Running title:
Sucrose transport into wood fibers

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Aspen SUCROSE TRANSPORTER 3 allocates carbon into wood fibers

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Reduction of a plasma membrane localised sucrose transporter decreases carbon allocation to secondary walls of wood fibers.
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Abstract
Wood formation in trees requires carbon import from the photosynthetic tissues. In several tree species, including *Populus* sp. the majority of this carbon is derived from sucrose transported in the phloem. The mechanism of radial sucrose transport from phloem to developing wood is not well understood. We investigated the role of active sucrose transport during secondary cell wall formation in hybrid aspen (*Populus tremula x tremuloides*). We show that RNAi mediated reduction of *PttSUT3* during secondary cell wall formation in developing wood caused thinner wood fiber walls accompanied by a reduction in cellulose and an increase in lignin. Sucrose content in the phloem and developing wood was not significantly changed. However, after $^{13}$CO$_2$ assimilation the *SUT3RNAi* lines contained more $^{13}$C than wild type in the sucrose containing extract of developing wood. Hence sucrose was transported into developing wood, but the sucrose derived carbon was not efficiently incorporated to wood fiber walls. A YFP:PttSUT3 fusion localized to plasma membrane suggesting that reduced sucrose import into developing wood fibers was the cause of the observed cell wall phenotype. The results show the importance of active sucrose transport for wood formation in a symplasmically phloem loading tree species, and identify PttSUT3 as a principal transporter for carbon delivery into secondary cell wall forming wood fibers.
Introduction

In trees the majority of the assimilated carbon dioxide accounting for biomass increase is deposited in the secondary cell walls of wood. In several tree species most of this carbon is derived from sucrose imported from leaves (Turgeon, 1996). Sucrose transport from leaves to wood occurs in the phloem sieve elements where a pressure flow mechanism is thought to drive the movement of solutes from source to sink tissues (Knoblauch and Peters, 2010; Münch, 1930). In Münch’s pressure flow model a high source tissue solute concentration causes an osmotic pressure driving the uptake of water and subsequent flow of water and solutes towards sink tissues with lower solute concentration and osmotic pressure. In most tree species including *Populus* sp., phloem loading is thought to occur passively through the symplasm (Davidson et al., 2011; Fu et al., 2011). In this process sucrose is thought to move passively by diffusion through plasmodesmata connecting mesophyll cells and into the phloem sieve element/companion cells of the source leaves. In addition to phloem loading in leaves the maintenance of the source to sink flow requires that the sink tissues are actively lowering the phloem solute concentration either through metabolism or export (Münch, 1930; Van Bel, 2003). It can therefore be hypothesised that active sucrose transport from the phloem into developing wood may be important for wood formation in sucrose transporting trees.

Our knowledge of the molecular mechanisms involved in the radial phloem to developing wood sucrose transport is very limited. In trees this transport of sucrose is thought to occur mainly through ray cells. Ray cells are symplasmically connected parenchyma cells, which have an important function in the nutrient exchange between phloem and wood (Van Bel, 1990). Symplasmic connections between phloem and ray cells appear to be rare in most investigated plant species (Van Bel, 1990). However, in *Populus x canadensis* wood symplasmic phloem to ray transport appeared to dominate (Sauter and Kloth, 1986). Sauter and Kloth (1986) estimated the minimum radial carbon flux rate from the rate of starch accumulation in ray cells, concluding that only a symplasmic radial carbon transport route could explain the observed starch accumulation rates (Sauter and Kloth, 1986). Additional support for the predominance
of the wood ray cell route in phloem unloading was provided by stem section autoradiographs of 14CO2 labelled 2-year-old spruce trees showing clear labelling of ray cells (Langenfeld-Heyser, 1987).

Export of solutes from ray cells to the developing wood is thought to occur primarily across so called contact pits located between ray cells and fibers (Barnett, 1981) (Van Bel, 1990). The mature pits are spherical porous openings crossing the cell walls of two neighbouring cells allowing solute transport between lignified cells, whereas developing pits have been observed to contain symplasmic connections in some species but not in others (Barnett, 1981). For example electron microscopy images of differentiating tracheids in Pinus radiata wood did not show symplasmic connections (Barnett and Harris, 1975), whereas clear connections were documented in the developing fiber pits of Aesculus hippocastanum (Barnett, 1981). Anatomical studies of developing pit structure in Populus are limited, but investigation of symplasmic connectivity in the developing wood of Populus tremula x tremuloides using symplasmic fluorescent tracers did not reveal connections between rays and developing vessels and fibers (Sokolowska and Zagorska-Marek, 2012). This observation suggested that during wood formation sucrose is exported across the ray cell plasma membrane and imported across the plasma membrane of the developing fibers and vessels. Thus, at least in Populus this step in the radial wood sucrose import pathway may involve active transport.

