov et al.,1997). Dörmann and Benning (1998) propose that the accumulation of UDP-galactose and possibly other metabolites could lead to an inhibition of cytosolic enzymes involved in the carbon flux from starch to sucrose. This would cause the observed accumulation of starch. The

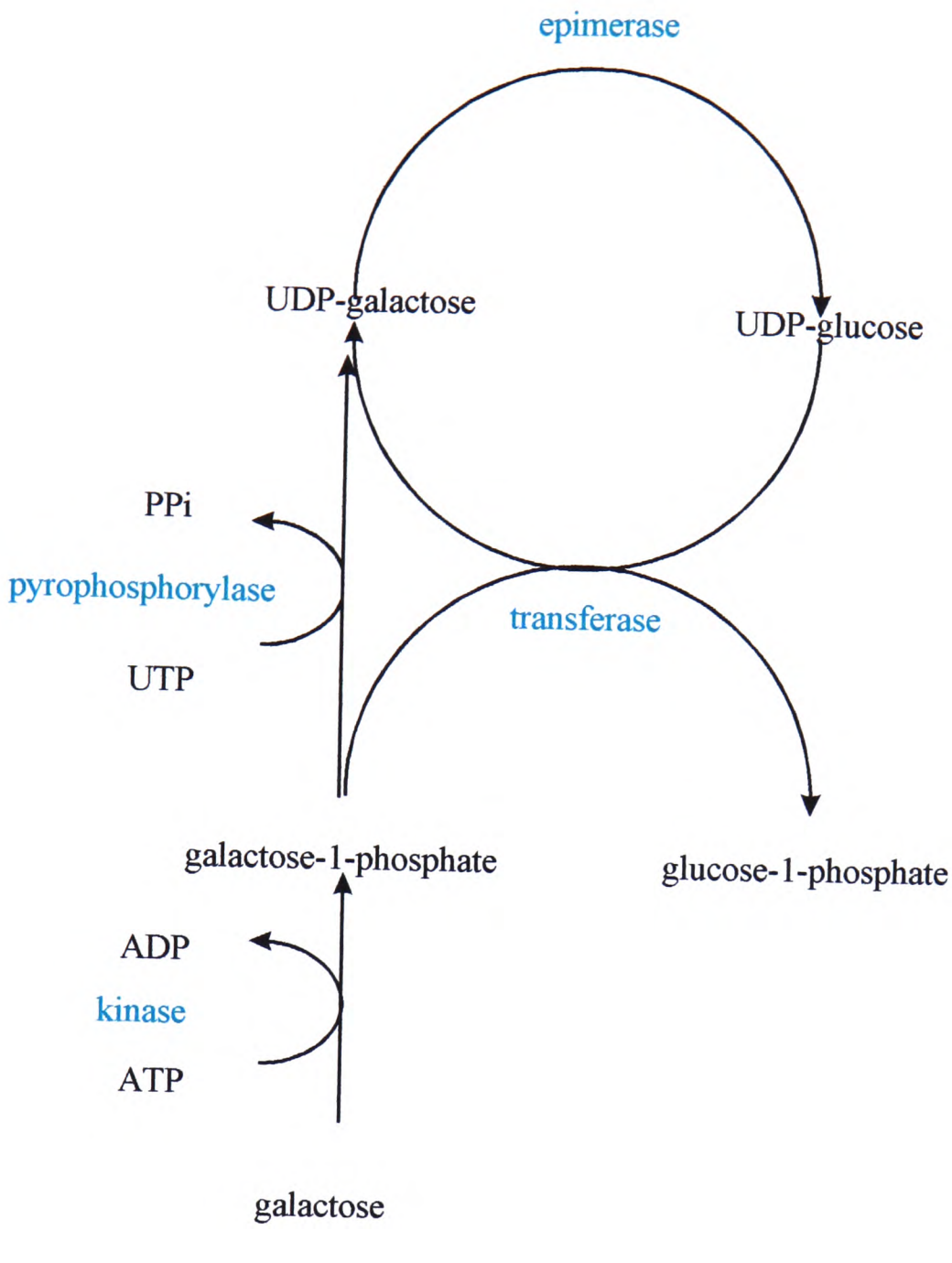


Figure 24: Schematic representation of galactose metabolism in plants. Enzymes are indicated in blue.

subsequent insufficient export of sucrose from source tissues could lead to the repressed growth.

As shown in figure 17 AtSTP1 knock out seedlings are insensitive to the toxic effects of exposure to 50 mM D-galactose. In section 5.3 it has been proposed that there is no difference in uptake of D-glucose between AtSTP1 knock out and wild type seedlings at 50 mM substrate concentration. Since AtSTP1 has a similar affinity for D-glucose and D-galactose and D-glucose and D-galactose have a similar effect on the *AtSTP1* mRNA level it can be hypothesised that the uptake of D-galactose at a 50 mM substrate concentration should be similar. To test this hypothesis the D-galactose and D-glucose concentrations in seedlings of the three genetic backgrounds after exposure to 50 mM galactose was determined (4.13).

These results indicate that the amount of D-galactose is different in the three genetic backgrounds. The lowest D-galactose level was found in AtSTP1 knock out seedlings and the highest level in AtSTP1 overexpressing seedlings. The amount of D-glucose increases in all three genetic backgrounds over time and reaches a similar level in all three genetic backgrounds at the end of the experiment. This indicates that D-galactose is converted into D-glucose in these seedlings. Theses observation can be interpreted in two different ways: First the observed D-galactose concentrations in the three different genetic backgrounds do reflect a difference in D-galactose uptake into these seedlings. This would contradict the hypothesis made before the experiment and would be difficult to explain in the context of the results obtained for D-glucose uptake (see 5.3). An alternative explanation for the observed changes in D-galactose and D-glucose levels is that the galactose uptake into wild type and AtSTP1 knock out seedlings occurs at a similar rate but the amount of D-

galactose turnover into UDP-glucose in the AtSTP1 knock out seedlings is greater than in wild type. Less D-galactose would accumulate in the AtSTP1 knock out plants. This would be in agreement with the observation that D-glucose initially accumulates faster and to a higher level in the AtSTP1 knock out seedlings compared to wild type seedlings. Therefore less intermediate metabolites of the D-galactose metabolism would accumulate in the AtSTP1 knock out seedlings. The accumulation of such metabolites has been suggested to cause the repressed growth phenotype caused by D-galactose. This would be in agreement with the observation that AtSTP1 knock out seedlings are less sensitive to this phenotypic effect. This hypothesis consequently leads to the following question:

How could the absence of AtSTP1 protein lead to a change in galactose metabolism?

It has been demonstrated that the mRNA level of the UDP-glucose epimerase, which is thought to be the rate-limiting enzyme in D-galactose metabolism is repressed by exogenously supplied D-galactose and D-glucose (Dormann and Benning, 1998). One could imagine that AtSTP1 could act as a sugar sensor. A sugars sensing function for plant hexose transporters has been proposed by various authors (Smeekens and Rook, 1997; Lalonde et al.,1999). The absence of a putative sugar sensor AtSTP1 would lead to the situation that in the presence of galactose, mRNA levels of UDP-galactose epimerase would not be down regulated and higher turnover of D-galactose achieved.

