LINKING CARBOHYDRATE CONTENT, GENE TRANSCRIPTS AND PROTEIN CONTENT FOR KEY ENZYMES INVOLVED IN CARBOHYDRATE METABOLISM IN POPLAR XYLEM ON A SEASONAL AND SPATIAL BASIS

by

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ABSTRACT

The goal of this study was to determine the seasonal and spatial correlation between the carbohydrate levels and gene transcripts of key enzymes involved in the carbohydrate metabolism in *Populus deltoides* xylem and twigs and *Populus balsamifera* twigs. The seasonal and spatial patterns in the carbohydrate variation was found to be consistent with the need to regulate starch storage so that it does not compete with xylem growth and yet can serve as a reserve for bud growth in the spring in both species. In the twigs of balsam poplar, a decline in starch during spring was observed to be associated with a lower transcript level for a key enzyme in starch synthesis than when starch levels were high. Low sucrose content was preceded by a low transcript level for a key sucrose synthesis enzyme. Transcripts for sucrose synthase that degrades sucrose for the growth processes of the vascular cambium were highest during the summer. However, the transcript level for β-amylase, an enzyme of starch degradation, was not always consistent with starch content. The growth and correlations observed with balsam poplar did not show good synchronization with the environment of Lubbock which could be due to a high degree of variation in the environment relative to its native region, Canada. The growth rings of *P.deltoides* showed high starch levels in the innermost ring and the starch levels decreased towards the outermost ring. Transcripts for the enzymes showed a positive correlation with that of carbohydrate levels. Since the storage of carbohydrate competes with the developing xylem of the stem via the activity of the vascular cambium during the growing season, regulation was expected. Accordingly, the correlations between the carbohydrates, gene transcripts and protein content of the enzymes were stumpy. Whether the transcript levels of these enzymes are being affected by the

regulation of small interfering RNAs was studied by other researchers and it was found that certain miRNAs were discovered to target SPS and SuSy gene transcripts. The eastern cottonwood which originally belonged to north Indiana did grow well with the environmental changes in Lubbock.

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CHAPTER I

INTRODUCTION TO THE STUDY, HYPOTHESIS, OBJECTIVES

1.1 General Introduction

Perennial plants are the principal producers in the terrestrial ecosystem. The secondary growth that is characteristic of "trees" is in fact a huge store house of biomass and a carbon sink (Lee et al., 2006) that represents chief industrial raw material. In recent times with decreasing natural reserves and growing demands for resources, it becomes quite indispensable to research more on these resources and find ways to recompense the demands. *Populus* species are one such resource that has now become a model forest tree for experimentation. Owing to their biological, economical, and ecological attributes, poplar trees are used for the production of several commercially important products such as paper, lumber, and furniture and very recently, it is targeted for biofuels like cellulosic ethanol. These woody plants have evolved a special arrangement of cells in their xylem (wood) to perform the functions of supporting a large crown and conducting water and inorganic nutrients to the crown. The development of the walls of these cells is critical to these functions and to the commercial uses of wood, making it important to study the pattern of carbohydrate metabolism in xylem that leads to cell wall synthesis during cellular differentiation. However, another function of xylem in trees is to store carbohydrates, primarily starch and soluble sugars. These carbohydrates are needed for a variety of functions associated with tree growth and survival during environmental stress. Therefore, an understanding of the controls on the metabolism of this storage

carbohydrate would lead the way toward manipulation of these reserves to favor better xylem and shoot development.

1.1.1 Carbohydrate transport, storage and utilization in trees

Parts of a plant that consist of chlorophyll pigments (chlorenchyma) like leaves and young bark, (Kozlowski, 1992) would act as the photosynthesizing "source" organs. Once synthesized, up to as much as 80% of the primary sugars will be translocated via the phloem to non-photosynthetic tissues, such as growing points and areas of carbohydrate storage. It was reported that the major transportable sugar from the source to sink tissues is sucrose in woody plants and other species (Haigler et al., 2000; Park et al., 2009; Winter and Huber, 2000; Kozlowski, 1992). This sucrose is the source of glucose and fructose (hexoses) used in various metabolic processes, such as the production of ATP and the building of new cell structure during growth. A major proportion of the sucrose is used for cambial growth and differentiation of the secondary xylem. Once this carbon is utilized in cell wall synthesis, it is permanently immobilized for the lifetime of the tree and unavailable for further growth in other parts of the plant. Sucrose in excess of that needed for xylem growth is available for starch synthesis. The excess of these carbohydrates (Guy et al., 1992) along with proteins and fats get deposited and stored in the parenchymatous cells of the xylem (wood) (Sauter and van Cleve, 1994) at different times during the year. The deposition and mobilization processes are dependent on environmental and physiological conditions of the tree. The stored carbohydrates can be used to protect the cells during the winter (cryostabilizing sugars, Kaplan and Guy, 2004; Guy et al., 1992) and as a source of material to develop

new leaves at the time of bud break and cambial growth in the spring (Sauter et al., 1994; Sauter and Wellenkamp, 1998; Zhi-Bin et al., 2006).

Carbohydrate is stored in the cells generally as starch (Geigenberger, 2003). The starch molecules are composed of two related types of polysaccharides: amylose and amylopectin (Pozueta-Romero et al., 1999; Smith et al., 1997; Witt and Sauter, 1995). Its digestion products can be utilized to form soluble sugars for metabolic processes in the cells or for translocation to other areas, such as apical and lateral meristems. Since this storage of carbohydrate as starch competes with the developing xylem cells of the stem during the growing season, the storage process should be highly down-regulated in the vicinity of the xylem development during the period when the vascular cambium is most active in the stem. In the autumn, when cambial activity is low, starch storage can proceed. Also, it would make sense that the further away from the active cambium, the greater may be starch synthesis. Figures 1 and 2 provide the basics for starch and sucrose metabolism in a heterotrophic plant cell.

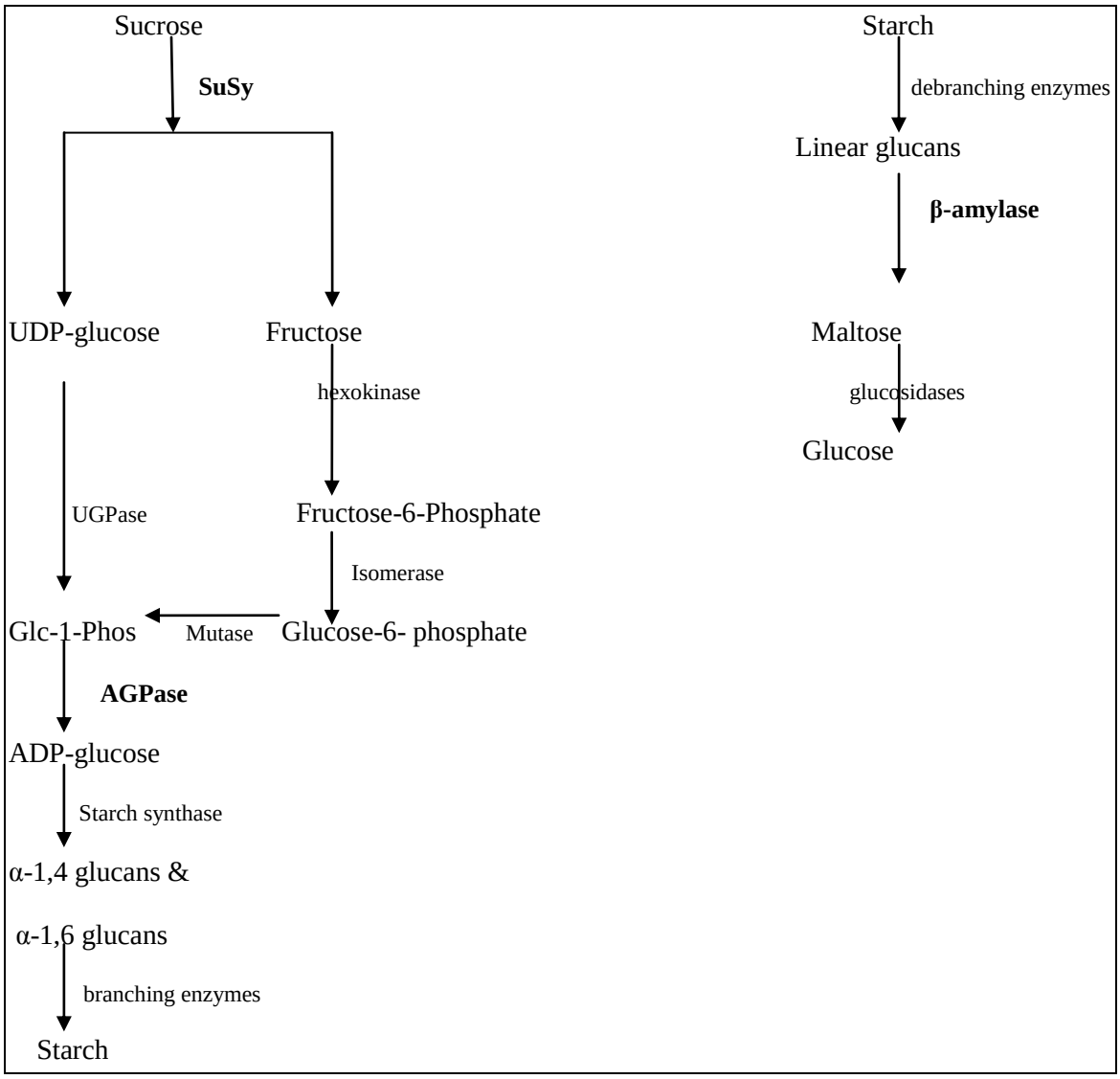

Figure 1. Major steps in starch metabolism pathway

(Abbreviations: AGPase- ADP glucose Pyrophosphorylase, SuSy- Sucrose Synthase)

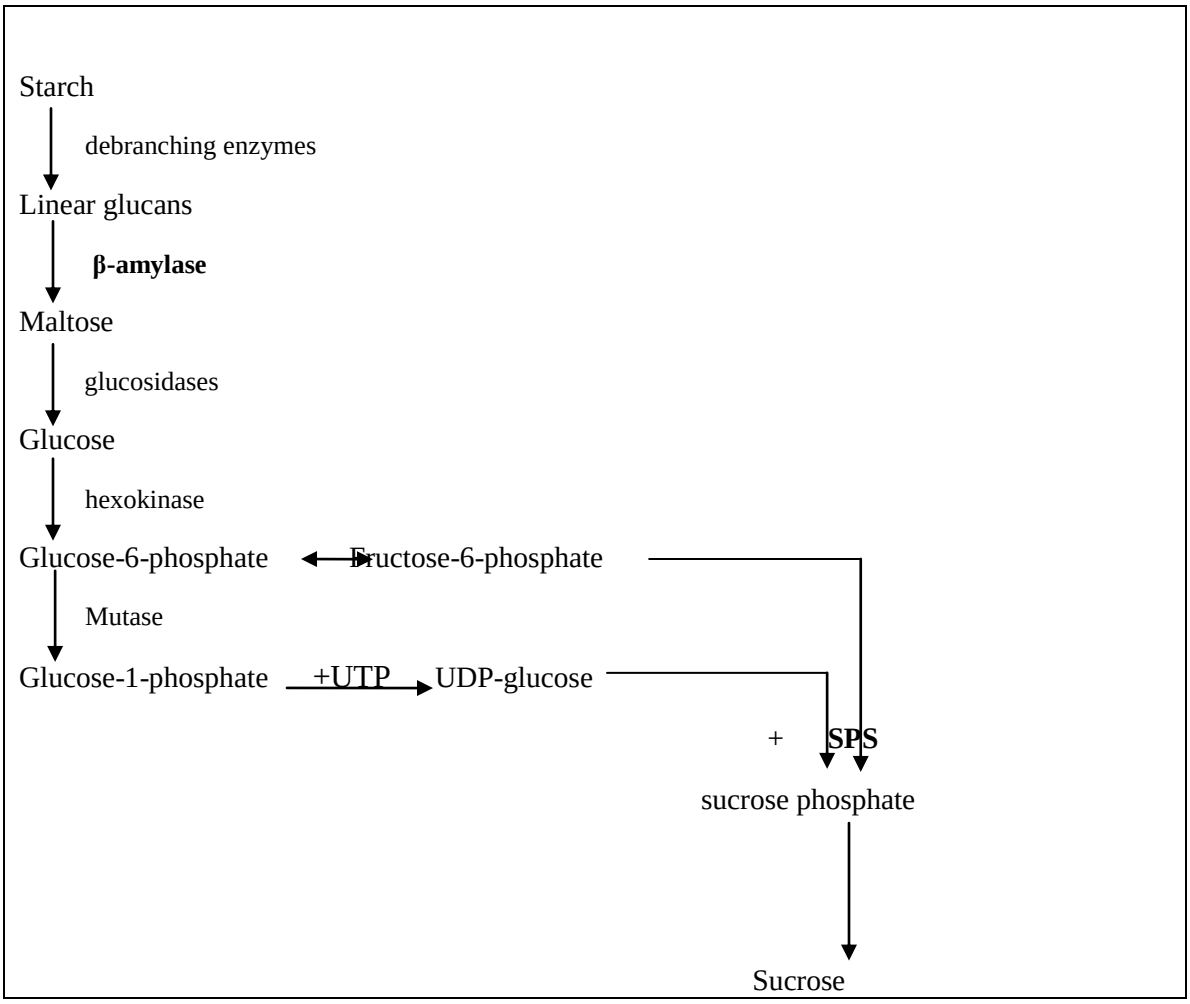

Figure 2. Major steps in sucrose biosynthetic pathway in heterotrophic cells.

(Abbreviations: SPS- Sucrose phosphate synthase, UTP-Uridine triphosphate, UDP-Uridine diphosphate).