Sucrose/H+ symporters (SUTs) facilitating active sucrose import into cytosol have been identified in several plant species (Kuhn and Grof, 2010; Lalonde et al., 2004). According to the current nomenclature SUTs are classified into groups I – IV based on their phylogenetic relationship (Sauer, 2007). The sequenced Populus trichocarpa genome encodes for five functional SUTs representing group II (PtSUT1 and PtSUT3), group III (PtSUT5 and PtSUT6) and group IV (PtSUT4) (Payyavula et al., 2011). Quantitative reverse transcriptase PCR (qPCR) analysis of SUT transcript levels in greenhouse grown Populus tremula x alba showed PtaSUT1 transcripts in phloem and roots, PtaSUT3 transcripts primarily in stems undergoing secondary growth, and PtaSUT4, PtaSUT5 and PtaSUT6 transcripts ubiquitously throughout the tree (Payyavula et al., 2011). Interestingly, microarray transcript profiling of different wood developmental stages in greenhouse grown Populus tremula x tremuloides...
showed an increase in *PttSUT1* and/or *PttSUT3* transcript levels during secondary cell wall formation (Hertzberg et al., 2001). The coding sequences of *PttSUT1* and *PttSUT3* are 90% identical, hence the cDNA microarray probe may have hybridised to both transcripts. The expression patterns of the other *PttSUTs* during wood formation is not known since the microarray used by Hertzberg et al., (2001) only included a probe for *PttSUT1/PttSUT3*.

Of the *Populus* *SUTs* only the *PtaSUT4* has been studied in detail. Similar to orthologous Group IV SUTs from barley and Arabidopsis (Endler et al., 2006; Schneider et al., 2012) a GFP:*PtaSUT4* fusion protein was localised to the vacular tonoplast membrane in tobacco protoplasts (Payyavula et al., 2011). In agreement with the qPCR results, *in situ* hybridisation of *PtaSUT4* transcripts in source leaf lamina and stem cross sections showed a clear signal supporting a role for *PtaSUT4* in sucrose transport in these tissues. Reduction of *PtaSUT4* expression in greenhouse grown *Populus tremula x alba* using a 35S promoter driven *SUT4RNAi* construct resulted in sucrose accumulation in source leaves, phloem and developing wood (Payyavula et al., 2011). No effect on wood anatomy or biosynthesis was reported for the *PtaSUT4RNAi* lines, but the lines did show a modest reduction in total shoot biomass (Payyavula et al., 2011). Payyavula et al. (2011) interpreted the *PtaSUT4RNAi* phenotype to reflect an important role of tonoplast sucrose transport in modulation of sucrose export from leaves as well as maintenance of sucrose homeostasis in sink tissues.

To explore the role of active sucrose transport in the radial phloem to wood pathway we first quantified transcript levels of all *Populus tremula* *SUTs* at different wood developmental stages from cambium to maturation/cell death. This analysis established that the previously observed increase in *PttSUT1* and/or *PttSUT3* transcript levels during secondary cell wall formation (Hertzberg et al., 2001) was due to *PttSUT3*. The group II SUTs, to which also *Populus* *SUT3* belongs, have been mainly associated with phloem loading and transport in apoplasmically loading species such as potato, tobacco and Arabidopsis (Burkle et al., 1998; Gottwald et al., 2000; Riesmeier et al., 1994). However the function of group II SUTs in symplasmic phloem loaders such as *Populus* sp. has not been investigated nor has the role of SUTs during secondary cell wall biosynthesis in wood. To examine the function of
Populus SUT3 during wood formation we generated transgenic *Populus tremula x tremuloides* SUT3RNAi lines where the RNAi construct was expressed under a promoter driving expression during secondary cell wall formation. Reduction of *PttSUT3* transcript levels in developing wood led to decreased carbon allocation to secondary cell walls as shown by thinner fiber cell walls, while at the same time the accumulation of $^{13}$C in developing wood after $^{13}$CO$_2$ supply was not reduced. Thus, our results supported a central role for *Populus* SUT3 in sucrose import to secondary cell wall forming wood fibers.

Results

Characterisation of SUT expression during wood formation

*Populus trichocarpa* genome encodes for five sucrose transporters (*PtSUT1, PtSUT3, PtSUT4, PtSUT5* and *PtSUT6*) (Payyavula et al., 2011). To investigate the expression of *Populus SUTs* at different stages of wood formation we analysed SUT transcript levels in cambium, early expansion, late expansion, secondary cell wall and maturation/cell death zones using qPCR. Samples were harvested from ca. 45-year-old *Populus tremula* trees on the 7th of July 2010 at Mullkälen, Sweden. Anatomical inspection and viability staining of wood showed that all trees were undergoing active wood formation (Figure S1). Total RNA from frozen microtome-cut sections corresponding to cambium, early expansion, late expansion, secondary cell wall and maturation/cell death zone was isolated from three independent trees. qPCR with gene specific primers showed *PtSUT1* transcript levels to be low in the cambium and below detection in the developing wood. *PtSUT4, PtSUT5* and *PtSUT6* transcript levels were stable across the developing wood, while *PtSUT3* transcripts were highest in the cambium, low in the early expansion zone and increased in late expansion zone to peak during secondary cell wall formation (Figure 1). The latter observation suggested a role for *PtSUT3* in sucrose transport to secondary cell wall forming cells.

*Populus* SUT3 is a plasma membrane localised sucrose transporter

*Populus* SUT3 is a functional sucrose transporter as shown by a previous study where *Populus tremula x alba* SUT3 complemented a yeast mutant deficient in sucrose
uptake from the growth media (Payyavula et al., 2011). In our work we used the hybrid aspen *Populus tremula x tremuloides* as a model system to investigate *PttSUT3* function during secondary cell wall biosynthesis. To determine the subcellular location of *PttSUT3* a *YFP:PttSUT3* fusion construct was transiently expressed in *Nicotiana benthamiana* leaf epidermis. YFP:SUT3 signal was not observed on the vacuole side of the chloroplasts (Figure 2). Furthermore plasmolysis experiments clearly showed the presence of YFP:PttSUT3 in Hechtian strands characteristic of plasma membrane localisation (Figure 2). Thus, similar to the other characterised dicot group II transporters (Sauer 2007), *PttSUT3* imports sucrose across the plasma membrane.