A further possible explanation for the decreased sensitivity of the AtSPT1 knock out seedlings again involves the proposal that AtSTP1 acts as a sugar sensor. In this hypothesis the lacking sugar signal from AtSTP1 does not directly influence galactose metabolism. Dörmann and Benning have suggested that the accumulation

of intermediate metabolites of D-galactose metabolism cause an inhibition of cytosolic enzymes involved in the carbon flux from starch to sucrose and thereby cause the phenotypic effects of D-galactose. The lack of a signal from a putative sugar sensor AtSTP1 could modulate activities of those enzymes and counterbalance the inhibitory effect of the intermediate metabolites.

The data presented in this thesis do not resolve the question why AtSTP1 knock out mutant displays a decreased sensitivity to 50 mM D-galactose. Further experiments are needed to address this question. If AtSTP1 should have a dual function as sugar transporter and sugar sensor it will be very difficult to separate effects caused by the lack of transport and effects caused by the lack of signalling function in the AtSTP1 knock-out mutant. This is illustrated by the ongoing debate whether the human glucose uniporter GLUT2 has a dual function as glucose transporter and glucose sensor (Schuit, 1996; Schuit, 1997; Antoine et al.,1997)

5.6 Possible physiological function of AtSTP1

The absence of AtSTP1 does not cause any obvious phenotypic effects when *Arabidopsis* is grown on soil under greenhouse conditions (4.7). Therefore it can be concluded that AtSTP1 is not an essential gene in *Arabidopsis*. This finding is not surprising since AtSTP1 belongs to a monosaccharide transporter family with an estimated number of 20 members (Buttner et al.,2000) and a certain redundancy between members of this family can be expected. It was quite surprising to observe that monosaccharide uptake into AtSTP1 knock out seedlings is reduced by up to

60% (Sherson et al. 2000). This suggests that AtSTP1 significantly contributes to monosaccharide uptake into seedlings. Whether in mature plants AtSTP1 contributes in a similar proportion to monosaccharide uptake is not known since uptake studies only have been performed on seedlings. *AtSTP1* mRNA is not specifically expressed in seedlings and is also found in the mature plant, which suggests that AtSTP1 also significantly contributes to monosaccharide uptake in the mature plant. No phenotypic effect was observed in AtSTP1 knock out seedlings when grown in the absence of carbohydrates. This suggests a 60% decrease in monosaccharide uptake in seedlings (Sherson et al. 2000) does not essentially interfere with the carbon partitioning in the seedlings. Therefore apoplastic uptake of monosaccharides does not play an important role in carbon partitioning in the seedling or the transport capacity is not limiting factor when decreased by 60%.

The *AtSTP1* mRNA level is decreased by the exogenously supplied monosaccharides. Therefore one possible physiological function of AtSTP1 could be to increase monosaccharide uptake into cells which suffer from carbon starvation. In experiments where the recovery of seedlings from carbon (light) starvation was analysed no difference between wild type plants and AtSTP1 knock out plants was observed (G. Wallace unpublished results). These results do not support the suggestion that the physiological role of AtSTP1 is to import monosaccharides specifically into cells under carbon starvation, but further investigation may be necessary.

AtSTP1 is expressed in every tissue of *Arabidopsis* that has been analysed so far. This is in contrast to the observation for the three so far analysed AtSTPs, which are expressed in specific tissues and /or in response to a specific stimulus (1.3.4). One

physiological role for AtSTP1 could be the general retrieval of monossacharides, which have leaked out of the cells by passive diffusion into the apoplast. The sugar regulation of AtSTP1 could ensure that cells which have the highest demand for monosaccharides would preferentially take up those monosaccharides.

In addition to that, the possibility that AtSTP1 has a dual function as a sugar sensor has been discussed (5.5) Other proposed sugar sensing mutants which have been identified in *Arabidopsis* (Nemeth et al.,1998; Zhou et al.,1998; Hellmann et al.,2000; Bhalerao et al.,1999; vanOosten et al.,1997) show a changed response to various substances including abscisic acid, high glucose concentrations, etheylne and proline. All of this substances have been tested but AtSTP1 knock out plants do not respond differently to wild type plants to those substances (Sherson et al. 2000). This is not indicating that AtSTP1 might not be a sugar sensor. Not every sugar sensing mutant shows an altered response every substance that causes an effect in another sugar sensing mutant. This reflects the possibility of several different sugar signalling pathways which act in parallel.

Recently it has been proposed that AtSTP1 is specifically expressed in guard cells and plays an important role in guard cell function (N. Sauer personal communication). Since mature guard cells are symplastically isolated (Palevitz and Hepler, 1985) and lack significant amounts of Calvin-cycle enzymes (Reckmann et al.,1990) carbon uptake across the plasma membrane is the only way to meet the carbon demand of the guard cells. It has been demonstrated that guard cells use sucrose as an osmoticum to regulate guard cell aperture at certain times (Talbott and Zeiger, 1998). We have been able to show *AtSTP1* mRNA in tissue like roots, which clearly do not have guard cells (Sherson et al.,2000). In addition to that the amount

of *AtSTP1* mRNA in total RNA extractions from leaves suggests that guard cells are not the only cells in leaves expressing AtSTP1. Analysis of guard cell function in AtSTP1 knock out mutants by porometry and wilting of leaves (S. Smith and S. Sherson unpublished results) show that guard cell function is not apparently altered. Therefore the suggestion that AtSTP1 is specifically expressed in guard cells and plays an important role in guard cell function is not supported.

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Appendix

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Monosaccharide/proton symporter AtSTP1 plays a major role in uptake and response of *Arabidopsis* seeds and seedlings to sugars

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Summary

The aim of this study was to investigate the *in vivo* properties and function of the high-affinity monosaccharide/proton symporter AtSTP1 of *Arabidopsis*. We isolated an *Atstp1* knock-out mutant and found that this plant grows and develops normally. The *AtSTP1* gene is expressed in germinating seeds and seedlings, with AtSTP1 activity found mainly in the seedling root. The rate of uptake of [^{14}C]-3-O-methylglucose and [^{14}C]-D-glucose is 60% less in *Atstp1* seedlings than in the wild type, showing that AtSTP1 is the major monosaccharide transporter in *Arabidopsis* seedlings. Transport of D-galactose and D-mannose is also up to 60% less in *Atstp1* seedlings compared to wild type, but transport of D-fructose, L-arabinose and sucrose is not reduced. Germination of *Atstp1* seed shows reduced sensitivity to D-mannose, demonstrating that AtSTP1 is active before germination. *Atstp1* seedlings grow effectively on concentrations of D-galactose that inhibit wild-type growth, even at up to 100 mM D-galactose, indicating that active transport by AtSTP1 plays a major role at very high concentrations of exogenous sugar. These findings provide insight into the physiological function of AtSTP1 and clearly establish its importance in the uptake of extracellular sugars by the embryo and in seedlings.