1.1.2 Starch metabolism in heterotrophic cells and the key enzymes involved

Starch synthesis from sucrose begins with the cleavage of sucrose (Winter and Huber, 2000; Haugh and Magel, 1998). Since the majority of the carbohydrate that is translocated in the phloem of many, especially woody, species, such as cotton is sucrose (Winter and Huber, 2000; Zimmerman and Zeigler, 1975; Fisher, 1978; Tarczynski et al., 1992), it must be metabolized to hexose to enter into other cellular metabolic processes of

the cell. Figure 1 illustrates the pathways for the synthesis of ADP-glucose, the substrate for starch synthesis, from sucrose in non-photosynthetic cells. In all non-photosynthetic starch storing organs, apart from endosperm of developing cereal grains, as soon as the sucrose enters the cytosol, it is cleaved to UDP-glucose and fructose by sucrose synthase (SuSy) in the cytosol (Smith, 2001; Pozueta-Romero et al., 1999; Schultz and Juvik, 2004). The UDP-glucose and the fructose, eventually, can be converted to glucose-1-phosphate (G-1-P), which can enter the plastid via a transporter on the inner envelope and be used by ADP-glucose pyrophosphorylase (AGPase) to form ADP-glucose (Smith, 1999; Smith, 2001; Geigenberger, 2003). Because AGPase is highly regulated, it is considered the major regulatory step in starch synthesis (Ballicora et al., 2004; Smith et al., 1997; Smith, 1990). The ADP-glucose is used by starch synthase for the synthesis of amylose, long chains of glucose that will lead to the development of starch. However, it must be noted that UDP-glucose is the substrate for cellulose synthesis. Therefore the two pathways, starch and cellulose synthesis, may compete for carbohydrate substrate in developing cells.

The starch that is stored in non-photosynthetic cells is catabolized to glucose, depending on particular seasonal or developmental carbohydrate demands (Stanley et al., 2005). A short diagram in Figure 1 illustrates the starch breakdown pathway. In this process, as a first step, debranching enzymes act on the starch breaking them to α1,4-glucans and α1,6-glucans. Thereafter amylases act on the glucan molecules to produce maltoses (Witt and Sauter, 1995). The α-glucosidases act on the maltose and maltotrioses while amyloglucosidases act on glucans to break them into glucose units (Witt and

Sauter, 1995). These glucose units could then enter the required metabolic processes at the time once it is phosphorylated.

Figure 2 illustrates major steps involved in sucrose metabolism in heterotrophic cells. In photosynthetic cells, hexose-phosphates are produced from triose phosphate produced by the Calvin-Benson cycle during the day and serve as starting points to make sucrose phosphate. In heterotrophic cells, catabolism of starch leads to glucose formation, which is phosphorylated and used to start sucrose synthesis. The Glucose-6-phosphate can be converted to Glucose-1-phosphate that is the substrate for UDP-glucose pyrophosphorylase to produce UDP-glucose, a substrate for sucrose phosphate synthase (SPS). The other substrate for SPS, Fructose-6-phosphate is synthesized from Glucose-6-phosphate (Winter and Huber, 2000; Geigenberger, 2003). The sucrose phosphate is dephosphorylated to sucrose by sucrose phosphate phosphatase.

Therefore, the major enzymes involved in sucrose metabolism are SPS and SuSy (Huber, 1996; Winter and Huber, 2000; Shrader, 2002). While SPS plays a prime role in sucrose synthesis in both photosynthetic and non-photosynthetic tissues (Huber, 1996; Shrader, 2002), SuSy is mainly involved in sucrose degradation than its synthesis (Shrader, 2002; Winter and Huber, 2002).

1.1.3 Seasonal variation of carbohydrate levels in poplar xylem

The ray parenchyma cells of poplar wood show remarkable changes in their carbohydrate status throughout the year (Sauter and van Cleve, 1994). Usually, the starch levels are high in the late summer and early autumn and low in the winter and during bud break (Table 1) (Witt and Sauter, 1994). Therefore, in xylem parenchyma, starch synthesis is greater than starch digestion (mobilization) during the summer and autumn,

whereas starch mobilization is high relative to synthesis during the winter and early spring. The starch is digested during the winter and again at bud break to increase the levels of soluble sugars, such as sucrose (Tables 1 and 2) (Schrader, 2002; Witt and Sauter, 1994; Guy et al., 1992). The increase in stored carbohydrate in the autumn is likely facilitated by a reduction in the growth rate of shoot and stem (Crespi et al, 1991; Winter and Huber, 2000). With more exposures to low temperatures during winter, the increase in soluble sugars, especially sucrose, is likely a mechanism of cryoprotection as part of the acclimation process to the cold (Guy et al., 1992). These seasonal patterns observed with sugars and starch offered a system to study the regulation of carbohydrate metabolism, in general, in xylem parenchyma cells. From previous research, the general pattern observed with starch and sugar metabolism in poplar wood in a year is indicated in Tables 1 and 2.

Table 1: Physiological state of starch metabolism throughout a year in poplar xylem

Time of the year	Physiological state of starch metabolism
January February	Starch completely digested
March April May	Starch resynthesis followed by mobilization
June July August September October	Starch deposition (Maximum deposition of starch in October)
November December	Starch digestion

Table 2.Physiological state of sugar metabolism throughout a year in poplar xylem

Time of the year	Physiological state of sugar metabolism
January February March	Increased sucrose levels until spring
April May	Sugar content is minimum
June July August	Sucrose levels remain low but increase as starch levels increase
September October November December	Sugars increase (High levels in November and December)

1.1.4 Seasonal variation of enzymes associated with carbohydrate metabolism in poplar

The four key enzymes of carbohydrate metabolism in xylem cells are sucrose synthase (SuSy EC 2.4.1.13), ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), β-amylase, and sucrose phosphate synthase (SPS, EC 2.4.1.14). They are involved in metabolism associated with starch synthesis (SuSy and AGPase) and starch mobilization to sucrose (β-amylase and SPS). Variations in the amounts of starch, sucrose, and hexose in xylem parenchyma cells of poplar and *Robinia* species correspond to variations in the extractable activities and, presumably, the levels of these enzymes involved in their synthesis and degradation at some points throughout the year (Sauter and Witt, 1993), but not at all times. How these putative changes in enzymes relate to the level of gene

expression for them and what factors control that gene expression are not always clear. The general pattern of the activity level of the four enzymes (AGPase, SPS, SuSy and amylase) involved in starch and sugar metabolism in a year are summarized in Table 3.

Table 3. Pattern of enzyme activity levels of the enzymes involved in starch and sucrose metabolism in poplar xylem, in a year.

Season	Activity level of the enzymes involved in starch and sucrose metabolism			
	AGPase	SPS	SuSy	Amylases
Spring	Increases	Decreases	High	
Summer	Max in May	Low	High (outer wood)	Low
Fall	Increases	Increases	Increases	Low
Winter	Low	Maximum	Maximum	Low

The gene transcripts for SuSy were found to be very high in the active cambial regions where high cell differentiation was occurring (Lee et al., 2006). In fact, the extractable activity of SuSy was remarkably high in the outer portion of the xylem, whereas its activity was lower in middle and inner areas of a 15 to 20 year-old stem of poplar (Shrader and Sauter, 2002). The high SuSy activity correlated more with the differentiation of xylem cells than with starch deposition (Shrader, 2002). Similarly, in a study of metabolically-active *Robinia* wood, SuSy gene transcripts, protein levels, and enzyme activity were found to be well correlated and highest in differentiating xylem in

the spring and summer, providing substantiation for the role of SuSy in contributing substrate for the synthesis of cell wall materials (Hauch and Magel, 1998). However, in the late autumn and the winter, a minor peak of extractable activity of SuSy occurred in inner and outer sections of the poplar stem, and *Robinia* exhibited an increase in SuSy activity in the sapwood-heartwood transition zone. This increase in SuSy activity corresponded to an increase in SPS activity (Hauch and Magel, 1998). It has also been reported that AGPase and starch synthase activities increase at this time, as well, possibly reflecting a final burst of starch synthesis before temperatures dropped considerably (Witt and Sauter, 1994). However, this late autumn period is when starch mobilization should be beginning. Such findings make it difficult to understand what control there may be on starch synthesis and mobilization.

The reported seasonal trends in SPS activity appear to correlate well with seasonal changes in sucrose and starch content of the xylem for poplar. The SPS activity was found to be high in the autumn and winter at the time of starch-to-sugar conversion and declined in spring during starch re-synthesis prior to final mobilization at bud break (Shrader, 2002). These results show that a "sugar cycle" is being followed by poplar wherein the starch and sugars might be inter-converted during summer and winter seasons. In summer, the activity of SPS remained at a very low level in poplar xylem (Shrader, 2002). In *Robinia*, cold-acclimated xylem collected in November or January showed higher rates of SPS than samples harvested in summer (Hauch and Magel, 1998). In November, however, the extractable SPS activity transiently increased in the inner sapwood.

Starch mobilization requires starch degradation by amylases. In *Arabidopsis* leaves, the beta form of amylases are predominantly responsible for degrading linear glucans of amyloses (long chains of glucose units linked at the number 1 and number 4 carbons) that can be derived from amylopectins (amylose chains linked together at the number 1 and the number 6 carbon) whose 1-6 linkages have been broken by debranching enzymes (Zeeman et al., 2004). In poplar xylem, β-amylase was the more important form of amylase involved in starch degradation (Witt and Sauter, 1995). This finding is a point of surprise, since β-amylase is inhibited by high concentrations of its product, maltose, which may occur in xylem parenchyma cells (Witt and Sauter, 1995). The activity of β-amylase was observed to be highest from March through April, decreasing rapidly after April (Witt and Sauter, 1995)

In a study conducted by Sauter and Witt (1994) with poplar, a sharp decrease of all these enzymes in the twigs was observed around the period of bud break in the spring. This reduction in the enzyme activity may have resulted from reduced protein synthesis due to gene regulation or increased protein degradation. Generally, in the phase from May to July the enzymes of starch synthesis, and sometimes sucrose synthesis, exhibited considerable activity, whereas the starch degrading enzymes did not follow this trend, leading to the predominance of synthesis pathway in the summer. In addition to the seasonal pattern to starch deposition and mobilization, it was previously discovered in my advisor's lab that starch accumulation varies spatially in poplar stems greater than a year old, being low during the summer in the new growth ring and high in the rings of previous years. Thus, in the regions of an active vascular cambium and xylem cell development, starch accumulation was low. The parenchymatous cells further away from

the active vascular cambium and region of cellular differentiation were found to be highly involved in the storage of metabolic products like carbohydrates (Uggla et al., 2000; Samuels et al., 2005). This finding is supported by the work done by Witt and Sauter (1994), and Shrader and Sauter (2002) with poplar species. Therefore, a spatial pattern with the level of starch and sugar deposition varying across the xylem may occur, but very little is known about the seasonal variation in this spatial variation. As with the seasonal pattern of starch metabolism, this spatial pattern may also offer a system to study the regulation of starch metabolism and that of carbohydrate metabolism, in general, in xylem parenchyma cells.

1.1.5 Regulation of key enzymes of starch and sucrose metabolism: AGPase, SPS, SuSy and β-amylase

It is my interest to determine whether there is any correlation between transcript and protein levels and the variations in enzyme activities associated with xylem starch metabolism found by other researchers. There is some information that environmental factors are involved in regulating gene expression for at least some of the key enzymes of starch and sucrose metabolism. For example, SPS gene transcription increases during water deficit (Geigenberger et al., 1999) in potato tubers. Also, prolonged exposure to suboptimal temperatures will increase SPS gene expression (Holaday et al., 1992; Guy et al., 1992). In addition, the levels of nitrate and phosphate were found to be inhibitors of AGPase gene expression, decreasing expression levels, whereas sucrose levels increased expression (Geigenberger, 2003). An increase in sucrose level increases SuSy gene expression, as well (Geigenberger, 2003).

However, it must be kept in mind that these four key enzymes, like many other enzymes of metabolism, are regulated by many factors. Sucrose synthase is controlled by post-translational modification via reversible protein phosphorylation and possibly redox modification (Pozueta-Romero, 1999). It has been shown that "in vitro phosphorylation of SuSy in maize leaves selectively activated the cleavage reaction by increasing the apparent affinity of the enzyme for sucrose and UDP" (Huber and Huber, 1996). The SuSy enzyme, along with an isozyme of fructokinase, is also inhibited by fructose (Dai et al., 2002; Schaffer and Petreikov, 1997). The AGPase in several species is regulated by the allosteric activator, 3-phosphoglyceric acid, and the inhibitor, inorganic phosphate (Pi) (Pozueta-Romero, 1999), and the control of starch biosynthesis is primarily affected by the ratio of these two effectors (Pozueta-Romero, 1999). Also, AGPase activity can be controlled through redox changes that affect its oligomerization (Teissen et al., 2002). Maltose, at a particular range of concentrations inhibits β-amylase activity, thereby affecting the starch catabolism (Witt and Sauter, 1995). Besides the importance of SuSy activity to carbohydrate metabolism in heterotrophic cells, SPS activity also plays a role in determining the levels of certain carbohydrates (Winter and Huber, 2000; Schrader and Sauter, 2002). Glucose-6-P (allosteric activator) and Pi (allosteric inhibitor) regulate the activity of SPS, and the effector sensitivity is altered by protein phosphorylation (Winter and Huber, 2000). Since SPS is the major enzyme in sugar synthesis, the amount of "carbon" directed into sucrose depends on the activity of SPS and thus SPS could be an indicator of the competence of that tissue for sucrose synthesis (Winter and Huber, 2000).

With these many factors controlling the enzyme activity, per se, the extent to which carbohydrate metabolism in xylem is controlled by gene expression and enzyme

level may be small. Only a strong correlation between the carbohydrate, transcript, and protein levels would be evidence supporting the idea that gene regulation is critical to seasonal and spatial patterns of carbohydrate metabolism in xylem parenchyma cells.