**SUT3RNAi increased the internode number and decreased the total leaf area**

To investigate the function of *PttSUT3* during secondary cell wall formation in wood we expressed a *SUT3RNAi* construct under the control of the *Populus trichocarpa GLYCOSYLTRANSFERASE43B* (*GT43B*) promoter. The *SUT3RNAi* sequence corresponds to a 140bp fragment of *PttSUT3* coding sequence (Table S1). The RNAi sequence is also similar to *PttSUT1* but not to the other *PttSUTs*. However *Populus SUT1* is not expressed in developing wood (Figure 1 and Payyavula et al. 2011), whereas *GT43B* is expressed during secondary cell wall formation while the expression in other tissues is low or absent (Figure S2) (Aspeborg et al., 2005; Lee et al., 2011). *In situ* hybridisation with a *GT43B* probe showed specific labelling of cells undergoing secondary cell wall biosynthesis in *Populus* (Zhou et al., 2007). The *GT43B* promoter driven *SUT3RNAi* is therefore designed to target *SUT3* in developing wood. Agrobacterium mediated transformation of stem pieces resulted in 18 independent *SUT3RNAi* transgenic lines. Three independent transgenic lines were selected for further characterization based on reduced *PttSUT3* transcript levels in the stems of *in vitro* grown trees. The lines were amplified from cuttings, named *SUT3RNAi* line 1, line 2 and line 3 and used in all subsequent experiments.

*SUT3RNAi* lines and the corresponding wild type (WT) were grown on soil under greenhouse conditions for eight weeks. qPCR analysis of *PttSUT3* transcript levels in developing wood showed that the expression of *PttSUT3* was reduced to 40 – 46% of WT in the transgenic trees (Figure 3a). The developing wood used for RNA isolation also included cambium where the *SUT3* expression is expected to be high (Figure 1),
but where the _GT43B::SUT3RNAi_ was expected not to be expressed. Hence the reduction in _PttSUT3_ expression during secondary cell wall formation may be more pronounced than indicated by the qPCR results.

The greenhouse grown two-month-old _SUT3RNAi_ lines showed a moderate but significant reduction in height as well as stem thickness compared to WT (Figure 4a and Table 1). There was also a clear decrease in the rate of leaf growth in the _SUT3RNAi_ lines resulting in ca. 50% reduction in final leaf size (Figure 4c). The difference in leaf size was less pronounced in young leaves, but started to increase after leaf number 7 counted from the top (Figure 4c). The linear leaf expansion phase had shifted from leaf 7 to leaf 13 in WT to leaf 9 to leaf 15 in _SUT3RNAi_ (Figure 4c). Hence the duration of the leaf expansion phase was similar between WT and _SUT3RNAi_, but the rate of leaf expansion was decreased resulting in smaller leaves. The average number of internodes per stem length increased from 0.3 in WT to 0.4 internodes per centimetre in the _SUT3RNAi_ lines (Table 1). Hence the effect of smaller leaves on the total photosynthetic area was partly alleviated by the increase in internode (leaf) number.

**_SUT3RNAi_ trees had thinner wood fiber walls**

Light microscopy and transmission electron microscopy (TEM) analyses were used to investigate the effect of _SUT3RNAi_ on wood anatomy. Visual inspection of transverse sections of stem 20cm above soil revealed no obvious change in the overall anatomy of _SUT3RNAi_ (Figure 5a). All _SUT3RNAi_ lines showed clearly thinner mature fiber walls compared to WT (Figure 5b). Quantification of the fiber wall area in four biological replicates per line confirmed this phenotype (Figure 5c). TEM images of mature fiber walls showed the difference in wall thickness to be due to thinner secondary cell wall (Figure 6).

Comparison of mature wood fiber walls between _SUT3RNAi_ and WT using cell specific Fourier transform/infrared (FT-IR) microspectroscopy combined with orthogonal projection to latent structures discriminant analysis (OPLS-DA) (Gorzsas et al., 2011), showed a difference in the fiber wall chemotype in all lines (Figure S3). The major differences in the FT-IR spectra of the _SUT3RNAi_ lines included a decrease in the intensity of the –C=O vibration at around 1740 cm\(^{-1}\) (Figure S3).
Since cellulose does not contain –C=O bonds, the source of this change is likely to be derived from hemicelluloses and/or pectins. Considering the relative amounts of major biopolymers in secondary cell walls, these changes are more likely to originate from hemicelluloses than pectins. This was further supported by the changes observed in spectral region of 900-1100 cm\(^{-1}\), which is dominated by various vibrations associated with carbohydrates. There was also a shift of the –C-O band from around 1250 cm\(^{-1}\) to 1210 cm\(^{-1}\) indicating to more flexible, possibly less cross-linked, cell wall polymers. Moreover, bands associated with lignin (aromatic -C=C- vibrations at 1510 and 1595 cm\(^{-1}\)) showed significant changes, also pointing towards a less cross-linked structure in the \textit{SUT3RNAi} lines (decreased 1510/1595 band ratio, (Zhong et al., 2000)). However, it is unclear from the FTIR spectra whether the observed cross-linking changes are attributable to lignin alone, or originate from changes in the cross-links between lignin and cell wall carbohydrates.

