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**An investigation of phenolic glycoside and condensed tannin homeostasis in *Populus* by salicyl alcohol feeding to cell cultures and by transgenic manipulation of the sucrose transporter, *PTSUT4*, in planta**

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AN INVESTIGATION OF PHENOLIC GLYCOSIDE AND CONDENSED TANNIN  
HOMEOSTASIS IN *POPULUS* BY SALICYL ALCOHOL FEEDING TO CELL  
CULTURES AND BY TRANSGENIC MANIPULATION OF THE SUCROSE  
TRANSPORTER, *PTSUT4*, IN *PLANTA*

By

RAJA SEKHAR PAYYAVULA

A DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of

**DOCTOR OF PHILOSOPHY**

(Forest Molecular Genetics and Biotechnology)

MICHIGAN TECHNOLOGICAL UNIVERSITY

2009

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This dissertation, “An investigation of phenolic glycoside and condensed tannin homeostasis in *Populus* by salicyl alcohol feeding to cell cultures and by transgenic manipulation of the sucrose transporter, *PtSUT4*, *in planta*” is hereby approved in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the field of Forest Molecular Genetics and Biotechnology.

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**An investigation of phenolic glycoside and condensed tannin homeostasis in *Populus* by salicyl alcohol feeding to cell cultures and by transgenic manipulation of the sucrose transporter, *PtSUT4*, in planta**

**ABSTRACT**

Secondary metabolites play an important role in plant protection against biotic and abiotic stress. In *Populus*, phenolic glycosides (PGs) and condensed tannins (CTs) are two such groups of compounds derived from the common phenylpropanoid pathway. The basal levels and the inducibility of PGs and CTs depend on genetic as well as environmental factors, such as soil nitrogen (N) level. Carbohydrate allocation, transport and sink strength also affect PG and CT levels. A negative correlation between the levels of PGs and CTs was observed in several studies. However, the molecular mechanism underlying such relation is not known. We used a cell culture system to understand negative correlation of PGs and CTs. Under normal culture conditions, neither salicin nor higher-order PGs accumulated in cell cultures. Several factors, such as hormones, light, organelles and precursors were discussed in the context of aspen suspension cells' inability to synthesize PGs. Salicin and its isomer, isosalicin, were detected in cell cultures fed with salicyl alcohol, salicylaldehyde and helicin. At higher levels (5 mM) of salicyl alcohol feeding, accumulation of salicins led to reduced CT production in the cells. Based on metabolic and gene expression data, the CT reduction in salicin-accumulating cells is partly a result of regulatory changes at the transcriptional level affecting carbon partitioning between growth processes, and phenylpropanoid CT biosynthesis. Based on molecular studies, the glycosyltransferases, GT1-2 and GT1-246,

may function in glycosylation of simple phenolics, such as salicyl alcohol in cell cultures. The uptake of such glycosides into vacuole may be mediated to some extent by tonoplast localized multidrug-resistance associated protein transporters, PtMRP1 and PtMRP6.

In *Populus*, sucrose is the common transported carbohydrate and its transport is possibly regulated by sucrose transporters (SUTs). SUTs are also capable of transporting simple PGs, such as salicin. Therefore, we characterized the SUT gene family in *Populus* and investigated, by transgenic analysis, the possible role of the most abundantly expressed member, *PtSUT4*, in PG-CT homeostasis using plants grown under varying nitrogen regimes. *PtSUT4* transgenic plants were phenotypically similar to the wildtype plants except that the leaf area-to-stem volume ratio was higher for transgenic plants. In *SUT4* transgenics, levels of non-structural carbohydrates, such as sucrose and starch, were altered in mature leaves. The levels of PGs and CTs were lower in green tissues of transgenic plants under N-replete, but were higher under N-depleted conditions, compared to the levels in wildtype plants. Based on our results, SUT4 partly regulates N-level dependent PG-CT homeostasis by differential carbohydrate allocation.

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## Chapter 1 Literature review

Plants produce primary metabolites such as nucleic acids, proteins and sugars that are common to all species and are vital for survival. They also produce secondary metabolites such as phenolics, whose abundance, structure and function differ among species (Buchanan et al., 2000; Galeotti et al., 2008; Lewinsohn and Gijzen, 2009; Ohkatsu et al., 2008). The secondary compounds were once considered to be byproducts as their specific functions were not known. It is now well understood that these compounds play a major role in many aspects of plant development and defense (reviewed in Seigler, 1998). Many secondary metabolites are derived from the phenylpropanoid pathway (Dixon and Paiva, 1995; Petersen, 2007). Chorismic acid, formed in the shikimate pathway from erythrose 4-phosphate and phosphoenolpyruvate, is the precursor for synthesis of phenylalanine, the starting compound in the phenylpropanoid pathway (Petersen, 2007; Weisshaar and Jenkins, 1998). Phenylalanine ammonia-lyase (PAL) catalyzes deamination of phenylalanine to form cinnamate, and cinnamate 4-hydroxylase (C4H) catalyses the conversion of cinnamic to *para*-coumaric acid. *P*-coumaric acid and subsequently formed hydroxycinnamates are utilized by different pathways for the formation of salicylic acid, salicin, and salicin-containing phenolic glycosides (PGs), flavonoids, condensed tannins (CTs), and lignin (Boerjan et al., 2003; Tsai et al., 2006a; Weisshaar and Jenkins, 1998). In some species, salicylic acid synthesis can also proceed from chorismic acid upstream of PAL (Gaille et al., 2003; van Tegelen et al., 1999; Wildermuth et al., 2001). Since phenolic derivatives are often

bioactive, a majority of these compounds occur as glycosylated conjugates and are stored in the vacuole (Harborne, 1980). PGs and CTs, the two major phenylpropanoid derivatives found in *Populus*, play a major role in defense and adaptation to biotic and abiotic stress (Bryant et al., 1993; Donaldson and Lindroth, 2007; Galeotti et al., 2008; Lavola, 1998; Lee et al., 2008).

### 1.1 Phenolic glycosides

The structures and abundance of PGs vary among different *Populus* genotypes (Erwin et al., 2001; Harding et al., 2005; Lindroth and Hwang, 1996; Orians et al., 2000; Osier and Lindroth, 2006). Salicin, salicortin, tremulacin and tremuloidin are the major PGs in *Populus tremuloides*, while salicortin and HCH (hydroxy cyclohexenone)-salicortin were identified from the leaves of *Populus fremontii* and its F<sub>1</sub> hybrids with *Populus angustifolia* (Lindroth and Pajutee, 1987; Rehill et al., 2005). In *Populus tremuloides*, salicortin and tremulacin contribute to more than 90% of the PGs (Lindroth and Pajutee, 1987). Salicin is composed of a phenol ring and a glucose molecule, whereas higher-order PGs, such as salicortin, tremulacin and tremuloidin, have an additional benzene ring and/or HCH ring (Kammerer et al., 2005). The stability and turnover rate of these compounds in plants is debated (Kleiner et al., 1999; Ruuhola and Julkunen-Tiitto, 2000). For example, Kleiner et al., (1999) showed that the specific activity of PGs was reduced by 38% within 48 h of <sup>14</sup>CO<sub>2</sub>-feeding to soil grown *Populus* plants. In contrast, studies of Ruuhola and Julkunen-Tiitto, (2000) using *in vitro* cultured *Salix* plantlets grown on 2-aminoindan-2-phosphonic acid (AIP), a PAL inhibitor, showed that the total PG turnover

was only 0.6% per day. The experimental methods, genotypes and time points might have contributed to the observed differences. In leaves, factors such as mechanical damage and subsequent exposure to foliar enzymes such as esterases and  $\beta$ -glucosidases, contribute to the stability of these compounds (Ruuhola et al., 2003). The stability of these compounds at least in *in vitro* depends on the complexity of the molecule where higher-order PGs are very labile and degrade to form salicin, catechol and 6-HCH (Lindroth and Pajutee, 1987; Ruuhola et al., 2003). The levels of these compounds and their inducibility depend more on the genotype than environmental and nutrient factors (Donaldson and Lindroth, 2007). Total PG levels generally vary among genotypes and accumulate up to 20% dry wt (Donaldson and Lindroth, 2007; Harding et al., 2005; Osier and Lindroth, 2006). The inducibility of these compounds in response to stress was negatively correlated with that of the constitutive PG levels as observed in defoliation studies using different *Populus* genotypes (Stevens and Lindroth, 2005).

The biosynthetic pathway to higher-order PGs has been proposed to start with salicin and/or salicylic acid, derived from PAL-independent or PAL-dependent pathways (Tsai et al., 2006a). Substantial proof for the existence of the PAL-dependent pathway comes from the radiolabel studies of Zenk, (1967) where salicin was formed by leaf feeding of phenylalanine derivatives such as cinnamic acid and *ortho*-coumaric acid. Recent evidence involving transgenic plants harboring the *NahG* gene encoding a bacterial salicylic acid hydrolase, along with the earlier label feeding studies argue that salicylic acid is probably not an intermediate in PG synthesis (Morse et al., 2007; Zenk, 1967). If it can be assumed that PGs are derived from the phenylpropanoid pathway, it still

must be recognized that glucose also comprises a substantial fraction (34 to 42%) of the carbon in higher-order PGs (Lindroth et al., 1987a; Pearl and Darling, 1971). So far, none of the genes or enzymes directly responsible for PG biosynthesis or homeostasis has been identified. In RNAi-mediated suppression lines of *p*-coumaroyl-CoA 3'-hydroxylase (C3'H) that catalyzes the 3'-hydroxylation of *p*-coumaroyl shikimate and *p*-coumaroyl quinate, PGs accumulated, but possibly as a secondary effect due to the disruption of lignin synthesis (Coleman et al., 2008). A similar increase in PGs as a result of altered lignin polymerization was also observed in *laccase* down-regulated plants (Ranocha et al., 2002).

Phenolic glycosides, especially salicortin and tremulacin, are important for poplar defense against generalist herbivores, such as gypsy moth (*Lymantria dispar*, Donaldson and Lindroth, 2007). In contrast, specialist herbivores, such as the cottonwood leaf beetle (*Chrysomela confluenta*), utilize these compounds as cues for oviposition, and convert PGs to salicylaldehyde which is toxic to its predators (Pasteels et al., 1983). PGs are also gaining recognition as chemotaxonomic markers in classification of *Salicaceae* (Julkunen-Tiitto, 1985; Rehill et al., 2005). Besides their importance to plants as defensive compounds, they also have medicinal properties which humans have exploited. An ancient traditional practice to cure aches and pains is to chew willow bark. It wasn't until the last century that the active ingredient in willow bark was found to be salicylic acid, a breakdown product of salicin that relieves pain. A brief review of the importance of salicylates in medicine and the discovery of acetyl salicylic acid, which we now use in the form of aspirin, is discussed elsewhere (Mahdi et al., 2006).



## 1.2 Condensed tannins

Another important branch from the phenylpropanoid pathway leads to the production of flavonoids and condensed tannins. *Populus* species, like all higher plants, contain different classes of flavonoids, such as chalcones, dihydrochalcones, flavanones, flavones, flavanonols, flavonols, anthocyanins and proanthocyanidin (CT) precursor flavan-3-ols. These compounds have different functions in plants, contributing to defense, pigmentation, stress response, signaling, pollen tube growth and root development (Donaldson and Lindroth, 2004; Feugey et al., 1999; Fischer et al., 2006; Schweitzer et al., 2004; Winkel-Shirley, 2002; Ylstra et al., 1992). The level of these foliar secondary compounds, such as CTs, has significant effects on leaf decomposition and nutrient cycling in the soil, especially as related to C and N mineralization (Gehring et al., 2006; Northup et al., 1998; Schweitzer et al., 2008). Some evidence exists that the levels of foliar CTs in *Populus* correlate with root growth (Fischer et al., 2006). A conceptual model has been developed that illustrates the complex roles of genes and environmental factors, such as herbivores and nutrient availability, on the synthesis of CTs, and the impact on the ecological interactions of above- and below-ground plant parts (Schweitzer et al., 2008).

The concentrations of flavonoids and CTs vary among clones of *Populus* and *Salix* species (Greenaway et al., 1991; Orians et al., 2000; Stevens and Lindroth, 2005). CT levels also vary among different vegetative tissues. For example, in the *P.tremula* × *P. alba* clone 717-1B4, CT concentrations are highest in elongating roots (20% dry wt), much lower in leaves and stems (<1% dry wt) and below detection in xylem (R.S.

Payyavula, unpublished). In other species of *Populus*, leaf CT abundance can be as high as 20% (Stevens and Lindroth, 2005; Stevens et al., 2007). Of the A, B and C rings of CTs, the A and C rings (60% C) are derivatives of phenylpropanoid, while the B ring (40% C) originates from malonyl-CoA, via acetyl-CoA synthesized from pyruvate or via beta-oxidation of fatty acids (Taiz and Zeiger, 1998). The phylogenetic organization of many of the phenylpropanoid and flavonoid pathway genes expressed in *Populus* tissues has been reported (Tsai et al., 2006b). The regulation of genes in this pathway has also been discussed in several review papers (Dixon and Paiva, 1995; Lanot et al., 2008; Lucheta et al., 2007; Weisshaar and Jenkins, 1998).

Substantial evidence exists for reciprocal levels of PGs and CTs. The tissue levels of CTs in *Populus* hybrids that accumulate both CT and PG often correlate negatively with the levels of PGs. PG levels are often higher in leaves collected from young *Populus* plants and the levels decrease during ontogenetic changes in growth of the trees over a several-year period (Donaldson et al., 2006b). In contrast, CT levels were lower in leaves from the younger plants and increased with plant age. Negative correlations of PGs and CTs have also been observed in *Salix* hybrids (Orians and Fritz, 1995; Orians et al., 2000). Several reports also presented growth tradeoff in relation to accumulation of secondary metabolites. *Populus* distributes up to 30% of the fixed C to the secondary metabolites, such as PGs and CTs that are useful for defense (Donaldson et al., 2006b; Harding et al., 2005; Kleiner et al., 1999). Besides substantial commitment of *Populus* for these compounds, the levels of these defensive compounds also increase in response to biotic (e.g., insect) and abiotic (e.g., nutrient) stress (Harding et al., 2005; Kleiner et al.,

1998; Osier and Lindroth, 2006; Ruuhola et al., 2001). Accumulation of high levels of these compounds especially under low nutrient conditions has a negative effect on growth (Donaldson and Lindroth, 2007). Also, a tradeoff between growth and the synthesis of salicylates was observed in the *in vitro* micropropagated *Salix* plantlets (Ruuhola and Julkunen-Tiitto, 2003). The synthesis of growth-compromising levels of these compounds may be necessary for defense, although it is an added cost to plant. A better understanding of the carbon partitioning to the secondary metabolite synthesis and of the PG biosynthetic pathway will be necessary for mechanistic investigation on the possible tradeoff among growth, PG and CT.

### **1.3 Sucrose and Sucrose transporters**

There is increasing evidence that the PG and CT accumulation depend on sink strength and the transported sucrose upon hydrolysis (Arnold and Schultz, 2002; Kleiner et al., 1999). In most plant species, sucrose is the major photosynthetic product transported from source to sink tissues. The imported sucrose, after hydrolysis by either invertases or sucrose synthases (SuSy), is channeled into glycolysis and the tricarboxylic acid cycle, through which primary and secondary metabolites are synthesized (Buchanan et al., 2000; Kleiner et al., 1999). Several studies revealed that sucrose, besides acting as a transport molecule, also acts as a signaling molecule in defense and carbohydrate synthesis and partitioning (Chiou and Bush, 1998; Gomez-Ariza et al., 2007; Rolland et al., 2002; Zhou et al., 2009). Many sucrose-regulated genes were also shown to be hexose-regulated, supporting the view that hydrolyzed sucrose delivers hexoses that

could act as signal molecules.

In mature leaves, sucrose loading into phloem occurs by either passive symplastic connections through plasmodesmata, or by energy-mediated apoplastic loading using sucrose transporters, also referred to as sucrose carriers (SUT/SUC). The first functional sucrose transporter was reported by Riesmeier et al., (1992) from spinach. In several of the model plant systems where sucrose transporters have been investigated, multiple proteins have been characterized (Sauer, 2007). For example, nine SUCs in *Arabidopsis* (*Arabidopsis Genome Initiative, 2000*), five in rice (*Oryza sativa*, Yu et al., 2002) and three in tomato (*Lycopersicon esculentum*, Barker et al., 2000) have been reported on. SUT/SUCs from different plant species were classified into three groups based on phylogenetic analysis (Reinders et al., 2008). Transporters from different groups can also be distinguished by their kinetic properties, including affinity ( $K_m$ ) for sucrose and capacity ( $V_{max}$ ) for sucrose transport (reviewed in Kuhn, 2003). While Group-1 transporters are involved in phloem loading of sucrose, the functions of Group-2 and Group-3 transporters are less well understood. The expression and activity of SUTs can be regulated by sugars such as glucose and sucrose, and by mechanical stimuli such as wounding (Chiou and Bush, 1998; Meyer et al., 2004). Besides sucrose, SUTs can also transport glycosylated phenolic derivatives such as salicin and helicin (Chandran et al., 2003; Sivitz et al., 2007). In several plant species, insertional mutants and antisense suppressed lines have been used to study the function of SUTs. Both carbohydrate transport and the levels of secondary metabolites are altered in *Arabidopsis*, tomato and potato (*Solanum tuberosum*) plants with suppressed Group-1 *SUT* expression

(Gottwald et al., 2000; Hackel et al., 2006; Riesmeier et al., 1994; Srivastava et al., 2008). These results support the involvement of SUTs in partitioning of the carbohydrate assimilates.

*Populus* is an ecologically and economically important tree species found throughout North America. The availability of the complete genome sequence and the presence of genotypic variation in growth rate and foliar abundance of PGs and CTs make *Populus* a commercially relevant model for studying the trade-off between secondary metabolism and growth. In this study, two different approaches, whole plant and cell culture, were used to understand PG-CT metabolism. In the first chapter, *SUT* gene family members in *Populus* were identified and characterized. An RNAi-mediated transgenic approach was used to investigate the role of the most abundantly expressed *SUT*, *PtSUT4*, on sucrose transport and PG homeostasis *in planta*. The effects on carbohydrate concentrations throughout the plant, on the expression of genes known to be important for carbohydrate utilization, and on PG and CT homeostasis were analyzed. In the second chapter, cell cultures were used in feeding experiments to investigate metabolic changes associated with glycosylation of phenolic substrates. The substrates used, salicyl alcohol and salicylaldehyde, are potential PG precursors *in planta*. Gene expression changes were analyzed using microarrays and QPCR. Gene expression and metabolite data was studied on general effects of feeding as well as on the sensitivity of carbon partitioning between phenylpropanoid branches to the feeding.

**Research objectives:**

1. Demonstrate the use of *Populus*-derived suspension cells for investigating effects of phenylpropanoid glycosylation on carbon partitioning relevant to growth, CT and PG homeostasis.
2. Characterize the *SUT* gene family in *Populus* and investigate SUT4 function in carbohydrate partitioning and PG-CT homeostasis *in planta*.

## Chapter 2 Metabolic and gene expression changes associated with salicyl alcohol feeding in aspen cell cultures

### ABSTRACT

Phenylpropanoid-derived phenolic glycosides (PGs) and condensed tannins (CTs) are the two major groups of secondary metabolites in *Populus*. PGs and CTs show reciprocity in their abundance. Although the biosynthesis of PGs is less well understood than that of CTs, certain phenolic derivatives, including salicin, are thought to be precursors for the synthesis of higher-order PGs, such as salicortin, tremulacin and tremuloidin. Salicin and higher-order PGs are not synthesized in cell cultures grown under standard conditions. Feeding with salicyl alcohol, salicylaldehyde and helicin, but not benzoic acid, benzyl alcohol, benzylaldehyde, salicylic acid, cinnamic acid and *O*-coumaric acid, led to synthesis of salicin and isosalicin in the cells. The highest levels of salicins were detected by salicyl alcohol feeding at the early stages of growth. Accompanying formation of salicins from salicyl alcohol was a decrease in CT levels by up to 30% and an increase in growth by up to 15%. The expression of genes encoding putative tonoplast localized sucrose transporter (*PtSUT4*), and sucrose hydrolytic enzymes, cytosolic sucrose synthases (*PtSuSYs 1, 2 and 3*) and vacuolar invertase (*PtVIN2*) was altered in salicyl alcohol-fed cultures. This suggests differential compartmentalization of sucrose hydrolysis and possibly differential utilization of sugars between carbon demanding pathways. Microarray analysis revealed an up-regulation of genes related to glycolysis and Krebs cycle pathways which utilize acetyl-CoA, a precursor for fatty acid and

malonic acid biosynthesis. As malonic acid contributes 40% of carbon skeleton of CTs, the amount of carbon allocated for CT synthesis might be reduced. The reduced expression of most phenylpropanoid and flavonoid pathway genes is consistent with reduced carbon flux for CT synthesis. Therefore, our data suggest that the reduced CTs in salicin-accumulating cells might be a result of competition for the carbon resources between growth, CT and salicin synthesis. In salicyl alcohol-fed cultures, two abundant glycosyltransferase transcripts, *PtGT1-2* and *PtGT1-246*, were further up-regulated, as well as genes encoding vacuolar ATP-binding cassette transporter proteins, *PtMRP1*, *PtMRP6*. Their possible roles during salicyl alcohol glycosylation and uptake of salicins into the vacuole are discussed.

## 2.1 Introduction

*Populus* trees and their close relatives in the family *Salicaceae* are capable of producing large quantities of phenolic glycosides (PGs) and condensed tannins (CTs) in their vegetative tissues (Donaldson et al., 2006a; Hwang and Lindroth, 1997; Osier and Lindroth, 2006; Ruuhola and Julkunen-Tiitto, 2003). PGs and CTs play a major role in defense and protection against biotic (e.g. insects, Bryant et al., 1993; Donaldson and Lindroth, 2007) and abiotic (e.g. UV-B radiation, Lavola, 1998) stress. PGs and CTs are thought to be derived from common phenylpropanoid precursors (Tsai et al., 2006a). Their abundance varies among species, genotypes or individuals (Harding et al., 2005; Orians et al., 2000; Ruuhola et al., 2001; Stevens et al., 2007). The biosynthesis of phenylpropanoid derivatives, such as flavonoids and CTs, is relatively well characterized



at the molecular and biochemical levels in herbaceous species, such as *Arabidopsis*, and in woody plants, such as *Populus* (Besseau et al., 2007; Matsui et al., 2004; Tsai et al., 2006a; Weisshaar and Jenkins, 1998). In contrast, the biosynthetic pathway of PGs is poorly understood. Salicin (*O*-hydroxymethyl phenyl  $\beta$ -D-glucoside) from *Salix* bark was the first PG-like substance to be isolated from a higher plant. Salicin is considered both a precursor and a degradation product of high-order PGs, such as salicortin and tremulacin (Julkunen-Tiitto and Sorsa, 2001; Lindroth and Pajutee, 1987; Pearl and Darling, 1971; Pierpoint, 1994; Ruuhola and Julkunen-Tiitto, 2003). At the present time however, salicin synthesis itself is also not well understood.