1.1.6 Small-RNA mediated regulation of enzymes in poplar

Besides direct regulation of the expression (transcription) of the genes encoding the enzymes of starch and sucrose metabolism, the transcript levels for enzymes can be regulated by microRNAs (miRNAs) and small interfering RNAs (siRNAs). These RNAs of approximately 20–24 nucleotides are non-coding RNAs that are negative regulators of transcript levels for some genes (Barakat et al., 2007; Jones-Rhoades and Bartel, 2006). Although miRNAs are usually conserved among many plant species, the non-conserved ones that are *Populus*-specific were shown to be involved in the regulation of electron transport, signal transduction processes, and secondary xylem formation (Barakat et al., 2007). Certain miRNA gene families were found to regulate transcripts for enzymes associated with xylem cell differentiation (Lu et al., 2005; Turner et al., 2007). The miRBase (miRBase, release 16) indicates that 234 miRNA genes and 33 miRNA families have been found in *Populus* to date (Li and Mao, 2007; Barakat et al., 2007). Using these miRNAs, sequences, one could determine whether any were complimentary to the coding sequences of AGPase, SPS, SuSy and β-amylase.

1.2 Hypotheses

From the previous research on carbohydrate studies and the activities of the enzymes involved in carbohydrate metabolism in poplar xylem parenchyma, it is understood that there is a typical physiological trend with respect to carbohydrate storage and metabolism

followed by these cells throughout a year. Studies have shown that at least for three of the enzymes of interest (AGPase, SuSy, SPS) are subjected to gene regulation, in general. My primary hypothesis was that the level of transcripts for the enzymes involved in starch and sucrose metabolism does follow the general trend in specific carbohydrate levels in poplar xylem seasonally and spatially across a stem. Therefore, the enzyme transcripts of SuSy and AGPase were expected to be high prior to the rise in starch in young twigs and in the inner regions of the xylem in multiyear stems where starch storage is high in the summer. Also the enzymes involved in starch degradation and sucrose synthesis (β-amylase and SPS) were expected to be high prior to the rise in sugar levels in young twigs or in the outer regions of xylem in multiyear old stems. I expected that the changes in transcript levels would be reflected in changes in the protein levels. Since there is at least one instance known where a miRNA is synthesized in response to low temperature in poplar xylem, I hypothesized that the transcripts for at least one enzyme may be subjected to known small interfering RNA regulation.

1.2.1 Specific Objectives

1. Determine whether the transcript changes for key enzymes (AGPase, SuSy, SPS, and β-amylase) of starch and sucrose metabolism correlate with the changes in starch and sucrose content of xylem parenchyma cells in young poplar stems on a seasonal and spatial basis.

Such transcript changes would provide support for transcriptional regulation of carbohydrate metabolism in poplar xylem. If the transcript levels of the enzymes associated with starch synthesis (AGPase and SuSy) were to increase and those of

sucrose synthesis (SPS) and starch mobilization (β-amylase) were to decrease at times when the level of starch increased, I would be able to accept my primary hypothesis with respect to starch synthesis. If the trends in these transcripts reversed during the early winter and/or at bud break when starch synthesis should be low and its mobilization increased, I would accept the hypothesis with respect to starch mobilization. Since the outer ring of wood is developing during the spring and summer of a multiyear poplar stem, with considerable cell differentiation, if I found that SuSy transcripts were high in that region during the summer, I would confirm the findings of other researchers studying poplar and *Robinia* xylem growth. On the other hand, if increased starch synthesis and high AGPase transcripts were observed in summer in the inner rings and a reverse trend is observed in winter, then my hypothesis would be better supported.

2. Determine whether the relative protein levels for SuSy, AGPase, and SPS change in accordance with the changes in transcript levels

A positive correlation between the transcripts and the final protein levels would strongly suggest that the major control of the levels of these enzymes is at the gene expression level. No correlation would indicate the presence of a good deal of translational regulation, reducing the protein synthesis, or regulation at the level of protein degradation.

3. Determine whether any transcripts for these enzymes could be controlled by small, interfering RNAs in vivo.

If I found that some sequences of small, interfering RNAs known from *Populus* had complimentarity to one or more regions of RNA for any of the enzymes of interest, it would suggest that the synthesis and likely the level of the enzyme(s) would be controlled by the presence of the interfering RNA.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Material

Two species of *Populus*, *Populus deltoides* and *Populus balsamifera*, were grown in 43-L pots with regular soil. These plants were placed outside the greenhouse at Texas Tech University, to expose them to natural environmental conditions (temperature, sunlight, humidity and winds) in Lubbock, TX. The *P. deltoides* plants were generated from stem cuttings or seeds collected in North Central Indiana and the *P. balsamifera* plants were generated from seed collected near Edmonton, Alberta, Canada. The plants were watered with de-ionized water each day and fertilized once a week with 500 mL of Hoagland's solution. The *P.deltoides* plants were four to five years old, whereas the *P. balsamifera* plants were two to three years old at the time of sampling.

2.2 Sampling procedures

Both the species were sampled at different times during the year. Sampling was done at the times when critical changes in carbohydrate metabolism were expected based on the literature. The date and time of sampling, physiological significance, and the prevailing weather conditions on the day of sampling are shown in Table 5. Sampling of twigs and/or multiyear stems was performed at: (a) the middle of the winter during the coldest temperatures (Dec.-Jan.); (b) at bud break; (c) in the early summer (June/early July); (d) late summer after shoot growth had ceased (September); and (e) in late autumn after leaf abscission. Since the stems of *P. balsamifera* did not attain a large enough diameter to

determine spatial patterns of transcripts for enzymes easily, multiyear stems were only sampled from *P. deltoides* plants.

Twigs from both species were sampled by cutting a section of ~10 cm in the center portion of the present year's growth and removing the bark to the vascular cambium with a sterile razorblade. The xylem was washed with sterile, autoclaved water, and thin sections were sliced. These sections were immediately placed into microfuge tubes and frozen in liquid nitrogen for storage at -80 $^{\circ}$C freezer. To study the seasonal and spatial variation in carbohydrate metabolism in multiyear stems, a sterile saw was used to cut the *P. deltoides* stem in cross-section approximately 20 cm above the soil line. The bark was peeled from an inner portion of the section. Slices through the stem were made at an angle to expose the growth rings. Thin sections of the wood were sliced from the outer portion of each ring with a sterile blade, washed with sterile water, and rapidly frozen in liquid nitrogen. The twig samples and each ring of wood were labelled and separately stored at -80 $^{\circ}$C. The outermost, youngest ring was designated the first annual ring.

Table 4. The date and time, physiological significance, and the weather conditions of the sampling day for *Populus deltoides* (weather conditions are taken from Weather History @ www.weather.org)

Date of Sampling	*P.deltoides*	Physiological significance of the sampling time	Average monthly Temp. (°C)	Weather
Feb 25 2009, 11am	Xylem growth rings	Bud opening	10.0	Clear
Jun 11 2009, 10 am	Xylem growth rings	High summer temperatures	26.6	Scattered cloudy
Dec 3 2009, 9 am	Xylem growth rings Young, mature twigs	Low winter temperatures	2.2	Overcast
Feb 08 2010, 9 am	Xylem growth rings Young, mature twigs	Bud opening time	4.4	Overcast
May 01 2010, 10 am	Xylem growth rings Young, mature twigs	Summer temperatures	21.1	Mostly cloudy
July 08 2010, 8 am	Xylem growth rings	High summer temperatures	25.5	Mostly cloudy

Table 5. The date and time, physiological significance and the weather conditions of the sampling day for *Populus balsamifera* (weather conditions are taken from Weather History @ www.weather.org)

Date of Sampling	*P.balsamifera*	Physiological significance of the sampling time	Average monthly Temp. (°C)	Weather
Oct 13 2008, 11 am	Young, mature twigs	Time of leaf fall	16.6	Cloudy
Feb 25 2009, 11 am	Young, mature twigs	Bud opening time	10.0	Clear
Apr 20 2009, 9 am	Young, mature twigs		16.1	Clear
Jun 11 2009, 11 am	Young, mature twigs	High summer temperatures	26.6	Scattered clouds
Sep 23 2009, 10 am	Young, mature twigs		21.6	Scattered clouds Clear
Nov 20 2009, 7 pm	Young, mature twigs	Low winter temperatures	11.6	Partly cloudy
Feb 15 2010, 10 am	Young , mature twigs		4.4	Mostly cloudy
May 01 2010, 9 am	Young, mature twigs	Summer temperatures	21.1	

2.3 Assays of Carbohydrate Content in the Xylem

The content of glucose, fructose, and sucrose in the xylem of twigs and the multiyear stems was measured to study their seasonal and spatial variation. Once these soluble carbohydrates were extracted from the xylem samples, the amount of starch remaining was determined following its digestion to glucose.

2.3.1 Extraction of the soluble sugars

The tubes with the frozen xylem samples were quickly weighed to determine the mass of the samples and then incubated in 1 mL of 80% ethanol at 75 $^{\circ}$C for approximately 1 h until the ethanol had almost evaporated. Then the tissues were allowed to cook in 0.5 mL of de-ionized water for about 1 h, followed by centrifugation 16,000 g for 5 min. The supernatants were pipetted into another tube, the level of the solution was marked and the tissues were stored in 80% ethanol in the freezer until assayed for its carbohydrate content. The supernatants contained the soluble sugars, such as glucose, fructose, and sucrose, and they were stored at -20 ^{o}C until they could be assayed for the sugars.

2.3.2 Digestion of insoluble starch

The starch content of the insoluble material remaining after soluble sugar extraction was determined by digesting the starch to glucose and assaying for the glucose. The tissues were placed into 0.5 mL of 100 mM acetate buffer, pH 6.0, that was then heated to 100 ^{o}C for 45 min to gelatinize the starch. After cooling to room temperature, the starch was digested by adding 0.1 mL of digestive enzyme solution to each tube (100 units of amyloglucosidase and 200 units of amylase in 0.1 mL of 100mM acetate buffer

pH 6.0) and incubating the digestion overnight at 55 °C on a slow shaker. The digestion was terminated by boiling the tubes for 1 minute. The tubes were centrifuged at 16,000 g, the supernatant was pipetted into a new labelled tube, and its volume marked and measured. These supernatants were stored at -20 °C till use for enzymatic assay of glucose content.

2.3.3 Enzymatic assay for the soluble and insoluble sugars

Sugars in the soluble fraction and the glucose from the digested starch fraction were determined using a spectrophotometer. The cuvettes were loaded with 990 µL of freshly made assay solution (50 mM HEPES, 5 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 0.4 mM NADP) and 10 µL of the soluble extract. For the measurement of glucose and fructose together in the soluble fraction, 2 µL of the assay solution containing 1 unit of glucose-6-phosphate dehydrogenase (G6PDH) and 1 unit of phosphoglucose isomerase (PGI) were added and the initial absorbance reading at 340 nm was taken. Two microliters of assay solution containing 1-2 units of hexokinase were added and the final absorbance was read once the reaction was completed. After a brief mixing, a new absorbance was taken as the baseline for the determination of sucrose. Five units of invertase were added and the absorbance was measured once the reaction was completed. To determine the glucose content in the digested starch sample, 990 µL of the assay solution and 10 µL of the sample solution were added to the cuvettes. Two microliters of the assay solution containing 1 unit of G6PDH were added, mixed, and the initial absorbance reading was taken. Then 2 µL of assay solution containing 2 units of hexokinase were added, mixed, and the final absorbance reading was taken once the

reaction was completed. The amount of a sugar in the extract aliquot assayed was calculated using Beer's Law and an extinction co-efficient of 6220 for NADPH.

2.4 RNA extraction and quantification

2.4.1 Total RNA Extraction

The total RNA was extracted from the xylem of twigs and multiyear stem sections using the Sigma-Aldrich Plant Total RNA extraction kit. The protocol, as given by Sigma Aldrich, was followed. The samples were ground to a fine powder in liquid nitrogen. To 100mg of powder, 500 µL of the lysis solution (10 µL of 2-mercaptoethanol in 1 mL of lysis solution) were added. The solution was then vortexed and incubated for 5 min to lyse the cells, followed by centrifugation at 16,000 g for 3 min and filtration of the lysate supernatant into a collection tube. The supernatant was centrifuged for 1 min and the clarified lysate was added to 500 µL of the binding-column solution and 700 µL of this mixture were pipetted onto the binding column followed by centrifugation. The RNA that was bound to the binding column was washed thrice with 500 µL of wash solution and dried by centrifugation at 16,000 g. The bound RNA was eluted by adding 50 µL of the elution solution directly to the binding column. Purified RNA was obtained by centrifugation at 16,000 g for 1 min. The RNA was stored at -80 $^{\circ}$C until its use for making cDNA.

2.4.2 Reverse transcription PCR

The cDNA was prepared from RNA by RT-PCR (Reverse Transcription PCR) using the kit and protocol by 'Bio-Rad i-script cDNA synthesis'(reverse transcriptase is RNaseH+ which provides greater sensitivity in qPCR and the primers are oligo dT and

random hexamers). The PCR reaction contained 4 µL of 5x iScript reaction mix, 1 µL of reverse transcriptase, and 1-2 µL of RNA template. The volume was made to 20 µL with nuclease-free water. The reaction mixture was subjected to PCR as follows: 5 min at 25° C, 30 min at 42 °C, 5 min at 85 °C, and hold at 4 °C. The cDNA was stored at -80 °C until its use for analysis.

2.4.3 cDNA amplification by the regular Polymerase Chain Reaction

Amplification of the reverse transcribed cDNA for the four enzymes was performed by regular PCR. The gene sequences for the four enzymes, AGPase, SPS, SuSy, and β-amylase, in poplar were obtained from the NCBI database (National Centre for Bio-informatics Institute (http://www.ncbi.nlm.nih.gov/) and the International *Populus* Genome Consortium (http://www.jgi.org *P*. *trichocarpa* database). The primers for the gene sequences were designed and obtained from "IDT", (Integrated DNA Technologies, http://www.idtdna.com/Scitools/Applications/Primerquest/). The accession numbers for the coding sequences, and the primer sequences for the four genes have been tabulated in Table 6. The primers were diluted 10 fold with sterile, de-ionized water (final concentration of the primers was 100 µM). The coding sequences and the primers used for these four enzymes were tabulated in Table 5. Ten microliters each of forward primer and reverse primer were added and made to 200 µL with water. The PCR reaction included 2 µL of template cDNA, 1 µL of primer mixture, 2 µL of dNTPs, 5 of 5X buffer 0.25 µL of Taq Polymerase, and 14.75 µL of deionized water (total reaction volume = 25 µL). The PCR cycle was programmed as follows: 95 °C for 2 min, 95 °C for 20 sec, 60 °C for 20 sec, 72 °C for 30 sec, go to step 2, 25 times, 72 °C for 5 min, and 4 °C for 24 hr.