To further investigate how cell wall biosynthesis was affected in \textit{SUT3RNAi} lines the proportions of the main wall polymers in extractive and starch free wood were quantified in line 1 and 3. Klason lignin analysis revealed a small but significant increase in the proportion of lignin in both RNAi lines (Figure 7a). Updegraff cellulose analysis showed a slight albeit statistically non-significant decrease in the proportion of cellulose for line 1 and 3 (Figure 7b). Analysis of cell wall monosugars revealed a significant decrease in total cell wall monosugars in line 1, which was due to a decrease in mannose and galacturonic acid (Figure 7c). However, this decrease was not observed in line 3. When estimating the amount of carbon allocated to wood it is often more meaningful to normalise cell wall data against wood volume rather than dry weight. Hence to estimate whether the amount of cellulose and lignin per volume of wood was reduced the wood bulk density was first measured and defined as dry weight per wet volume. Wood density was decreased in line 1 by approx. 12% and in line 3 by approx. 7% compared to WT (Table 2). Using the density values to derive the cellulose and lignin amounts per volume of wood revealed a clear cellulose reduction in both transgenic lines while lignin was slightly increased (Figure 7d).

Differences in wood density may be caused by changes in overall lumen to cell wall ratio. Such changes may for example occur as a result of altered vessel to fiber ratio, differences in cell number or the morphology of xylem cells. No difference in the
number of fibers or in the relative fiber area between WT and SUT3RNAi line 1 and 3 was observed (Table 2). Hence the reduced wood density in SUT3RNAi was most likely due to a reduction in the fiber cell wall thickness and a corresponding increase in fiber lumen size.

In summary the SUT3RNAi reduced the wood density and thickness of the secondary cell walls of wood fibers, and this was accompanied by a decrease in cellulose and an increase in lignin. In addition the FT-IR analysis indicated to a changed fiber wall structure in all transgenic lines.

SUT3RNAi had no effect on CO2 assimilation
Reduced level of Group II SUT transcripts have been associated with reduced photosynthesis in tobacco (Burkle et al., 1998). Several reports have also shown a positive correlation between sink strength and photosynthetic efficiency of source leaves in several plant species including soybean, sugar cane and Populus (Clough et al., 1981; Coleman et al., 2008; McCormick et al., 2006). These observations raised the possibility that the SUT3RNAi wood phenotype could be due to general carbon limitation. To investigate this we measured the source leaf CO2 assimilation rate under different photosynthetic active light intensities. No difference between SUT3RNAi lines and WT was observed (Figure S4) establishing that reduced photosynthetic efficiency was not the cause of the observed wood phenotype. However, it remained possible that the reduction in source leaf size in SUT3RNAi (Figure 4) may have reduced the amount of carbon available for wood biosynthesis.

Specific reduction of carbon allocation to fiber walls in SUT3RNAi
To further elucidate how SUT3RNAi was inhibiting carbon allocation to wood secondary cell walls we first measured the levels of sucrose, glucose and fructose in phloem and developing wood. No significant difference in any of the sugars was observed in the phloem (Figure 8a). In developing wood line 3 exhibited decreased glucose and fructose levels (Figure 8b), which could suggest reduced carbon flux from sucrose to the hexose pools. Line 1 showed an increase in the sucrose level in developing wood, and although not significant this may explain why no reduction in the total glucose and fructose pool was observed in this line. When the sucrose to
hexose ratio was compared, all of the *SUT3RNAi* lines showed an increase in developing wood (Figure 8c).

Analysis of the soluble sugar pool sizes gives limited information on the status of carbon flux into developing wood. Therefore to gain a better understanding of how *SUT3RNAi* is affecting carbon allocation at the whole tree level we developed a $^{13}$CO$_2$ isotope labelling system to study carbon allocation from photosynthetic tissues to developing wood. In this experiment eight-week-old greenhouse grown trees were placed in sealed transparent plastic tent with controlled temperature and supplied a pulse of $^{13}$CO$_2$ followed by sampling in ambient CO$_2$. After $^{13}$CO$_2$ assimilation part of the $^{13}$C is exported to the developing wood, presumably mostly in the form of sucrose. Hence the measurement of $^{13}$C amount in developing wood after $^{13}$CO$_2$ assimilation can be used to estimate carbon import to developing wood. To do this the amount of $^{13}$C in the total ethanol soluble fraction of developing wood was quantified using elemental analyzer-isotope ratio mass spectrometer (EA-IRMS). We first established a suitable $^{13}$CO$_2$ incubation time and the time it took for $^{13}$C isotope label to reach the developing wood in WT trees. A 4-hour $^{13}$CO$_2$ pulse and sampling at 0, 4, 8 and 12 hours showed that the $^{13}$C was detected in the developing wood after 4 hours and continued to accumulate at least until 12 hours (data not shown). An 8-hour timeframe was therefore deemed suitable for comparison of $^{13}$C translocation rate between WT and *SUT3RNAi* trees.

*SUT3RNAi* and WT trees were given a 4-hour pulse of $^{13}$CO$_2$, and samples were harvested at 0, 4 and 8 hours. Interestingly all of the *SUT3RNAi* lines accumulated more $^{13}$C in the soluble fraction of developing wood compared to WT. This $^{13}$C accumulation was apparent in line 1 and 3 after 4 hours and in all lines after 8 hours, with lines 1 and 3 showing significant label accumulation (Figure 9).