Multiple biosynthetic pathways for salicin have been proposed (Pierpoint, 1994; Pridham and Saltmarsh, 1963; Ruuhola and Julkunen-Tiitto, 2003; Zenk, 1967), resulting in a number of proposed precursors. Radiolabel studies suggested that, in *Salix* (*Salix purpurea*) leaves, salicin synthesis proceeds from the phenylpropanoid pathway, via intermediates such as *O*-coumaric acid, benzyl derivatives, salicylaldehyde and helicin (Zenk, 1967). Salicin was also synthesized from salicyl alcohol that may itself be derived from *O*-coumaric acid (Zenk, 1967). In contrast, fed salicyl alcohol accumulated only as isosalicin (*O*-hydroxybenzyl- $\beta$ -D-glucoside) in leaves of sunflower (*Helianthus annuus*, Zenk, 1967) and seedling of broad beans (*Vicia faba*) and maize (*Zea mays*, Pridham and Saltmarsh, 1963). In cell cultures, salicyl alcohol was converted into salicin and isosalicin in *Salix*, *Gardenia* (*Gardenia jasminoides*) and *Lithospermum* (*Lithospermum erythrorhizon*), and into isosalicin in tobacco (*Nicotiana tabacum*), *Datura* (*Datura innoxia*), *Duboisia* (*Duboisia myoporoides*) and several other species (Mizukami et

al., 1986; Shimoda et al., 2002; Tabata et al., 1976). Salicylaldehyde accumulated as salicin and isosalicin in cell cultures of *Salix* (Dombrowski and Alferman, 1992), but as isosalicin in *Datura* (Tabata et al., 1976). These results suggest that salicyl alcohol- and salicylaldehyde-mediated salicin synthesis is species- and tissue-specific. In cell cultures of all the above species, neither salicin nor isosalicin has been observed without precursor feeding.

The synthesis of phenolics and their subsequent glycosylation demand substantial amounts of C which otherwise can be used for growth. For example, growth was increased by inhibition of endogenous synthesis of PGs in micropropagated plantlets of *Salix* after feeding 2-aminoindan 2-phosphonic acid (AIP), a specific inhibitor of phenylalanine ammonia-lyase (PAL) involved in phenylpropanoid pathway (Ruuhola and Julkunen-Tiitto, 2003). A negative correlation was observed in *Populus* and *Salix*, where fast growing plants accumulated lower levels of defensive compounds than did slow growing plants and *vice versa* (Hwang and Lindroth, 1997; McDonald et al., 1999; Nichols-Orians et al., 1993). Besides genetic factors, environmental factors such as soil nutrient contents also play a role in growth and defense tradeoff. For example, application of N fertilizer increased growth and reduced secondary metabolite levels and *vice versa* (Donaldson et al., 2006a; Hakulinen et al., 1995; Osier and Lindroth, 2006; Ruohomaki et al., 1996). A negative correlation was also observed in levels of PGs and CTs. For example, F<sub>1</sub> hybrids of *Salix* that accumulate high levels of PGs accumulate low levels of CTs (Orians and Fritz, 1995; Orians et al., 2000). Developmental shifts between CTs and PGs was observed in *Populus* (Donaldson et al., 2006b). In spite of several

correlative results, the molecular mechanisms underlying the metabolic competition between growth and secondary metabolites and within secondary metabolites are not known.

In heterotrophic cell cultures, growth and metabolism depends on the sugars, typically sucrose, supplied in the media. Sucrose imported into the cells is utilized in different metabolic pathways, but only upon its hydrolysis into hexoses (Koch, 2004; Sturm, 1999). The hexoses are also utilized for glycosylation of the phenolics, which are potentially toxic to the cells at high levels. Glycosyl transferases (GT) catalyzes the transfer of sugar molecules to the phenolics (Ross et al., 2001). In plants, the GTs that are involved in the glycosylation of simple phenolics belong to family 1 (Gachon et al., 2005). Exogenously fed phenolics are glycosylated in the cytoplasm and are transported into vacuoles by class- and plant species-specific mechanisms (Dean et al., 2005; Rea, 2007; Walczak and Dean, 2000).

Since genes specific to PG biosynthesis have not yet been identified, a transgenic approach is not currently feasible. Cell culture provides a convenient, albeit artificial, means to manipulate PG accumulation by feeding precursors under relatively uniform conditions. A challenge with woody species is their general recalcitrance to cell suspension cultures. We have previously identified a single aspen (*Populus tremuloides*) line (L4) that is amenable to suspension culture from a screen of over 300 seeds (Tsai, unpublished data). Salicin does not accumulate to detectable levels in *Populus* cell cultures grown under normal culture conditions (preliminary work). Cell cultures

were fed with potential salicin precursors in order to address if salicin accumulation and sequestration interfere with CT homeostasis. Microarrays were used to determine changes in gene expression across broad categories of metabolic pathways, and to help understand the metabolic consequences of PG accumulation. Investigation of metabolic shifts associated with PG homeostasis in simple cell culture systems may shed light on whole plant level investigation of PG regulation.

## **2.2 Materials and methods**

### **2.2.1 Cell cultures**

Leaves from greenhouse-grown *Populus* genotype PtL-4 were disinfected by surface-sterilizing in 20% bleach for 20 min, rinsed with water and cultured on semi-solid woody plant medium (WPM, Lloyd and McCown, 1980) supplemented with 2.2 mg l<sup>-1</sup> of 2,4-dichlorophenoxy acetic acid (2,4-D) and 3% sucrose for callus induction. Calli (~5 gm) were used to establish suspension cultures in 30 ml of liquid WPM medium containing 2.2 mg l<sup>-1</sup> of 2,4-D with 3% sucrose in a 125 ml flask covered with aluminum foil, and maintained in an orbital shaker at 120 rpm in the dark at 25 °C. Cells were subcultured at 11-day intervals by inoculating 5 ml culture to 30 ml fresh medium. Cell cultures were sieved with a 750 µm mesh at 2-month intervals to select uniform sized cells. Experiments were conducted after 2 subcultures of sieved cells to ensure uniformity. Cell growth was regularly monitored during the study period by measuring percent settled cell volume using a Nephlo flask. When needed, the amount of inoculum was adjusted such that the settled cell volume was near 20% at the time of subculture.

Phenolic compounds, including salicin, cinnamic acid, *O*-coumaric acid, benzoic acid, benzyl alcohol, benzylaldehyde, salicylic acid, salicyl alcohol and salicylaldehyde, were used in the feeding studies. The stocks of these compounds prepared in either ddH<sub>2</sub>O or dimethyl sulfoxide (DMSO) were filter-sterilized (0.45 µm pore size) and administered to the cultures in early log phase, typically 5 days after subculture unless otherwise mentioned. The concentration of the phenolics used in this study varied between 0.2 to 5 mM as specified in the results. Cells were harvested at regular intervals by low vacuum filtration after three rinses with 25 ml of 50 mM sodium chloride followed by three rinses with water, snap frozen in liquid nitrogen and stored at -80 °C.

### **2.2.2 PG & CT extraction and quantitative estimation**

Freeze-dried cell samples were analyzed for PG and CT as described by Harding et al (2005) with slight modifications. Briefly, 5 mg of each sample were extracted in 800 µl of cold methanol for 20 min in a cold ultrasonic bath and centrifuged at 15,000 g for 5 min. The methanol extract was used to estimate glucose-conjugates (salicin, isosalicin, cinnamoyl-glucoside, *O*-coumaroyl-glucoside, salicyloyl-glucoside, benzyl alcohol-glucoside, and benzoyl-glucoside) by using HPLC-UV/MS (Hewlett-Packard 1100 Series, Agilent Technologies, Palo Alto, CA) equipped with an Eclipse XBD-C18 column (5 µm, 2.1 X 150 mm). Methanolic extracts (5 µl) was injected into the column and eluted at a flow rate of 0.2 ml/min using solvents A (10 mM formic acid, pH 3.4) and B (100% Acetonitrile) according to the following gradient: 0 to 15 min, 0% to 70% B, 15 to 17 min, 70% to 100% B, 17 to 19 min, 100% to 0% B and 19 to 30 min 0% B.

Compounds were identified by UV-absorbance (274 nm) and mass spectral data. Concentrations of both salicin and isosalicin were estimated by calibration curves developed using authentic salicin (Sigma St. Louis, MO).

Soluble and residue-bound CTs were estimated according to Porter et al., (1986). Briefly, a dried aliquot of the methanol extract (125  $\mu$ l) was used to estimate methanol-extracted CTs. Both dried extract aliquot and the pellet were resuspended in 250  $\mu$ l methanol, 750  $\mu$ l butanol:HCl (95:5) and 25  $\mu$ l 2% ferric-ammonium-sulfate in 2 M HCl, and heated for 20 min at 95°C. CTs were quantified using a 96-well plate reader (SpectraMax, Molecular Devices, Sunnyvale, CA) by comparing their absorbance at 550 nm (Porter et al., 1986; Tiarks et al., 1992) with that of purified aspen leaf CT.

### **2.2.3 RNA extraction and DNase treatment**

RNA was extracted from frozen cells using the CTAB (cetyltrimethylammonium bromide) method (Chang et al., 1993). Briefly, in a Oakridge tube containing 15 ml of CTAB buffer (2M NaCl, 25 mM EDTA, 0.1 M Tris-HCl (pH 9.0), 2% w/v PVP (K-30), 2% w/v CTAB) containing 2%  $\beta$ -mercaptoethanol, the finely ground sample was added, vortexed for 1 min and incubated for 15 min at 65 °C with intermittent mixing. To this tube, 10 ml of chloroform:isoamyl alcohol (24:1) was added, vortexed and centrifuged for 10 min at 15,000 g to separate the two phases. Chloroform:isoamyl alcohol extraction was repeated twice before precipitating RNA from the aqueous phase (~12 ml) with 4 ml of 8M LiCl. The samples were incubated on ice for 4 hours and centrifuged at 4 °C for 15

min at 15,000 g. The sample was resuspended in 500  $\mu$ l of ddH<sub>2</sub>O and reprecipitated with 170  $\mu$ l of 8 M LiCl as above. After the second precipitation, the pellet was dissolved in 300  $\mu$ l of ddH<sub>2</sub>O, with 30  $\mu$ l of 3M sodium acetate (pH 5.0) and 800  $\mu$ l of 95% ethanol, incubated at -80 °C for 30 min and centrifuged at 4 °C for 15 min at 14,000 g. The pellet was then washed with 1 ml of 70% ethanol, air dried and dissolved in 50-100  $\mu$ l of ddH<sub>2</sub>O, depending on the pellet size. RNA quantity was assessed by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and integrity was assessed by running 400 ng RNA on a 1% agarose gel. RNA was treated with Turbo DNase, according to the manufacturer's instructions (Ambion INC, Austin, TX) to remove the contaminating genomic DNA. Briefly, 25  $\mu$ g of RNA were incubated with 5  $\mu$ l of DNase buffer (10X) and 1  $\mu$ l of DNase enzyme (2 U/ $\mu$ l) in a 50  $\mu$ l reaction for 30 min at 37 °C. The DNase enzyme was then inactivated by adding 5  $\mu$ l of DNase inactivation reagent. The mixture was incubated for 2 min at room temperature and centrifuged at 10,000 g for 1.5 min. The supernatant was collected as DNA-free total RNA.

## **2.2.4 Microarrays**

### ***2.2.4.1 cDNA synthesis and aminoallyl labeling***

For microarray hybridization, cDNA synthesis was carried out with 10  $\mu$ g of DNA-free total RNA, 0.5  $\mu$ l of RNase inhibitor (40 U/ $\mu$ l, Ambion), 0.5  $\mu$ l of 5 $\times$  first strand buffer, 2  $\mu$ l of amine-modified random primers (2  $\mu$ g/ $\mu$ l, Sigma), 3  $\mu$ l of mRNA spike mix (Lucidea Universal ScoreCard, Amersham, Piscataway, NJ), and ddH<sub>2</sub>O to a final

volume of 19  $\mu$ l. To denature the RNA secondary structure, the reaction mixture was incubated for 5 min at 65 °C, and cooled on ice. Remaining reagents, which include 5.5  $\mu$ l of 5 $\times$  first strand buffer, 2.5  $\mu$ l of 0.1 M DTT, 0.5  $\mu$ l RNase inhibitor, 0.6  $\mu$ l of 50 $\times$  nucleotide mix (prepared by mixing 5  $\mu$ l each of 100 mM dATP, dCTP and dGTP, and 3  $\mu$ l of dTTP and 2  $\mu$ l of aminoallyl-labeled dUTP), and 2.0  $\mu$ l superscript III RT (200 U/ $\mu$ l, Invitrogen, Carlsbad, CA), were added and the tube was incubated for 10 min at room temperature, followed by 4 hours at 46 °C. Following the reaction, RNA was hydrolyzed with 10  $\mu$ l of 1 M NaOH, and 10  $\mu$ l of 0.5M EDTA and incubating for 15 min at 65 °C. The reaction was neutralized by adding 12  $\mu$ l of 1 M HCl and 20  $\mu$ l of 100 mM sodium acetate (pH 5.2).

#### ***2.2.4.2 cDNA purification***

The cDNA was purified using a QIAquick column (Qiagen, Valencia, CA) as per manufacturer's protocol. Briefly, cDNA was diluted with 425  $\mu$ l of Qiagen buffer PB, and the mixture transferred to a QIAquick column and centrifuged for 1 min at 13,000 g. The flow through was passed through the same column twice prior to disposal. The column was washed twice with 600  $\mu$ l of 80% ethanol wash buffer and spun at 13,000 rpm for 1 min. The flow through was discarded and the column was recentrifuged. cDNA was then eluted twice, each with 30  $\mu$ l of 4 mM phosphate buffer with centrifugation at 13,000 g for 1 min. After estimating the quantity of cDNA with a NanoDrop, 2  $\mu$ g of cDNA was aliquoted into amber microcentrifuge tubes, vacuum dried and stored at -20°C until used.



#### ***2.2.4.3 cDNA-Cy dye coupling and dye labeled target purification***

Cy3 or Cy5 dye (Amersham, Piscataway, NJ) was dissolved in 10  $\mu$ l of 0.1 M sodium carbonate buffer (pH 9.0), and mixed with 2  $\mu$ g of dried aminoallyl-labeled cDNA in the amber microfuge tube. The labeling reaction was dark-incubated at room temperature on a shaker for 20 min, and the reaction was then quenched by adding 35  $\mu$ l of 100 mM sodium acetate (pH 5.2) and 250  $\mu$ l of the Qiagen PB buffer. The Cy dye-labeled cDNA was purified using a QIAquick column (Qiagen), washed and eluted twice, each with 30  $\mu$ l of Qiagen EB elution buffer. The Cy dye-labeled cDNA concentration was estimated using a NanoDrop and the amount of dye incorporation (pmol) and frequency of incorporation (FOI) was calculated. Finally, aliquots of 50 pmol Cy dye-labeled cDNA were vacuum dried, and stored at -20 °C until used.

#### ***2.2.4.4 Array hybridization***

The experiment consisted of three biological and two technical (dye swap) replicates. The microarray slides used for this study contained replicate subarrays of 6705 elements representing 6313 previously characterized aspen expressed sequence tags (ESTs, Ranjan et al., 2004). The construction of aspen cDNA microarray was described in Harding et al., (2005). Briefly, the cloned cDNAs were PCR amplified using M13 forward and reverse primers. The dried, ethanol precipitated PCR products were resuspended in 20  $\mu$ l of Corning Pronto! spotting solution (Fisher, Hanover Park, IL) and transferred to 384-well plates for spotting. cDNAs were spotted onto amino silane glass slides (Corning UltraGAPS, Fisher) at the Genomics Technology Support Facility of Michigan State

University. To monitor target labeling and hybridization efficiency, various positive and negative controls (Lucidea Universal ScoreCard, Amersham) were also included on the slide.

The spotted cDNA was immobilized onto the slide by UV cross-linking (150 mJ) and by snap baking (1 min at 120 °C). Immediately prior to hybridization, the slide was soaked in a petri dish containing prehybridization solution (5× SSC, 0.1% SDS and 1% BSA) for 15 min on an orbital shaker, rinsed 20 times in a falcon tube containing ddH<sub>2</sub>O followed by rinsing in ethanol for a few seconds, and dried under a heavy stream of Hepa-filtered air using a microarray Air Jet. Equal amounts (50 pmole) of Cy3- and Cy5- labeled cDNA from control and salicyl alcohol-fed cultures were suspended in 55 µl of hybridization buffer (50% formamide, 5× SSC, 0.4% SDS and 0.1% BSA). The mixture was denatured at 42 °C for 5 min, centrifuged briefly, applied to the slide and covered with a piece of parafilm. Hybridization was carried out for 36 hours in a hybridization oven (Boekel Scientific, Feasterville, PA) at 38.5 °C with humidity maintained by wet paper towels soaked in the hybridization buffer. The slides were then rinsed three times separately in beakers containing 500 ml of 1× SSC and 0.2% SDS (42 °C), followed by 0.1× SSC and 0.2% SDS, and finally in 0.1× SSC before drying the slides using a microarray Air Jet.

#### ***2.2.4.5 GenePix Image acquisition and analysis***

The slides were scanned at 532 and 635 nm for Cy3 and Cy5, respectively, with a Genepix 4000B scanner (Axon Instruments, Union City, CA) and the florescence signal

intensity was quantified using the GenePix Pro 5.1 software (Axon Instruments). Spots with signal intensities in both channels greater than two standard deviations from the background signal were flagged as present.

LOWLESS (locally weighted linear regression) normalization (Cleveland and Devlin, 1988; Yang et al., 2002) of the data was performed using GeneSpring 6.2 software (Silicon Genetics, Redwood City, CA). Hybridization signals satisfying the criteria mentioned below were used for expression analysis. Spots must be present in four of the six replicates (4,149 spots); coefficient of variation (CV) among samples less than 35% (3,957 spots); and raw hybridization signal more than 100 in at least four of the six replicates (1,812 spots). Among these were 938 differentially expressed genes based on the *t*-test with a false discovery rate for multiple testing correction at  $P = 0.05$  using the Benjamini and Hochberg algorithm (Benjamini and Hochberg, 1995). A threshold ratio cutoff of 1.3 was used to further narrow the list of differentially expressed genes to 540.

### **2.2.5 Q-PCR expression analysis**

cDNA was synthesized using total RNA (2  $\mu$ g), anchored oligo(dT)20 primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Relative transcript abundance was analyzed by Q-PCR in a 12.5  $\mu$ l reaction volume using cDNA (from 2.5 ng of total RNA), gene-specific primers and ABSolute QPCR SYBR Green Mix (Abgene, Rochester, NY, USA) with ROX as an internal reference. Amplification was carried out as follows: 15 min at 95 °C followed by 40 cycles of 15 sec at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, using the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA,

USA). Three biological and two technical replicates were used for each determination. A zero-template reaction was included as the negative control. Relative expression of the genes was calculated by the  $\Delta C_T$  method by normalizing the expression levels of target genes to the expression mean of two housekeeping genes, ubiquitin-conjugating enzyme E2 and elongation factor 1- $\beta$  (Tsai et al., 2006a). Specificity of amplification was assessed by dissociation curve analysis using the MxPro software (Stratagene). The primer sequences are presented in Appendix A (sucrose transporters, sucrose synthases, invertases, glucosyl transferases and multi-drug resistance associated proteins) or in Tsai et al., 2006a (phenylpropanoid and flavonoid pathways genes).

## **2.3 Results**

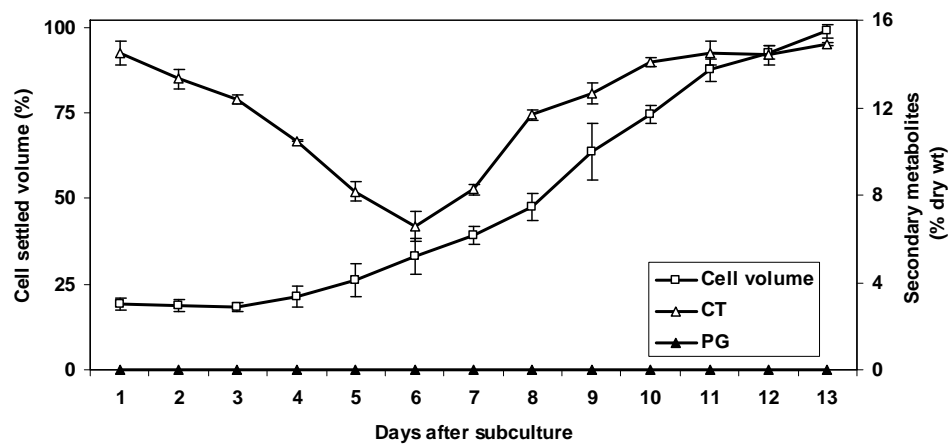
### **2.3.1 Cell culture system characterization**

The baseline growth pattern of cell cultures established from the aspen L4 genotype is presented in Figure 2-1. Growth of the cells was monitored by estimating settled cell volume (%) using a Nephlo flask. After subculturing into fresh medium, cells showed a characteristic growth curve with a 4-day lag phase, followed by an exponential phase, before entering into the stationary phase around day 12 (Figure 2-1). An 11-day interval was therefore chosen for routine subcultures, and the cell volume typically increased by ~400% at the end of the culture cycle. This pattern of cell growth was stable for more than 40 passages during the experimental period.

To establish the basal levels of CT and PG accumulation during the culture cycle, cells were harvested at regular intervals and analyzed for CTs and PGs. At the start of the culture, CT concentrations averaged 14.4% dry wt, but decreased gradually through the lag and early log phases to a low of 6.5%, and then increased rapidly thereafter, reaching 15% at the end of the culture cycle (Figure 2-1.). The initially high CT content corresponded with the CT level at the end of the culture cycle. As CT turnover is considered to be slow (Kleiner et al., 1999), the decrease in CT content during the early growth cycle was most likely from dilution, due to rapid cell proliferation with little or no new CT synthesis. Under normal culture conditions, the L4 cell line does not accumulate salicin or higher-order PGs.

### **2.3.2 Feeding of potential salicin precursors**

The absence of detectable levels of PGs in the aspen cell cultures may be attributed to the absence of possible PG precursors and/or spatiotemporal regulation of metabolic activities at the intracellular level. To identify possible lesions in the PG biosynthetic pathway, a collection of putative PG precursors, including salicin, the simplest PG, salicylic acid, salicyl alcohol, salicylaldehyde, helicin, benzoic acid, benzyl alcohol, benzylaldehyde, cinnamic acid and *O*-coumaric acid (Zenk, 1967) were fed to the cell cultures. Phenolic acids were fed at 0.2 mM, since higher feeding levels led to discoloration of cells, suggestive of toxicity, in preliminary trials. Alcohols, aldehydes, and glucosides were fed at 1 mM. Feeding was conducted 5 days after subculturing, and cells were harvested 24 h and 48 h after feeding for chemical analysis. Higher-order PGs



**Figure 2-1.** Growth and secondary metabolite levels in *Populus* L4 cell suspensions grown under normal culture conditions.