Nanodrop and Southern blot were performed to quantify the DNA and check quality of DNA respectively.

Table 6. The NCBI accession numbers and primer sequences for the four enzymes under study.

Enzyme	NCBI Accession number	Source organism	Forward and Reverse primers
ADP-Glucose Pyrophosphorylase	XM_002321178	*Populus trichocarpa*	F: TGA CAA TGT GCA AGA AGC AGC CAG R: AGA TCA CAG TTC CGC TGG GAA TCA
Sucrose Phosphate Synthase	XM_002324585	*Populus trichocarpa*	F: AGG CAG GTT TAG CCC AAC TCG TTA R: GTC TTC TGC CAC GTG TTG CTG TTT
Sucrose Synthase	XM_002324100	*Populus trichocarpa*	F: TTG TTG AGG AGT TGC GTG TTGCTG R:TGAAAGAGTTGGGCGAG GGAAAGA
β-Amylase	XM_002311670	*Populus trichocarpa*	F: ATT TCC TGG AAT CGG AGC CTT CCA R: TCT TCC GGC CAG TTG TTG TAC TCA
18 S primer (control gene)			F: AAA TAC CGC GGC GCT GGC A R: CGG CTA CCA CAT CCA AGG AA

2.4.4 Quantitative Real Time PCR

Quantification of genes (DNA) for the four enzymes was performed by quantitative Real Time PCR. The PCR reactions included 23 μL of Syber Green Master Mix (for 10 PCR reactions, 125 μL of Syber Green concentrate added to 105 μL of deionized water), 1 μL of cDNA, and 1 μL of the forward and reverse primer mixture. A total reaction volume of 25 μL was pipetted into each well of the PCR plate. The Real Time PCR was programmed in the computer connected to the Real time PCR unit. The level of gene expression in a particular period of time was studied using a mathematical model to detect fold changes between samples, called DDCt (delta delta Ct). 18S ribosomal RNA was used as the control. Comparisons of expression levels were made on a seasonal and spatial basis for the xylem of one-year twigs and multiyear stems.

2.5 Protein Analysis

To compliment the gene expression analysis and to check the relative changes in protein levels, protein quantification was performed by Western blot. The antibodies for these enzymes were obtained from the following researchers: Anti-SPS and anti-SuSy antibodies were provided by Dr. Steven C. Huber, USDA Plant Physiologist and Professor of Plant Biology and Crop Sciences at University of Illinois, IL. Anti-AGPase antibody was provided by Dr.Thomas W. Okita, Institute of Biological Chemistry, Washington State University, Washington.

2.5.1 Total protein extraction

The procedure for protein extraction as described in Lunn et al. (2003) was followed. The frozen xylem samples were ground to a fine powder at liquid nitrogen

temperature followed by the addition of ice cold extraction buffer. The extraction buffer was contained 50 mM Tricine-KOH, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM benzamide, 1 mM benzamidine, 5 mM ε-aminocaproic acid, 10 µM leupeptin, 10 µM antipain, and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The crude extract was centrifuged at 11,600*g* for 1 min, and the supernatant was desalted by running it through a 3-mL column of sephadex G 25 M equilibrated with the extraction buffer minus PVPP.

2.5.2 Protein concentration (Bradford) Assay

Prior to loading the protein samples on SDS-PAGE, the concentration of protein was measured by the method of Bradford (Bradford, 1976). A standard curve was prepared by assaying known concentrations of bovine serum albumin (BSA).

2.5.3 SDS-PAGE

The extracted proteins of all the samples were mixed with sample loading buffer (0.5 M Tris, 10% SDS, 1mL glycerol, 1 mL Bromophenol blue, and 1 mL DTT) such that the concentration of protein in all the samples was same. The proteins were denatured by the SDS present in the sample loading buffer. The boiled extract was then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5% separating slab gel and a minigel system, at 40 mA for 5 h. Gels of low percentage acrylamide were chosen, since the enzymes of interest are of high molecular weight. Equivalent concentrations of protein from xylem samples were loaded in each lane. Ready-made gels were purchased from Biorad (Biorad.com). Molecular weight markers were used for molecular mass determination.

2.5.4 Analyses of the relative protein concentration using western blots

The procedure for Western blot was followed as given by Crespi et al (1991). Once the SDS-PAGE had separated the proteins, the gel was prepared for western blot. Invitrogen's iblot dry blotting system was used to transfer the proteins from the gel onto the nitrocellulose membrane. The blotting sandwich was assembled as follows: anode stack, gel, water soaked filter papers, cathode stack, and water absorbent (sponge). The transfer process was pre-programmed and required 7 min.

The nitrocellulose membrane was immediately transferred into the blocking buffer and left overnight at 4 ^{o}C to mask all of the nonspecific protein binding sites. The blocking buffer consisted of phosphate buffered saline (PBS: 20mM sodium phosphate, 150 mM sodium chloride, pH 7.2, 2% bovine albumin) containing 5% (mass/volume) non-fat milk powder. The blocked membranes were washed with PBS + 0.1% Tween 20 for 15 min followed by incubation with the primary antibody to the enzyme (antibodies were diluted 1: 2000 with PBS) for 2 h at room temperature. The immunoblots were washed of excess primary antibody using PBS + 0.1% Tween 20 followed by incubation with secondary antibody (horse radish peroxidase-conjugated secondary antibody diluted 1:4000 with PBS) for 2 h at room temperature. The blots were washed of excess secondary antibody with PBS + 0.1% Tween 20 followed by their exposure to DAB solution (1 mg/ml of DAB [3,3´-diaminobenzidine tetrahydrochloride] in 50 mM Tris-HCl, pH 7.2, and 0.2% hydrogen peroxide). The position of the protein of interest on the membrane was detected by the development of a brown color change produced due to the oxidation of hydrogen peroxide by DAB.

Quantification of the proteins on the blots was done by measuring the intensities of the bands using Image J, a software program developed by NIH (National Institute of Health).

2.6 Statistical Analyses

The mean values (± standard deviation) for replicates within each month or sampling time were analyzed for significant differences with that of the other seasons. All analyses for the seasonal variations were carried out with one way ANOVA at 95% confidence, followed by turkey's post hoc test.

Two-way ANOVA, followed by Bonferroni's post tests were conducted to look at the spatial variation of the carbohydrates, transcripts and protein content in the growth rings. For all analyses, $P \leq 0.05$ was considered significant.

2.7 Bioinformatics

NCBI (National Centre for Biotechnology Information- www.ncbi.gov) website was used for the Bioinformatics study. In order to check for other proteins that might belong to same family as the proteins under study (AGPase, SPS, SuSy, β-Amylase), BLASTx (Basic Local Alignment Search Tool) was performed. In BLASTx, the amino acid sequences of these proteins were compared against translated nucleotide sequences of poplar sequences in the NCBI database. This tool was used to determine if there were any proteins that belonged to the same gene family. For the identification of known small RNAs that regulate the gene transcripts for the enzymes under study (AGPase, SPS, SuSy and beta-Amylase), a bioinformatics software tool by name Batch RNA22 developed by IBM, was used, where all the known miRNAs in *Populus trichocarpa*, taken from the

miRBASE were aligned with the gene sequence of each enzyme in order to check for any complimentary miRNAs that might regulate the expression of these genes.

CHAPTER III

RESULTS

3.1 Correlations between carbohydrate content and gene transcript level of enzymes involved in carbohydrate metabolism in *Populus deltoides* and *Populus balsamifera*

3.1.1 Storage carbohydrates and transcripts of associated enzymes in balsam poplar twigs in a year

The twigs of *Populus balsamifera* were sampled at prominent time points of its life cycle. Seasonal variations in the carbohydrate and gene expression level were studied in relation to these phenological phases. Sampling during February and March (spring) was done as bud break and leaf development was conspicuous. Twigs were sampled in April, May and June (summer) to observe the trends with the onset of summer temperatures and photoperiod. In balsam poplar the twigs were observed to transition from the active growing phase into the dormant phase as early as June. No twig growth was observed after June until the following spring. In September (late summer) the leaves were still visibly green, with visible signs of leaf senescence occurring in October. The November (winter) samples were used to study the impact of low temperatures and photoperiod.

Over the sampling period of 2009 and 2010, the xylem of the balsam poplar twigs showed a definite trend in the variation of starch over the seasons (Fig. 3a). The differences in starch levels were significant over a broad range of time points. The February and March samples were taken from twigs that grew in the previous growing

season. Later samples were of the present year's growth. It was as the growth of the twigs slowed in May and June that the starch content reached its highest levels. The lowest levels measured occurred in September, October and November. Starch content in the summer (June 2009 and May 2010) was 200% more than the content in September, October and November. This finding is interesting, since the September sampling was performed before any marked change in growth temperatures had occurred.

Transcripts for the enzyme AGPase showed trends parallel to the trends in starch levels from February to May and June (Fig. 3b). The lowest transcript levels measured for AGPase occurred in February. Whether in the old twig immediately after bud break (March) or in the new, rapidly-growing twig (April), the AGPase transcript levels had increased significantly 3 to 4 fold over February levels. A further increase was noted in May 2010, but the transcript levels had declined by June of 2009 from the levels in April of that year. By the sampling time in June, the growth flush had ceased and the buds were fully developed. We also noted some anomalies in the transcript levels relative to the starch levels, particularly when the twigs were dormant with low starch content and moderately high transcript levels in September, October, and November.

The seasonal variation in SuSy gene transcripts showed a clear trend, being highest during the dormant phase with exposure to low temperatures (November and February) (Fig 3c). The SuSy transcript levels were generally lower during the warmer months, but the twigs that were starting to grow in March 2010 also had low levels of SuSy transcripts. The maximum level measured occurred in November 2009 and was five-fold higher than the lowest level measured in March 2010. The very high transcript levels in November of

2009 coincided with low temperatures and high soluble sugar (sucrose, glucose, and fructose) content (Fig 4a) as leaf fall occurred, signifying the positive relationship between the level of sugars and SuSy gene expression.

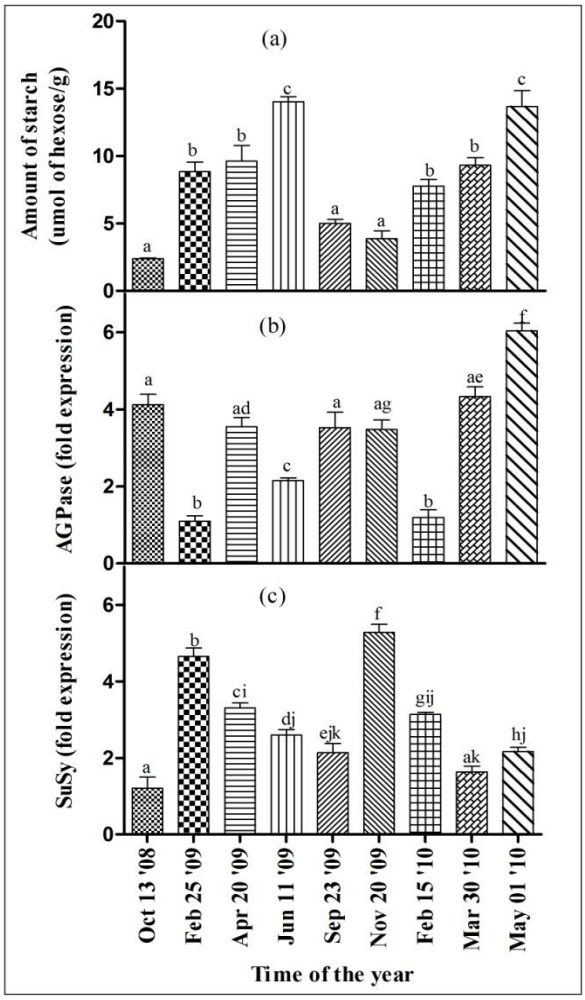

Figure 3. (from top to bottom) The content of starch (a) followed by the relative transcript levels for AGPase (b) and SuSy (c) in the xylem of one-year twigs of *Populus balsamifera* growing outside in pots at Texas Tech greenhouse. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was

calculated relative to 18s rRNA. Values are the means ± standard deviation and n = 3 (biological replication units).

Sucrose and hexose contents (total soluble sugars analyzed) also varied significantly over the seasons (Fig. 4a). The highest sugar levels measured occurred in the colder periods (November and February) when the twigs were dormant, whereas the lowest amounts were noted for growing twigs or twigs in sampled during warm periods. However, during one period of active growth (April 2009), the twigs did have a high level of these sugars. The maximum level of sugars measured (November 2010) was 300% more than the minimum level (June 2010). It is noteworthy that a strong increase in the sugar levels was observed from June to September of 2009, while the leaves remained green and low temperatures were not experienced. It was in this period that starch levels decreased dramatically (Fig. 3 a).

No significant differences in the measured SPS gene transcript levels occurred for much of the sampling period (Fig. 4b). However, from September 2009 to May 2010, a trend toward an increase in the late autumn (November) followed by a significant decrease for the late winter and spring (the growing period) was detected. The maximum SPS levels in November coincided with the high level of soluble sugars and high SuSy transcript levels (Fig. 4c) during the same period. Also, relatively high SPS transcript levels during February and September of 2009 correlated with high sugar levels during these periods.

The variation in the transcript level of the starch digesting enzyme, β-Amylase, roughly correlated positively with the levels of sugars in 2009 (Fig. 4c). However, the transcript levels were high in growing twigs that had moderate or low sugar levels. The

maximum transcript level observed in March 2010 was five-fold higher than the lowest levels in June 2009.