In summary the unchanged total sucrose pool in developing wood and the $^{13}$C accumulation in the ethanol soluble fraction suggested that sucrose transport into the developing wood of the *SUT3RNAi* lines was not limiting. Instead the incorporation of carbon from soluble fraction into secondary cell walls was reduced as shown by the thinner wood fiber walls. A likely explanation for this observation is that the sucrose
import into the secondary cell wall forming cells was inhibited in the SUT3RNAi lines.

**Discussion**

Molecular mechanisms of carbon allocation to wood are poorly understood, but are likely to involve active transport of sugars. Sucrose is the main transported form of carbon in several trees and sucrose transporters (SUTs) responsible for the proton gradient driven transport of sucrose have been identified from several tree species including *Betula pendula* (Wright et al., 2000), *Juglans regia* (Decourteix et al., 2006) and *Populus* sp. (Payyavula et al., 2011). Here we investigated the role of SUTs in carbon import into secondary cell wall forming wood fibers of aspen.

Transcript profiling of the SUT gene family across developing wood indicated a role for *PtSUT3* in cambium and during secondary cell wall formation (Figure 1). The high cambial *PtSUT3* transcript levels correlated with fast dividing cambial cells requiring sucrose for the biogenesis of new cells. In the early and late expansion zone xylem cells undergo primary wall biosynthesis and turgor pressure driven growth (Mellerowicz et al., 2001) during this stage the *PtSUT3* transcripts levels decreased relative to cambium. A clear increase in *PtSUT3* transcript levels was observed during secondary cell wall formation when majority of the wood biomass is formed, and the levels decreased again towards the maturation and cell death zone (Figure 1). Thus, *PtSUT3* transcript levels in developing wood correlated with carbon demand for radial growth.

To investigate the function of PtSUT3 during secondary cell wall biosynthesis in wood we used the promoter of *GT43B* to drive the expression of a SUT3RNAi construct. We reasoned that the use of *GT43B* promoter for SUT3RNAi expression was likely to prevent substantial effects on cambial PtSUT3 expression, which may have led to severe growth phenotypes. Three independent SUT3RNAi lines with 40 – 46% remaining PtSUT3 transcript in developing wood were selected for characterisation. The reduction in PtSUT3 transcripts correlated with reduced fiber wall area in all SUT3RNAi lines (Figure 5). Thinner secondary walls of fibers are a reliable indicator of reduced carbon allocation to wood. A similar phenotype was
observed in transgenic hybrid aspen with reduced wood fructokinase activity, which led to a reduction in cellulose (Roach et al., 2012). We also observed reduced cross-linking of fiber wall polymers in SUT3RNAi by FT-IR, possibly caused by changes in the cell wall polymer proportions.

Quantification of extractive free wood polymer proportions per dry weight showed a significant increase in lignin accompanied by a tendency to decreased cellulose in SUT3RNAi (Figure 7a and 7b). The changes in the proportions of wood cell wall polymers were most likely reflecting the primary effect of SUT3RNAi on the carbohydrate fraction of secondary cell walls – the amount of lignin per gram dry weight increased when cellulose decreased. The nature of cell wall changes became more obvious when the decrease in the SUT3RNAi wood density was taken into consideration. Per volume of wood SUT3RNAi lines contained clearly less cellulose whereas lignin content was modestly increased (Figure 7d). Carbon for lignin biosynthesis is also derived from the imported sucrose, however lignification continues in the maturation/cell death zone where SUT3 expression is low and SUT3RNAi is expected not to be active (Figure 1 and Figure S2). At this stage carbon for lignin may be supplied by ray cells, which remain alive in the maturation/cell death zone. This hypothesis is supported by recent evidence from Zinnia elegans cell culture and Arabidopsis experiments showing that lignification continues after cell death and that this process is supported by the adjacent living parenchymatic cells (Pesquet et al., 2013).

In addition to the wood fiber wall phenotypes the height and diameter growth of SUT3RNAi lines was modestly affected (Table 1). All lines also showed a clear increase in the average number of internodes per centimetre of stem (from 0.3 to 0.4) accompanied by ca. 50% decrease in mature leaf size (Table 1 and Figure 4). Although the SUT3RNAi was expected to be primarily active in secondary cell wall forming cells, a direct effect on young leaves and shoot tip cannot be excluded. A general sink role for the walnut tree (Juglans regia) group II SUT (JrSUT1) was postulated based on high JrSUT1 transcript levels in diverse sink tissues such as roots, female flowers and stem while the transcripts were barely detected in source leaves (Decourteix et al., 2006). However, qPCR analysis detected no PtaSUT1 transcripts in shoot tip and developing leaves, and only very low levels of PtaSUT3 arguing against
a function of *Populus* group II SUTs in these sink tissues (Payyavula et al., 2011). Hence it is possible that the leaf area and number was influenced indirectly by the reduction of *PttSUT3* in developing wood. In this context it is interesting that woody species transport significant amounts of carbon in xylem, for example 9 – 28% of the carbon delivered to leaves in 3-month-old *Populus* trees over a diurnal cycle was derived from sugars transported by the transpiration stream (Mayrhofer et al., 2004). It is possible that *SUT3RNAi* reduced the amount of carbon transported to shoot tips and growing leaves in the transpiration stream. However, further experiments are needed to investigate how *SUT3RNAi* affected internode number and leaf area.