Data represents the mean  $\pm$  SE of three biological replicates.

were not detected during any of the feeding experiments. The glucoside salicin was readily taken up and detected primarily as unaltered salicin in the cell extracts, although a slow conversion to isosalicin continued throughout the 48 hr feeding period (Figure 2-2). Feeding of the unglycosylated form of salicin, salicyl alcohol, resulted in the accumulation of both salicin and its isomer, isosalicin, in a near one:two ratio. The glycosylated aldehyde, helicin, was reduced to salicin and isosalicin, which accumulated in a one:one ratio. When the unglycosylated form of helicin, salicylaldehyde, was fed, it also was recovered as salicin and isosalicin in a one:one ratio (Figure 2-2). Feeding with benzoic acid, salicylic acid, cinnamic acid and *O*-coumaric acid led to accumulation of their respective glucosides (Table 2-1). Benzyl alcohol glucoside was formed when either benzyl alcohol or benzaldehyde were fed (Table 2-1).

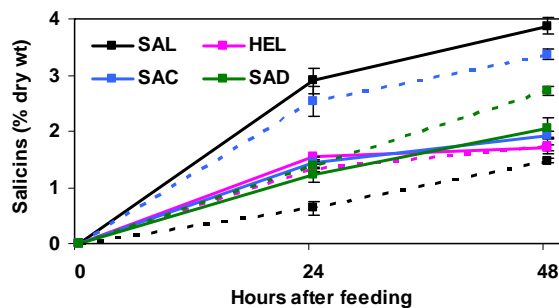
### **2.3.3 Dose-dependent effects of salicyl alcohol feeding on accumulation of salicin, isosalicin and CTs**

Both PG and CT are derived from the phenylpropanoid pathway (Tsai et al., 2006a) with a reported negative correlation in their accumulation (Orians et al., 2000). We therefore examined whether accumulation of salicin and isosalicin (hereafter referred to as the salicins) in salicyl alcohol-fed cultures affects CT production that would exemplify metabolic competition. Cells were fed with varying levels of salicyl alcohol (0, 1, 5 and 10 mM) and sampled over a 4-day period for salicin, isosalicin and CT analysis. A dose-dependent accumulation of the salicins was observed following salicyl alcohol feeding (Figure 2-3 A). Accumulation of the salicins plateaued 24 h and 48 h after feeding

**Table 2-1.** Feeding precursors (mM) and their corresponding products identified based on HPLC-UV/MS analysis using retention time and/or *m/z* ratio.

<b>Precursors</b>	<b>mM fed</b>	<b>Products formed</b>
Salicin	1	Salicin and Isosalicin
Helicin	1	Salicin and Isosalicin
Salicyl alcohol	1	Salicin and Isosalicin
Salicyl aldehyde	1	Salicin and Isosalicin
Salicylic acid	0.2	Salicyloyl-glucoside
Benzyl alcohol	1	Benzyl alcohol-glucoside
Benzyl aldehyde	1	Benzyl alcohol-glucoside
Benzoic acid	0.2	Benzoyl-glucoside
Cinnamic acid	0.2	Cinnamoyl-glucoside
<i>O</i> -Coumaric acid	0.2	<i>O</i> -coumaroyl-glucoside





**Figure 2-2.** Levels of salicin and isosalicin formed over a 48 h period in cultures fed with 1 mM salicin (SAL), helicin (HEL), salicyl alcohol (SAC) and salicylaldehyde (SAD).

Solid and dotted lines represent salicin and isosalicin levels, respectively.

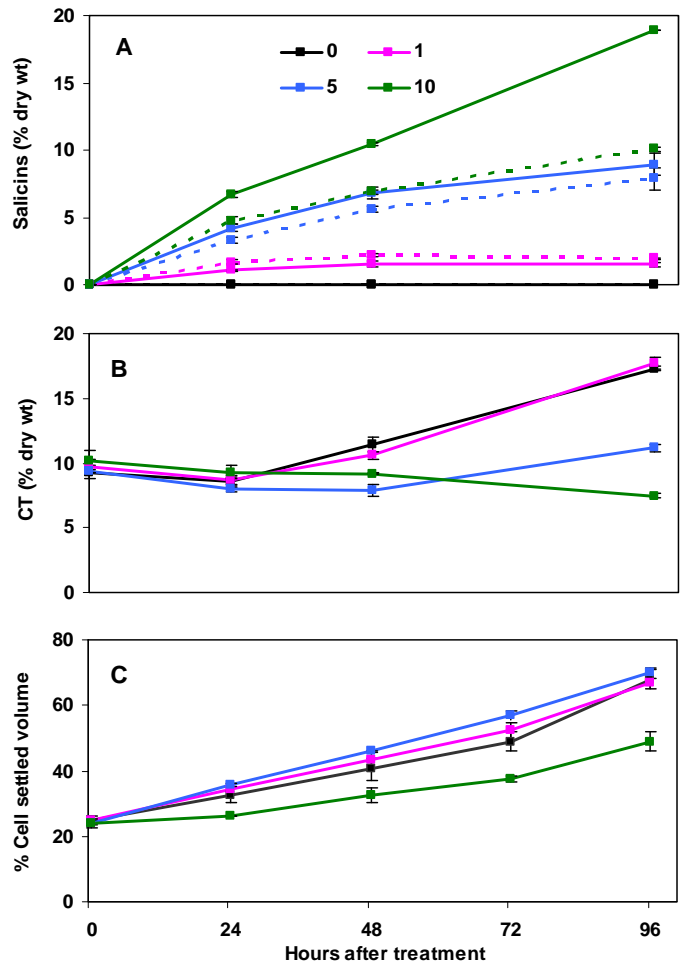
Data represents the mean  $\pm$  standard deviation of two biological replicates.

with 1 mM and 5 mM salicyl alcohol, respectively, but continued to increase with 10 mM feeding over the 4-day period. The total salicins detected at the end of the experimental period were 3, 17 and 29% in cells fed with 1, 5, and 10 mM salicyl alcohol, respectively. The growth of the cell cultures was stimulated by 6-7% with 1mM and 9-15% with 5 mM, but reduced by 20-28% with 10 mM salicyl alcohol feeding (Figure 2-3 C). At 5 mM feeding, the efficiency of salicyl alcohol conversion at the end of the 4-day period was at least 60%, as 175  $\mu$ moles equivalent of 5 mM salicyl alcohol was converted to 100  $\mu$ moles of salicins.

CT levels showed a dose-dependent negative response to salicyl alcohol feeding. At 1 mM, no difference was observed in CT levels as compared to unfed cultures (Figure 2-3 B), with the CT levels decreasing at 24 h (corresponding to 6 days after subculture, Figure 2-1), followed by a steady increase through the end of the 96 h period. In cultures fed with 5 mM salicyl alcohol, CT levels did not increase until after 48 h (or 7 days after subculture). At 10 mM, cells were no longer able to maintain the basal CT level observed at the time of feeding. The reduction in CT levels in the cells accumulating salicins supports a metabolic competition of salicins and CTs.

#### **2.3.4 Effect of culture stage on levels of salicin, isosalicin and CT upon salicyl alcohol feeding**

As shown in Figure 2-1, active CT synthesis in the aspen cell suspensions typically resumed by the mid-exponential phase, i.e. 6 days after subculturing. To examine

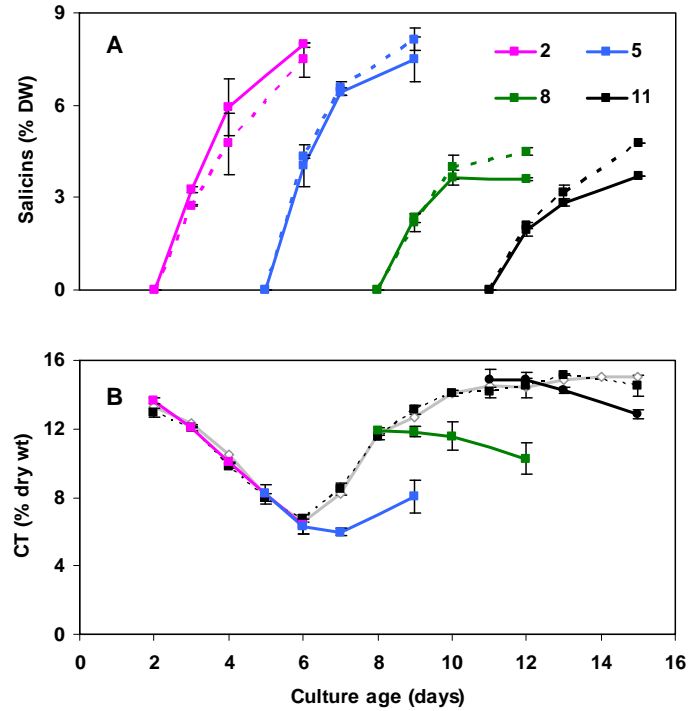


**Figure 2-3.** Effect of salicyl alcohol concentration (0, 1, 5, 10 mM) on (A) salicin and isosalicin formation, (B) CT levels and growth (C) in aspen cell cultures.

Salicyl alcohol was fed to 5-day old cultures and samples were analyzed at regular intervals over a 96 h period. In panel A, solid and dotted lines represent salicin and isosalicin levels, respectively. Data represents the mean  $\pm$  standard deviation of two biological replicates.

whether growth phase plays a role in cells' capacity to accumulate the various secondary metabolites, cell suspensions were fed with 5 mM salicyl alcohol to 2-, 5-, 8-, or 11-day old cultures. Salicin, isosalicin and CT levels were measured over a 96 h period following feeding. In general, cells at the lag or early exponential phase, when the CT levels were in decline, have a higher capacity to accumulate the salicins than cells at the mid-exponential or stationary phases with active CT synthesis (Figure 2-4 A). In all cases, the salicins increased steadily over the experimental period, and reached ~15% in cells fed at the earlier growth phases (2- and 5-day old) and ~8% in cells fed at the later stages (8- and 11-day old).

Salicyl alcohol feeding to cells at the lag phase (2-day old) had no effect on basal CT accumulation during the 4-day experimental period (Figure 2-4 B), consistent with the idea that CT synthesis was not yet active at this stage. Feeding of salicyl alcohol to 5-day old cells delayed the onset of CT accumulation (Figure 2-3, Figure 2-4 B). At a later stage of cell growth (8- and 11-day) when CT was accumulating, salicyl alcohol feeding reduced the rate of accumulation. In sum, CT levels were reduced by 38%, 30% and 10% at the end of the 96 h period when salicyl alcohol was fed to 5-, 8- and 11-day old cultures, respectively, relative to the unfed controls. The results provide an additional line of support for metabolic competition between CTs and salicins in actively growing cultures.



**Figure 2-4.** Effects of culture age (2-, 5-, 8-, and 11-day old) on (A) salicin and isosalicin formation and (B) CT accumulation following 5 mM salicyl alcohol feeding.

In panel A, solid and dotted lines represent salicin and isosalicin levels, respectively. In panel B, solid grey line represent background CT levels from Figure 2-1, and dotted black line represents CT levels from control cultures of this experiment. Data represents the mean  $\pm$  standard deviation of two biological replicates.

### 2.3.5 Microarray analysis

The observed reciprocal regulation of salicin and CT accumulation in cell cultures suggested that salicyl alcohol feeding affected both carbohydrate utilization and carbon flux through the phenylpropanoid pathway. To assess the breadth of the metabolic response, global gene expression using the 7K aspen EST array was analyzed in 5-day old cultures, after 48 h of feeding salicyl alcohol and where growth and CT synthesis are more active. Of the 1,812 ESTs that passed a series of quality control measures, expression of 938 ESTs representing 884 non-redundant genes was found to be significantly altered (with a false discovery rate  $p < 0.05$ ) in salicyl alcohol-fed cells relative to the control. However, only 300 and 240 ESTs were up- and down-regulated, respectively, by more than 1.3-fold. Stress-related transcripts, such as glutathione S transferase (GST), peroxidase, dehydrin, thaumatin-like protein, and germin-like protein, were among the most up-regulated genes in the salicyl alcohol-fed cells (Table 2-2). The most highly up-regulated EST (MTU6CR.P6.H02) corresponds to a peroxidase. From our other analyses, this gene was poorly expressed in vegetative tissues, except cell cultures, and was greatly induced in methyl jasmonate-treated cells as well as in gypsy moth-fed aspen leaves (Tsai et al., unpublished data). Many of the down-regulated genes were associated with phenylpropanoid and flavonoid biosynthesis, including phenylalanine ammonia-lyases (*PAL1* and *PAL2*), 4-coumarate:CoA ligase, caffeoyl-CoA *O*-methyltransferase, *S*-adenosylmethionine synthase, cinnamoyl-CoA reductase, and chalcone isomerase. These results are in line with the reduced CT accumulation in salicyl alcohol-treated cells.

**Table 2-2.** List of representative non-redundant genes differentially regulated in salicyl alcohol-fed cultures compared to unfed cultures.

Putative function	JGI Gene Model	SAC/C	P-value
<b>Cell rescue and defense</b>			
Chitinase, glycosyl hydrolase family 18	estExt_Genewise1_v1.C_LG_VI1054	0.67	0.050
Plant disease resistance response protein	eugene3.00031873	0.74	0.028
Chitinase putative, class I, glycosyl hydrolase family 19	gw1.XIV.3121.1	1.48	0.001
Thaumatin-like protein, PR-5b precursor	estExt_fgenes4_pg.C_LG_I0902	1.62	0.011
Carbohydrate oxidase, antifungal	gw1.I.5965.1	1.81	0.001
Germin-like protein	estExt_fgenes4_pm.C_LG_XIII0003	2.09	0.015
Pollen Ole e 1 allergen and extensin	grail3.0046017801	2.62	0.006
Dehydrin, stress-induced	estExt_fgenes4_pg.C_LG_V1612	3.13	< 0.001
Glutathione S-transferase GST 18	grail3.0036009801	4.37	< 0.001
Peroxidase	estExt_fgenes4_pm.C_870009	8.18	< 0.001
<b>Energy metabolism</b>			
Light-harvesting complex I protein	grail3.0012036701	0.63	0.034
NADP/FAD dependent oxidoreductase	gw1.X.6680.1	0.63	0.027
Chlorophyll a/b binding protein 4	eugene3.00110470	0.69	0.017
Photosystem II core complex proteins psbY	estExt_Genewise1_v1.C_LG_X5024	0.70	0.018
Photosystem I reaction center subunit V	eugene3.00013110	0.73	0.032
NADP-isocitrate dehydrogenase	grail3.0038019202	1.30	0.023
Phosphoglycerate kinase	estExt_fgenes4_pm.C_LG_VIII0335	1.36	0.025
Phosphoenolpyruvate carboxylase	estExt_Genewise1_v1.C_1460016	1.37	0.005
Transaldolase	grail3.0008017101	1.44	0.015
Fructose-1,6-bisphosphatase	eugene3.00101874	1.45	0.007
Glyceraldehyde 3-phosphate dehydrogenase	estExt_fgenes4_pg.C_LG_X0484	1.52	0.023
Enolase	eugene3.00151093	1.59	0.031
Aldehyde dehydrogenase 1	estExt_fgenes4_pm.C_LG_II0915	1.72	0.003
Aconitase	gw1.XIV.3318.1	1.74	0.005
Quinone oxidoreductase	estExt_Genewise1_v1.C_LG_XI2337	1.76	< 0.001
Malic enzyme	estExt_Genewise1_v1.C_LG_XVIII25	1.86	< 0.001
Malate dehydrogenase	fgenes4_pg.C_LG_XV000664	2.02	< 0.001
<b>General metabolism</b>			
5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	estExt_fgenes4_pg.C_LG_XIII0289	0.60	0.002
Glutamate-1-semialdehyde 2,1-aminomutase	eugene3.00150799	0.63	0.018
Glutamate decarboxylase 1	grail3.0044016002	0.66	0.018
Serine hydroxymethyltransferase 2	grail3.0003095602	0.71	0.012
UDP-glucuronosyl and UDP-glucosyl transferase	eugene3.00060083	1.31	0.028
Sucrose synthase	estExt_fgenes4_pg.C_280066	1.34	0.015
Dehydroquinase shikimate dehydrogenase	estExt_Genewise1_v1.C_700420	1.46	0.006
CDP-alcohol phosphatidyltransferase/Phosphatidylglycerol-phosphate synthase	gw1.X.2456.1	1.57	0.002
Asparagine synthase (glutamine-hydrolyzing)	eugene3.00110945	1.69	0.001
Vacuolar invertase	estExt_fgenes4_pg.C_LG_III0902	2.22	0.006
<b>Secondary metabolism</b>			
Phenylalanine ammonia-lyase 1	estExt_Genewise1_v1.C_280661	0.43	0.010
Caffeoyl-CoA O-methyltransferase	grail3.0001059501	0.43	0.002
S-adenosylmethionine synthase	grail3.0050014702	0.57	0.001
Cinnamoyl-CoA reductase	estExt_fgenes4_kg.C_LG_III0056	0.60	0.002
Phenylalanine ammonia-lyase 2	fgenes4_pg.C_LG_X002043	0.67	0.042
Chalcone isomerase, putative	estExt_fgenes4_pg.C_LG_XVI0075	0.71	0.006
Leucoanthocyanidin reductase	grail3.0010045601	0.72	0.010
4-coumarate-CoA ligase 1	grail3.0100002702	0.82	0.013
Glucosyltransferase	gw1.1772.3.1	1.82	< 0.001
Polyphenol oxidase	eugene3.00110271	1.92	0.001
Cytochrome P450 81B1	gw1.40.1024.1	2.23	0.002

General metabolism comprised the predominant functional category of the differentially expressed genes. The expression of sucrose hydrolyzing genes such as SuSy and a vacuolar invertase was up-regulated in salicyl alcohol-fed cultures (Table 2-2). Energy metabolism comprised 6% of the differentially expressed genes. Several genes related to photosynthesis that could have an unknown function in dark grown cells were down-regulated. The expression of glycolysis pathway genes, including glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase, and of tricarboxylic acid (TCA) cycle genes, including malate dehydrogenase, aconitase and NADP-isocitrate dehydrogenase, were up-regulated (Table 2-2).

### **2.3.6 Real-time PCR analysis**

QPCR was used to enable higher resolution analysis of the gene expression changes observed in microarrays. Because of the limited coverage of the EST array, we expanded the analysis to include many more of the genes associated with the flavonoid biosynthetic pathway (Tsai et al., 2006a). We also included gene families associated with sucrose transport and hydrolysis (sucrose transporters, invertase and sucrose synthase), and the glycosylation (glycosyl transferases) and transport (multidrug-resistance associated proteins) of simple phenolics (Bowles et al., 2005; Gachon et al., 2005; Koch, 2004; Lu et al., 1998; Reinders et al., 2008). Data for genes with extremely low transcript levels were not shown.

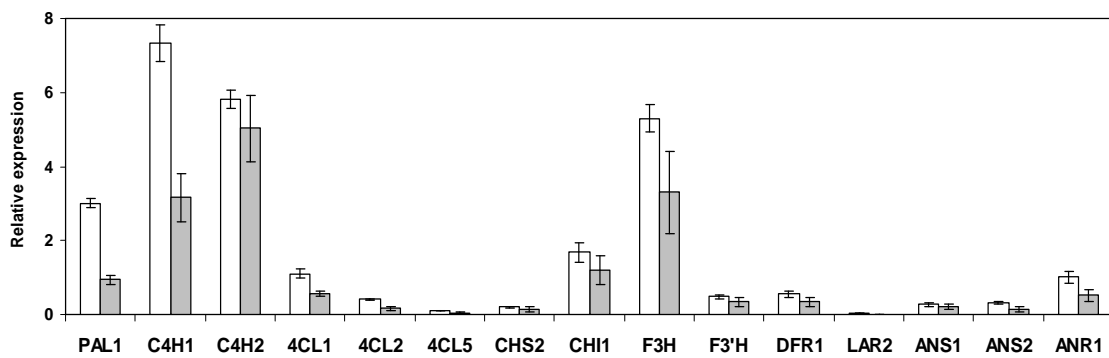


### **2.3.6.1 Phenylpropanoid and flavonoid genes**

QPCR analysis confirmed the microarray data in that there was a trend toward down-regulated expression of most phenylpropanoid and flavonoid pathway genes as a result of salicyl alcohol feeding (Figure 2-5). Among the more highly expressed genes, transcript levels of *PtPAL1*, and *PtC4H1* and the early flavonoid pathway genes, *PtCHI1* and *PtF3H*, decreased (Figure 2-5). Expression of several of the other less abundant genes, such as *Pt4CL1*, *Pt4CL2*, *PtF3H*, *PtF3'H*, *PtANS2* and *PtANR1* was also reduced. Although the degree of reduction was small in some cases (20-30%), the trend towards down-regulation was consistent throughout the pathway. Like the microarray results, QPCR data suggest that the reduced CTs in the salicyl alcohol-fed cultures could be a result of reduced C flux through the phenylpropanoid/flavonoid pathway.

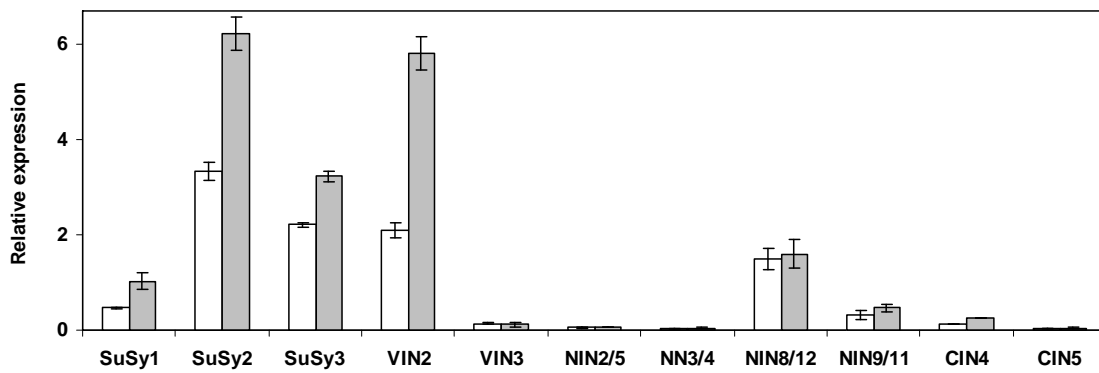
### **2.3.6.2 Sugar metabolism genes**

In heterotrophic cell cultures, exogenously supplied sucrose is critical for cellular metabolism but only upon its hydrolysis. Hydrolysis of sucrose is carried out by sucrose synthases (SuSy) and invertases (Koch, 2004; Sturm, 1999). A number of cytosolic SuSy, cell wall invertases (CIN), vacuolar invertases (VIN), and cytosolic, neutral invertases (*NIN*) have been annotated in the poplar genome (Bocock et al., 2008; Tuskan et al., 2006). *PtSuSy2*, *PtSuSy3*, *PtVIN2* and *PtNIN8/12* are the isoforms that are most strongly expressed in cell cultures (Figure 2-6). The expression of *PtSuSy1* and *PtSuSy2* was up-regulated 2-fold, and that of *PtSuSy3* by 40% in salicyl alcohol-fed cultures. The expression of *PtVIN2* was also up-regulated 2-fold, while expression of neutral invertase did not change. Transcript levels of the other genes were low in both control and salicyl



**Figure 2-5.** Q-PCR expression analysis of phenylpropanoid and flavonoid pathway genes in control (white bars) and salicyl alcohol-fed (gray bars) cultures.