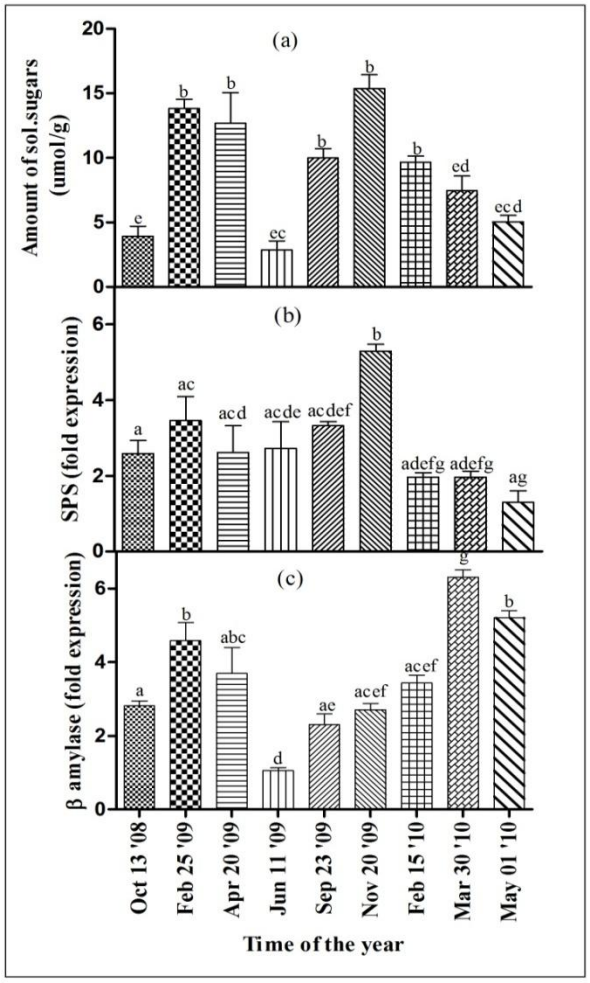

Figure 4. (from top to bottom) The content of soluble sugars (sucrose and hexose) (a) followed by the relative transcript levels for SPS (b) and β-amylase (c) in the xylem of one-year twigs of *Populus balsamifera* growing outside in pots at Texas Tech greenhouse. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was calculated relative to 18s rRNA. Values are the means ± standard deviation and n = 3.

3.1.2 Xylem carbohydrate content and gene transcript level in one-year twigs of *P. deltoides* during dormant and growth periods

Populus deltoides twigs showed a completely different timing of phenological events as compared to *P. balsamifera*. Bud burst and leaf development were observed in early to mid-April, and the active growth phase persisted until October. Leaf senescence occurred in late October into November. The twigs were sampled in December and February to study the impact of low temperatures and short day lengths during the dormant phase on carbohydrate and transcript levels for enzymes of carbohydrate metabolism. Sampling in May, June and July was aimed to study the carbohydrates and gene expression levels during the time of the active growth period. Hereafter, trends were broadly compared between dormant phase (winter- December 2009 and February 2010) and actively growing (summer- May and July 2010) time points. The winter samples represent twigs that had completed their first year of growth, whereas the summer samples represent growing twigs.

The carbohydrates in *P. deltoide*s twigs showed a noticeable trend with changing environmental conditions and the physiological state of the plants (Fig. 5 and Fig. 6). The starch levels were significantly lower in the winter than in the summer, as expected (Fig. 5a). An approximately three-fold increase in starch content was observed from winter to summer. As with starch levels, a clear and statistically significant increase (more than three-fold) in AGPase transcript levels from winter (February) to early summer (May) was observed (Fig. 5b). Although more data are needed, the peak transcript levels may occur in the early summer, followed by a decline until the following spring. The SuSy transcript levels showed no significant differences between winter and summer months

(Fig. 5c). The levels remained almost the same at all time points except for a small decrease from May to July of 2010. At least in the summer, SuSy transcript levels do not correlate with starch content.

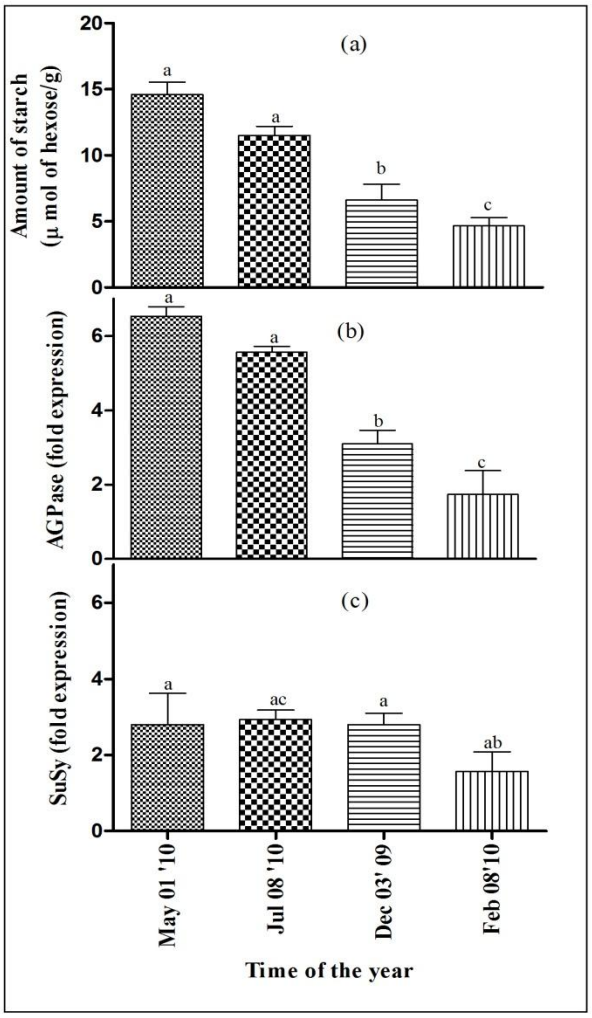

Figure 5. The content of starch (a) and the relative transcript levels for AGPase (b) and SuSy (c) in the xylem of three-year old twigs of *Populus deltoides* growing outside in pots at the Texas Tech greenhouse. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was calculated

relative to 18s rRNA.Values are the means ± standard deviation. n = 3 (biological replication units).

Soluble sugar levels in *P. deltoides* twigs varied only slightly from winter to summer (Fig. 6a), contrary to the results obtained for *P. balsamifera* twigs (Fig. 5a). However, a significant, two-fold decrease in SPS transcript levels occurred from winter to summer (Fig. 6b). Also, there was a trend toward an increase in transcript level for β-amylase from early winter.

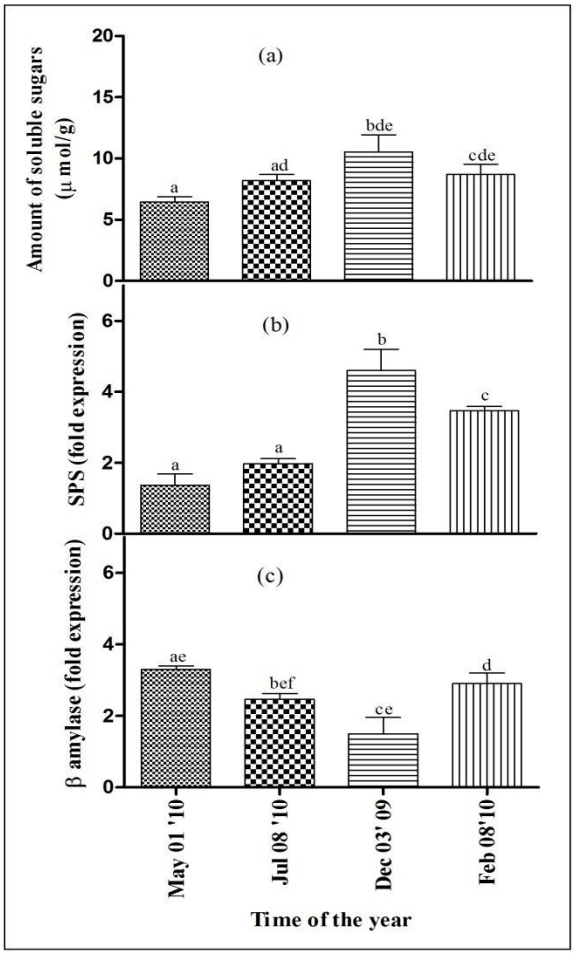

Figure 6. The content of soluble sugars (sucrose and hexose), a) and the relative transcript levels for SPS (b), and β-amylase (c) in the xylem of twigs of *Populus deltoides* growing outside in pots at the Texas Tech greenhouse. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was calculated relative to 18s rRNA.Values are the means ± standard deviation. n = 3 (biological replication units).

3.1.3 Carbohydrate content and the relative transcript level of enzymes associated with carbohydrate metabolism, in the annual rings of *Populus deltoides* multiyear stems.

Xylem samples taken at six times during the years 2009 and 2010 have been combined into two broad categories: (1) growing period (summer: June 2009, May and July 2010); (2) dormant period (winter: Feb and Dec 2009, and Feb 2010) (Fig. 7). As observed for the twigs (Fig. 5), the starch levels were lower in the dormant period than in the growing period. There was a tendency for the starch content to increase from the outside ring to the inner-most ring during both time periods (7a). However, no significant differences were found for the relative AGPase transcript levels across the stem in the summer and winter samples (Fig. 7b). In contrast, the SuSy transcript level in the inner-most ring was significantly higher than the levels in the middle and outer rings in the summer, and the lowest in the winter period (Fig. 7c). The SuSy transcript level in the inner ring was about 65% of the level in the middle and outer rings in the winter (Fig. 7c).

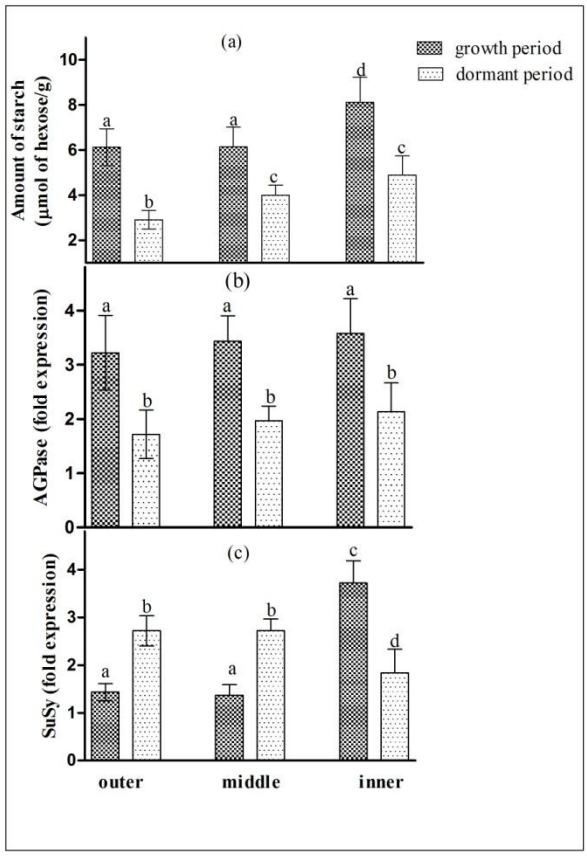

Figure 7. The content of starch (a), followed by the level of AGPase (b) and SuSy (c) transcripts in growth rings of *Populus deltoides* xylem during growth period and dormant period. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was calculated relative to 18s rRNA.Values are the means ± standard deviation, n = 3 (biological replication units).

There were no seasonal differences in soluble sugars in the multiyear stem of *P. deltoides* (Fig. 8a). In both the growing and dormant periods, the sucrose and hexose levels in the outer and middle growth rings of the *P. deltoides* stems were not significantly different, but they were significantly greater than the level in the inner ring (Fig. 8a). Although the transcript levels for SPS were different between dormant and

growing periods, they were not significantly different across the stem for each sampling period (Fig. 8b). In the case of the transcript levels for β-amylase, they were higher in the dormant period than in the growing period except for the inner-most ring. During the growing period, the transcript levels increased from the outer to the inner ring (Fig. 8c).

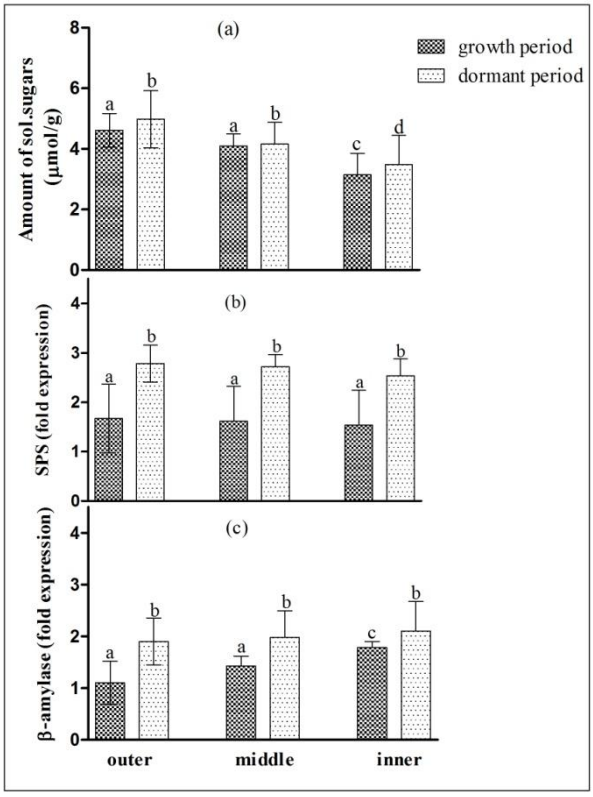

Figure 8. Soluble sugar content (a), followed by the level of SPS (b) and β-amylase (c) transcripts in growth rings of *Populus deltoides* xylem during growth phase and dormant phase. For the analysis of transcript levels, RNA isolated from the twig xylem was analyzed using RT-PCR and the expression was calculated relative to 18s rRNA.Values are the means ± standard deviation. n = 3 (biological replication units).