The shoot growth phenotypes complicated the interpretation of *SUT3RNAi* effect since a decrease in photosynthetic area may have reduced the amount of carbon available for wood secondary cell wall biosynthesis. However, we did not observe significant differences in sucrose levels in the developing wood (Figure 8), suggesting that sucrose was not limiting. Reduced group II *SUT* expression has been shown to result in reduced CO₂ assimilation in tobacco (Burkle et al., 1998) raising the possibility that the carbon flux to wood may have been reduced in *SUT3RNAi* even though the wood sucrose pool size was similar to WT. However *SUT3RNAi* had no effect on the rate of CO₂ assimilation (Figure S4), and the ^13^CO₂ pulse-chase experiments showed increased ^13^C accumulation in developing wood in the *SUT3RNAi* lines showing that sucrose movement from photosynthetic tissues into developing wood was not constrained (Figure 9). Together with the observed thinner fiber walls and reduced wood density the accumulation of ^13^C in the soluble extract of the *SUT3RNAi* wood was likely due to reduced sucrose transport into secondary cell wall forming cells.

Lateral sucrose transport in developing wood is thought to occur symplasmically in the ray cells as discussed in the introduction. Solute transport in rays has been hypothesised to be facilitated by cytoplasmic streaming propelled by cytoskeletal components including microtubules, microfilaments and myosin (Chaffey and Barlow 2001), and may therefore be independent of SUT activity. The YFP:*PttSUT3* localised to the plasma membrane in epidermal cells of tobacco leaves in a transient expression assay (Figure 2) supporting a model where PttSUT3 is responsible for sucrose import across the plasma membrane during secondary cell wall formation in
developing wood. The mechanism of sucrose export from plant cells was recently revealed by the identification of sucrose efflux carriers (SWEETs) in Arabidopsis (Chen et al., 2012). *Populus* genome encodes for 31 SWEETs some of which are expressed in developing wood (Mahboubi and Niittylä, unpublished). Hence the current model of apoplasmic sucrose transport between neighbouring cells (Braun 2012) suggested that the *Populus* SWEETs are responsible for sucrose export from ray cells followed by SUT3 mediated sucrose import into fibers undergoing secondary cell wall formation. In the future it will be important to localize the PttSUT3 in developing wood to test this hypothesis. The possibility of symplasmic connections between ray cells and developing vessels/fibers also needs further investigation to assess the possible contribution of symplasmic transport.

Nonetheless our results suggested that symplasmic transport or apoplastic sucrose cleavage followed by hexose sugar import into the secondary cell wall forming wood fibers were unable to compensate for the reduction in PttSUT3 levels. Hence based on the presented data it is concluded that PttSUT3 imports carbon into the secondary cell wall forming wood fibers making this SUT a critical component in the wood formation of trees.

**Materials and Methods**

**Plant material and growth conditions**

Transgenic and wild type hybrid aspen (*Populus tremula × tremuloides*) trees were micro propagated and grown *in vitro* for four weeks and then transferred to soil. Trees were grown in the greenhouse at 20/15°C (light/dark) with 50-70% humidity and 18 h light / 6 h dark photoperiod. Approximately 1 litre of 0.2% Rika-S fertiliser (N/P/K 7:1:5, Weibulls Horto, [http://www.weibullshorto.se](http://www.weibullshorto.se)) was applied every seven days. Trees were harvested after 10 weeks in the greenhouse and samples frozen in liquid nitrogen and stored in -80°C. Developing wood used for gene expression, soluble sugar and 13C isotope analyses was obtained by peeling the bark and scraping the wood tissues from the bottom third of the stem excluding the section 0 - 20cm above soil. Phloem sample for sugar analysis was collected by scraping the inside of the
pealed bark from the same stem section. The remaining wood was cleaved to remove the pith, freeze-dried and used for wet chemistry analysis. Sections for anatomy, fiber wall area and FT-IR measurements were prepared from stem 20cm above soil.

Samples for SUT family transcript level analysis in different wood developmental zones were harvested from three 45-year-old aspen (Populus tremula) trees on the 7th of July 2010 at Mullkälen, Sweden. Trees were cut with a motor saw and stem pieces frozen in liquid nitrogen. Sections were made from different wood developmental zones (cambial, early expansion, late expansion, developing xylem and maturation/cell death zone) as described previously (Hertzberg et al., 2001).

**SUT3RNAi vector construction and hybrid aspen transformation**
The RNAi cassette was created in the pBluescript vector using a 140bp fragment of PttSUT3 in sense and anti-sense orientations. The RNAi cassette was then inserted into the pENTR-D-TOPO entry vector (Invitrogen, www.invitrogen.com), and moved into the pK2GW7 destination vector (Karimi et al., 2002) where the 35S promoter was replaced by a 1490bp promoter fragment of Populus trichocarpa GLYCOSYLTRANSFERASE43B. Hybrid aspen was transformed as described in (Nilsson et al., 1992).

**Subcellular localization of PttSUT3**
The coding sequence of PttSUT3 was cloned into pB7WGY2 binary vector (Karimi et al., 2002). Transient tobacco leaf agrobacterium mediated transformation was performed as described in (Sparkes et al., 2006), and YFP fluorescence imaged by Leica SP2 confocal microscope (http://www.leica-microsystems.com).

**Growth analysis**
For stem height the distance from the bottom of the stem to the shoot tip was measured. The stem diameter was measured 20 cm above soil. The number of internodes was counted from the first visible internode to the internode 20 cm above soil. The leaf area was measured using Image J software (rsbweb.nih.gov) from scaled leaf photographs.