Data represents the mean  $\pm$  SE of three biological replicates.



**Figure 2-6.** Q-PCR expression analysis of invertase and sucrose synthase gene family members in control (white bars) and salicyl alcohol-fed (gray bars) cultures.

Data represents the mean  $\pm$  SE of three biological replicates.

alcohol-fed cultures. The results suggest that when salicyl alcohol was fed to the cultures, sucrose hydrolysis increased in the cytosol and in the vacuole.

### **2.3.6.3 Glycosyltransferase genes**

The glycosylation of small phenolic molecules is typically catalyzed by members of the GT family 1 (Bowles et al., 2006). Of the 326 GT1 family members annotated in the *Populus* genome (Geisler-Lee et al., 2006), 47 genes classified into several sub-families are expressed in the aspen cell cultures, based on Affimetrix microarray analysis (Harding et al., unpublished data). We further narrowed this list of candidate genes for QPCR analysis, focusing on members of the GT1 sub-families B, D, E and L. In *Arabidopsis*, the GT1 members capable of forming glucose esters of phenolics (such as salicylates and benzoates) were restricted to sub-family L, while those forming *O*-glucosides of phenolics were dispersed among the five sub-families B, D, E, F and L (Lim et al., 2002). Sub-family F is represented by a single *Populus* gene that is poorly expressed in cell culture, and, therefore, it was not included in the QPCR analysis. As references, we also included members from sub-families G, J and M that are well-expressed in leaves and showed a positive relationship between transcript abundance and PG concentrations in several *Populus* clones subjected to various treatments (e.g., N-stress, wounding, Babst et al., unpublished data).

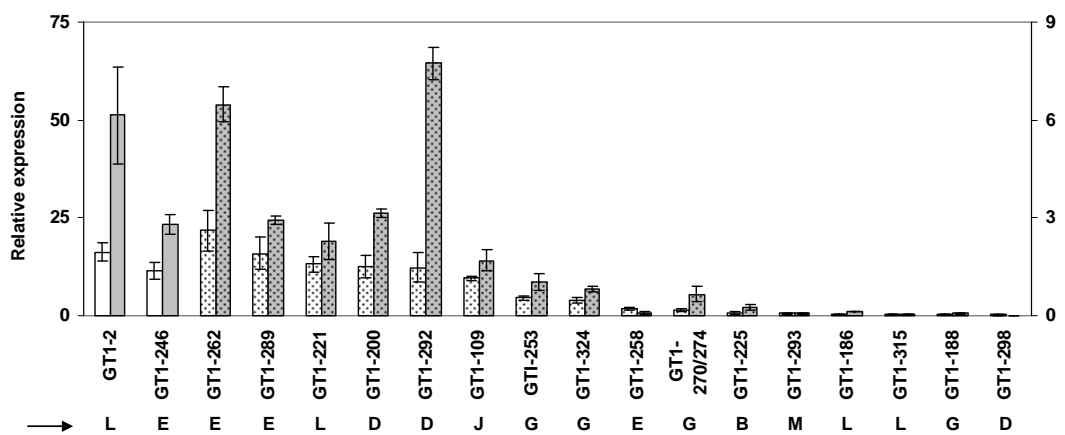
Altogether, QPCR was performed for 18 GT1 genes. The 10 well-expressed genes were from sub-families D, E and L (Figure 2-7). The remaining eight are expressed at very low

levels. The expression of the two most abundant GTs, *GTI-2* and *GTI-246* from sub-families L and E respectively, was up-regulated 2-3 fold by salicyl alcohol feeding. The expression of eight other moderately expressed GTs increased during the feeding, with GTs from sub-families D and E up-regulated more than 2-fold. The strong expression of sub-family L and E members, *GTI-2* and *GTI-246*, respectively in unfed cultures and their inducibility by salicyl alcohol-feeding suggest their involvement in the synthesis of salicins.

#### **2.3.6.4 Possible phenolic transporters**

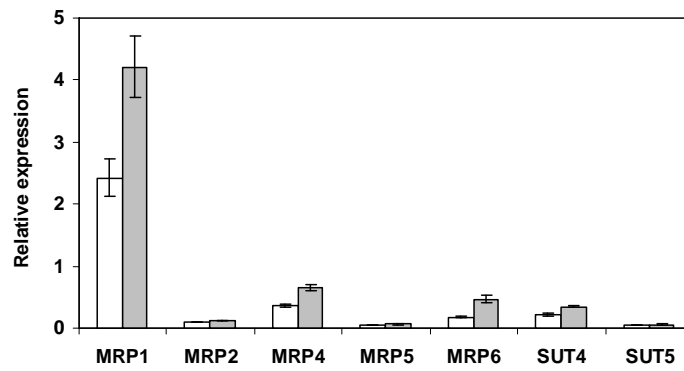
Phenolics and other xenobiotics glycosylated in cytoplasm are commonly transported into the vacuole. This is thought to be mediated by ATP-binding cassette (ABC) transporters or H<sup>+</sup> antiporters, depending on the compound being compartmentalized and the plant species (Dean et al., 2005; Dean et al., 2003; Rea, 2007). Microarray expression results showed a slight up-regulation of a Mg-ATP-dependent glutathione conjugate pump, known as MRP transporter, in salicyl alcohol-fed cultures (supplemental data). Therefore, we expanded our studies to the four putative tonoplast-localized and two plasma membrane-localized MRP transporters in *Populus*, based on the orthologs from *Arabidopsis* and maize (Kolukisaoglu et al., 2002; Rea, 2007). *PtMRP1* was the most abundant transcript and was up-regulated by 70% in salicyl alcohol-fed cultures (Figure 2-8). The expression of *PtMRP4* and *PtMRP6* was lower in unfed cultures, but was also up-regulated by 2.6 and 1.8 fold, respectively. *PtMRP2* and *PtMRP5* are poorly expressed and showed little change compared to control.

SUTs from several plant species are also capable of transporting a wide range of  $\alpha$ - and  $\beta$ -phenolic glucosides, besides sucrose, across biological membranes (Chandran et al., 2003; Reinders et al., 2008; Sivitz et al., 2007). *LeSUT4*, a tonoplast-localized transporter, has been suggested to be involved in the export of sucrose and possibly of phenolic glucosides from vacuole into cytoplasm (Reinders et al., 2008). Additional scenarios for how various SUTs function have been posited in review papers (Lalonde et al., 1999; Williams et al., 2000). Therefore, we examined the expression of the six *Populus SUT* gene family members in control and salicyl alcohol-fed cultures. *PtSUT4* was the most abundant transcript and its expression was up-regulated ~1.5-fold in cultures fed with salicyl alcohol (Figure 2-8). Expression of *PtSUT5* was very low while that of the remaining four SUTs was not observed in control or salicyl alcohol-fed cells. Together, these results suggest the possible involvement of *PtMRP1*, *PtMRP6* and *PtSUT4* in the transport of glycosides into and out of vacuole.



**Figure 2-7.** Q-PCR expression analysis of GT-1 gene family members in control (white bars) and salicyl alcohol-fed (gray bars) cultures.

The arrow at the bottom points to the subgroup of the GTs. Data represents the mean  $\pm$  SE of three biological replicates. Refer to the right-hand side axis for dotted bars.



**Figure 2-8.** Q-PCR expression analysis of *MRP* transporters and *SUT* genes in control (white bars) and salicyl alcohol-fed (gray bars) cultures.

Data represents the mean  $\pm$  SE of three biological replicates.



## 2.4 Discussion

### 2.4.1 Salicin synthesis in cell cultures

PGs and CTs are the two major phenylpropanoid derivatives detected in abundant quantities in *Populus* plants (Donaldson and Lindroth, 2007; Harding et al., 2005). Here, we report that CTs were also abundant in cell cultures, but that salicin and higher-order PGs were not detected (Figure 2-1). Since both CT and PG originate from different branches of the phenylpropanoid pathway, we conducted a series of experiments in search of a lesion in the PG biosynthetic branch. In general, phenolic precursors were all glycosylated upon suspension cell feeding. During the feeding experiments, it was evident that PG (salicin) precursor feeding interfered with CT biosynthesis. Several experiments were then conducted to investigate the relationship between PG precursor feeding, CT accrual and cell growth.

Several factors can be considered in analyzing the inability of the aspen suspension cells to synthesize PG. In cultured suspension cells of *Vanilla*, PAL activity and synthesis of phenylpropanoid derivatives were severely inhibited when grown in media supplemented with 2,4-D, but not with naphthalene acetic acid (NAA, Funk and Brodelius, 1990). In our study, the aspen cell cultures were also maintained in 2,4-D-containing media. However, high levels of *PAL* expression (Figure 2-5) and CT accumulation (Figure 2-1) were detected, making it unlikely that 2,4-D inhibited the phenylpropanoid pathway. It is possible that specific branches of the phenylpropanoid pathway are differentially regulated by 2,4-D. In *Populus* cell cultures, secondary metabolites such as anthocyanins

can be induced by feeding auxins (NAA and IAA), vitamins (riboflavin) or high levels of sucrose (5%, Matsumoto et al., 1973; Verma et al., 2000). However, in none of these experiments were anthocyanins induced without light, suggesting light as a key factor in synthesis of certain secondary metabolites (Matsumoto et al., 1973; Verma et al., 2000). In the present study, salicin or higher-order PGs were not detected in light-grown calli or heterotrophic cell cultures, but were present in the leaves of the aspen L4 line from which the cell cultures were initiated. It is possible that other factors needed for PG synthesis were not optimal under the callus and cell culture conditions used. Several secondary metabolites, such as quinolizidine alkaloid in *Lupinus* (Wink and Hartmann, 1980) and trigonelline (N methyl nicotinic acid) in tobacco (Ikemeyer and Barz, 1989) are synthesized only in phototrophic cells but not in heterotrophic cells. The shikimate pathway upstream of phenylpropanoid metabolism is localized in the chloroplast (Herrmann and Weaver, 1999). We reasoned that lack of plastids may hinder PG synthesis in dark-grown cells. Efforts to establish photoautotrophic cells, however, did not produce vigorous callus or cell suspension cultures.

Interestingly, salicin and isosalicin were detected in salicyl alcohol-, salicylaldehyde- and helicin-fed cultures. In contrast, the feeding of benzoates or phenylpropanoid precursors thought to be important for PG synthesis did not result in PG accumulation. Isotope feeding of leaf tissues with PG precursors such as cinnamic acid and benzoates resulted in salicortin labeling, and competition studies suggested that benzoic acid is an intermediate in salicin synthesis in poplar leaves (B. babst, unpublished, Zenk, 1967). Feeding of benzoic acid also induced accumulation of higher-order PGs in different

tissues of *Salix* (Ruuholta and Julkunen-Tiitto, 2003), confirming their importance in PG synthesis. It is possible that downstream product formation was not observed in the aspen cell cultures we used because the substrates fed to the cells were rapidly glycosylated and sequestered. The compartmentalization differs between cultured cells and intact tissue could be one of several possible contributing factors to failure of cells to make PGs, thus making this system not convenient for higher-order PG biosynthetic pathway elucidation.

#### **2.4.2 Metabolic competition between salicin and CTs in salicyl alcohol-fed cultures**

In cell cultures, feeding of 5 mM salicyl alcohol, especially during the exponential and stationary growth phases, led to accumulation of salicins with concomitant reduction of CTs by up to 35% at 96 h. The fact that salicins could not accumulate to the maximum levels following salicyl alcohol feeding to actively growing and CT-synthesizing cells, and that these cells were no longer able to maintain the normal trajectory of CT increases, further supports a dynamic mode of competition dictated by cell growth and metabolic status. The reduction in CTs is unlikely because of competition for intermediates, as salicins and CTs do not share common intermediates in cell cultures. A metabolic competition may exist in 5 mM salicyl alcohol-fed cultures for the sugar resources required for glycosylation of salicyl alcohol, growth and CT synthesis. The common sugar utilized by most GTs for glycosylation is UDP-glucose (Hostel, 1981; Jones and Vogt, 2001). In heterotrophic cells, UDP-glucose is derived from sucrose via SuSy-mediated hydrolysis or via phosphorylation of glucose released from invertase-hydrolyzed sucrose (Doehlert, 1990; Karnil and Aloni, 2002; Koch, 2004). Up-regulation

of *PtVIN2* and several *PtSuSy* genes indicates participation of multiple sub-cellular compartments during salicyl alcohol glycosylation. Gene expression for the Group 3 sucrose transporter *PtSUT4* (see Chapter 2) was also up-regulated. Based on the proposed model for SUT4 function (Chapter 2), orthologs of SUT4 mediate the efflux of sucrose and other phenolic glycosides from the vacuole into the cytosol (Reinders et al., 2008). Therefore, increased expression of *PtSUT4* supports the increased efflux of sucrose from the vacuole for SuSy-mediated hydrolysis in the cytosol. However, it is not clear that this is the major route for the provisioning of UDP-glucose in our experiments because *PtVIN2* expression also was sharply up-regulated (Figure 2-6, Table 2-2). In salicyl alcohol-fed cultures, several key genes involved in glycolysis and Krebs cycle pathway are up-regulated. However, these genes may have other functions in the cells besides involving in Krebs cycle. The hexoses not utilized for salicyl alcohol glycosylation can be utilized as intermediates in glycolysis. The conversion of pyruvate, the end product of glycolysis into acetyl-CoA, is the starting for Krebs cycle (Fernie et al., 2004). The increased Krebs cycle demands increased acetyl-CoA. Acetyl-CoA is also an intermediate in the synthesis of fatty acids and malonic acid pathway (Gueguen et al., 2000). Forty percents of the C skeleton of CTs is derived from malonic acid (Taiz and Zeiger, 1998). It may be possible that the C that otherwise can be used for CT synthesis is driven into Krebs cycle, thus possibly resulting in reduced CT in salicyl alcohol-fed cultures.

The differential compartmentalization of the sugar hydrolysis might affect sugar utilization for different pathways. The partitioning of sugars to growth is likely higher in salicyl alcohol-fed cultures because of the observed stimulated growth at 24 h and 48

h after feeding 1 and 5 mM salicyl alcohol. In addition, because the fed salicyl alcohol is toxic, substantial amount of C has to be diverted for its glycosylation. Thus, reduction in sugars could be a possibility of reduced CTs in salicyl alcohol-fed cultures. The observed reduced expression of phenylpropanoid and flavonoid pathway genes (Figure 2-5, Table 2-2) is also consistent with the reduced CT levels in salicyl alcohol-fed cultures. Thus, simple resource competition could have reduced C for CT synthesis supporting the observation that PGs and CTs show metabolic reciprocity in their abundance. Here we show that such reciprocity is also true in cell cultures. Alternatively, in these cultures, substantial amount of C could be diverted for the synthesis of proteins that are required for the sequestration of salicin into vacuole as reported in *Medicago* cells accumulating triterpenes after elicitation (Broeckling et al., 2005).

Normally, glycosylation is associated with sequestration or inactivation of toxins, reaction intermediates, or hormones etc (Gachon et al., 2005; Kim et al., 2009; Kita et al., 2000). Several of the characterized GTs show broad substrate specificity, while single substrate specific GTs were also reported (Hostel, 1981; Lim et al., 2005; Lim et al., 2002; Meßner et al., 2003). In this study, two conversions were observed in the feeding experiments; glycosylation and isomerization. Glycosylation to yield multiple glucoside isomers may be mediated by regio-specific glucosyltransferases as reported (Sato and Hasegawa, 1971; Sato and Hasegawa, 1972). In general, the findings from the various cell culture systems examined are consistent with our observation that isosalicin increased in relation to salicin during the feeding period, and that the process may be mediated by glycosyltransferases. As shown in Figure 2-7, most of the well-expressed

GTs were up-regulated in salicyl alcohol-fed cultures. The two most abundantly expressed genes, GT1-246 and GT1-2, are members of sub-families E and L, respectively. Substrate specificity differs within and among sub-family members, as two of three sub-family E GTs of *Arabidopsis* glycosylate sinapyl and coniferyl alcohols preferentially, while the third one glycosylates sinapyl and coniferyl aldehydes only (Lim et al., 2005). In general, GTs that form esters from aglycones are restricted to sub-family L, while those that form *O*-glucosides are distributed among different families, including L and E (Lim et al., 2002). The L-family protein FaGT2 from strawberry (*Fragaria x ananassa*) glycosylates phenol-carboxylic acid aglycones to form glucose esters, but not *O*-glucosides (Lunkenbein et al., 2006). The two abundant and salicyl alcohol-induced GTs, *GT1-2* and *GT1-246* are most likely candidates for salicin and isosalicin formation from salicyl alcohol, although we cannot exclude the possible involvement of other weakly expressed GTs.

After glycosylation, the glycosides are transported into the vacuole for storage (Dean et al., 2005; Dean et al., 2003; Rea, 2007). It has been recently reported that these glycosides can be exported from the vacuole in response to defense signals (Farag et al., 2008; Naoumkina et al., 2007; Rea, 2007). An ABC transporter in cell cultures of soybean, and a H<sup>+</sup> antiporter in tobacco are involved in the uptake of salicylate glucosides (Dean et al., 2005; Dean et al., 2003). A sub-group of tonoplast-localized ABC transporters in *Arabidopsis*, AtMRP1 and AtMRP2 (multidrug resistance proteins), are also capable of transporting xenobiotics, herbicides and anthocyanins into the vacuole (Lu et al., 1998; Lu et al., 1997). As mentioned earlier, orthologs of PtSUT4 are also

capable of transporting salicin (Reinders et al., 2008). The up-regulation of putative tonoplast localized MRP transporters, *PtMRP1* and *PtMRP6* suggests their involvement in the uptake of salicins from cytoplasm into vacuole. Salicin uptake was also shown to be mediated by SUTs (Reinders et al., 2008). The up-regulation of *PtSUT4* in salicyl alcohol-fed cultures supported its involvement in the transport of salicins from vacuole into cytoplasm, as an alternative function, presumably for transglucosylation i.e. conversion of salicin to isosalicin which occur in cytoplasm. However, involvement of unidentified H<sup>+</sup> transporters cannot be ruled out.

Our cell culture studies provided additional information related to PG-CT metabolic reciprocity. Additional work such as gene silencing is needed to confirm the involvement of our predicted genes in salicin and isosalicin synthesis and transport. Our gene expression studies were limited only to one salicyl alcohol feeding condition. A better understanding of the class or classes of GTs involved in salicin formation and of the transporters involved in uptake of salicins may be achieved by using different levels of salicyl alcohol, alternative time points or other aglycones such as salicylaldehyde.

## Chapter 3 Sucrose transporter (SUT) gene family in *Populus* and PtSUT4-mediated regulation of non-structural carbohydrates and phenylpropanoids

### ABSTRACT

Sucrose is the most abundant sugar found in the transport stream of *Populus*. In this and several closely related temperate tree taxa, the mechanism of sucrose loading into the phloem is thought to be passive symplastic, and to be facilitated by sucrose transporters (SUT). In addition to their function in sucrose transport, certain SUT proteins transport phenylpropanoid-glycosides, at least one of which comprises an important carbon sink in *Populus*. The objective of this study was to characterize the *Populus* SUT gene family and to investigate the effect of *SUT* suppression on carbohydrate and secondary metabolite homeostasis in transgenic *Populus*. Six *SUTs* are found in the *Populus* genome and they can be classified into three groups based on phylogenetic analysis. The expression of Group-1 members *PtSUT1/2* and *PtSUT3* is restricted to vascular tissues in leaves and stems. *PtSUT4* (Group-3) and *PtSUT5* (Group-2) are the most abundantly expressed isoforms, and their transcripts were found in all tissues examined. *PtSUT3*, *PtSUT4* and *PtSUT5* encode functional transporters based on yeast complementation experiments. To investigate SUT function *in planta*, transgenic poplar plants with suppressed *SUT4* expression were produced and grown under two regimes of N nutrition. *SUT4* was chosen because of its abundant expression in all the tissues and because of its correlation with PGs under N stress in the preliminary studies. Transgenic plants exhibited higher ratios of leaf area-to-plant height and leaf area-to-stem mass than



wildtype plants whether grown under N-replete or low N nutrient conditions. Sucrose and glucose levels were higher in source leaves of *SUT4* transgenics, regardless of N treatment. Foliar starch accrual was not affected by *SUT4* suppression during N-replete growth. However, the foliar starch increase caused by N-deficiency in wildtype plants did not occur in *SUT4* transgenics. Phenolic glycoside (PG) levels were reduced in leaves and primary stems of *SUT4* plants. Under N deficiency, PGs decreased in wildtype, but not in *SUT4* plants. Sucrose synthase (*SuSy*) gene expression was reduced in source leaves of *SUT4* transformants. Plant-wide, the expression of *SuSy* and invertase genes was slightly elevated in stems and roots, but slightly suppressed in leaves of *SUT4* transgenics. The plant-wide distribution of phenylalanine ammonia-lyase gene transcripts was also affected by *SUT4* down-regulation. In *Populus* it appeared that *PtSUT4* at least partly regulates N-dependent C partitioning between sugars and phenylpropanoids.

### **3.1 Introduction**

Sucrose is the predominant transport form of photoassimilate in many herbaceous annual, including *Arabidopsis* (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*), and woody perennial (e.g., *Populus*) species (Haritatos et al., 2000; Konishi et al., 2004). Sucrose synthesized in the cytoplasm from Calvin cycle triose phosphates is stored in the vacuole or is exported to sink organs via the phloem (Buchanan et al., 2000). Transport sucrose is actively loaded into the phloem apoplastically as in *Arabidopsis* (Gottwald et al., 2000), or moves there symplastically via plasmodesmata (Lalonde et al., 1999; Turgeon, 2000). In the close *Populus* relative, *Salix*, and many other temperate tree taxa,

symplastic transfer to phloem is not thought to involve polymer trapping as in the cucurbits (Turgeon, 2006; Turgeon and Medville, 1998). In passive symplastic transporters, the mechanism regulating sucrose movement into the phloem is not well understood but may partly be regulated by the distribution of sucrose between vacuole and mesophyll (Turgeon and Medville, 1998). Plasmolytic studies indicated that an apoplastic component may contribute to sucrose transport in *Populus* (Russin and Evert, 1985), but in *Salix* at least, there is no evidence for apoplastic phloem loading (Turgeon and Medville, 1998).