Keywords: monosaccharide transporter, mutant, sugar response, germination, seedling growth, *Arabidopsis thaliana*.

Introduction

Monosaccharide/proton symporters have been described at the molecular level in several different plant species (Harrison, 1996; Roitsch and Tanner, 1994; Sauer and Stadler, 1993; Sauer and Tanner, 1989; Sauer *et al.*, 1990). *Arabidopsis thaliana* contains a family of at least 14 genes encoding putative sugar transport proteins (STPs) of this type (Büttner *et al.*, 2000), which complicates studies of their biological functions and of their transport characteristics *in vivo*. AtSTP1 has been functionally characterized in *Schizosaccharomyces pombe* and *Xenopus* oocytes, and shown to be a high affinity monosaccharide/proton symporter capable of transporting several monosaccharides but not fructose (Boorer *et al.*, 1994; Stolz *et al.*, 1994). The *AtSTP1* mRNA is reported to be most abundant in leaves and is also found in other organs including stems, flowers and roots (Sauer *et al.*, 1990), but no specific function for this protein has been proposed. AtSTP2 is also a high-affinity monosaccharide transporter, but is found specifically in developing pollen. It is hypothesized that it could have a role in uptake of glucose derived from callose degradation during pollen maturation (Truernit *et al.*, 1999). AtSTP3 is a low-affinity transporter found in leaves (Büttner *et al.*, 2000). AtSTP4 is another high-affinity transporter and increases in amount in wounded leaves, implying a role in response to pathogen attack (Truernit *et al.*, 1996). The properties and functions of other putative AtSTPs have yet

to be investigated, but it can be speculated that some such transporters will have important roles in hexose uptake and regulation of metabolism in sink tissues where sucrose is hydrolysed in the apoplast (Sturm and Tang, 1999; Weber *et al.*, 1997). It is apparent, therefore, that AtSTPs have a wide range of specific and distinct functions in many different tissues of the plant.

There have been many studies of sugar uptake by plant cells and tissues, which have characterized the specificity and kinetic properties of the uptake systems of those cells. Typically, high-affinity, saturable, proton-coupled transport of sucrose and hexoses is observed, together with a non-saturable component believed to represent passive diffusion, but the relative contributions of these components varies widely between studies (Komor *et al.*, 1981; Lin *et al.*, 1984; Ruan *et al.*, 1997). In no case has it been possible to assess the contribution made by a specific transporter to the sugar uptake by plant cells or tissues, because the means to selectively eliminate or inactivate individual transporters has not been available. Understanding the factors that regulate sugar transport into plant cells is not only important for understanding apoplast–symplast metabolic interactions, but also for understanding the responses of plant cells to extracellular sugars. In recent years, several simple screens have been adopted for the isolation of mutant seedlings which respond differently to exogenous sugars, with the aim of isolating sugar-sensing mutants (Pego *et al.*, 2000). Such screens include the use of D-mannose, which is inhibitory to plant metabolism and seed germination (Pego *et al.*, 1999) and very high concentrations of glucose or sucrose to inhibit seedling growth and development (Németh *et al.*, 1998; Zhou *et al.*, 1998). In order to understand how such screens work, it is important to know the pathways of sugar uptake by seedlings, particularly since sugar transporters are implicated in sensing functions (Lalonde *et al.*, 1999).

As an approach to studying the properties of specific STPs and to understanding their functions, we have searched for mutants in which *AtSTP* genes are disrupted by T-DNA insertions (Krysan *et al.*, 1999). This strategy only requires knowledge of the sequence of the target gene and can identify plants which are hemizygous for the T-DNA, so avoiding the problem of mutations which are lethal when homozygous. Using this approach we have isolated a mutant lacking AtSTP1, allowing for the first time direct analysis of its activity and substrate specificity *in planta*. While growth of the mutant is not obviously impaired, it exhibits altered responses to exogenous sugars.