**Transcript analysis**
Quantitative real time PCR was used for transcript abundance analysis. To assess the transcript level of all hybrid aspen SUTs along the wood developmental zones, tangential sections were made from different wood developmental stages using a cryotome. Total RNA was extracted from the sections using miRNeasy Micro Kit (Qiagen, www.qiagen.com) with on column DNase (Qiagen) treatment. 100 ng of total RNA was amplified using MessageAmp™ Premier RNA Amplification Kit (Ambion, http://www.invitrogen.com/ambion). Concentration of amplified RNA was adjusted to 250 ng/µL. To analyze the SUT3 transcript abundance in developing wood of greenhouse grown WT and SUT3RNAi lines developing wood including the tissue from cambium to cell death/maturation zone was scraped into liquid nitrogen and homogenised. RNA was isolated from the homogenised scrapings using Plant RNAeasy kit (Qiagen). qPCR was performed using CFX96 real time system (Bio-Rad, http://www.bio-rad.com/), and double stranded DNA detected with SYBR®Green (Bio-Rad). Based on the stability of expression 26S proteasome transcript level was used for normalisation in the wood developmental series. Actin and ubiquitin transcript levels were used as reference genes in total developing wood samples. Relative transcript level was calculated using ΔCq method (Pfaffl, 2001). Gene specific primers used for SUT transcript analysis are listed in Table S1.

**Wood anatomy and wet chemical analysis**

For wood anatomy analysis 1mm cross-sections were prepared from the middle of the internode located 20 cm above ground. Sections were fixed in 3% v/v glutaraldehyde containing 2% v/v paraformaldehyde in sodium cacodylate buffer (0.1 M, pH 7.2). Fixed sections were then dehydrated through ethanol series and embedded in LR white resin (www.agarscientific.com). Cross sections (0.5 µm) were cut using a microtome and the sections stained with toluidine blue. Mature wood cell wall images were recorded approximately 1 mm from the cambium using Leica DMLB light microscope and Leica DC300 camera (www.leicamicrosystems.com). Fiber cell wall area was calculated from the images using ImageJ software (rsbweb.nih.gov). Fiber cell number in mature wood was counted from the same images.

For cell wall polymer analysis freeze-dried mature wood was ground to powder using a ball mill. Extractive free wood was then prepared by extraction with 70% ethanol
for 30 minutes at 95°C, followed by chloroform:methanol (1:1) for 5 minutes at room temperature and two washes with acetone. To remove starch the wood powder was then treated overnight at 37°C with 1000U of α-amylase (Roche, http://www.roche.com) in 0.1 M potassium phosphate buffer, pH7. Crystalline cellulose content was determined using the Updegraff method (Updegraff, 1969) followed by Anthrone assay for detection of released glucose (Melvin, 1953). Lignin content was measured by the Klason lignin method (Fengel and Wegener, 2003). For quantification of hemicellulose sugars 0.5 mg of extractive free wood was methanolysed using 2 M HCL/methanol followed by tri-sil reagent (1,1,3,3,3-Hexamethyldisilazane + Trimethylchlorosilane + pyridine, 3:1:9) derivatisation using Sylon HTP kit (SUPELCO, http://www.sigmaaldrich.com) as described in (Sweeley et al., 1963). The monosugar contents were then determined on a gas chromatography-mass spectrometer Agilent 7890A/5975C (Agilent technologies, www.agilent.com).

**Wood density measurement**

Wood density was measured on an oven-dry weight per wet volume basis. Wood pieces were placed in water on a XA105 analytical balance with precision of 0.01 mg (Mettler Toledo, www.mt.com) and pushed under water to measure the sample volume based on displaced water weight. Samples were then oven dried at 102°C for 24 hours and weighed again to obtain the dry mass weight. The wood density was then estimated from the dry weight to wet volume ratio.

**Soluble sugar analysis**

Glucose, fructose and sucrose were extracted and measured as previously described in (Stitt et al., 1989) and (Roach et al., 2012).

**13CO2 labelling and 13C analysis**

Two-month-old trees were enclosed in a transparent tent and labelled with 0.3 moles of 13CO2 for 4 hours at 25°C and relative humidity of 65% to 75 %. The CO2 concentration inside the chamber was between 400-500 ppm as determined by a WMA-4 CO2 analyzer (www.ppsystems.com) calibrated for 13CO2. The labelled trees were harvested and the developing wood scraped and ground in liquid nitrogen.
Ethanol soluble extract from the developing wood was dried and analysed by Elemental Analyser Isotope Ratio mass spectrometer (www.thermofisher.com) to determine the $^{13}$C content.

**Photosynthesis rate measurement**

Light-response curve of photosynthesis rate was measured for WT and SUT3RNAi lines at different photon irradiances (0, 50, 100, 300, 700, 1200, 1500 and 1800 μmol s$^{-1}$ m$^{-2}$) using Licor portable gas exchange system (LI-COR 6400, http://www.licor.com).

**Acknowledgements**

We would like to thank Kjell Olofsson and Lenore Johansson for help with sample preparation and microscopy, Junko Takahashi-Schmidt for help with the wood wet chemistry analysis and Stephanie Robert for assistance with the confocal microscope.