Sucrose transporter proteins (SUT/SUCs) that facilitate phloem-loading, long-distance transport and intracellular compartmentalization have been characterized from a number of model plant systems, including *Arabidopsis* (Sivitz et al., 2007; Srivastava et al., 2008), rice (*Oryza sativa*, Aoki et al., 2003) and tomato (*Lycopersicon esculentum*, Hackel et al., 2006). Multiple *SUT/SUC* genes encoding functional proteins with distinct properties are generally expressed in each species (ArabidopsisGenomeInitiative, 2000; Turgeon, 2006; Yu et al., 2002). Based on sequence phylogeny, the known SUT/SUCs fall into three groups (Kuhn, 2003; Reinders et al., 2008; Sivitz et al., 2007). In general, the transporters in Group-1 (e.g., AtSUC1, AtSUC2, AtSUC9) exhibit a high affinity (low  $K_m$ ) for sucrose (Chandran et al., 2003; Sivitz et al., 2007). Several Group-1 proteins (AtSUC2, LeSUT1, NtSUT1, StSUT1) have been localized at the plasma membrane of phloem companion cells in leaves and stems (Schmitt et al., 2008; Stadler and Sauer, 1996). Group-2 transporters (e.g., AtSUC3, PmSUC3 and HvSUT1) generally exhibit a moderate affinity for sucrose (Barth et al., 2003; Schulze et al., 2000; Sivitz

et al., 2005). Several Group-2 transporters have been localized at the plasma membrane of phloem sieve elements. The Group-3 transporters, e.g., LjSUT4, StSUT4 and AtSUC4, also exhibit low affinity for sucrose (Reinders et al., 2008; Weise et al., 2000). Members of this group are localized at the tonoplast (AtSUC4, HvSUT2 and LjSUT4) of leaf mesophyll cells and roots (Endler et al., 2006; Reinders et al., 2008) or at the plasma membrane (StSUT4, Chincinska et al., 2008). An exception to the general patterns of sucrose affinity described above was observed in grape (*Vitis vinifera*), where Group-1 transporters exhibited a lower affinity ( $K_m$  8.0-10.5 mM) for sucrose than Group-2 and Group-3 transporters ( $K_m$  0.9-1.4 mM, Manning et al., 2001; Zhang et al., 2008). SUTs from all three groups exhibit *in vitro* transport activity for maltose, various  $\alpha$ - and  $\beta$ -phenyl glucosides, salicin and helicin at rates similar to that of sucrose (Reinders et al., 2008; Sivitz et al., 2007; Sivitz et al., 2005).

Gene silencing has been used to understand the function of various SUT/SUCs. On the basis of high sugar/starch accumulation in source leaves of mutant or SUT-suppressed lines, Group-1 transporters (*AtSUC2*, *NtSUT1*, *LeSUT1* and *StSUT1*) are confirmed to play a central role in active phloem loading of sucrose (Burkle et al., 1998; Gottwald et al., 2000; Hackel et al., 2006; Riesmeier et al., 1994; Srivastava et al., 2008). So far, gene silencing has been tested on only one Group-3 transporter, a plasma membrane-localized *StSUT4* in potato (*Solanum tuberosum*). The diurnal pattern of sugar accumulation and export was altered in source leaves of the silenced plants. Those plants also displayed early flowering, increased tuber yield, and reduced expression of gibberellic acid and

ethylene biosynthetic genes (Chincinska et al., 2008). To date, no mutant or transgenic manipulation studies have been reported for the tonoplast-localized SUTs.

In addition to direct effects on sugar metabolism, altered SUT expression has been reported to affect phenylpropanoid metabolism. Secondary metabolites such as anthocyanins accumulate abnormally in source leaves of *AtSUC2* mutants and *StSUT1* antisense lines (Gottwald et al., 2000; Riesmeier et al., 1994; Srivastava et al., 2008). Microarray analysis of the *AtSUC2* mutants revealed a several-fold up-regulation of phenylpropanoid and flavonoid pathway genes, consistent with the increased anthocyanin levels (Lloyd and Zakhleniuk, 2004; Riesmeier et al., 1994). Anthocyanins accumulate in hypocotyls and cotyledons to higher levels when *Arabidopsis* is fed sucrose and maltose than when the sucrose analog turanose is fed (Solfanelli et al., 2006; Teng et al., 2005). Anthocyanins do not accumulate when the sucrose analog palatinose is fed (Solfanelli et al., 2006; Teng et al., 2005). In accordance with a role for SUT in the response, SUT proteins do not transport palatinose (Chandran et al., 2003; Sivitz et al., 2007). Anthocyanins also do not accumulate when *AtSUC1* mutants are grown on sucrose media which again supports the involvement of SUT for anthocyanin accumulation (Reinders et al., 2008). Either sucrose or maltose feeding can induce the expression of defense related genes such as hydroxyproline-rich glycoprotein (HRGP) in soybean, and the induction is suppressed when *p*-chloromercuribenzenesulfonate (PCMBS) is used to inhibit transport (Ahn and Lee, 2003).

SUT/SUC may affect secondary metabolism directly via their affinity for glycosylated secondary metabolites such as salicin (Chandran et al., 2003; Reinders et al., 2008), or indirectly via participation in sugar transport (Sauer, 2007). Whereas high levels of secondary metabolites can be induced in herbaceous model species, they are constitutively abundant in leaves of *Populus*, *Salix* and other woody perennials (Lindroth et al., 1987a; Orians et al., 2000; Ruuhola et al., 2001). The phenolic glycosides (PGs) characteristic of these taxa are thought to be derived from salicin, a putative SUT substrate (Chandran et al., 2003; Pierpoint, 1994; Reinders et al., 2008; Ruuhola and Julkunen-Tiitto, 2003). PG concentrations exceeding 25% leaf dry weight are not uncommon in aspen and cottonwood (Donaldson and Lindroth, 2007; Harding et al., 2005; Lindroth and Hwang, 1996). Based on  $^{14}\text{CO}_2$  tracer studies in *Populus*, 30% of photoassimilate are rapidly partitioned into non-structural phenylpropanoids, including PGs and anthocyanidin-derived condensed tannins (CTs, Kleiner et al., 1999). Concentrations of these non-structural phenylpropanoids are also known to vary substantially during the growing season and in response to soil nutrient availability and atmospheric  $\text{CO}_2$  (Hakulinen et al., 1995; Kleiner et al., 1998; Lindroth et al., 1987b; McDonald et al., 1999; Stevens et al., 2007). Here we characterize the *PtSUT* gene family and investigate its importance to the interface between carbohydrate and secondary metabolism in *Populus*.

## 3.2 Materials and Methods

### 3.2.1 Plant materials

*Populus tremula* × *Populus alba* clone 717-1B4 was used in this study unless otherwise mentioned. *In vitro* micropropagated plants were hardened in a mist chamber and transferred to either soil or hydroponics tubs. Pot fertilization and hydroponic nutrient composition were as reported previously (Harding et al., 2005). The first fully unfurled leaf 2 cm in length was considered leaf plastocron index 0 (LPI-0, Larson and Isebrands, 1971). The following tissues were analyzed: shoot tip (ST, apical to LPI-0); young leaf (YL, LPI-2); mature leaf (ML, LPI5); primary stem (PS, stem internodes 0-3); secondary stem (SS, stem internodes 7-9); root (RT, the distal-most 6-8 cm of rapidly elongating primary roots excluding the 1-cm tip/cap); developing phloem (PH) and xylem (XY) were collected from stem internodes 10-15. Male and female catkins and pollen were collected from field grown flowering *P. tremuloides* trees. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until use.

### 3.2.2 Gene and protein sequence analysis

*SUT* gene models were identified from the sequenced *Populus trichocarpa* genome (Tuskan et al., 2006) by BlastP with published SUT proteins. Best predicted models were chosen by manually examining all available computational gene model predictions at each locus in the JGI *Populus trichocarpa* genome portal v1.1. The primary structure analysis of SUT proteins was carried out using the ExPASy proteomic server (<http://us.expasy.org/>). Multiple sequence alignment and similarity calculation were

performed using the GCG (Genetics Computer Group, Madison, WI) software. Phylogenetic analysis of protein sequences was performed with MEGA package v3.1 (Kumar et al., 2004) using the minimum-evolution algorithm. Rooted trees were generated from the bootstrap test with 1,000 replicates, using the Poisson correction (homogenous patterns) and the complete deletion option for handling alignment gaps.

### **3.2.3 Quantitative dual target PCR**

*PtSUT1/2* gene copy number was estimated using QD-PCR (Kihara et al., 2006) in a 10 µl reaction containing the following: 20 ng of genomic DNA, 0.35 U of RedTaq polymerase (Sigma), 100 µM dNTPs, and 0.5 µM primers. The primers used in this study were designed using the PrimerQuest online primer design tool (<http://www.idtdna.com>) and are listed in Appendix A. Known single-copy genes in the *Populus* genome, *PtSUT3*, *PtC4HL1*, were chosen as the reference genes. Primers of reference and target genes were designed such that the GC content (50%) and T<sub>m</sub> (60 °C) values are similar. To minimize effects of amplicon size on band intensity estimation, two target gene (*PtSUT1/2*) and reference gene (*PtSUT3*, *PtC4HL*) primer sets were used. The target gene primers were designed to amplify ~500 bp and the reference gene primers, *PtSUT3*, 420 bp, and *PtC4HL1*, 680 bp.

### **3.2.4 RNA extraction**

RNA was extracted from frozen tissues using the CTAB (cetyltrimethylammonium bromide) method (Chang et al., 1993). To the finely ground sample, 1 ml of CTAB

buffer containing 2%  $\beta$ -mercaptoethanol was added, vortexed for 1 min and incubated for 15 min at 65 °C with intermittent mixing. To this tube, 900  $\mu$ l of chloroform:isoamyl alcohol (24:1) were added, vortexed and centrifuged for 10 min at 10,000 g to separate the two phases. RNA in the aqueous phase was precipitated using 330  $\mu$ l of 8M LiCl. The samples were incubated on ice for 2 hours and centrifuged at 4 °C for 15 min at 14,000 g. The remaining steps for RNA extraction were performed as described in Chapter 2.

### **3.2.5 cDNA synthesis and Q-PCR expression analysis**

cDNA synthesis and Q-PCR were performed as described in Chapter 2.

### **3.2.6 Yeast complementation**

Functional analysis of the *Populus* SUTs was carried out by yeast complementation experiments using the SUSY7/ura3 mutant strain (Riesmeier et al., 1992; Weise et al., 2000). Full-length coding sequences of *PtSUT3*, *PtSUT4* and *PtSUT5* was cloned into the yeast shuttle vector PDR196 (Rentsch et al., 1995), between the *plasma membrane H<sup>+</sup>-ATPase* (PMA1) promoter and the *alcohol dehydrogenase* (ADH) terminator. The resultant plasmids were transformed into the SUSY7/ura3 strain using the lithium acetate method (Gietz and Woods, 2002). Transformants were selected on uracil-deficient medium and screened by PCR using a combination of gene-specific and vector primers. The transformed and untransformed mutants were cultured on yeast media (1.7 gm l<sup>-1</sup> yeast nitrogen base without amino acids and 500 mg l<sup>-1</sup> ammonium sulfate) supplemented with either 2% glucose or 2% sucrose as the sole carbon source for 3 days.



### **3.2.7 RNAi construct development and plant transformation**

A 200 bp *SUT4* fragment amplified from the 3'-region of the *Populus* cDNA was ligated to an *XcmI* digested pGFPm-T vector (Luo et al., 2008) and transformed into TOP10 competent *Escherichia coli* cells. Subcloning of the *SUT4* fragment and development of an inverted repeat in the pGSA1285 ([www.chromdb.org](http://www.chromdb.org)) binary vector backbone was performed as described previously (Luo et al., 2008). The binary vector was transformed into *Agrobacterium* strain C58-pMP90 using the freeze and thaw method (Holsters et al., 1978). Transformed colonies were selected on LB media supplemented with chloramphenicol (170 mg l<sup>-1</sup>), and were PCR confirmed. Positive *Agrobacterium* strain was used to transform *Populus* leaf pieces by co-cultivation as previously described (Ma et al., 2004). Transformed plants were selected from kanamycin (100 mg l<sup>-1</sup>) media and confirmed by PCR using a combination of gene-specific and pGSA1285 vector primers. Fully rooted wildtype and *SUT4* transgenic plants were moved from culture boxes to the mist chamber in a greenhouse for acclimation.

### **3.2.8 Hydroponic nitrogen treatment studies**

Hydroponically-grown, untransformed and *SUT4* transformed plants (~15 cm in height) were distributed into small, 10-L hydroponics tubs 8 days before nutrient treatments began. After the 8-day acclimation period, the plants were maintained either with full-strength (2.5 mM N, referred to as N-replete) or with 5% of full strength N (0.125 mM N, referred to as low-N or stressed). For each treatment, 7-10 individual plants were used. Nutrient treatments were continued for 2 weeks before the plants were harvested.

Nutrients were changed every 2 days to maintain the N treatment levels.

### **3.2.9 Sugar and starch extraction and quantification**

Sucrose, glucose and fructose were estimated as described (Sherma and Zulick, 1996). Briefly, 20 mg of freeze-dried tissue sample was extracted three times in 800  $\mu$ l of 80% methanol at 95°C. The supernatants were combined for fructose, glucose and sucrose estimation and the pellet was saved for starch analysis. The three sugars were resolved by HPTLC with a Linomat5 applicator (Camag, Muttenz, Switzerland). Methanol extract (2  $\mu$ l) was loaded onto HPTLC Merck silica gel 60 F254 plates (Fisher, Hanover Park, IL) impregnated with 0.10 M sodium bisulfate and 10 mM citrate buffer (pH 4.8). The plates were developed three times with the mobile phase acetonitrile:H<sub>2</sub>O (85:15) in a horizontal TLC plate developing chamber equilibrated for 10 min. After the third development, plates were sprayed with 1-naphthol-sulfuric acid reagent (180 mM 1-naphthol dissolved in 75% ethanol containing 10% sulfuric acid) and heated for 10 min at 100-110°C. The sugars were quantified by scanning the plate at 515 nm using a CAMAG™ TLC scanner. Peak areas were integrated using WINCATS™ software (Camag) and sucrose, glucose and fructose were quantified using authentic standards.

Starch was estimated by an enzymatic method (Chow and Landhausser, 2004; Fox et al., 2001). The pellet was dispersed into 720  $\mu$ l of 0.1 N NaOH and incubated at 50°C for 30 min with intermittent mixing. The suspension was neutralized with 800  $\mu$ l of 0.1 N acetic acid, and starch was digested by adding 1 U each of  $\alpha$ -amylase (from *Aspergillus oryzae*,

Sigma chemicals, St Louis, MO) and *amyloglucosidase* (from *Aspergillus niger*, Sigma) in 180  $\mu$ l of 0.05 M sodium acetate buffer (pH 5.1) and incubated for 24 h at 50°C. Equal volumes of enzyme digest and 33 mM *p*-hydroxy benzoic acid hydrazite solution in alkaline diluent (0.5 M trisodium citrate, 0.1 M calcium chloride and 0.5 M sodium hydroxide) were combined, boiled for 4 min, cooled on ice for 4 min and absorbance measured at 415 nm. Starch was then estimated based on the standard curve developed using glucose.

### **3.2.10 PG and CT extraction and quantification**

Freeze-dried samples were analyzed for PG and CT as described (Harding et al., 2005) with slight modifications. Briefly, 5 mg of freeze dried tissue was extracted in 500  $\mu$ l of cold methanol (4°C-10°C) for 20 min in an ultrasonic bath and centrifuged at 15,000 g for 5 min. For PG quantification, 2  $\mu$ l of the methanol extract was loaded onto Merck silica gel 60 F254 silica plates (Fisher) and developed in ethyl acetate:methanol:water (77:13:10, v/v, Meier et al., 1987). The plates were scanned at a wavelength of 270 nm using a CAMAG™ TLC scanner (Camag). Peak areas were integrated using WINCATS™ software (Camag) and PGs quantified using authentic salicin, salicortin and tremulacin standards (provided by Rick Lindroth, Univ. of Wisconsin, Madison). Soluble and insoluble CTs were extracted and quantified as described in Chapter 2. Total CT levels were based on estimates of soluble and insoluble CTs.

### 3.3 Results

#### 3.3.1 The *Populus SUT* gene family

Six *PtSUT* gene sequences were identified in the *P. trichocarpa* genome (Tuskan et al., 2006). While *PtSUT3*, *PtSUT4*, *PtSUT5* and *PtSUT6* are located on linkage groups (LG) 19, 2, 8 and 10, respectively, *PtSUT1* and *PtSUT2* are located on scaffolds that have not been assembled to any chromosome (Table 3-1). There is a large gap in the *P. trichocarpa SUT2* sequence upstream of the stop codon, but the rest of the coding region sequence is nearly identical to that of *PtSUT1* (99% identity at the nucleotide and amino acid levels). We estimated the copy number of *PtSUT1/2* in the *Populus* genome by quantitative dual-target PCR (Kihara et al., 2006). As shown in Figure 3-1, the amplified band of the target gene(s), *PtSUT1/2*, was more intense than those of the single-copy reference genes, *PtSUT3* and cinnamate 4-hydroxylase-like (PtC4HL, Tsai et al., 2006a). To determine whether distinct *PtSUT1* and *PtSUT2* genes exist, a 1kb genome fragment near the 3' region of *PtSUT1* and *PtSUT2* sequence was amplified from *P.tremula* × *P. alba* using a primer pair designed to amplify both *PtSUT1* and *PtSUT2*, and the products were cloned and sequenced. Figure 3-2 shows sequence alignment of the 1kb-fragments for *PtSUT1* and *PtSUT2* from *P. trichocarpa* and *PtSUT1x* and *PtSUT1y* from *P. tremula* × *P. alba*. Of the 18 clones sequenced, *PtSUT1x* was represented in 14 and *PtSUT1y* in 4 clones. Because of the observed sequence differences between *PtSUT1x* and *PtSUT1y*, the presence of two highly identical genes, *PtSUT1* and *PtSUT2* in the *Populus* genome is supported.

Sequence and gene structure analyses revealed that the six *PtSUT* genes belong to three distinct groups. *PtSUT1/2* and *PtSUT3* are highly similar to each other, exhibiting 90% identity at the nucleotide level and 92% similarity at the amino acid level (Table 3-2). They also share a conserved gene structure, with 3 exons and 4 introns each (Figure 3-3). *PtSUT4*, shares 55-63% similarity with the other SUT isoforms at the coding sequence and amino acid levels, and contains 5 exons. *PtSUT5* and *PtSUT6* are highly similar to each other (91% nucleotide identity and 93% amino acid similarity, Table 3-2). They each contain 14 exons, and their predicted polypeptides are relatively large, ~65 KDa versus 55-57 KDa for the other SUT proteins. In sharp contrast to the other *PtSUT* isoforms which have theoretical pI's >9.0, *PtSUT5* and *PtSUT6* have acidic theoretical pI's of 6.0-6.2. The 12 transmembrane domains characteristic of SUT/SUCs (Sauer, 2007) are predicted for all six PtSUTs.

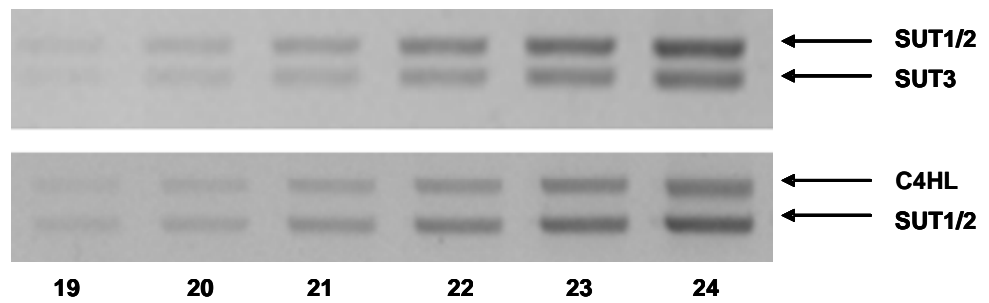
### **3.3.2 Phylogenetic analysis of SUT proteins**

Phylogenetic analysis of SUT proteins from several plant species was carried out using SpSUT from *Schizosaccharomyces pombe* (Reinders and Ward, 2001) as the outgroup. As shown in Figure 3-4, plant SUTs formed three distinct groups. Group-1 consisted of SUTs exclusively from dicots, and included three *Populus* (PtSUT1-PtSUT3) and seven *Arabidopsis* isoforms. Within Group-1, a sub-clade included SUTs predominantly from perennial species, such as *Populus*, castor bean (*Ricinus communis*), rubber (*Hevea brasiliensis*), grape (*Vitis vinefera*) and walnut (*Juglans regia*). Group-2 and Group-3 included SUTs from both monocots and dicots. Within Group-2, SUTs from monocots

**Table 3-1.** *SUT* gene models identified from the *P. trichocarpa* genome (version 1.1).

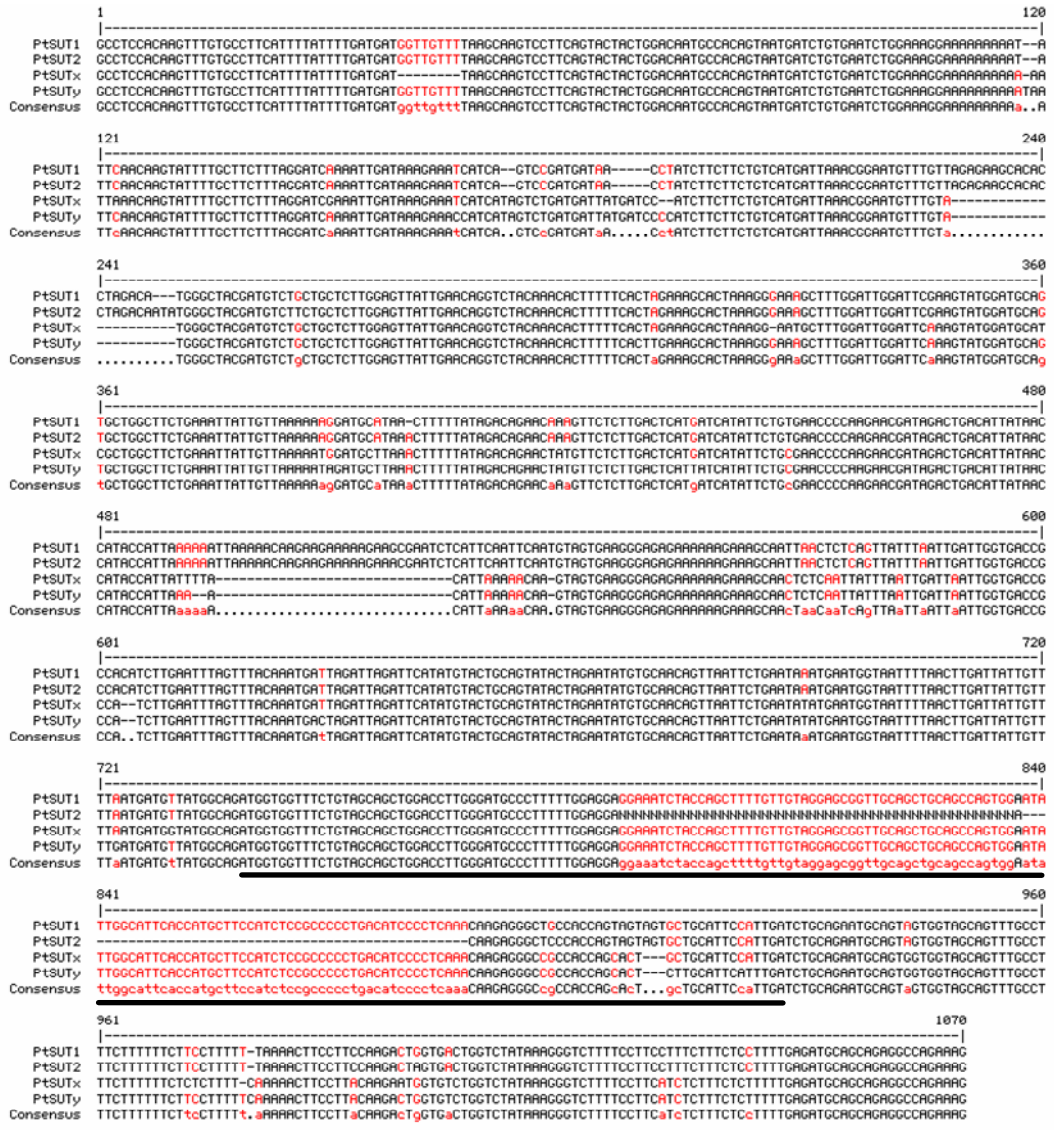
([http://genome.jgi-psf.org/Poptr1\\_1/Poptr1\\_1.home.html](http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html)).