Results

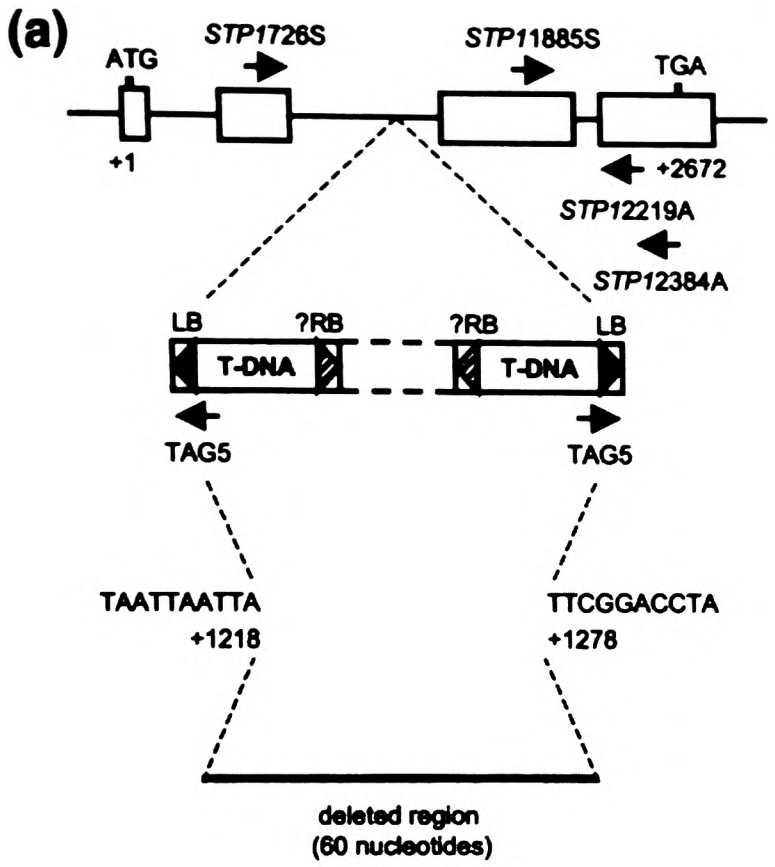
Isolation of an Atstp1 *mutant plant*

In order to find mutants with disruptions in genes encoding monosaccharide transporters of the AtSTP family, collections of T-DNA-tagged plants were screened using PCR with combinations of T-DNA- and gene-specific primers (Krysan *et al.*, 1999). A T-DNA insertion was found in the *AtSTP1* gene in a plant from the collection of INRA-Versailles (Bechtold *et al.*, 1993; Bouchez *et al.*, 1993). Unique PCR products were detected using a primer from the T-DNA left border in combination with gene-specific primers from both 5′ and 3′ ends of the *AtSTP1* gene, while primers from the T-DNA right border failed to generate any unique products. This indication that the T-DNA exists as an inverted repeat was confirmed by DNA sequence analysis, which further showed that the T-DNA had inserted into intron 2 of the *AtSTP1* gene, and that approximately 60 nucleotides had been deleted in the process (Figure 1a). Genomic Southern blot analysis using a T-DNA left border probe indicated the presence of at least two T-DNA copies within the *AtSTP1* gene, but their precise organization was not determined. Previous reports suggest that complex T-DNA insertion events of this sort are not uncommon (McKinney *et al.*, 1995). Following backcrossing to wild type, segregation analysis of kanamycin resistance indicated the presence of a single T-DNA insertion site in this plant (232 resistant : 74 sensitive; $\chi^2 = 0.109$; $P > 0.5$). Furthermore, Southern blotting experiments with backcross progeny did not detect any segregation of T-DNA sequences, indicating the absence of T-DNA sequences unlinked to *AtSTP1* (results not shown). Complementation studies using an *AtSTP1* cDNA subsequently proved that properties of the *Atstp1* mutant are a consequence of T-DNA insertion in the *AtSTP1* gene. Plants homozygous for the T-DNA insertion in *AtSTP1* were obtained with the expected frequency, and these plants had growth phenotypes which were not apparently different to the wild type, indicating that *AtSTP1* is not an essential gene. In the course of isolating the homozygous *Atstp1* mutant, a wild-type segregant was isolated from the same transformant to serve as a control in subsequent experiments.

Insertion of two or more copies of a 14 kbp T-DNA sequence into the middle of the *AtSTP1* gene would be expected to completely disrupt expression. To confirm this, RNA was isolated from leaves of wild-type and *Atstp1* mutant plants, and analysed by Northern blot hybridization. *AtSTP1* gene expression is observed clearly in leaves of the wild type, but in the *Atstp1* mutant plants no mRNA is detected (Figure 1b).

Hexose transport is impaired in the mutant

In order to establish a simple experimental system for the analysis of sugar transport in *Atstp1*, we chose to examine germinating seeds and seedlings. They are rapidly and reproducibly obtained, free of any contaminating organism, by means of axenic culture on agar, and preliminary



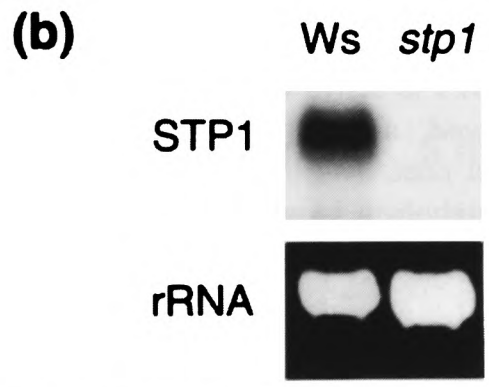


Figure 1. The *Atstp1* gene is interrupted by a T-DNA insertion which abolishes gene expression.
(a) Structure of the *AtSTP1* gene, comprising four exons (open boxes), three introns, start codon (ATG at nucleotide +1) and stop codon (TGA at nucleotide +2672). Positions of primers used in screening and sequencing are also shown: *STP1* primers are gene-specific; A indicates antisense orientation, S indicates sense orientation relative to coding region; TAG5 is a T-DNA-specific primer. LB indicates the T-DNA left border; RB the T-DNA right border. Sequence from the insertion site shows that the T-DNA is located between nucleotides +1218 and +1278 within intron 2, and that the intervening 60 nucleotides are deleted.
(b) Northern blot hybridization of total RNA (10 μg per lane) isolated from rosette leaves of mature wild-type (Ws) and *Atstp1* plants. Ethidium bromide-stained rRNA is also shown.

experiments indicated that *Atstp1* seedlings behaved differently in response to exogenous sugars. Firstly we demonstrated that *AtSTP1* is expressed in wild-type seedlings by isolating RNA at different stages of seedling growth for analysis by Northern blotting. The result (Figure 2) shows that *AtSTP1* mRNA increases in amount appreciably up to 2 days' growth and then declines slowly during the following 5 days. No signal was detected in *Atstp1* seedlings (result not shown). Seven-day-old seedlings were chosen as the most convenient for analysis. They were submerged in radioactive sugar solution (≈50 μM) for up to 30 min, then removed, washed completely, and uptake of sugar quantified. Using this system we could show that the rate of uptake of either D-glucose (Figure 3a) or 3-O-methylglucose (not shown) was linear during the experiment, and uptake rate into *Atstp1* was approximately 60% less than that of the wild type (Figure 3a,b). Carbonylcyanide m-chlorophenyl hydrazone (CCCP) at 100 μM reduced uptake of D-glucose by 87% in the wild type (12.5 and 1.6 nmol g^{-1} h^{-1}, respectively) and by 89% in *Atstp1* (4.8 and 0.53 nmol g^{-1} h^{-1}, respectively), confirming that uptake was proton-driven. We conclude that AtSTP1 is the major high-affinity glucose transporter of such seedlings. Furthermore, when the glucose concentration was increased from 50 μM to 1 mM, uptake by *Atstp1* seedlings was still 60% less than in the wild type (not shown), indicating that there is no low-affinity glucose transporter active in such seedlings.

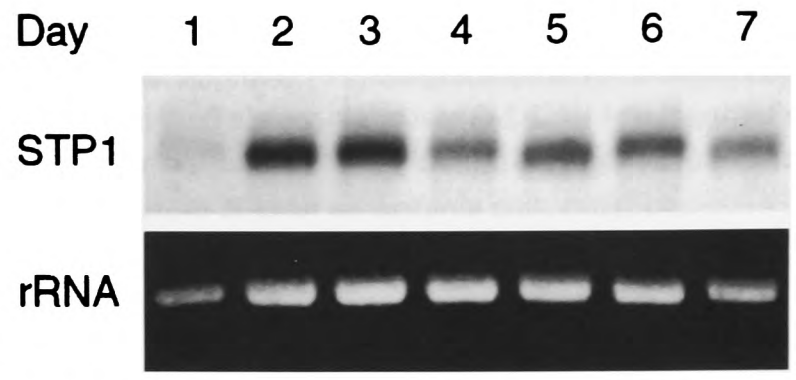
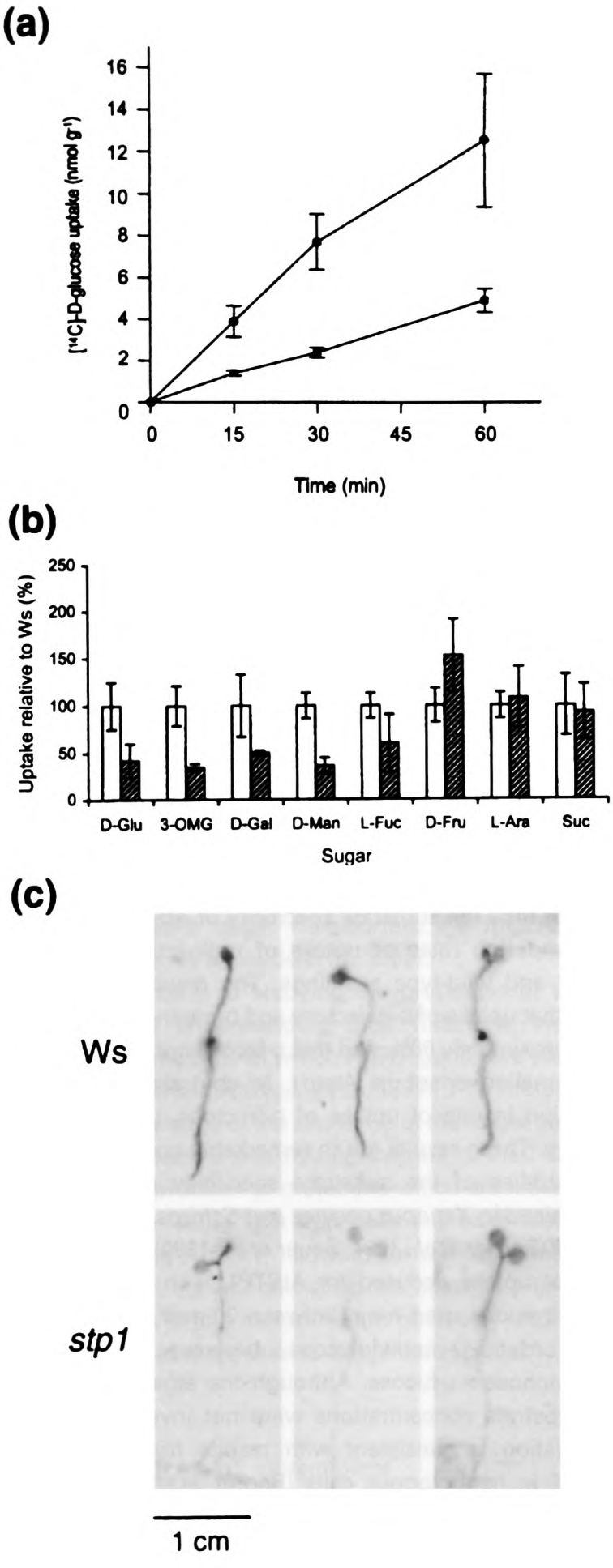