**Figure legends**

**Figure 1.** PttSUT transcript levels during wood development. A, Illustration of wood developmental zones defined as, cambium, early expansion, late expansion, secondary cell wall and maturation adapted from Hertzberg et al. (2001). B, Relative transcript abundance of PttSUTs in different developmental zones of developing wood. Error bars represent standard error of mean (SEM) of three biological replicates.

Figure 3. *PttSUT3* transcript level in developing wood of the WT and SUT3RNAi lines. Error bars represent SEM of four biological replicates. Asterisks indicate P value comparison with WT. *P < 0.05 (Student’s t-test).

Figure 4. Phenotype of SUT3RNAi lines. A, Two-month-old greenhouse grown WT and SUT3RNAi trees. B, Comparison of WT and SUT3RNAi leaves. C, Leaf area in WT and SUT3RNAi. Error bars represent SEM of three biological replicates.

Figure 5. Wood anatomy and fiber wall area in WT and SUT3RNAi lines. A and B, Light microscopy images of representative stem sections. C, Fiber cell wall area of mature xylem fiber cells. Error bars represent SEM of four biological replicates. Asterisks indicate P value comparison with WT. *P < 0.05 (Student’s t-test).

Figure 6. Transmission electron microscopy images of representative mature wood fiber walls in WT (A) SUT3RNAi-1 (B), SUT3RNAi-2 (C), SUT3RNAi-3 (D). Cell wall layers are indicated as middle lamella (ML), S1 and S2.

Figure 7. Analysis of extractive and starch free wood in WT and SUT3RNAi lines. A, Acid insoluble lignin. B, Crystalline cellulose. C, Cell wall mono-sugars. D, Estimation of cellulose and lignin content per volume of wood. Error bars represent SEM of four biological replicates. Asterisks indicate P value comparison with WT. *P < 0.05 (Student’s t-test).

Figure 8. Analysis of sucrose, glucose and fructose content. A, in phloem and B, developing wood of WT and SUT3RNAi lines. C, Sucrose to hexose ratio in developing wood. Error bars represent SEM of four biological replicates. Asterisks indicate P value comparison with WT. *P < 0.05 (Student’s t-test).

Figure 9. Accumulation of $^{13}$C in the ethanol soluble fraction of developing wood in two-month-old trees labeled with $^{13}$CO$_2$. $^{13}$CO$_2$ was supplied for 4 hours and samples were harvested at 0, 4 and 8 hours after the start of the labeling. Error bars represent
SEM of three biological replicates. Asterisks indicate $P$ value comparison with WT.

* $P < 0.05$ (Student’s $t$-test).

SUPPLEMENTAL DATA

**Figure S1.** Anatomy and viability staining of developing wood used for transcript profiling of *PtSUTs*.

**Figure S2.** Expression of *GT43B* in hybrid aspen.

**Figure S3.** FT-IR analysis of mature wood fiber walls.

**Figure S4.** Analysis of photosynthesis rate in WT and *SUT3RNAi*.

**Table S1.** List of primers and the *SUT3RNAi* sequence.

REFERENCES


Chen LQ, Qu XQ, Hou BH, Sosso D, Osorio S, Fernie AR and Frommer WB (2012) Sucrose Efflux Mediated by SWEET Proteins as a Key Step for Phloem Transport. Science 335: 207-211


Muñch E (1930) Die Stoffbewegungen in der Pflanze. Gustav Fischer, Jena, Germany


Table 1. Diameter, height, internode and leaf number of WT and SUT3RNAi lines at harvest. Mean ± SEM. n = 7 biological replicates. Student’s T-test $P$ values are denoted as (*) < 0.05 and (**) < 0.01.

<table>
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<tr>
<th></th>
<th>Wild Type</th>
<th>SUT3RNAi-1</th>
<th>SUT3RNAi-2</th>
<th>SUT3RNAi-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem diameter (mm)</td>
<td>12.3 ± 0.3</td>
<td>11.3 ± 0.4*</td>
<td>10.6 ± 0.1**</td>
<td>11.3 ± 0.3*</td>
</tr>
<tr>
<td>Stem height (cm)</td>
<td>178 ± 1.7</td>
<td>167 ± 2.3 **</td>
<td>153 ± 2.8**</td>
<td>161 ± 1.7**</td>
</tr>
<tr>
<td>Internode number</td>
<td>58 ± 2</td>
<td>67 ± 2 **</td>
<td>59 ± 3</td>
<td>63 ± 2**</td>
</tr>
<tr>
<td>Average number of internodes per cm</td>
<td>0.3 ± 0.005</td>
<td>0.4 ± 0.007**</td>
<td>0.4 ± 0.004**</td>
<td>0.4 ± 0.004**</td>
</tr>
</tbody>
</table>
Table 2. Wood bulk density, fiber area and number of fiber cells per 10000 μm² in WT and SUT3RNAi lines. Mean ± SEM. n = 4 biological replicates. Student’s T-test P values are denoted as (*) < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
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<th>SUT3RNAi-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density (kg/m³)</td>
<td>295 ± 6</td>
<td>260 ± 11*</td>
<td>275 ± 10</td>
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<tr>
<td>Fiber area of total area (%)</td>
<td>80 ± 0.9</td>
<td>78 ± 0.6</td>
<td>79 ± 0.4</td>
</tr>
<tr>
<td>Number of fiber cells per 10000 μm²</td>
<td>58 ± 3</td>
<td>58 ± 3</td>
<td>59 ± 3</td>
</tr>
</tbody>
</table>
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