<b>Gene</b>	<b>Gene model</b>	<b>Location</b>
<b>PtSUT1</b>	eugene3.00410059	Poptr1_1/scaffold_41:677823-681235
<b>PtSUT2</b>	estExt_Genewise1_v1.C_4590006	Poptr1_1/scaffold_459:41195-44407
<b>PtSUT3</b>	estExt_Genewise1Plus.C_LG_XIX2107	Poptr1_1/LG_XIX:9549420-9553188
<b>PtSUT4</b>	estExt_fgenesh4_pm.C_LG_II0488	Poptr1_1/LG_II:7765116-7776023
<b>PtSUT5</b>	fgenesh4_pg.C_LG_VIII001323	Poptr1_1/LG_VIII:9982945-9989473
<b>PtSUT6</b>	fgenesh4_pg.C_LG_X000861	Poptr1_1/LG_X:10053327-10060018



**Figure 3-1.** Estimation of *PtSUT1/2* gene copy number using QD-PCR.

*PtSUT1/2*, and single-copy *PtSUT3* and *PtC4HL* gene fragments were amplified from 20 ng of genomic DNA. The bands for the respective gene fragments are indicated by arrows.



**Figure 3-2.** Sequence alignment of the 1-kb fragment of *PtSUT1* and *PtSUT2* from the *P. trichocarpa* genome database, and *PtSUT1x* and *PtSUT1y* from the clones of *P. tremula* × *P. alba*.

Sequence variations are shown in red color letters. The underlined sequence represents the last exon of the genes.





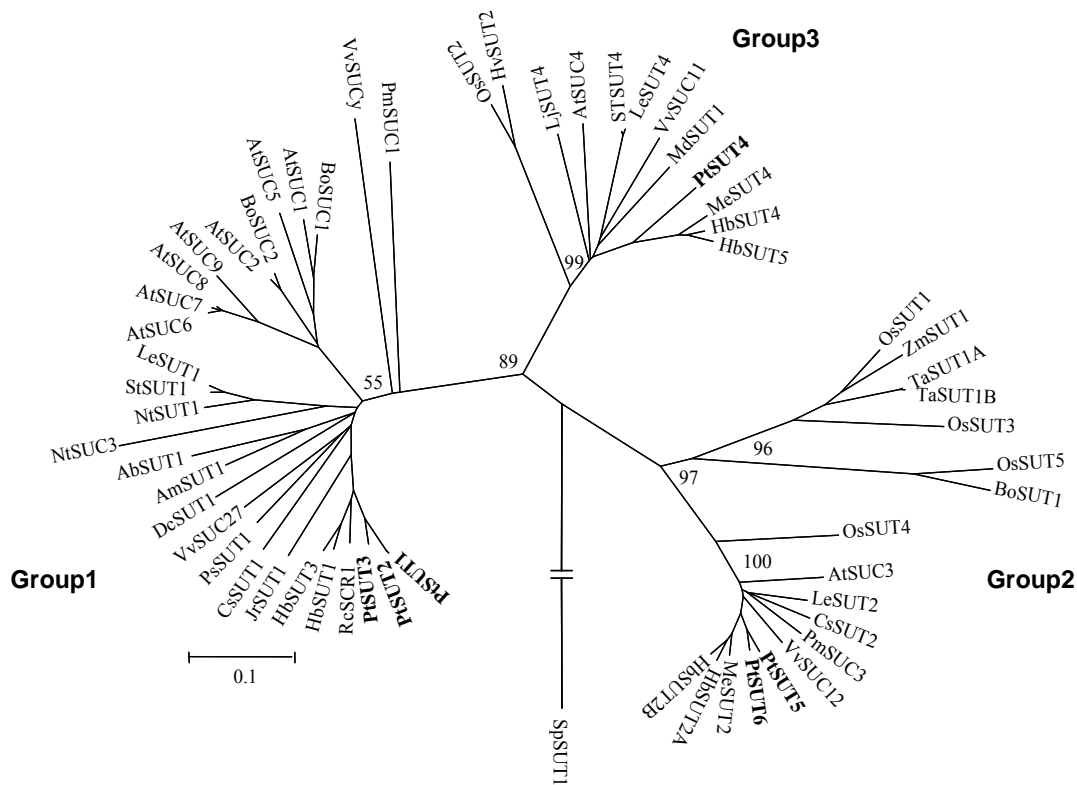
**Figure 3-3.** Schematic diagram comparing the *Populus* SUT gene structures.

Gene structures were predicted using spidey (<http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/>). Group-1, Group-2 and Group-3 members have 3, 4 and 13 introns, respectively.

**Table 3-2.** Properties of *Populus* SUT proteins as deduced from the coding sequence.

	Size		pI	% similarity (coding sequence/amino acid) to				
	a.a	kDa		PtSUT2	PtSUT3	PtSUT4	PtSUT5	PtSUT6
<b>PtSUT1</b>	535	56.8	9.2	99/99	90/92	55/63	54/57	54/57
<b>PtSUT3</b>	535	57.0	9.0			60/63	54/56	53/57
<b>PtSUT4</b>	510	55.3	9.1				56/58	55/56
<b>PtSUT5</b>	605	65.0	6.0					91/93
<b>PtSUT6</b>	602	64.5	6.2					

The size and the pI were calculated using the ExPASy proteomic server ([http://expasy.org/cgi-bin/pi\\_tool](http://expasy.org/cgi-bin/pi_tool)). Amino acid sequence similarity was determined using GCG (Genetic computer group, Madison, WI).



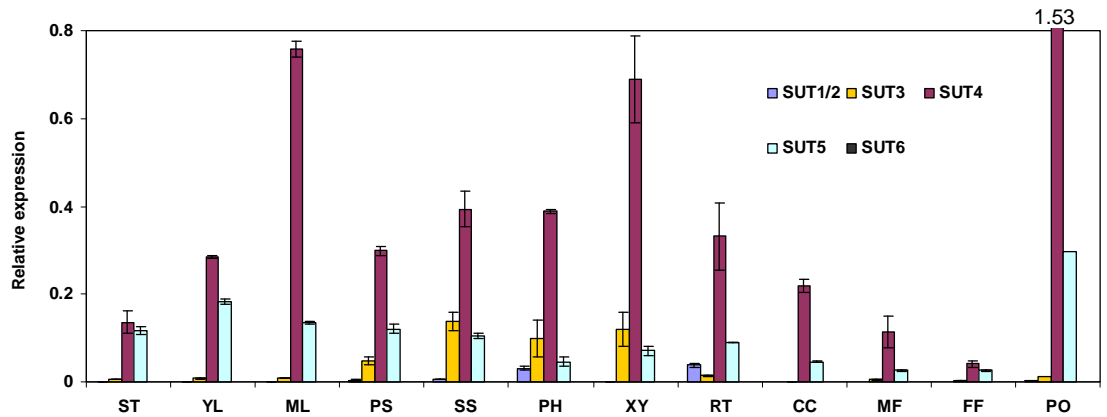
**Figure 3-4.** Minimum-evolution tree of 58 SUT protein sequences from several plant species.

The sucrose transporter SpSUT1 (*Schizosaccharomyces pombe*, CAB16264) was used as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Populus* SUTs are indicated by boldface. GenBank accession numbers of the sequences included are given below. AbSUT1 (*Asarina barclaiana*; AAF04294), AmSUT1 (*Alonsoa meridionalis*; AAF04295), AtSUC1 (At1g71880), AtSUC2 (At1g22710), AtSUC3 (At2g02860), AtSUC4 (At1g09960), AtSUC5 (At1g71890), AtSUC6 (At5g43610), AtSUC7 (At1g66570), AtSUC8 (At2g14670), AtSUC9 (At5g06170), BoSUC1 (*Brassica oleracea*; AAL58071), BoSUC2 (AAL58072), BoSUT1 (*Bambusa oldhamii*; AAY43226), CsSUT1 (*Citrus sinensis*; AAM29150), CsSUT2 (AAM29153), DcSUT1 (*Daucus carota*; BAA89458), HbSUT1 (*Hevea brasiliensis*; ABJ51933), HbSUT2a (*H. brasiliensis*; ABJ51934), HbSUT2b (ABJ51932), HbSUT3 (ABK60190), HbSUT4 (ABK60191), HbSUT5 (ABK60189), HvSUT2 (*Hardium vulgare*; CAB75881), JrSUT1 (*Juglans regia*; AAU11810), LeSUT1 (*Lycopersicon esculentum*; X82275), LeSUT2 (AAG12987), LeSUT4 (AAG09270), LjSUT4 (*Lotus japonicus*; CAD61275), MdSUT1 (*Malus domestica*; AAR17700), MeSUT2 (*Manihot esculenta*; ABA08445), MeSUT4 (ABA08443), NtSUT1 (*Nicotiana tabacum*; X82276), NtSUT3 (AAD34610), OsSUT1 (*Oryza sativa*; AAF90181), OsSUT2 (AAN15219), OsSUT3 (BAB68368), OsSUT4 (BAC67164), OsSUT5 (BAC67165), PmSUC1 (*Plantago major*; CAI59556), PmSUC3 (CAD58887), PsSUT1 (*Pisum sativum*; AAD41024), RcSCR1 (*Ricinus communis*; CAA83436), StSUT1 (*Solanum tuberosum*; CAA48915), StSUT4 (AAG25923), TaSUT1A (*Triticum aestivum*; AAM13408), TaSUT1B (AAM13409), VvSUCy (*Vitis vinifera*; AAL32020), VvSUC11 (AAF08329), VvSUC12 (AAF08330), VvSUC27 (AAF08331), and ZmSUT1 (*Zea mays*; BAA83501). Protein sequences for PtSUTs were deduced from the gene models presented in Table 3.1.

formed a distinct sub-clade. PtSUT5 and PtSUT6 clustered in the dicot sub-clade of Group-2, along with AtSUC3. Group-3 contained PtSUT4 and tonoplast localized AtSUC4, LjSUT4 and HvSUT4.

### **3.3.3 Expression of PtSUT genes in *Populus***

Plant-wide expression patterns of the six *PtSUT* genes were compared by quantitative RT-PCR. Primers were designed to amplify specific genes or, in the case of *PtSUT1/2*, the gene pair (Table 3-2). The Group-3 transporter *PtSUT4* was ubiquitously expressed, and its transcripts were more abundant than those of the other *SUT* genes in all tissues examined (Figure 3-5). *PtSUT4* expression was strongest in pollen, followed by mature leaves and xylem. The Group-2 member *PtSUT5* was also expressed in all tissues. Its expression in shoot tips and young leaves was similar to that of *PtSUT4*, but was comparatively low in all other tissues. *PtSUT6* transcripts were not detected in any of the tissues analyzed (Figure 3-5). In contrast to Groups- 2 and 3, expression of Group-1 members was more restricted. *PtSUT1/2* was weakly expressed in phloem and roots, but barely detected in other tissues (Figure 3-5). Transcript levels of *PtSUT3* were most readily detected in phloem and xylem of shoots, although very weak expression was observed in leaves, root and pollen tissues. It appears that, in *Populus*, transcription of Group-1 SUTs was primarily restricted to the vascular system.



**Figure 3-5.** Tissues expression patterns of *Populus SUTs*.

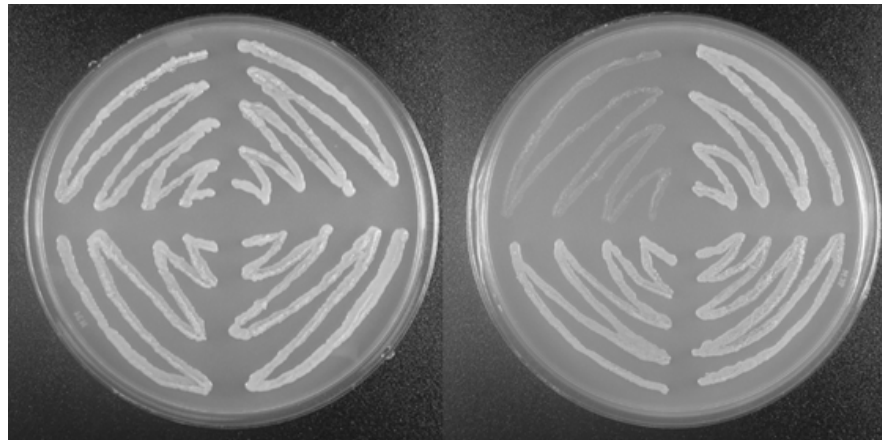
Expression of *SUTs* in shoot tips (ST), young leaves (YL), mature leaves (ML), primary stems (PS), secondary stems (SS), phloem (PH), xylem (XY), cell cultures (CC), roots (RT), male flowers (MF), female flowers (FF) and pollen (PO). Values are the means  $\pm$  SE of three biological replicates for all tissues except pollen where only one replicate was used.

### 3.3.4 Yeast complementation

For functional analysis of the *Populus* SUTs, yeast complementation experiments were conducted using the *Saccharomyces cerevisiae* *SUSY7/ura3* mutant strain (Riesmeier et al., 1992; Weise et al., 2000). This mutant lacks the ability to transport and hydrolyze exogenously supplied sucrose and does not grow on media supplemented with sucrose as the sole carbon source. Transformation of the *SUSY7/ura3* yeast mutant strain with a functional SUT enables it to grow on sucrose media (Riesmeier et al., 1992). The coding sequences of the three most abundantly expressed isoforms, *PtSUT3*, *PtSUT4* and *PtSUT5*, representing Group-1, Group-3 and Group-2, respectively, were tested. Both untransformed and *SUT*-transformed mutants grew on glucose media (Figure 3-6, left), but only *SUT* transformed mutants grew on sucrose media (Figure 3-6, right). These results indicate that *PtSUT3*, *PtSUT4* and *PtSUT5* encode functional transporters.

### 3.3.5 Transgenic manipulation of *PtSUT4* by RNAi

The strong expression of *PtSUT4* in all tissues analyzed suggests that it plays a major role in carbohydrate transport and/or compartmentalization in *Populus*. To investigate the function of *PtSUT4* in planta, RNAi-mediated gene silencing was performed. *PtSUT4* expression was suppressed by 50-90% in different tissues of four independent transgenic poplar lines (Figure 3-7A). Transcript levels of *PtSUT5*, also ubiquitously expressed, were found to differ little between transgenic and wildtype plants, confirming specificity of *PtSUT4* targeting by the RNAi construct (Figure 3-7B). Based on these results, transgenic line G exhibiting the highest level of *PtSUT4* suppression overall was



**Figure 3-6.** Complementation of yeast mutant (SUSY/ura3) with PtSUTs.

Transformed yeast was cultured on minimal media supplemented with either glucose (left) or sucrose (right). PtSUT3-, PtSUT4- or PtSUT5-transformed and untransformed mutants are arranged clockwise from top-right.

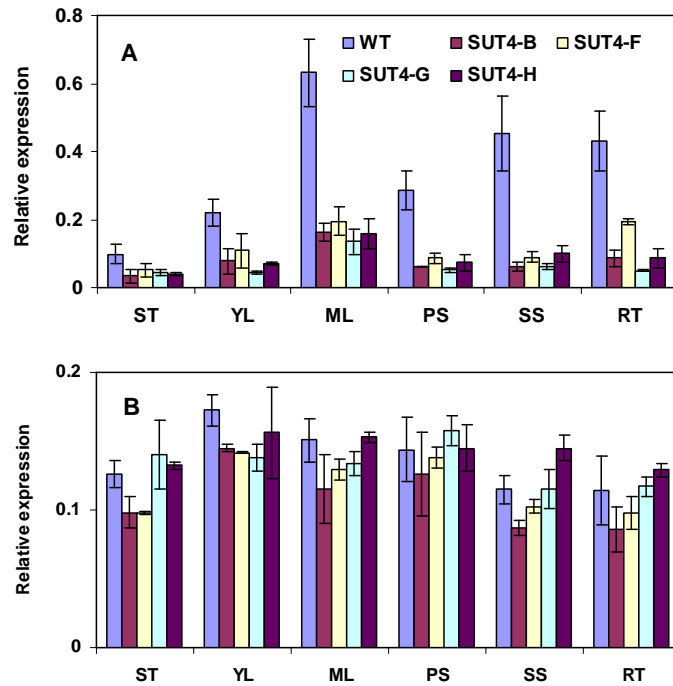
propagated for further analysis.

In fast-growing early successional taxa such as *Populus*, decreases in nutrient availability under conditions that otherwise favor photosynthetic carbon fixation result in elevated foliar abundance of starch and C-rich defensive compounds, and reduced levels of sugars (Bryant et al., 1983; Hemming and Lindroth, 1999; Jones and Hartley, 1999; Osier and Lindroth, 2006). Therefore, we used N deficiency to investigate SUT4 function under conditions expected to perturb carbohydrate, PG and CT homeostasis in *Populus*. The foliar N content in young and mature leaves of wildtype and transgenics was reduced by 60-65% under N-depleted condition. Plant growth was monitored in 3-week-old hydroponically-maintained plants. Although leaf and stem growth did not appear to differ between wildtype and transgenic plants, the ratio of leaf area to an index of stem volume (mid-height diameter x stem length) was significantly greater in transgenic than wildtype plants, regardless of N nutritional regime (Figure 3-8, Table 3-3).

### **3.3.6 Non-structural carbohydrates**

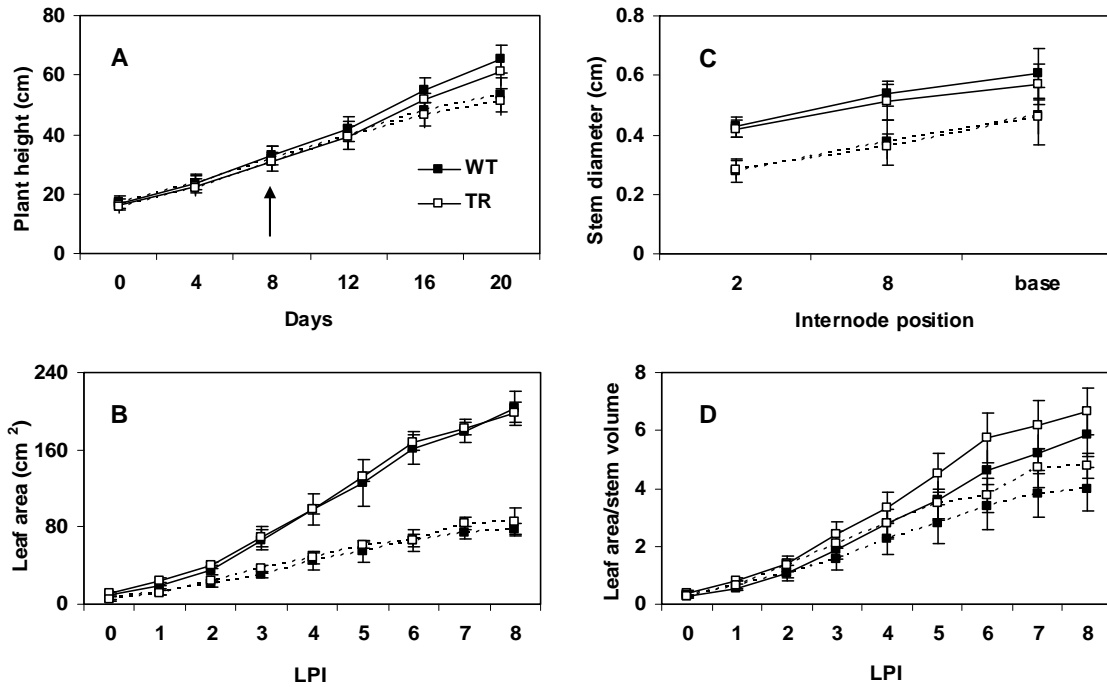
To assess the effects of *PtSUT4* down-regulation on carbohydrate metabolism, tissue levels of sucrose, glucose, fructose and starch were analyzed. Sucrose concentrations were significantly elevated in fully expanded source leaves of transgenic plants compared to wildtype controls at both LPI-5 (mature leaves, Figure 3-9) and LPI-8 (AppendixC). In other organs, concentrations of all three sugars did not differ between wildtype and transgenic plants (Figure 3-9). Glucose and fructose were not detected in shoot tips or





**Figure 3-7.** Transcript levels of *PtSUT4* (A) and *PtSUT5* (B) in various tissues of wildtype (WT) and four transgenic lines (SUT4-B, F, G and H).

Tissue abbreviations are as in Figure 3-5. The data represents the means  $\pm$  SE of 2-3 biological replicates.



**Figure 3-8.** Growth characteristics of *SUT4* transgenic and wildtype plants.

A. Plant height of wildtype (WT) and transgenics (TR) measured at 4-day intervals during the experimental period. B. Area of leaves at different positions of the stem. C. Stem diameter at internode 2, internode 8 and near the base, and D. Ratio of leaf area/final stem volume calculated for each LPI. Plants were grown with either full strength N (2.5 mM, solid lines) or with 5% of full strength N (0.125 mM, dotted lines). The arrow in panel A points to the day at which N stress was started. The data represents the means  $\pm$  standard deviation (n = 4 plants).