Figure 2. Expression of the *AtSTP1* gene in wild-type seedlings.
Northern blot hybridization of total RNA (2.5 μg per lane) isolated from wild type seedlings 1–7 days post-germination. Ethidium bromide-stained rRNA is also shown.

In view of these findings, we were able to investigate for the first time the substrate specificity of AtSTP1 *in planta*, by comparing rates of uptake of radioactive sugars by *Atstp1* and wild-type seedlings. The results (Figure 3b) show that uptake of D-galactose and D-mannose is reduced by approximately 60%, and that L-fucose uptake is reduced to a smaller extent in *Atstp1*. In contrast, there is no reduction in rate of uptake of D-fructose, L-arabinose or sucrose. These results are in remarkably good agreement with studies of the substrate specificity of AtSTP1 as determined in *Xenopus* oocytes and *Schizosaccharomyces pombe* (Boorer *et al.*, 1994; Sauer *et al.*, 1990). The absolute rates of uptake deduced for AtSTP1, with sugars at the concentrations used here (between 20 and 100 μM), were in the order 3-O-methylglucose > D-glucose > D-galactose > D-mannose > L-fucose. Although the effects of varying the substrate concentrations were not investigated, this observation is consistent with results from studies of AtSTP1 in heterologous cells (Boorer *et al.*, 1994; Sauer *et al.*, 1990). It is not known which other proteins are responsible for hexose uptake by *Arabidopsis* seedlings.

The distribution of AtSTP1 activity in 7-day-old seedlings was deduced by autoradiography of wild type and mutant after uptake of [^{14}C]-3-O-methylglucose (which is metabolized slowly and presumably not transported within the seedling). It was found that sugar is taken up by all organs of wild-type seedlings, but disruption of *AtSTP1* results in a dramatic reduction of uptake into roots and, to a lesser extent, into hypocotyl and cotyledons (Figure 3c). Apparently AtSTP1 is relatively more active in the root than in the shoot of such seedlings.



Sugars affect seed germination and seedling growth

The growth and development of *Atstp1* mutant plants was studied under a range of growth conditions, but no morphological phenotype was apparent. In order to reveal possible consequences of a null mutation in *AtSTP1*, attention was focused on seed germination and seedling growth, where AtSTP1 plays a major role in hexose transport. No differences in seed germination and seedling growth rate were detected. Seeds were then germinated in the presence of D-mannose to determine if the reduced rate of uptake would be reflected in reduced sensitivity to this sugar. Germination of *Atstp1* was found to be significantly more tolerant of D-mannose than wild type (Figure 4a,b), and the mutant is therefore a *mig* (mannose-insensitive germination) mutant (Pego *et al.*, 2000). This result shows that AtSTP1 is active in the embryo before germination is observed, since the radicle does not emerge from the seed coat. The inhibitory effect of D-mannose on plant metabolism is well documented, and reported to act through a hexokinase-dependent step (Pego *et al.*, 1999).

Seedlings were next grown in the presence of increasing concentrations of D-galactose, which is also toxic to cells of many plant species. It was found that *Atstp1* is much more tolerant of D-galactose than the wild type, particularly with respect to root growth (Figure 5a–c). The toxic effect of D-galactose on plants has been well documented (Maretzki and Thom, 1978; Yamamoto *et al.*, 1988) and it is proposed that UDP-glucose epimerase is a rate-limiting step in D-galactose metabolism leading to sequestration of uridine nucleotides and inhibition of growth (Dörmann and Benning, 1998). Remarkably, *Atstp1* seedlings show

Figure 3. Uptake of sugars by wild-type and *Atstp1* seedlings.
(a) Seven-day-old wild type (●) and *Atstp1* (■) seedlings were incubated in [^{14}C]-D-glucose for 15, 30 and 60 min intervals, then washed and the uptake of radioactivity determined. Results shown are the means of three replicates at each time point with standard deviations.
(b) The uptake was determined of different ^{14}C-labelled sugars into wild-type (white bars) and *Atstp1* (hatched bars) 7-day-old seedlings during a 30 min incubation. The values for wild type were defined as 100% and the relative rates of sugar uptake by *Atstp1* calculated accordingly. Results shown are the means of three independent experiments, each with three replicates per treatment, with standard deviations.
(c) Autoradiographs of 7-day-old seedlings which have taken up [^{14}C]-3-O-methylglucose. Three representative seedlings are shown for both wild type (Ws) and *Atstp1*.

increased tolerance at up to 100 mM D-galactose. This suggests that even at such high concentrations, AtSTP1 plays a major role in uptake, and therefore passive diffusion is relatively minor. Extremely high concentrations of D-glucose (330 mM) have been used to isolate glucose-insensitive (*gin*) mutants (Zhou *et al.*, 1998). Germination and seedling growth rate of *Atstp1* did not differ appreciably from the wild type at 330 mM D-glucose. Therefore *Atstp1* is not a *gin* mutant (results not shown), and presumably AtSTP1 does not play a significant role in glucose uptake at such an extreme concentration.