3.2 Correlation between the carbohydrate level and protein content of the enzymes

3.2.1 Western blots of AGPase, SuSy and SPS proteins in twigs of *Populus balsamifera* and *Populus deltoides*

The protein bands for AGPase, SuSy, and SPS (Examples in Fig. 9) were quantified.

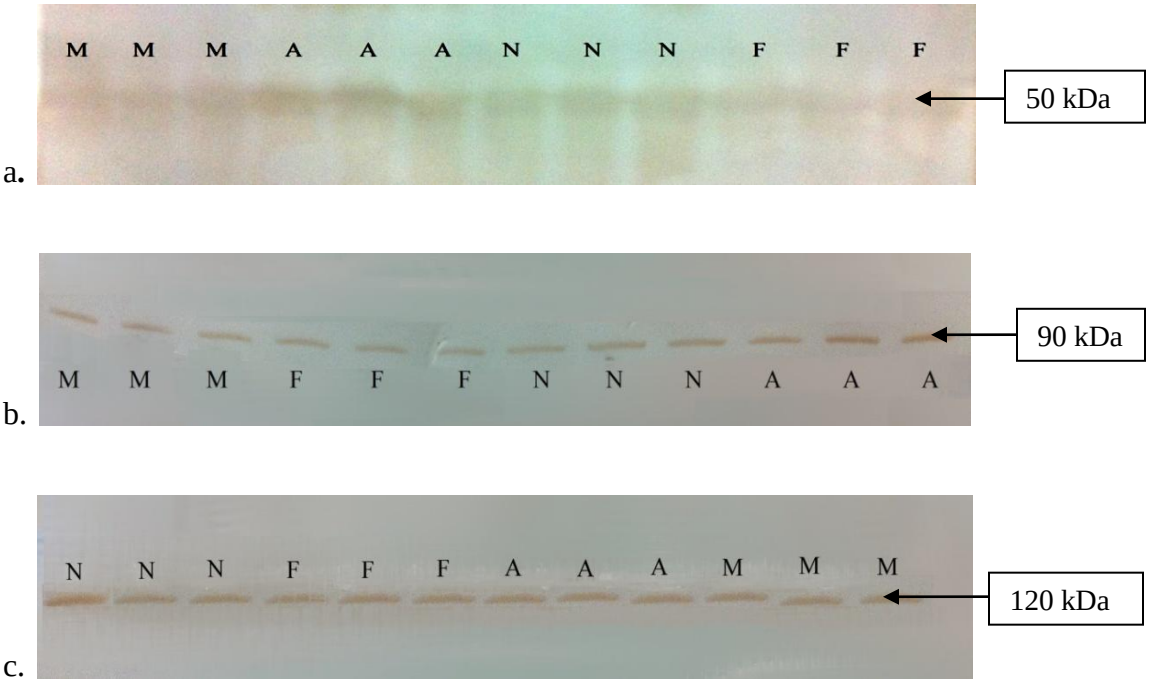

Figure 9. Western blots of AGPase, SuSy and SPS protein in the twigs of poplar. a) AGPase protein bands from three twig samples of *Populus balsamifera* taken in May 2010 (M), April 2009 (A), November 2009 (N) and February 2010 (F). 40 µg of total protein was loaded in each lane. Molecular weight of the band was found to be 50 kDa. b) SuSy protein bands from three twig samples of *Populus balsamifera* taken in May 2010 (M), February 2010 (F), November 2009 (N) and April 2009 (A). 30µg of total

protein was loaded in each lane. Molecular weight of the band was observed to be approximately 90 kDa. c) SPS protein bands from three twig samples of (a) *Populus balsamifera* taken in November 2009 (N), February 2010 (F), April 2009 (A) and May 2010 (M). 35µg of total protein was loaded in each lane. Molecular weight of the band was found to be 120 kDa.

3.2.2 Relative level of carbohydrate, transcript and enzymes involved in carbohydrate metabolism in *Populus balsamifera* twigs

The correlations of carbohydrate content with the relative protein levels for these enzymes are presented in Figures 10-12 for *P. balsamifera*. A relatively high level of starch coincided with a high amount of AGPase transcript and protein level in the twigs in the growing period (April and May) as compared to the dormant period (November and February) (Fig. 10). No significant variation with time in the level of AGPase protein was observed in *P. balsamifera* twigs, although a slight decrease in the level was observed in the dormant twigs (Fig. 10c).

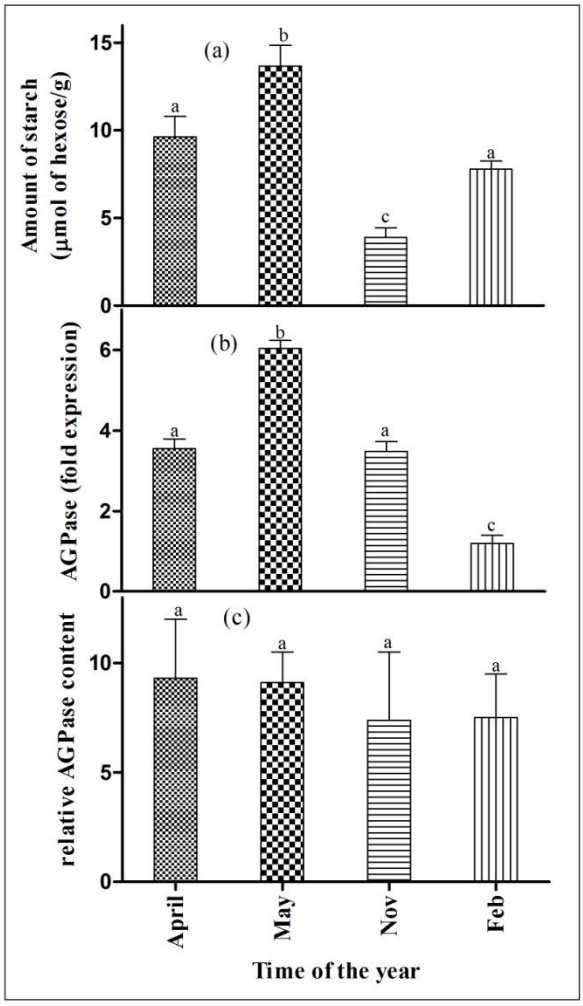

Figure 10. Amount of starch content (a) followed by the relative level of AGPase transcript (b) and protein content (c) in the twigs of *Populus balsamifera* over seasons. Values are means± one standard deviation and n = 3 (biological replication unit).

The variation in starch content was not paralleled by the SuSy expression and protein content during both the growing and dormant time periods in balsam poplar twigs (Fig. 11). However, relative transcript levels for SuSy were correlated with the enzyme's protein content. The growing twigs of balsam poplar (April-July) had significantly lower

amounts of SuSy protein in their xylem than did the xylem of dormant twigs (November-February) (Fig. 11c).

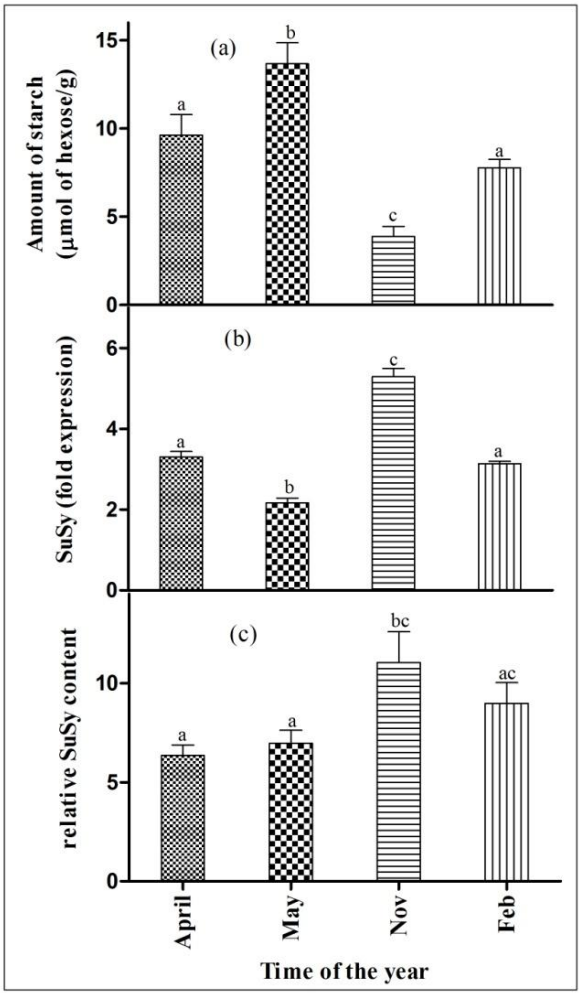

Figure 11. Amount of starch content (a) followed by the relative level of SuSy transcript (b) and protein content (c) in the twigs of *Populus balsamifera* over seasons. Values are means± one standard deviation and n = 3 (biological replication unit).

A high level of soluble sugar content during the dormant period (November) correlated well with the level of SPS transcripts and protein content (Fig. 12). The balsam poplar twigs had a significant, three-fold higher level of SPS protein in the early to

middle portion of the dormant period (November-December) than the level in the growing period (April-July) (Fig. 12c).

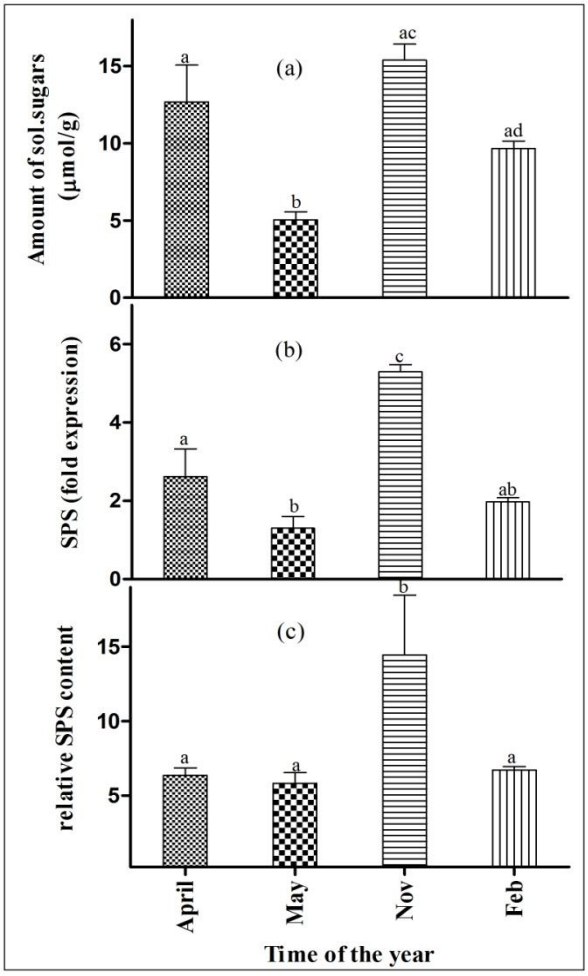

Figure 12. Soluble sugar content (a) followed by the relative level of SPS transcript (b) and protein content (c) in the twigs of *Populus balsamifera* over seasons. Values are means± one standard deviation and n = 3 (biological replication unit).

3.2.3 Relative level of carbohydrate, transcript and enzymes involved in carbohydrate metabolism in *Populus deltoides* twigs

In the twigs of *Populus deltoides*, a high amount of starch coincided with the high level of AGPase transcripts and protein in the growing period (Fig.13). However, no significant difference in the level of AGPase protein was observed.

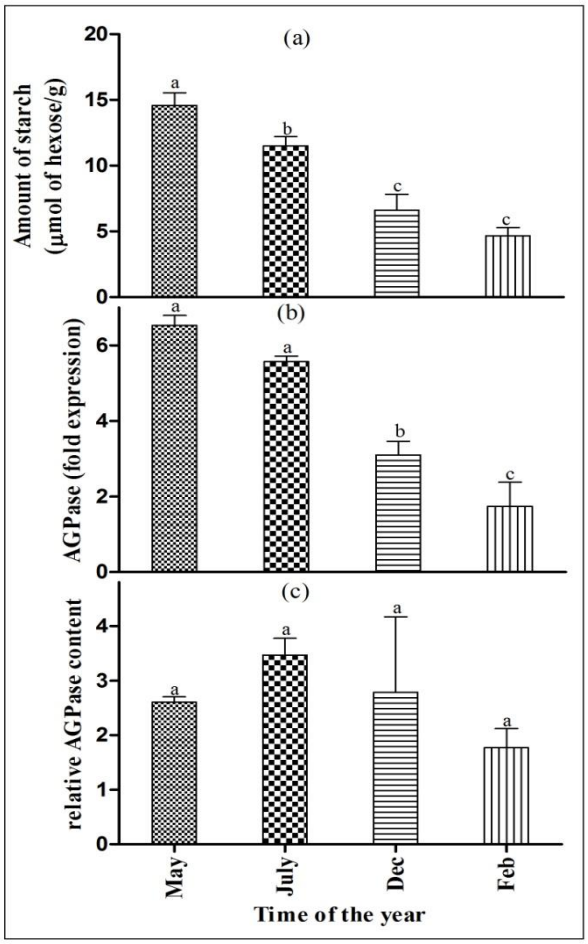

Figure 13. Amount of starch content (a) followed by the relative level of AGPase transcript (b) and protein content (c) in the twigs of *Populus deltoides* over seasons. Values are means± one standard deviation and n = 3 (biological replication unit).

The growing twigs of *P. deltoides* (April-July) had lower SuSy protein in their xylem than did the xylem of dormant twigs of (November-February) (Fig. 14). Because of a large variation in the data the differences in means were not significant.