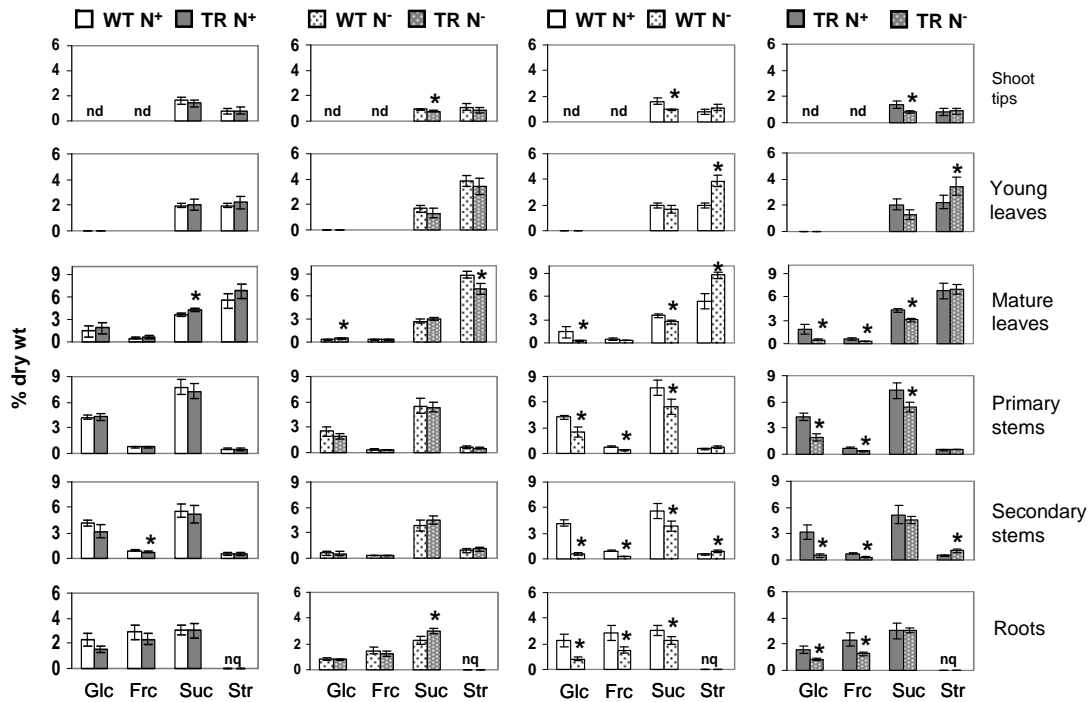
**Table 3-3.** Three-way ANOVA for leaf area/stem biomass ratio.

<b>Source of Variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Genotype	1	21.35	21.35	19.535	<0.001
N-level	1	42.018	42.018	38.445	<0.001
Leaf position	8	901.66	112.707	103.125	<0.001
Genotype x N-level	1	0.518	0.518	0.474	0.492
Genotype x leaf position	8	6.833	0.854	0.781	0.619
N-level x leaf position	8	34.703	4.338	3.969	<0.001
Genotype x N-level x leaf position	8	0.9	0.112	0.103	0.999
Residual	261	285.252	1.093		
Total	296	1328.108	4.487		

The parameters were genotypes (wildtype and transgenic), N levels (2.5 mM and 0.125 mM), and leaf positions (LPI-0 through LPI-8). df denotes degrees of freedom, SS for sum of squares, MS for mean sum of squares, *F* for Fisher-test, a ratio of between and within treatment variance and *P* for probability for statistical significance.

young leaves, presumably due to the high growth demands of those organs. Total (glucose+fructose+sucrose) sugar concentrations were relatively low in shoot tips and young leaves ( $\leq 2\%$ ), but higher in other sink-like organs, ranging between 8.1-12.7% in roots and stems of both wildtype and transgenic plants. Starch was more abundant in leaves than in stems (Figure 3-9). Starch level increased with leaf age from 0.8% in shoot tips, to 1.9% in young leaves and 5.4% in mature leaves. No significant difference in starch levels was observed between wildtype and transgenic plants in any of the tissues examined.

Low-N nutrition caused soluble sugar concentrations to decrease comparably in wildtype and transgenic plants, in all organs analyzed (Figure 3-9). As was the case with N-replete plants, sucrose and glucose concentrations were higher in source leaves of N-deficient transgenic than of N-deficient wildtype plants (Figure 3-9). The effects of low N feeding on starch differed markedly between transgenic and wildtype plants (Figure 3-9). In transgenic plants, only young leaves and secondary stem exhibited starch increases under N stress, while no increase was observed in mature leaves. By contrast, starch levels nearly doubled in young and mature leaves of wildtype plants.



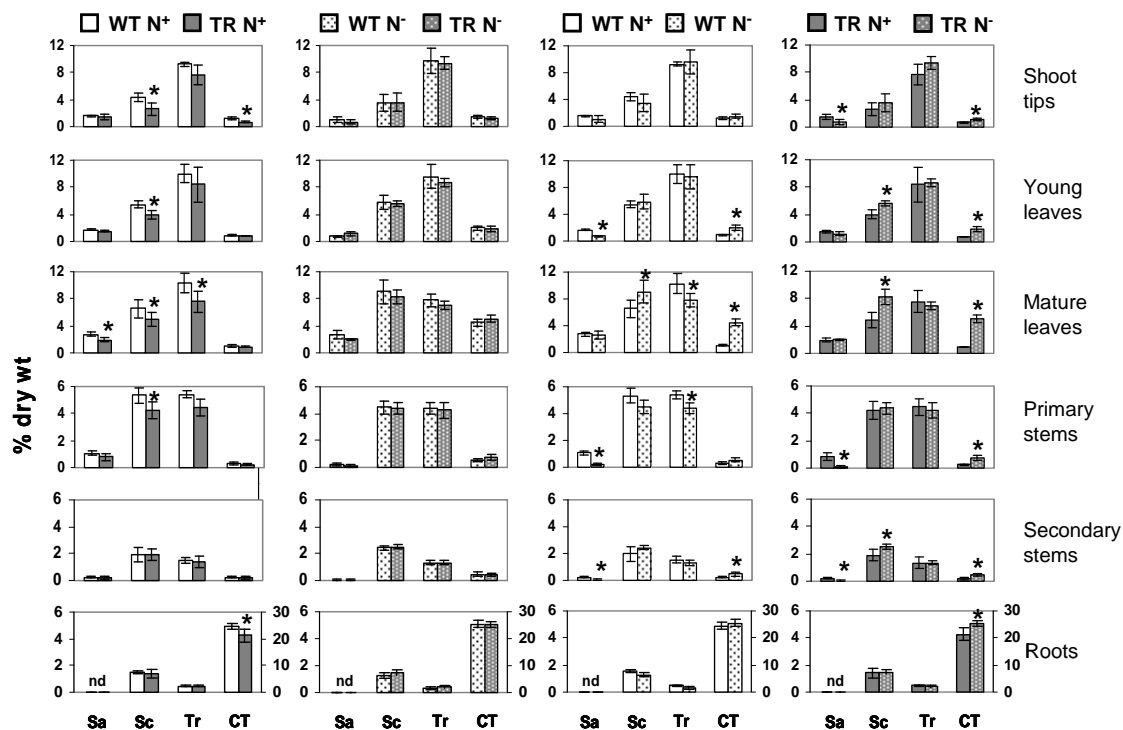
**Figure 3-9.** Non-structural carbohydrate levels in wildtype and transgenics plants.

Percent dry weight of glucose (Glc), fructose (Frc) sucrose (Suc) and starch (Str) in tissues of wildtype (WT) and SUT4 transgenic (TR) plants grown in full strength, 2.5 mM nitrogen (N+) or 0.125 mM nitrogen, (N-) nutrient solution. The data represent means  $\pm$  standard deviation (n = 4 plants). Differences between means were tested for significance using the *t-test* for two samples assuming equal variances. In the graphs, nd and nq indicates not-detected and not quantified, respectively. \* indicates significant difference ( $p = 0.05$ ) between genotypes or between N treatments.

### 3.3.7 Non-structural phenylpropanoids

There is evidence that accrual of both PG and CT demands substantial amounts of carbohydrates (Arnold et al., 2004; Arnold and Schultz, 2002; Kleiner et al., 1999). Therefore, the effect of *PtSUT4* down-regulation on the levels of PGs and CTs was investigated. The two higher-order PGs salicortin and tremulacin predominated in all tissues analyzed (Figure 3-10). Salicortin concentrations increased with leaf maturation, and tremulacin concentrations remained constant with leaf maturation in both genotypes. Down regulation of *SUT4* resulted in the decreased abundance of both salicortin and tremulacin in young shoot organs, including shoot tips, young leaves, mature leaves and primary stems (Figure 3-10). PG level was typically lower in secondary stem and root tissues, and their abundance did not change in the transgenic plants. In general, CT abundance was not affected in the transgenics, although small decreases were noted in the shoot tips and roots, organs where CTs normally accumulate (Figure 3-10).

Low N feeding resulted in salicortin increases in shoot tips and primary stems of transgenic plants and decreases in those organs in wildtype plants (Figure 3-10). Salicortin increased in mature leaves of both genotypes, but the increase was larger in the transgenics. Tremulacin decreased in nearly all shoot organs of the wildtype plants, but was sustained at N-replete levels of leaves and stems in the transgenics. Overall, there was a clear trend towards enhanced partitioning into the PG pool in transgenic compared to wildtype plants during low N growth. As a result of low N feeding, CT levels increased significantly in all shoot tissues in both wildtype and transgenic plants (Figure 3-10).



**Figure 3-10.** Non-structural phenylpropanoid levels in wildtype and transgenics plants.

Levels (% dry weight) of salicin (Sa), salicortin (Sc) tremulacin (Tr) and condensed tannins (CT) in tissues of wildtype (WT) and SUT4 transgenic (TR) plants grown in full strength, 2.5 mM nitrogen (N+) or 0.125 mM nitrogen, (N-) nutrient solution. The data represent means  $\pm$  standard deviation (n = 4 plants). Differences between means were tested for significance using the *t-test* for two samples assuming equal variances. In the graphs, nd indicates not-detected. Note the use of 2<sup>nd</sup> y-axis for root CT levels. \* indicates significant difference ( $p = 0.05$ ) between genotypes or between N treatments.

### 3.3.8 Gene expression studies

#### 2.3.8.1 Sucrose synthases and invertases

The expression of the entire suite of six sucrose synthases and 16 invertases annotated in the *Populus* genome (Bocock et al., 2008; Tuskan et al., 2006) and associated with sucrose hydrolysis was analyzed. As shown in Figure 3-11, expression could be quantified for 4 paralogous pairs of neutral invertases (*NIN*), two vacuolar invertases (*VIN*), one cytosolic invertase (*CIN*), and three sucrose synthases (*SuSy*). *SuSy1* and *SuSy2*, the predominantly expressed *SuSy* genes in mature leaves, were significantly down-regulated there in transgenic plants (Figure 3-11 B, Figure 3-12 A). There was a consistent tendency towards down regulation of the other sucrose hydrolysis-related genes in leaves and shoot tips of transgenic compared to wildtype plants. In contrast, a consistent tendency toward up regulation of those genes was observed in primary stems. That tendency persisted for *SuSy* and several of the neutral invertase genes in secondary stem and roots of the transgenics.

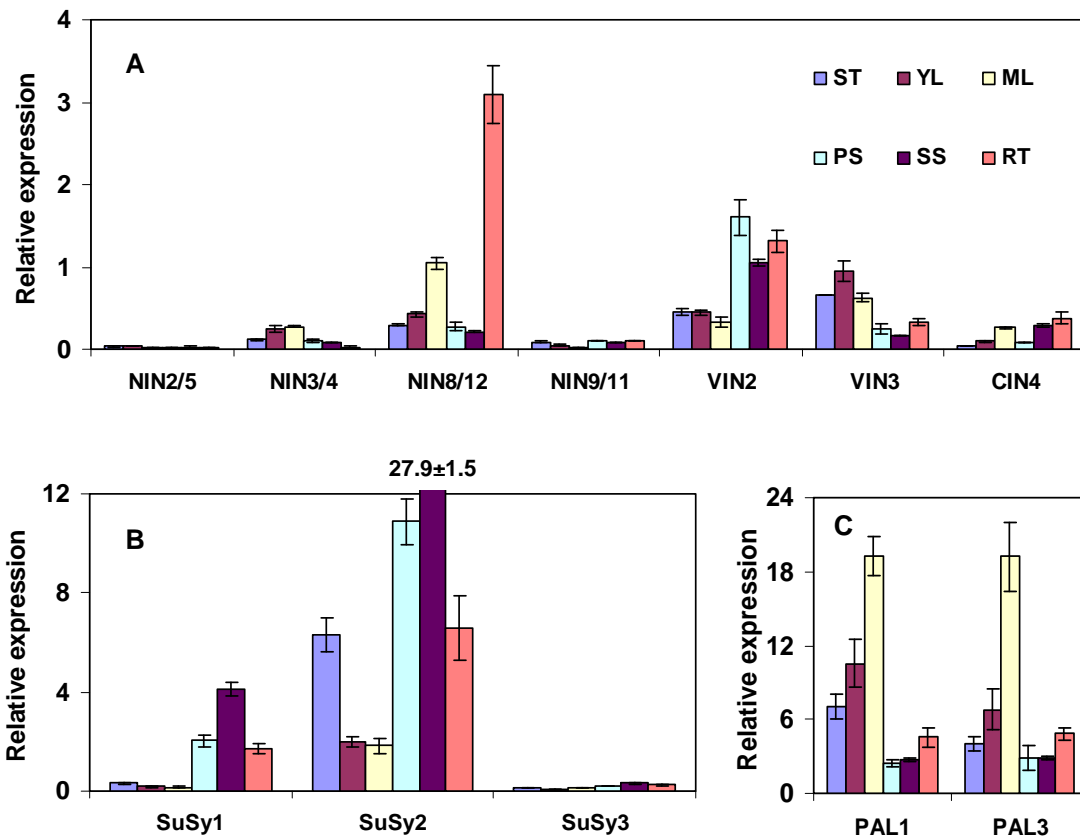
In response to N limitation, *PtSUT4* exhibited an upward trend in young and mature leaves and primary stems of wildtype plants (Figure 3-12 C), but such response was either low (young leaves) or the reverse was true (mature leaves) in the *SUT4* transgenic plants (Figure 3-12 D). N-limitation resulted in substantially greater up-regulation of *SuSy* in mature leaves of transgenics than of wildtype plants (Figure 3-12 C and D). In transgenic plants, in contrast to the trend observed under N-replete where *SuSy* transcripts were lower, *SuSy* transcript levels were higher under low N condition (Figure 3-12 A and



B). In addition, several weakly mature leaf-expressed invertases (*NIN2/5* and *NIN3/4*) and *SUT3* exhibited a tendency toward up-regulation during N stress in transgenic mature leaves (Figure 3-12 B). In most organs, expression of the relatively strongly expressed *PtVIN3* was reduced in wildtype but was up-regulated in transgenic plants in response to N stress. In shoot tips, most of the *SuSy* and invertase genes exhibited a trend towards down-regulation in N-limited wildtype plants and towards up-regulation in N-limited transgenic plants.

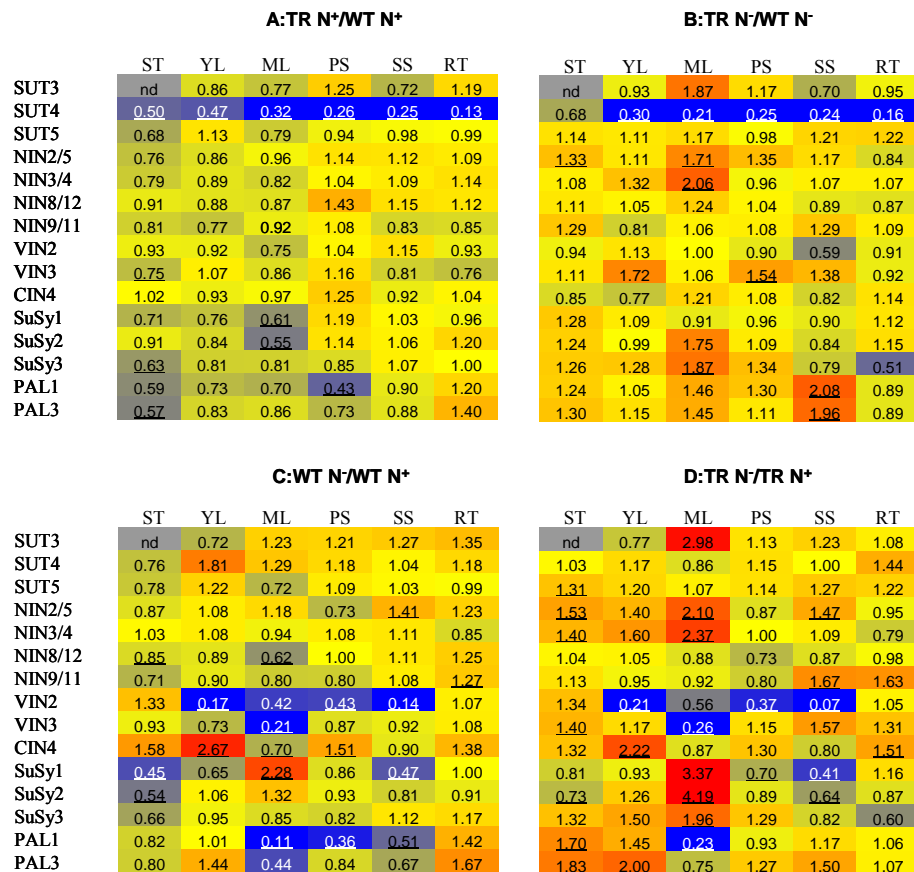
### **2.3.8.2 Phenylalanine ammonia-lyases**

The effects of *PtSUT4* down-regulation on PG and CT accumulation were further investigated by analyzing expression of phenylalanine ammonia-lyase (PAL). The PAL protein regulates the entry of phenylalanine into the phenylpropanoid pathway for synthesis of a variety of products, including PG and CT (Dixon and Paiva, 1995; Tsai et al., 2006a). *PtPAL1* and *PtPAL3* were well expressed in all tissues, but expression was clearly strongest in mature leaves (Figure 3-11 C). Both *PtPAL1* and *PtPAL3* were less well expressed in shoot organs of transgenic than of wildtype plants (Figure 3-12 A). This pattern changed during N-limitation. In general, *PAL* expression either increased more or decreased less in shoot organs of transgenic than of wildtype plants (Figure 3-12 B). The result, during N-stress, was that *PAL* expression was much higher in transgenic than wildtype plants in mature leaves and in major shoot sink organs like shoot tips and secondary stems. At the same time, expression of *PAL* in roots became lower in transgenic than wildtype plants. Generally speaking, the contrasting *PAL* expression



**Figure 3-11.** Q-PCR expression analysis of sucrose synthase (*SuSy*), invertase (*INV*) and phenylalanine ammonia-lyase genes (*PAL*) in various tissues.

Expression patterns of A. neutral (*NIN*), vacuolar (*VIN*) and cell wall (*CIN*) invertases; B. sucrose synthases (*SuSy*); C. phenylalanine ammonia-lyases (*PAL*) in different tissues of *Populus*. Tissue abbreviations are as in Figure 3-5. The data represent the means  $\pm$  standard error from three biological replicates.



**Figure 3-12.** Heatmaps of *SUT*, *INV*, *SuSy* and *PAL* gene expression in shoot and root tissues.

Data was converted into gene expression ratios, TR/WT at N<sup>+</sup> (A), TR/WT at N<sup>-</sup> (B), WT N<sup>-</sup>/WT N<sup>+</sup> (C), TR N<sup>-</sup>/TR N<sup>+</sup> (D). Tissue abbreviations are as in Figure 3-5. Data were visualized using the heatmapper plus program available at [http://bar.utoronto.ca/ntools/cgi-bin/ntools\\_heatmapper\\_plus.cgi](http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi). Yellow color indicates no change in gene expression while red and blue indicate up and down-regulation, respectively. nd indicates below quantifiable levels. Underline denotes statistical significance at  $P = 0.05$  for the differences between numerator and denominator used to calculate the ratios in each panel.

responses by the two genotypes to N-stress correlated with the observed trends in PG abundance (Figure 3-10, Figure 3-12).

### **3.4 Discussion**

The *Populus* SUTs were found to cluster into the three phylogenetic groups established for higher plant SUT genes. Within Group-1, SUTs from woody perennials, such as *Populus*, cassava (*Manihot esculenta*), rubber tree, walnut, grape and castor bean form a distinct subclade. The most ubiquitous and abundantly expressed *SUT* gene in *Populus* encodes the Group-3 transporter *PtSUT4* (Figure 3-4). Several Group-3 transporters such as *AtSUC4*, *HvSUT2* and *LjSUT4* are tonoplast localized (Endler et al., 2006; Reinders et al., 2008) and, like *PtSUT4*, are capable of sucrose transport across the plasma membrane in yeast complementation experiments (Reinders et al., 2008; Weise et al., 2000). No conserved peptide signal has been reported for any of the vacuolar targeting proteins.

#### **3.4.1 Group-1 members are vascular-localized in *Populus***

Group-1 *PtSUT1/2* and *PtSUT3* exhibited a pattern of stem vascular tissue expression that differs from that of other Group-1 members. These include SUTs from apoplastic (*AtSUC2*, *LeSUT1*, *StSUT1*, *NtSUT1*, *PsSUT1*) or both symplastic and apoplastic (*AmSUT1*) loading herbaceous species (Barker et al., 2000; Knop et al., 2001; Riesmeier et al., 1993; Riesmeier et al., 1994; Stadler et al., 1999; Tegeder et al., 1999). In all

these species, the Group-1 *SUTs* are well expressed in source leaves, where phloem loading occurs. Similarly, Group-1 transporters of fruit-bearing woody perennials, including *VvSUC27* from grape (Davies et al., 1999) and *CsSUT1* from citrus (*Citrus sinensis*) are well expressed in both leaf and stem tissues (Li et al., 2003). However, the Group-1 *VvSUC27* protein has a lower affinity for sucrose than other Group-1 *SUTs*, and is better expressed in expanding than in mature source leaves (Davies et al., 1999). The plant-wide distribution of Group-1 *PtSUT* gene transcripts was similar to that of Group-1 *SUT* genes in castor bean and walnut (Bick et al., 1998; Decourteix et al., 2006). The walnut Group-1 *SUT*, *JrSUT1* is exclusively stem localized and thought to have a role in the distribution of sucrose between xylem vessels and parenchyma cells for osmotic pressure regulation during seasonal freeze-thaw cycles (Alves et al., 2004; Decourteix et al., 2006). Sucrose enters the xylem sap during freeze-thaw cycles in *Populus* and *Salix* as well (Sauter, 1983; Sauter, 1988). Therefore, preferential vascular expression of the Group-1 *PtSUTs*, like that of the walnut Group-1 *SUT*, may be consistent with a specialized function during seasonal freeze-thaw cycles.