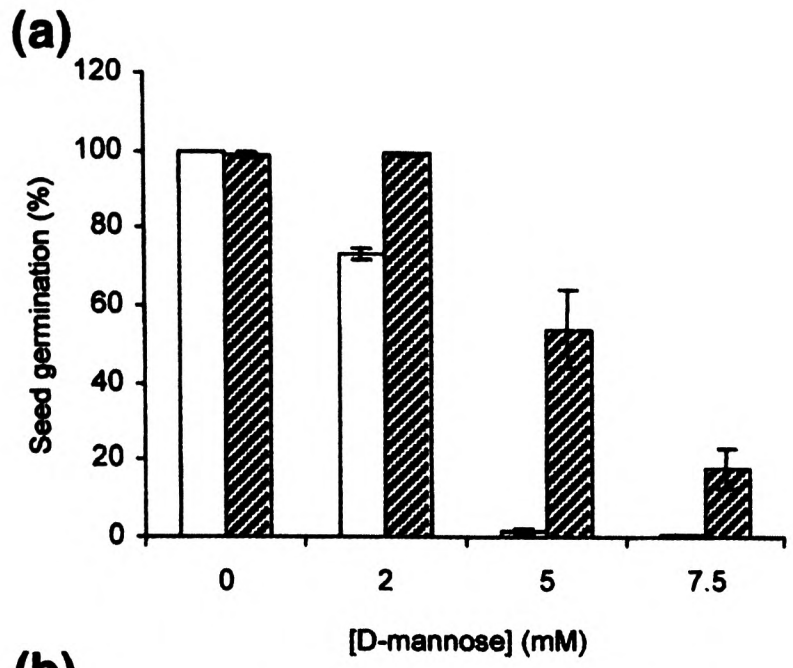


Figure 4. Effect of D-mannose on seed germination.
(a) Seeds (wild type and *Atstp1*) were placed on D-mannose medium (in the range 0–7.5 mM) and incubated in continuous light for 7 days, after which the percentage of germinated seeds was determined. Germination was defined as the emergence of at least 1 mm of radicle from the seed coat (Pego *et al.*, 1999). Results shown are the means of three replicates, each with 200 seedlings, with standard deviations.
(b) Representative plates showing wild-type (Ws) and *Atstp1* seed germination and seedling growth on 5 mM mannose.

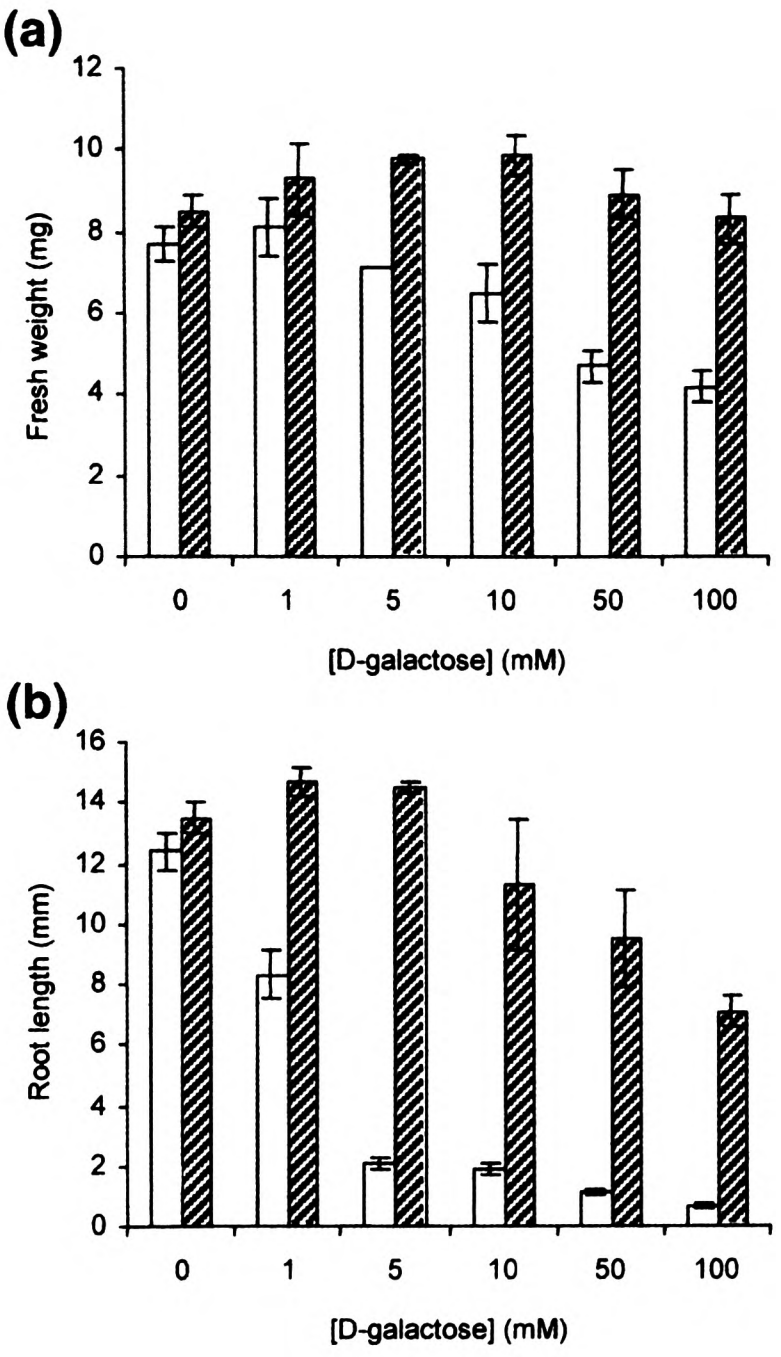


Figure 5. Effect of D-galactose on seedling growth.
(a) Seeds (wild-type and *Atstp1*) were germinated on D-galactose medium (in the range 0–100 mM), seedlings grown for 7 days in continuous light, and their fresh weights determined. Results shown are the means of three replicates, each with 20 seedlings, with standard deviations.
(b) Root lengths of seedlings described in (a).
(c) Representative seedlings of wild type (Ws) and *Atstp1* on 0 mM and 50 mM D-galactose medium.

Table 1. Complementation of *Atstp1* by transformation with a 35S::*AtSTP1* cDNA construct

	Hygromycin resistance			Root length on 50 mM D-galactose			Germination on 5 mM D-mannose		
Line	R	S	χ^2 (P)	<2 mm	>2 mm	χ^2 (P)	+	-	%
Ws	0	98		93	0		2	104	1.9
stp1	0	102		0	88		68	36	65.4
stp1 35S-STP1 α	104	39	0.39 (>0.5)	77	20	0.99 (>0.1)	4	97	4.0
stp1 35S-STP1 β	140	50	0.18 (>0.5)	80	17	2.89 (>0.05)	4	96	4.0

Progeny of hygromycin-resistant transformants were germinated on medium containing hygromycin B (40 μg ml^{-1}), D-galactose (50 mM) or D-mannose (5 mM). In the presence of hygromycin the ratio of resistant (R) to sensitive (S) individuals was used to estimate the number of segregating transgenic loci. In the presence of D-galactose the phenotypes were compared with wild type (<2 mm root growth after 7 days) and stp1 (>2 mm root growth after 7 days). In the presence of D-mannose, percentage germination was determined and compared with the wild-type and stp1 controls. Where the segregation approximated a 3 : 1 ratio a χ^2 value based on the expected ratio was calculated as shown.