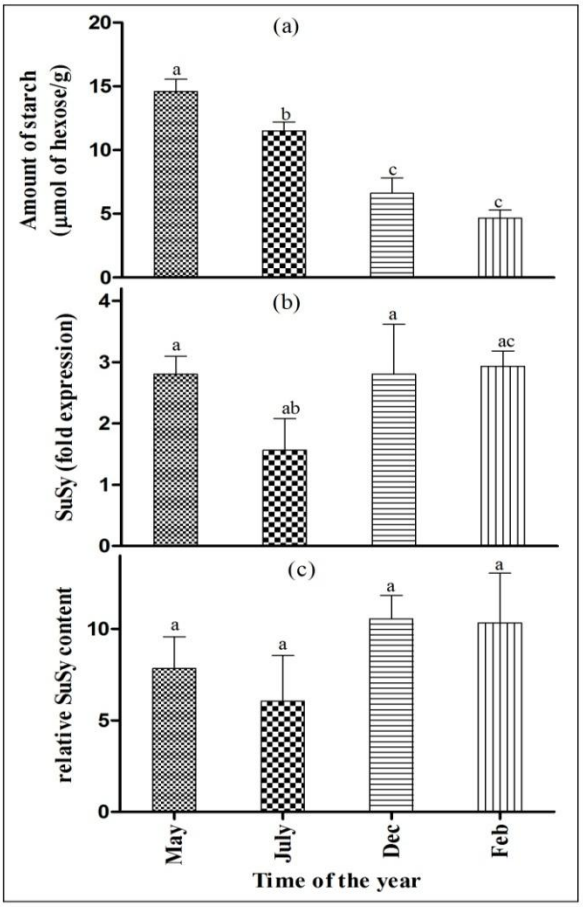

Figure 14. Amount of starch content (a) followed by the relative level of SuSy transcript (b) and protein content (c) in the twigs of *Populus deltoides* over seasons. Values are means± one standard deviation and n = 3 (biological replication unit).

Significantly higher soluble sugar levels in the dormant period coincided with a high level of SPS transcript and protein in *P. deltoides* twigs as compared to the levels in

the growth phase (Fig. 15). Though the protein level was higher in the dormant period, no significant variation was observed within the time points during both of the phases (growth and dormant) (Fig. 15c).

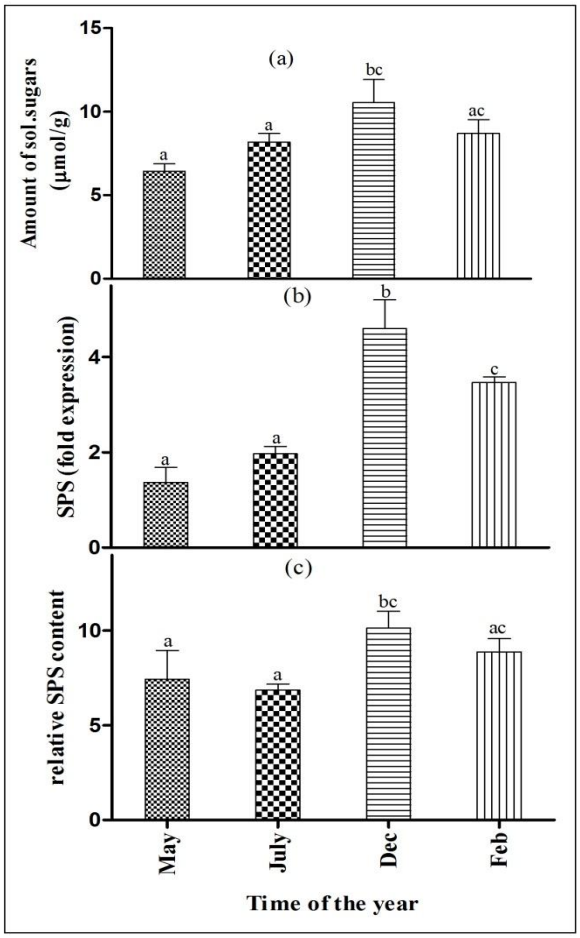

Figure 15. Soluble sugar content (a) followed by the relative level of SPS transcript (b) and protein content (c) in the twigs of *Populus deltoides* over seasons. Values are means ± standard deviation and n = 3 (biological replication unit).

3.2.4 Western blots of AGPase, SuSy and SPS proteins in the 3-4 year old stem of *Populus deltoides*

The protein bands of AGPase, SuSy, and SPS (Fig. 16) from extracts of three growth rings in the *P. deltoides* multiyear stem were quantified.

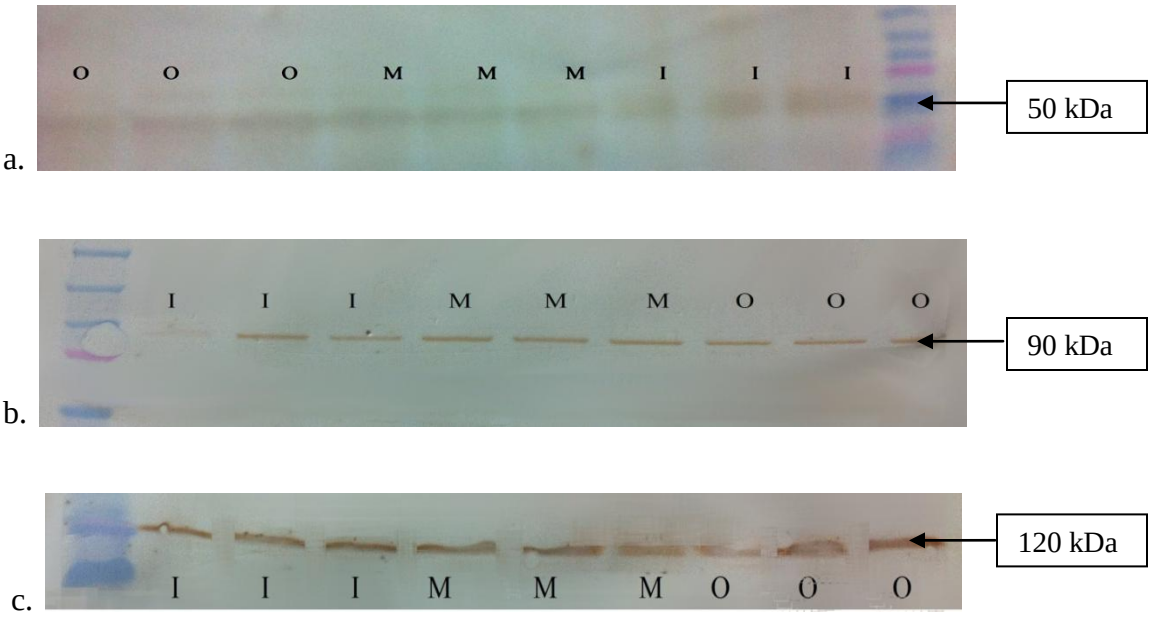

Figure 16**.** Western blots showing marker and three replicates each of AGPase sampled in July 2009 (a), SuSy sampled in December 2010 (b) and SPS sampled in December 2010 (c), from *Populus deltoides* growth rings- outer most (O), middle (M) and inner rings (I). 30µg, 27µg and 35µg of total protein was loaded in a, b and c respectively. Molecular weights were found to be 50 kDa, 90 kDa and 120 kDa for AGPase, SuSy and SPS.

3.2.5 Relative level of carbohydrate, transcript and enzymes involved in carbohydrate metabolism in *Populus deltoides* growth rings

During both the growth and the dormant periods, the increasing trend of starch levels from outer ring towards the inner ring did not coincide with trends in AGPase

transcript and protein levels (Fig. 17). In the summer, the inner and middle rings contained high levels of AGPase, but the mean protein level for the outer ring was considerably lower (Fig. 17c). However, the variability among the plants sampled was great, and the differences were not significant.

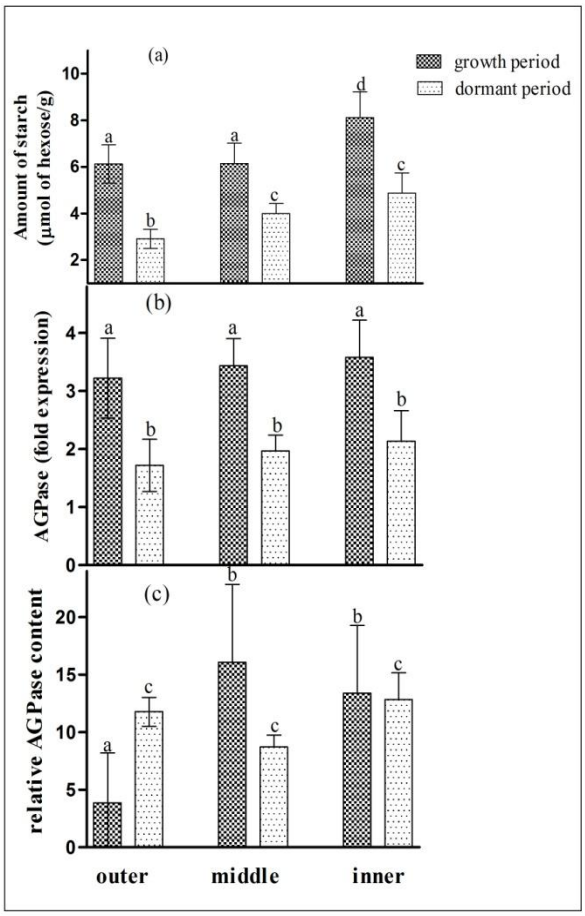

Figure 17. Amount of starch content (a) followed by relative level of AGPase transcript (b) and AGPase protein content (c) in the growth rings of *P.deltoides* stem during the growth period (May, June and July) and dormant period (November, December and February). Values are means± standard deviation. n=3 (biological replication units).

High starch levels in the inner most ring during the growth period coincided with a high level of SuSy transcript but not with SuSy protein (Fig. 18). No significant variation was observed in SuSy protein levels from the outer, middle and inner rings during both phases In fact, SuSy levels were high in the winter and in the summer throughout the 3-year-old stems (Fig. 18c).

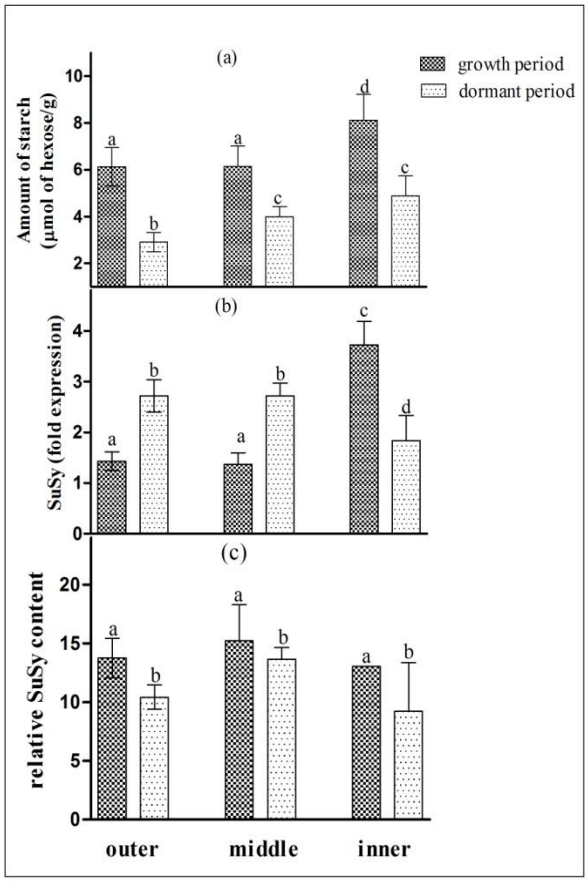

Figure 18. Amount of starch content (a) followed by relative level of SuSy transcript (b) and SuSy protein content (c) in the growth rings of *P.deltoides* stem during the growth period (May, June and July) and dormant period (November, December and February). Values are means± standard deviation. n=3 (biological replication units).

The soluble sugars were high in the outermost ring and decreased towards the inner ring, no correlations were observed between soluble sugar content and SPS protein content during both growing and dormant periods (Fig. 19). Also, no significant spatial difference in the amount of SPS protein was observed across the growth rings in *P. deltoides* xylem during either of the seasons (Fig. 19c).

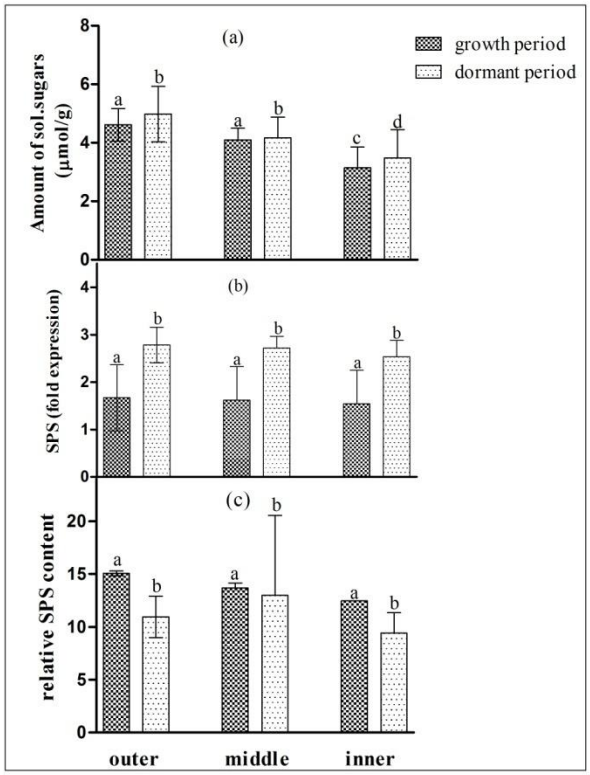

Figure 19. Amount of soluble sugar content (a) followed by relative level of SPS transcript (b) and SPS protein content in the growth rings of *P.deltoides* stem during the growth period (May, June and July) and dormant period (November, December and February). Values are means± standard deviation. n=3 (biological replication units).

3.3 Bioinformatics

Using Batch RNA22 software developed by IBM, I aligned all of the miRNAs discovered in *Populus trichocarpa* to date (taken from the miRBase database) with the gene coding sequences of the AGPase, SPS, SuSy and β-amylase enzymes. The sequence of the miRNA, ptc-miR478a, aligned well with the SuSy gene transcript. The gene sequences for AGPase and β-amylase did not align well with any known miRNA sequences.