### **3.4.2 Group-3 *PtSUT4* exhibits an unusual expression pattern in *Populus***

So far, a small number of Group-3 *SUT/SUC* proteins, *AtSUC4*, *StSUT4*, *LjSUT4*, *LeSUT4*, and *VvSUC11*, have been characterized, with only *StSUT4* being subjected to transgenic manipulation (Chincinska et al., 2008; Davies et al., 1999; Manning et al., 2001; Reinders et al., 2008; Weise et al., 2000). While herbaceous Group-3 *SUT* proteins (*AtSUC4*, *LeSUT4* and *LjSUT4*) exhibit a low affinity for sucrose, *VvSUC11* from the

woody perennial (grape) exhibits an affinity as high as that of Group-1 transporters from the other species (Manning et al., 2001). However, *VvSUC11* is not expressed in stems. From the limited number of studies, it is unusual for Group-3 transporters that are well expressed in leaf tissues to also be well expressed in stem tissues, as appears to be the case for *PtSUT4*. Whereas the Group-3 grape *SUT* is not expressed in stems, the low-sucrose-affinity Group-1 SUT, *VvSUC27*, is very strongly expressed there (Davies et al., 1999). In *Populus*, *PtSUT4* is far more strongly expressed in stems than is the Group-1 PtSUT cluster (Figure 3-5). With the exceptions of grape, and now *Populus*, Group-3 *SUT* transcript levels are higher in sink leaves, where sucrose unloading is likely to predominate, than in source tissues, where loading occurs (Chincinska et al., 2008; Weise et al., 2000). In short, the expression pattern of *PtSUT4* overlays those of the high-affinity Group-1 and Group-3 transporters of herbaceous species and grape, respectively (Barker et al., 2000; Burkle et al., 1998; Davies et al., 1999; Riesmeier et al., 1993; Truernit and Sauer, 1995).

Although mutation or down-regulation of abundantly expressed Group-1 SUTs in other species (i.e., *AtSUC2*, *StSUC1*, *NtSUC1*) can result in severe alterations to plant growth and carbohydrate utilization (Gottwald et al., 2000; Hackel et al., 2006; Riesmeier et al., 1994; Srivastava et al., 2008), down-regulation of *PtSUT4* in *Populus* resulted in modest growth changes and increased accumulation of sugars and starch in mature leaves, but by no more than 25% over the levels in wildtype plants (Figure 3-9). The expression of *SUT4* was reduced by 68% in mature leaves, whereas similar transcript level reductions of *StSUT1*, *NtSUT1* and *LeSUT1* in antisense lines severely altered plant growth

(Burkle et al., 1998; Hackel et al., 2006; Riesmeier et al., 1994). Thus, the effects of *PtSUT4* manipulation were comparatively mild. The reduction in *PtSUT4* transcripts was not compensated by increased expression of other *PtSUTs* (Figure 3-12 A). In addition, the only other SUT gene to exhibit significant transcript levels in mature leaves is *PtSUT5*, which encodes a SUT protein that is highly distinct from PtSUT4 (Figure 3-5, Table 3-2). These results, along with the other reports of severe repercussions from SUT gene down-regulation, are consistent with the interpretation that PtSUT4 and PtSUT5 proteins somehow have complementary roles in sucrose export from source leaves.

### **3.4.3 Carbohydrate and phenylpropanoid metabolism are altered in *PtSUT4* transgenics**

The increased level of sucrose, glucose and starch in source leaves may be attributed to a number of factors, including increased photosynthetic rates and carbohydrate synthesis, inefficient utilization or inefficient export of the photosynthates. There is not strong support for the idea that photosynthesis increased in the transgenics, as photosynthesis was reduced in *NtSUT1* and *StSUT1* down-regulated plants (Burkle et al., 1998; Riesmeier et al., 1994). The increased accumulation of sugars in mature leaves coupled with the decrease in sink tissues, such as shoot tips and roots, suggests that sucrose export from source leaves was less efficient in transgenic than wildtype plants. The increased leaf:stem growth ratios in transgenic plants lends support to the idea that sucrose export was indeed reduced, and that there were negative consequences to stem growth. An alternative point is that the plants may be exporting at higher rates during the dark cycle

as observed in *StSUT4* silenced lines (Chincinska et al., 2008). However, this interpretation is less easily reconciled with the increased leaf-to-stem growth ratio we observed. The reduced *INV* and *SuSy* gene expression in mature leaves of transgenics is consistent with the idea that sucrose hydrolysis decreased. At the same time, mature leaf glucose concentrations increased in the transgenic plants (Figure 3-9). This indicates that sucrose hydrolysis provided glucose in excess of source-leaf demand, and that *SuSy* expression may have been negatively regulated by the accumulated hexoses as reported for maize *SuSy*, *Shrunken1* (Xu et al., 1996). The finding that glucose concentrations increased despite evidence for reduced sucrose catabolism suggests that utilization of glucose for hexose-demanding activities, including in *Populus*, glycosylation of phenylpropanoid-skeletons for PG synthesis, decreased. Exemplifying the magnitude of the demand, glucose comprises 42% and 34% of the mass of the PGs salicortin and tremulacin, respectively (Kammerer et al., 2005; Pearl and Darling, 1971). These are the two most abundant phenolics found in leaf tissues of the 717 genotype used in the experiments. Interestingly, PG content decreased and expression of *PAL* gene family members specifically associated with the synthesis of non-structural phenylpropanoids in leaves of *Populus* (Kao et al., 2002) was down-regulated in the transgenics (Figure 3-10, Figure 3-11).

During N-limited growth, the concentration of PGs was similar in wildtype and transgenic plants for every organ compared (Figure 3-10). In addition, PG levels were comparable to those in N-replete wildtype plants despite conspicuous decreases in the mature leaf expression of the relevant *PAL* genes due to N deficiency. Clearly, *PAL*



gene expression was not a limiting factor, while RNAi down-regulation of *PtSUT4* appeared to constitute a lesion to PG homeostasis during N-replete growth. An important observation is that salicin levels were severely depleted in primary and secondary stems of all plants during N-stress (Figure 3-10). Although not yet proven unequivocally to be a precursor in the biosynthesis of the higher-order PGs salicortin and tremulacin, a salicin moiety constitutes the core of those PGs (Lindroth and Pajutee, 1987; Pearl and Darling, 1971). If the depletion in salicin represents a decrease in the stem contribution to plant PG biosynthesis during N stress, it is possible to formulate a hypothesis to rationalize a role for *PtSUT4* in total plant PG homeostasis.

According to such a hypothesis, *PtSUT4* participates in the transport of sucrose to the stems which actively synthesize PG during N-replete growth. Functional consequences of reduced *PtSUT4* gene expression, including plant-wide shifts in the relative concentrations of sucrose between source and sink organs, and a reduction in stem volume growth relative to leaf area growth, were documented (Figure 3-8 D, Figure 3-9). The plant-wide differential in PG concentration between wildtype and transgenic plants during N-replete growth can therefore be attributed to limited transport of sucrose to sink organs, including stems, to support PG synthesis. In the N-deficient plants, carbohydrate reserves in stems probably became limited as lignin accrual increased (Appendix D), perhaps at the expense of carbon skeletons for the biosynthesis of non-structural phenylpropanoid PGs. Competition for phenylpropanoid substrates by PG and lignin biosynthesis has been alluded to in reports on the down-regulation of lignin accrual in *Populus* (Coleman et al., 2008; Ranocha et al., 2002). As a result, the burden of PG

synthesis shifted more completely to source organs in closer proximity to the point of sucrose biosynthesis and supply. The shift in PG biosynthesis away from stem during low N growth removed the dependence of plant PG homeostasis on PtSUT4 transport function, and, according to the model hypothesized, removed the basis for the PG differential between wildtype and transgenic plants during N-replete growth.

Although a number of Group-3 SUTs are known to be localized in the tonoplast (Endler et al., 2006; Reinders et al., 2008), only the LeSUT4 of *Lotus japonicus* has been characterized in some depth (Reinders et al., 2008). It is tonoplast localized and appears to function in the export of sucrose from the vacuole into the cytoplasm and possibly for long distance transport. Evidence for close association of SuSy with SUT proteins in the tonoplast has also been reported (Etxeberria and Gonzalez, 2003). In the present work, *PtSUT4*, *SuSy*, and *NIN* gene expression increased in mature leaves during N-stress (Figure 3-12). These increases, along with the decrease in expression of *PtVIN2* are consistent with the idea that cytosolic sucrose hydrolyzing activity increased in relation to vacuolar sucrose hydrolyzing activity during N-stress. In addition, based on the emerging picture of Group-3 transporter function, PtSUT4 appeared to participate in the export of vacuolar sucrose during N-stress. Such a role would be distinct from the regulation of cytosolic sucrose concentration to facilitate long-distance symplastic transport of sucrose during N-replete conditions. With regard to distinct roles for PtSUT4 in N-replete and N-limited plants, starch levels increased in mature leaves of wildtype source leaves during N stress, and they did not in transgenic mature leaves. Whether the vacuolar sucrose was used for starch biosynthesis during N-stress in this case was not determined.

However, *SuSy* involvement in cytosolic starch synthesis has been reported (Munoz et al., 2005; Munoz et al., 2006). *SuSy* gene expression was sharply up-regulated in both *Populus* genotypes during N stress, but starch levels only increased in wildtype mature leaves (Figure 3-12, Figure 3-9). This is consistent with the idea that compromised *PtSUT4* function interfered with the utilization of vacuolar sucrose for starch synthesis during N stress. Overall, participation of *PtSUT4* in mature leaf starch storage is also consistent with reduced transport of sucrose from mature leaves during N stress.

It appears that *PtSUT4* is functionally integrated with changes in carbohydrate partitioning and compartmentalization that contribute to the regulation of long-distance sucrose transport in *Populus*. Alterations in its expression affect both constitutive plant defense and growth of *Populus*. It is less likely that *PtSUT4* is the transporter that regulates the long-distance movement of salicin, a putative PG precursor, even though SUTs are capable of transporting salicin, and despite findings that salicin occurs in phloem sap exudates collected from *Populus* leaves (Chandran et al., 2003; Gould et al., 2007; Reinders et al., 2008; Sivitz et al., 2007).

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**Appendix A.** List of primers used in the study. F and R denote forward and reverse primers, respectively.

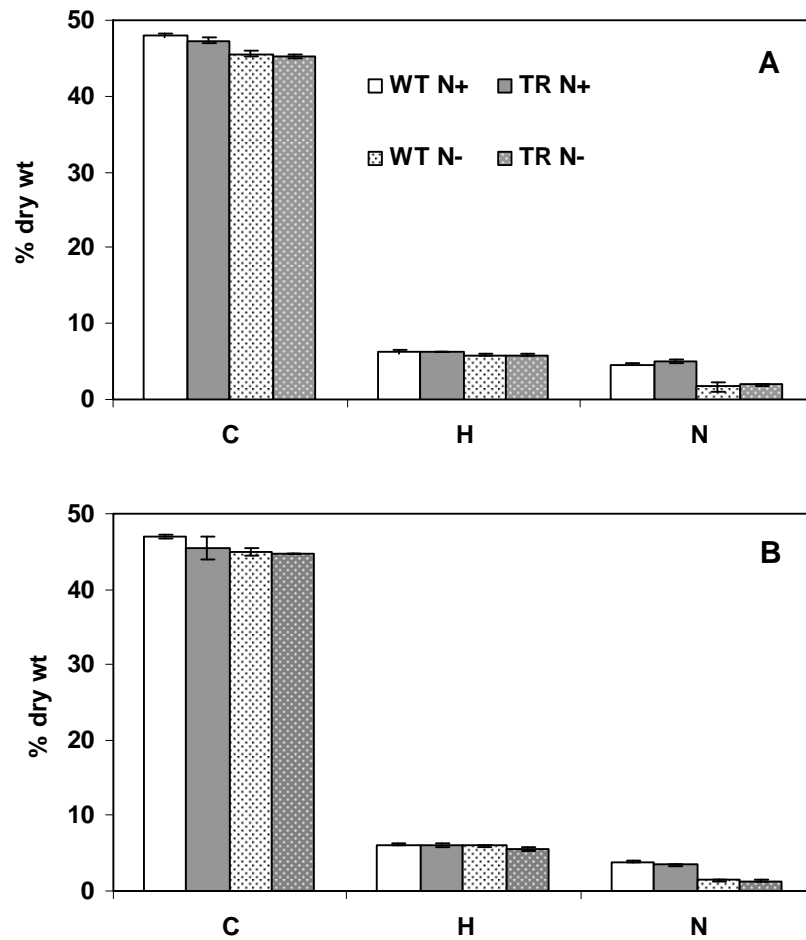
Gene		Sequence
GT1-225	F	AGACTGTTGGTGGAGGAATTGGGA
	R	ATTCCTYGATGAGCCCGTTCAAGT
GT1-200	F	TGGTGCCCTGGAATGGTCWAGATA
	R	CTGCGGATTCTTGCTTGCTTGTC
GT1-292/297	F	ACTGCAGGGAARCCMATGRTTACA
	R	TCCACCTTCTTCRACAGCCTTCCT
GT1-298	F	TGAAGATTGGTGTRAGGGTTGGGT
	R	AGCCCTCTTCTTCCACTGCTTTGT
GT1-246/245/249	F	AGTGCTGAACCATCCATCGGTAGG
GT1-246	R	CTGTGGTTCGCTCCCTAATCAACT
GT1-258	F	GCTCGCAAGTGGAAGAACCACAAA
	R	TCACGTGGCAGAGAGCACMTTATT
GT1-262	F	CTGCCGTAAGGTTTCTTGGAACGA
	R	ACCTTCTTCTCCACCTCATCAGCA
GT1-289	F	TTGCTACATGGCCAATGCATGCTG
	R	CACACGATSTAACCAAGTGKAYGAGG
GT1-188	F	TGMCTATGCTTTGTTGGCCKTCCT
	R	WTGACAARAGCACTTCCGTCACCA
GT1-255/253	F	TTGGTGTCCACAAGAGGAAGTKCT
GT1-253	R	TGAACCATTAGGGACAGTAGCCTC
GT1-270/274	F	CCTATGCTTTGTTGGCCATTGCT
	R	AAGTTCATGGATGATGAACCACCGGG
GT1-324	F	CCCATGATTTGCTGGCCCTTCTTT
	R	GCAATTCACTGACCAGCTTCTCCA
GT1-109	F	CTGTGCCATTCTGCTGTTGGAGGT
	R	AGCTCTCTTCCGATTGCTTTCAC
GT1-186	F	GGAGMGAAGGGCGAAGAGATGARA
	R	ACATCRCAGTCCARAGAAGRAAGG
GT1-228/216/2	F	AATGCCAAGYTGATTGMAGACGTG
GT1-2	R	ACCCRTCCATCATAACAACCAAGC
GT1-221	F	AACACTTGAGGCTTTGAGCTTGGG
	R	AGCTTCCACCTTCATCCATAGCCA
GT1-315/316	F	CCTAAGGCAGTGAAATGAAGSAG
GT1-315	R	CCAGTTCADCSACTTYCTCRHTGG
GT1-293	F	TGGGTGGAATTCTGTCTTGAAGC
	R	CCCTTGACCTTGCTAGCTTTCCTT
MRP1	F	ATATCCAGAGATGGGTGGTGGTCA
	R	CAGGTACTTGCAAGTAAGTTGCTACC
MRP2/3	F	AGGAAGCGCATTCTCTAGGATGGT
MRP2	R	AGCAAGATGCATAAGACACGACA
MRP3	R	GAGACTAACAACCAGGGCSAGTT
MRP4	F	AGTTGATTTCRAAACTGAYGCTG
	R	AAACAGYGATGGCCTCTCAAGCAA
MRP5	F	GTGCTTGATGAAGCRACRGCATCT
	R	ATCCRGTAYGCYACTTGACCTTGA
MRP6	F	CACAGTAGCTCACMGGATACCRAC
	F	CTTCAATGYGATTCTGCWRCRTGT

Appendix A Continued.

Gene		Sequence
<b>Q-PCR</b>		
NIN2/5	F	TGCTTTGGCYGAGAAGAGACTTMA
	R	GGCCACTHGTGTTAAGYCCACAAA
NIN3/4	F	AACAAGCACRYCTGTTCCAGACAT
	R	TCTGWCCACGMTTTCCTCCTGGRT
NIN8/12	F	TTTATGGTTGCTGACTGCTGCRTG
	R	CCAGCATCATYTTTGGCACCAGT
NIN9/11	F	GCTTCTCACMGCRCATGCATMAA
	R	TCYTCGAGTGCCACCRTRCCCAA
CIN1/2/3	F	AGAAYTGCCATCWCATCYAGGGTT
CIN1/2	R	TTCATTGCGTGGATTCTCYCCC
CIN3	R	GTACGGGACAATTCATCATCCATTG
CIN4	F	GCTATTCAWGAAGAAGCTCGCCTG
	R	AGACACTAAAGCAGACTGCAGAGG
CIN5	F	TTTCGTAGACATGGATCCTCGCCA
	R	TGGCTTCCTTCGTTTCGTGGTAGA
VIN1/2	F	TTATCCGACGASGGCAATMTATGG
VIN1	R	GGTACAGATGGATGCAAATTAGGT
VIN2	R	CGAAGGCABTGCTACTGTTKTTCA
VIN3	F	AGGCCACACTCAAGATTTGGGA
	R	TCTCAYGTGGTTGCCCTCAAGGT
SUSY1	F	GAACCTTGATCGTCTTGAGAGYCG
	R	GGTTCTGTCTCCMAACYGAAACCA
SUSY2	F	CAACCTYGATCAYCGTGAGAGCCG
	R	ACCATTATTCTGGACCCGGAACCC
SUSY3	F	TATCTGATGCTGGGCTKCAACGGA
	R	TGCCRGTCMTCGATTGACAAAGGT
SUSY4/5	F	CAATCAAGGTGGCCCAGCAGAAAT
SUSY4	R	GTAGATGCGTTGGAGACCAGTTGC
SUSY5	R	ATAGATGCGTTGAAGACCAGCTGC
SUSY6	F	TGGATCCCGGACACTGGAATAAGT
	R	TCTGAGRCTGGTGTGTTGASCTTCT
SUT1/2/3	F	TGGTKTCTGTAGCRRSTGGACCTT
SUT1/2	R	ACCAGTCACCAGTCTTGGAAGGAA
SUT3	R	GGAATGCAKCAAGTGACAGYCMTTT
SUT4	F	ATCCTTGGGACTTGGACAAGGGTT
	R	TGATCGAGGAATACYCAAGATGGC
SUT5/6	F	ATACCAGCSTTYGTTCTGGCWTCT
SUT5	R	TAGCATGCTCCTGTCCTTGACAATYA
SUT6	R	TCCTYGACRATTACATGTTGGCTC
EF1B	F	AAGAGACAAGAAGGCAGCA
	R	CTAACCGCCTTCTCCAACAC
UBCc	F	CTGAAGAAGGAGATGACARCMCCA
	R	GCATCCCTTCAACACAGTTTCAMG

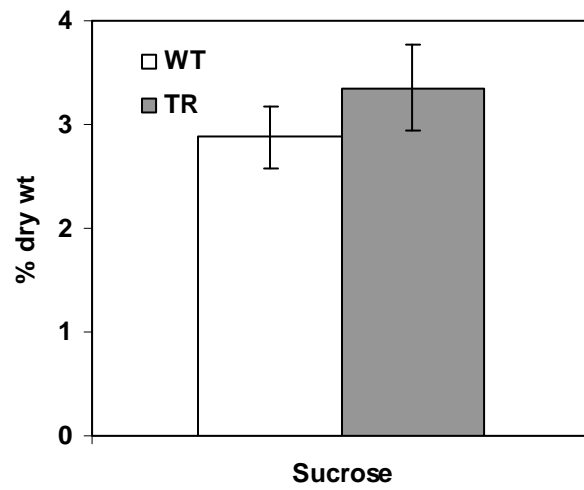
## Appendix A. Continued.

Gene		Sequence
<b>Yeast complementation</b>		
SUT1/2/3	F	ATGGAGAGTGGAGTTAGAAAAGAA
SUT3	R	TCAATGGAATGCAKCACTGACAGYC
SUT4	F	CTAGCTAGCATGTCAGTCGCTAATCCAGAGCC
	R	CCGCTCGAGTCATGAGAAGACCATGGGCTTTTGAAC
SUT5/6	F	GCTCTAGATGGAGTCGGCRCCGATTCGGGTA
SUT5	R	CCGCTCGAGTAGCATGCTCCTGTCCTTGACAATCA
PDR196	F	CTATCAACCTCGTTGATAAAT
	R	AGGTAGACAAGCCGACAACCTTG
<b>QD-PCR</b>		
SUT1/2	F	CACTCCTTGTCAACTTGCCACACA
	R	GCGGCATAGCCAATGAGAAACACA
SUT1/2	F	ACAACAACCTCCTGCAACAAACCC
	R	GCTGTACGAGTCTTCTTGTGATCCGT
SUT1/2/3	F	TTTCTAACACYTCKGGTGCTGGC
SUT3	R	GCTGGGAATAGGATWAACCATGACYG
C4HL	F	TTCATGGGAGCAGGAAATGGACC
	R	ACTYCCCTTTAGGACGGCTCTGAT

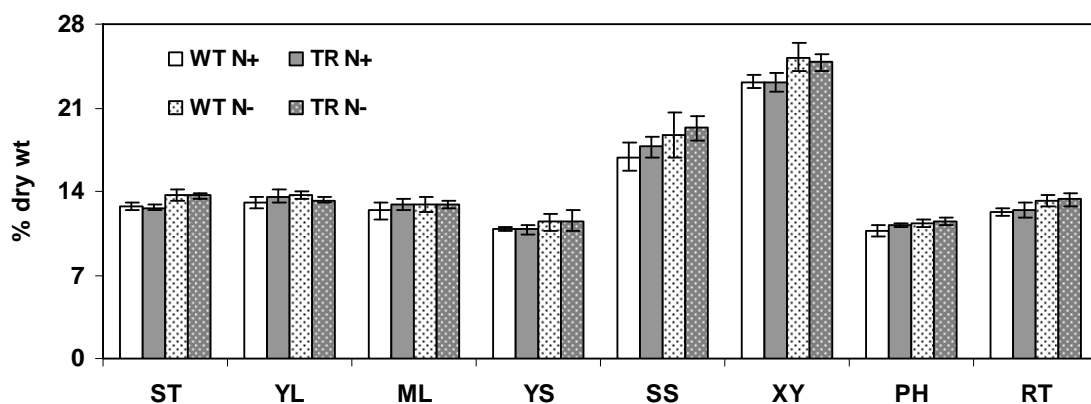


**Appendix B.** Foliar carbon (C), hydrogen (H) and nitrogen (N) levels in wildtype and transgenic leaves

Levels of C, H and N in young (A) and mature (B) leaves of wildtype (WT) and SUT4 transgenic (TR) plants grown in full-strength, 2.5 mM nitrogen (N+) or 5%, 0.125 mM nitrogen, (N-). The data represents means  $\pm$  standard deviation (n = 4 plants).



**Appendix C.** Sucrose levels on percent dry weight basis in LPI8 of wildtype (WT) and transgenic (TR) plants.



**Appendix D.** Structural phenylpropanoid (lignin) content in wildtype and transgenic plants.

Lignin content in different tissues of wildtype (WT) and SUT4 transgenic (TR) plants grown in full-strength, 2.5 mM nitrogen (N+) or 5%, 0.125 mM nitrogen, (N-). Tissue abbreviations are as in Figure 3-5. The data represents means  $\pm$  standard deviation (n = 4 plants).