Transgenic reconstruction restores Atstp1 *to wild-type phenotype*

To confirm that the phenotypes seen in *Atstp1* are a result of the T-DNA insertion in the *AtSTP1* gene and not due to another unknown mutation, transformation with a 35S::AtSTP1 cDNA was performed. Seeds from two independent primary transformants were plated on 50 mM D-galactose and on 5 mM D-mannose. Restoration of D-galactose and D-mannose sensitivity was observed with the expected frequency, which correlated with inheritance of the hygromycin resistance gene used for the transformation experiment (Table 1).

Discussion

We show here that *AtSTP1* is a nonessential gene, and lack of AtSTP1 does not result in any obvious growth or morphological phenotype under greenhouse or growth-room conditions. However, our results now provide important new information about the physiological function of AtSTP1. First, the mannose-insensitive germination of *Atstp1* seed shows clearly that AtSTP1 is normally active in the seed during imbibition. Since radicle emergence depends on disruption of cells of the endosperm and inner seed coat (Bewley and Black, 1994), we propose that AtSTP1 could provide a means for the embryo to take up monosaccharides liberated by hydrolysis of the walls of such cells. Presumably the amounts of D-mannose and D-galactose taken up by the embryo, from the mixture of monosaccharides produced within the seed in this way, are too low to be toxic. After germination, AtSTP1 activity is seen mainly in the seedling root and at this stage it may have a different function. It may be responsible for the uptake of glucose derived from sucrose after transport from the cotyledons to the root and hydrolysis in the apoplast. The fructose simultaneously released from sucrose would be taken up by a different route since it is not a substrate for AtSTP1. Other hypotheses are that AtSTP1 functions in the retrieval of glucose that may leak from cells, or that it recovers monosaccharides liberated by cell-wall turnover during root growth. The monosaccharide composition of the *Arabidopsis* primary cell wall (Reiter *et al.*, 1997) is consistent with the substrate specificity of AtSTP1.

The lack of an obvious phenotype in *Atstp1* seeds and seedlings may be explained by the fact that an appreciable amount of hexose transport activity remains, even in the absence of AtSTP1. A full understanding of AtSTP1 function in seeds and seedlings will require identification and characterization of the other sugar transporters active at these stages of growth. Furthermore, it will be necessary to identify those cells in which AtSTP1 is localized. Immunolocalization of AtSTP1, *in situ* hybridization to detect AtSTP1 mRNA, and GUS reporter gene studies have so far all failed to localize *AtSTP1* gene expression in the root (R. Stadler and N. Sauer, unpublished results), presumably because the level of expression is too low. Further studies are required to address this important question. In addition, it will be necessary to study AtSTP1 activity in mature leaves (Sauer *et al.*, 1990), where it may have a different physiological function to that of seeds and seedlings. Recent studies indicate that *AtSTP1* is expressed in the guard cells of leaves (R. Stadler and N. Sauer, unpublished results).

Our results indicate that AtSTP1 makes a major contribution to the response of *Arabidopsis* seeds and seedlings to exogenous D-mannose and to D-galactose at concentrations up to 100 mM, but apparently not to glucose at 330 mM. The finding that *Atstp1* shows reduced sensitivity to D-galactose at concentrations up to 100 mM is striking, because it indicates that active transport by AtSTP1 plays a major role in uptake at very high concentrations of hexose. In numerous other studies with plant cells, the concentration dependence of hexose uptake has been shown to be

biphasic, with saturable energy-dependent transport at low concentrations (typically below 20 mM) and a non-saturable diffusional component at higher concentrations (Lin *et al.*, 1984; Ruan *et al.*, 1997 and references therein). In *Xenopus* oocytes AtSTP1 has a K_m for 3-O-methylglucose of 60 μM (Boorer *et al.*, 1992), and in *Schizosaccharomyces pombe* the K_m for glucose and 3-O-methylglucose is ≈20 μM and 100 μM, respectively (Sauer *et al.*, 1990). Based on these considerations, we would expect AtSTP1 to make a very small, perhaps insignificant, contribution to hexose uptake at 100 mM, but this is apparently not the case for D-galactose. We conclude that in *Arabidopsis* seeds and seedlings under normal physiological conditions, hexose uptake across the plasma membrane is entirely AtSTP-dependent because diffusional uptake is negligible.

Our observation of the tolerance of *Atstp1* seedlings to exogenous sugars is particularly pertinent to numerous current studies that employ genetic screens to select mutant plants with altered responses to sugars (Pego *et al.*, 2000). A large number of *mig* mutants have been isolated (Pego *et al.*, 2000). It has previously been proposed that the toxic effect of D-mannose on plant cells is the result of ATP depletion or sequestration of phosphate as mannose-6-phosphate. However, Pego *et al.* (1999) argue that this is not the case in *Arabidopsis* seeds because ATP is not apparently depleted and addition of inorganic phosphate does not relieve inhibition of germination, whereas addition of D-glucose does. Based on our results, we would argue that addition of D-glucose would probably compete with D-mannose for transport by AtSTP1, resulting in less D-mannose uptake and less toxicity. Pego *et al.* (1999) suggest that the effects of D-mannose could be manifested through the activity of hexokinase, which is proposed to be a key component of a sugar-sensing system in plants (Jang and Sheen, 1997; Smeekens and Rook, 1997). The molecular characterization of *mig* mutants is ongoing, but some are mutated in genes required for response to abscisic acid (Huijser *et al.*, 2000; Laby *et al.*, 2000). We find no change in sensitivity of *Atstp1* to abscisic acid (results not shown). The results presented here clearly show that *Atstp1* is a *mig* mutant and that other monosaccharide transporter mutants are likely to be found in such screens.

Finally, there is indirect evidence to suggest that hexose transporters in plants may have a sugar-signalling function in addition to a transport function (Lalonde *et al.*, 1999). In mammals, glucose transporters are similarly implicated, but it is very difficult to distinguish signalling and transport functions (Antoine *et al.*, 1997). In yeast, there are two hexose-transporter-like proteins in the plasma membrane, which act as glucose sensors rather than transporters (Ozcan *et al.*, 1998). So far no such sensor proteins have been found in plants or animals. Therefore STP proteins should be considered as potential candidates for dual sugar transport and signalling proteins. The *Atstp1* mutant that we have isolated is the first such mutant from a higher plant, and now provides the opportunity to study such potential functions in detail.