CHAPTER IV

DISCUSSION

4.1. Carbohydrates, gene expression and protein studies in the twigs of *Populus balsamifera* and *Populus deltoides*

4.1.1 Morphological and phenological differences in *Populus deltoides* and *Populus balsamifera* plants

The three- to four-year-old *P. balsamifera* plants growing outside of the greenhouse at Texas Tech University, Lubbock, TX were approximately 1 to 1.5 m tall with slender twigs. Their size was considerably smaller than what would be expected if they had grown in their native range in Canada. I expected that their physiology and phenology, such as the timing of bud growth, bud dormancy, and leaf abscission, would follow the seasonal changes in weather rather closely. However, bud burst and leafing out occurred in March while twig growth cessation occurred as early as late May or early June. The leaves remained green all summer and finally senesced in October with colder temperatures. Interestingly, in balsam poplar twigs grown in Lubbock, TX, there was a good correlation between the annual trends in carbohydrate content of the xylem and the gene transcripts for enzymes of carbohydrate metabolism with these physiological changes but not as much with the environmental changes. In the case of *P. deltoides* grown along with the *P. balsamifera* plants, they grew to a height of about 2 to 2.5 m with heavy twigs and larger stem diameters than the balsam poplars had. These plants had a fairly different timing of the phenological processes that were more closely linked

to periods without any frosts than for balsam poplar. Bud burst occurred in late April and growth cessation (bud dormancy) started in early October. My research was aimed at studying the physiological response in terms of the annual trends in key carbohydrate levels and gene expression for the enzymes associated with the carbohydrate metabolism in the xylem parenchyma cells of these two species, which have substantially different latitudinal ranges, while growing them in a location close to the range of the southern species.

4.1.2 Balsam poplar carbohydrate metabolism had poor synchronization of growth with the environmental changes in Lubbock

In balsam poplar twigs, the variation in the level of starches and soluble sugars (glucose plus fructose and sucrose) followed a definite pattern across their developmental stages. During the growth flush and early in the dormancy period starting in early June, the starch levels increased. During this period, average temperature was increasing, and the parenchymatous ray cells were carbohydrate sinks for starch deposition (Witt and Sauter, 1994). What is interesting is that xylem starch levels decreased and soluble sugars increased from June to September during the period of bud dormancy long before the temperatures started to decrease. I propose that, at least for *P. balsamifera*, factors other than a lowering of temperature stimulate starch mobilization in twigs whose buds have become dormant. One possible factor could be photoperiod, since in June in Lubbock, the photoperiod was 14 h whereas it would be 16 h in western Alberta, Canada from where the seed came. Perhaps the longest photoperiod in Lubbock, TX was still short for *P. balsamifera* from Alberta, causing bud dormancy and leading to starch mobilization in the warmest part of the growing season (June to September). The starch level remained

low at least through November with soluble sugar levels rising, supporting the fact that the increase of sugars in winter is a significant cold adaptation process (Guy et al., 1992; Holaday et al., 1992) However, in the winter before average temperatures remained above freezing and bud break occurred, starch levels started to rise in the dormant twigs with a concomitant decrease in soluble sugar levels. These carbohydrate changes were rather consistent with the general trends observed for poplar by Sauter et al. (1994) at those times of the year. Also, the changes in starch were accompanied by reciprocal changes in soluble sugars, suggesting a link between the two types of carbohydrates.

Looking at the expression levels for AGPase, correlations were observed with changing physiological states of poplar xylem in twigs. Though AGPase transcripts levels did not necessarily follow expected patterns with respect to the seasons, they were high when starch levels were rising (April 2009, March and May 2010). Also, generally AGPase protein levels were slightly higher in the growth period when starch levels were high than in winter (Figure 10a). However, low starch levels of the old twig xylem in September and November were accompanied by moderately high AGPase transcript levels. One explanation for these high transcript levels could be that, long before low temperatures were experienced, these twigs had entered into the next phase of physiology, in which starch synthesis would be increasing once AGPase protein levels rose. At first glance, the very low AGPase transcript abundance in February 2009 and 2010 does not support this idea, but by February, the coldest part of the winter season had been experienced by the dormant twigs. The low temperatures may have suppressed the transcript levels. However, note that protein levels were as high as in November (Fig.

10). With warmer temperatures and bud break in March, the older twig section increased its AGPase transcripts in the xylem for future starch synthesis.

The SuSy transcript and enzyme levels (Figures 4 and 11), which could result in increased monosaccharides for energy requirements, cellulose synthesis, and/or starch synthesis, were high during the colder periods (February and November) when twigs were dormant. When twigs were dormant during the warmer periods (June and September), transcript levels for SuSy were about half of what they were during low temperature conditions. These data suggest that temperature, more than physiological state of the xylem, controls transcript and protein levels for SuSy. The higher enzyme levels in winter may have assisted in maintaining high hexose levels at that time.

Throughout 2009, the SPS transcript levels of xylem appeared to be uncoupled to the physiological state of the cells and the temperature patterns, remaining high whether the twigs were growing or dormant (Figure 5). However, the dormant twigs in November of 2009 did exhibit a spike in soluble sugars accompanied by a spike in SPS transcripts and protein (Figure 12). Then as starch levels rose and soluble sugar levels fell in dormant twigs from after November, SPS transcripts also fell. Therefore, a loose correlation of SPS transcripts and protein levels was observed and may be stronger in certain years for unknown reasons. Schrader and Sauter (2002) observed a large amount of SPS protein and high enzyme activity during autumn and winter of poplars growing in their native range in Europe. The transcript levels for β-amylase rose during the dormant period, reaching the highest level in the old twig as the buds began growing in March. In 2010, the trend in transcript abundance lagged behind the trend in soluble sugars and was

peaking as starch content was increasing. The trends in β-amylase transcripts appeared to be linked more to the physiological state of the twigs than to temperature, per se.

4.1.3 The carbohydrate status in *Populus deltoides* twigs varied according to the environmental changes

Unlike the twigs of balsam poplar, the trends observed in *P. deltoides* were more consistent with the environmental conditions in Lubbock, TX. In *P. deltoides* twigs, the starch levels were low during its dormant time of the year (December and February) when soluble sugar levels were higher than in the summer growth phase. Starch storage clearly occurred in the xylem of the growing twigs in the late spring and summer months. The balsam poplar twigs were observed to have already started their dormant phase by this time (late June). The phenology of *P. deltoides* was timed according to the environmental conditions of northern Indiana (14 h day length in June) from where the stem cuttings and seeds were taken. The Lubbock, TX conditions were close enough to those of the home range of *P. deltoides* to allow better co-ordination with photoperiod and temperature.

4.1.4 Correlation between the carbohydrate status and gene expression in *Populus deltoides* twigs

The trends in AGPase transcript levels followed that of the starch in both the growing and dormant times, but the actual AGPase protein level was rather constant from the dormant stage through the early portion of the growth phase. Perhaps there is a need for high transcript levels in the growing period to maintain AGPase protein levels. However, in the case of SuSy and SPS, changes in transcript levels were associated with

similar relative changes in protein content for these enzymes. Moderate SuSy transcript levels during the times of high SPS mRNA and soluble sugars were apparent, and it could be due to increased hexoses for other cellular processes during cold acclimation.

Therefore, in the case of the twigs of these two poplar species, my primary hypothesis which stated that there might be a possible correlation between the expression level of the enzymes of starch and sucrose metabolism with starch and sugar levels, was supported by the results for a major part.

4.2 Carbohydrates, gene expression and protein studies in *Populus deltoides* stem

The 3-4 year old stems of *P.deltoides* served as a good model to study the spatial variation in the carbohydrate metabolism. The annual rings were very conspicuous in the stem. The cambial tissue is active during the growing period. Therefore, secondary growth in poplar stems initiated during the growing season and cambial dormancy occurred as the environmental conditions altered. From the above discussed trends and the morphological observations in *Populus deltoides* twigs over seasons, it was assumed that the cambial activity in the stem also persisted from March to October, and that it was dormant the rest of the year. However, I did not perform any direct measurements of cambial activity.

Previous studies showed that the new, growing annual ring (outermost ring) engaged in a high rate of sucrose metabolism for new cell formation from cambial activity and their differentiation, whereas the inner rings contained mature parenchyma

cells where the metabolic products (protein, oils, sugars and carbohydrates) were stored in preparation for survival in unfavorable conditions (Uggla et al., 2000; Samuels et al., 2005). In my study, for the most part, it was observed that the inner ring had higher levels of starch than the outer most rings (Fig. 7), while the sugars were higher in the outermost rings than in the inner rings. This trend is also in accordance with the one observed by Shrader and Sauter (2002).

4.2.1 Correlation between the starch content and the gene expression of starch metabolizing enzymes in *Populus deltoides* stem

During both the growing (summer) and dormant (winter) phases, high starch levels, AGPase transcripts, and AGPase protein (Figs. 7 and 13) in the inner ring of the multiyear P. deltoides stems, confirm the active participation of this enzyme in starch synthesis and accumulation in the fully developed cells. In the process of starch synthesis in heterotrophic cells like the xylem parenchyma, cleavage of sucrose to fructose and glucose is indeed required (Pozueta-Romero et al., 1999). High SuSy expression in the same position (inner ring) where high starches accumulated suggests that SuSy activity might have contributed more to the formation of sugars directed towards starch synthesis (Fig. 8). High SuSy expression in the growth phase in the outer ring was expected as the demand for sugars would be high. Although these transcript levels may be sufficient for cell growth and maturation, they were lower than the level in the inner ring. My transcript findings were opposite to the SuSy activity data of Shrader and Sauter (2002) from multiyear stems of *Populus X canadensis* Moench (*robusta*) trees during the growing season. However, the higher transcript levels in the outer rings relative to that for the inner ring during the dormant, winter period coincides well with the SuSy activity data

from the study by Shrader and Sauter (2002). Thus, the high SuSy protein levels throughout the entire stem during the growing period in my study (Fig. 18) coincide better with the data from Shrader and Sauter (2002) than do the transcript levels.

4.2.2 Correlation between the soluble sugars and the gene expression of sucrose metabolizing enzymes in *Populus deltoides* stem

The relationships between the spatial and seasonal trends in soluble sugars, SPS and β-amylase transcripts, and SPS protein levels were not always consistent (Fig. 8 and 19). High sugars and SPS protein level in the outer-most ring during the growth period supported high cell differentiation activity of the newly synthesized cells (Shrader and Sauter, 2002). However, these parameters were also substantial in the outer-most ring in the dormant period, as well. Throughout both growing and dormant periods, there was a trend toward lower soluble sugars in the inner portion of the stem, but SPS transcript and protein levels were basically the same across the stem. Also, β-amylase transcripts were highest in the region where soluble sugars were the lowest during the growing period. Nonetheless, the β-amylase expression levels were higher in the dormant period than in the growing period, consistent with the need for more soluble sugars at that period in the xylem parenchyma cells.

In total, all the above trends observed with the carbohydrates and gene expression would propose that the xylem of *Populus deltoides* showed good synchronization of its metabolic patterns with environment in Lubbock. Such correlations support the second half of my hypothesis which stated that the enzymes involved in starch synthesis would be high prior to the rise of starches in the inner ring and that the transcripts of the

enzymes involved in starch degradation and sugar synthesis will be high prior to the rise in sugar levels in the outer regions of xylem in multiyear old stems. Though the correlation between the carbohydrates and the transcript and protein levels was clear over wide range of temperature and time interval as in summer and winter, it was not overwhelming the rest of the year in both the twigs and the stems (Appendix (Figure 15)).

4.3 Regulation of the gene transcripts for the enzymes (AGPase, SPS, SuSy, Beta-amylase) by miRNAs

As discovered recently, small, interfering RNAs may be involved in the regulation of xylem development by environmental factors, such as temperature (Ko et al., 2006). Ko et al. (2006) discovered that a specific miRNA (*miR166*) was synthesized during low temperatures in poplar and was associated with the down-regulation of transcripts of a gene associated with secondary growth, but evidence for the presence of miRNAs targeting any of the genes in my study has not been presented in the literature. From my communication with one researcher, Dr. Magdy S. Alabady from University of Illinois, Urbana-Champaign (Department of Crop Science), I was told that he discovered certain miRNAs that targeted the gene transcripts of the enzymes that I studied. My work using bioinformatics tools (Batch RNA22 developed by IIM) to check for sequence alignments of known miRNAs in *Populus trichocarpa* (taken from the miRBase) with the coding sequences of these enzymes has showed that ptc-miRNA478a had a noticeable alignment with the SuSy gene. Therefore my second hypothesis which stated that transcripts for at least one enzyme would be regulated by small interfering RNAs can be accepted.

4.4 Conclusion

Since balsam poplar (*P. balsamifera*) is native to regions of high latitude, its genetic and physiological signaling was found to be unsynchronized with the environmental conditions of Lubbock. However, when examining parameters associated with the metabolism of xylem carbohydrate on the basis of developmental stages (growth and dormancy), there was a good relationship between carbohydrate metabolism and transcript and protein levels for most enzymes studied. However, a better a correlation of these features and changes in temperatures with the seasons was noted for P. deltoides, whose natural range extends into the western Texas region. The correlation of carbohydrates and gene transcripts was fair in the growth rings of the multiyear stem of *P. deltoides* but was not consistent with the protein content of the enzymes.

The results from the analysis of *P. balsamifera* twigs suggest that photoperiod may control some aspects of starch metabolism, whereas sucrose metabolism via SuSy may be controlled by temperature. My finding that ptc-miR478a may be involved in SuSy transcript levels is consistent with the low temperature control of the development of xylem and xylem dormancy by *miR166*. Further research on the involvement of miRNAs and their targets in poplar xylem metabolism would help understand the regulation of the patterns of carbohydrate metabolism so that manipulations of the metabolic processes for commercial purposes could be achieved.

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