Experimental procedures

DNA isolation and pooling strategy

T-DNA-transformed *Arabidopsis thaliana* populations were obtained from the Nottingham Arabidopsis Stock Centre (6500 lines [Wassilewskija ecotype, Ws] donated by Dr Kenneth Feldmann and 5000 lines [Columbia ecotype, Col] donated by Dr Thomas Jack), from E.I. Dupont de Nemours (7100 lines [Ws] donated by Dr Kenneth Feldmann) and from INRA-Versailles (7900 lines [Ws]) as pools of 100 lines. Plant material was prepared from each pool as described by Krysan *et al.* (1996), except that 2% (w/v) glucose in the liquid culture medium was replaced by sucrose, and the flasks containing the sterilized seeds were placed at 4°C for 24 h before transferring to a shaker under continuous light. DNA preparations were originally performed as described by Krysan *et al.* (1996), however, subsequent use of a DNeasy plant maxi kit (Qiagen, Hilden, Germany) was preferred as this significantly enhanced the sensitivity of PCR reactions. Following spectrophotometric quantification and visualization on an ethidium bromide stained gel against a standard, a fraction of each preparation was adjusted to 10 ng μl^{-1} to give a working stock solution. DNA superpools were generated by combining aliquots from between seven and 10 pools (700–1000 lines) for use in the initial screening experiments.

*PCR amplification and detection of T-DNA/*AtSTP1 *junction sequences*

The positions of PCR primers used to screen for a T-DNA insertion in the At*STP1* gene are shown in Figure 1(a), with *STP1*1885S (5′-TTCTTTCAACAGCTAACCGGAATCA-3′) and *STP1*2384A (5′-ACTCTGCGCCGCCGACCTTATCTCC-3′) being used in initial experiments. *STP1*726S (5′-GTATCTTGCTTGGTTTCGGTATCGG-3′) and *STP1*2219A (5′-CTTGGCCCCTATGCAAGCTGCAACC-3′) were designed later and were used to confirm the presence of the T-DNA and to obtain junction fragments for DNA sequence analysis. PCR was performed in 50 μl reactions containing 50 ng of the superpool DNA in 1 × PCR buffer (50 mM Tris–HCl pH 8.3, 500 μg ml^{-1} BSA, 0.5% (w/v) Ficoll, 1% (w/v) sucrose, 30 mM KCl, 3 mM $MgCl_2$, 1 mM tartrazine), 1 unit of Taq polymerase (Pluthero, 1993), 10 pmol of either the left-border or right-border primer, 10 pmol of either the sense (S) or antisense (A) *STP1* gene-specific primer, and 0.1 mM dNTP (Sigma, Poole, Dorset, UK). Each reaction was overlaid with mineral oil (40 μl). Control reactions on wild-type DNA were always performed and were spiked with the complementary gene-specific primer (positive controls) or not (negative controls). The PCR protocol began with a 30 sec incubation at 94°C, followed by 35 cycles of 94°C, 30 sec; 55°C, 30 sec; 68°C, 2 min; and ended with a 2 min incubation at 68°C. The product (20 μl) was run on a 0.8% (w/v) agarose gel and transferred to positively charged nylon membranes (Hybond N+ Amersham, Braunschweig, Germany) using standard protocols (Sambrook *et al.*, 1989). Full-length *AtSTP1* cDNA was labelled using the random primer method and used to hybridize the

membranes according to the manufacturer's instructions. Filters were exposed to X-ray film for 30 min, 3 h, and overnight to check for signal specificity.

Sequencing T-DNA AtSTP1 *PCR products*

The T-DNA *AtSTP1* junctions were PCR-amplified using the gene-specific primers *STP1*726S and *STP1*2219A with the T-DNA left-border primer TAG5 (5′-CTACAAATTGCCTTTTCTTATCGAC-3′). PCR products were purified using a QIAquick gel extraction kit (Qiagen) and subjected to cycle sequencing (Perkin Elmer ABI Prism 377 DNA sequencer). The sequencing reactions were primed with the T-DNA left-border primer.

Identifying Atstp1 *homozygote and wild-type segregants*

DNA samples from young rosette leaves of individual F_1 plants were prepared according to Edwards *et al.* (1991). PCR reactions were performed on 1 μl aliquots of each sample using *STP1*726S and *STP1*2219A, absence of a product indicating the presence of homozygous T-DNA. Wild-type segregants were identified as those failing to give a PCR product with TAG5 and *STP1*2219A primers, and one such plant was used as the Ws control throughout.

Northern analysis

Total RNA was isolated using an RNeasy plant mini kit (Qiagen). The RNA was separated by electrophoresis through formaldehyde-containing agarose gels and transferred to positively charged nylon membranes (Hybond N$^+$, Amersham) using standard protocols (Sambrook *et al.*, 1989). The hybridization probe was a ^{32}P-labelled 500 bp *Sac*I fragment from the 5′ end of the *AtSTP1* cDNA.

Sugar uptake studies

Seedlings were grown in continuous light (30 μmol m^{-2} sec^{-1}) on vertical agar plates containing 1 × Gamborg's B5 medium, 0.5% w/v MES, 0.8% agar pH 5.7. After 7 days, 5–10 mg of tissue (five to ten seedlings) was immersed in 0.2 ml Gamborg's B5 medium, vacuum infiltrated using a Venturi water pump for 3 min, and left at room temperature (20°C) for 30 min to equilibrate. 0.1 μCi of the [^{14}C]-labelled sugar to be tested (55–300 mCi mmol^{-1}) was added in 10 μl water and incubated at room temperature for up to 30 min. The label was removed and the tissue washed five times (1 min each) with 1 ml ice-cold Gamborg's B5 medium containing 1 mM of the unlabelled sugar. Finally, the samples were extracted twice with 1 ml 80% (v/v) ethanol, and the amount of label in the combined soluble extracts (in excess of 90% of the label) and in the tissue determined by liquid scintillation counting. For autoradiography, seedlings were immersed in 1 ml Gamborg's B5 medium containing 2 μCi [^{14}C]-3-O-methylglucose (specific activity 55 mCi mmol^{-1}), vacuum infiltrated, and incubated for 5 min. Following five washes in 1 l ice-cold water, seedlings were positioned on acetate sheets, covered with cling-film and freeze-dried. Samples were exposed to X-ray film for 2 weeks.

Growth tests

Basal growth medium (see Sugar uptake studies) was supplemented with different sugars (D-glucose, D-galactose and D-mannose) at the concentrations indicated. Abscisic acid was tested at 0.01, 0.1 and 1 μM in the absence of sugars. In all cases, agar plates were stored at 4°C for 48 h prior to transfer to dark or continuous light (30 μmol m^{-2} sec^{-1}) at 23°C. Seedling growth was recorded after an additional 7 days.

Construction of transgenic plants

The *AtSTP1* cDNA and terminator sequences were ligated into pBI121 (Clontech, Basingstoke, Hampshire, UK) downstream of the CaMV 35S promoter (R. Stadler and N. Sauer, unpublished results). An *Eco*RI–*Hin*dIII partial digest was used to transfer the entire chimeric gene into pGreen0229 (Hellens *et al.*, 2000). *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) was used to treat *Atstp1* plants by vacuum infiltration (Bechtold *et al.*, 1993). Transformants were selected on 40 μg ml^{-1} hygromycin B.

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