AN INVESTIGATION OF THE PHYSIOLOGICAL ROLES AND ENZYMATIC

PROPERTIES OF INVERTASES IN TOBACCO AND HYBRID POPLAR

by

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Abstract

Plant invertases (EC 3.2.1.26) represent a multi-gene family of β-fructofuranosidases that perform integral roles in several biochemical processes. The central importance of this family of enzymes to plant growth and development has made them a primary target of investigation in plant biology. Research has principally focused on sink-source interactions, and the potential to increase sink capacity in several economically important crop species, including potato and tomato. However, studies exploring the impacts of invertase mis-regulation on cellulose and lignin, the two most abundant biopolymers on earth, had not been conducted. Consequently, we investigated the effects of overexpressing yeast-derived invertases in tobacco and hybrid poplar. Transgenic tobacco expressing the yeast-derived invertases showed reduced height and interference in sink-source metabolism. In addition, some transgenic lines showed significant changes in cellulose and lignin content, providing evidence that sink capacity can be altered via the overexpression of this class of enzyme. In contrast, hybrid poplar expressing foreign invertase genes showed no visible phenotype, with only minor changes to the structural polymers cellulose and lignin, suggesting the mechanism of carbohydrate transport differs between tobacco and hybrid poplar. However, there was evidence for post-translational modification of the foreign invertases in hybrid poplar, which may also explain the difference in phenotypes observed. We suggest that the yeast-derived invertases may not be the most effective target to alter sink biopolymers, and that mis-regulating endogenous invertases may be a more suitable alternative. Consequently, we identified three cell-wall invertase genes in hybrid poplar and investigated their spatial and temporal expression profiles during the complete first year of growth. In addition, we heterologously expressed and characterized two hybrid poplar cell-wall invertase genes involved in vegetative growth. Collectively, the expression and functional characterization data suggest that one floral-specific and two vegetative cell-wall invertases exist in hybrid poplar. Of the two vegetative cell-wall invertases, one (*PaxgINV1*) appears to be involved in processes relating to dormancy, while the other (*PaxgINV2*) appears to be involved in phloem unloading and the seasonal reallocation of carbohydrate. We therefore hypothesize that *PaxgINV2* may be a suitable target for future mis-regulation studies aimed at altering sink capacity.

Table of Contents

List of Tables

List of Figures

Acknowledgements

Co-authorship Statement

Chapter 2: Thomas Canam was involved with the design of the research, performing research, data analysis and manuscript preparation. Shawn D. Mansfield, David D. Ellis and Malcolm M. Campbell were involved with identification and design of the research program and manuscript preparation. Ji-Young Park and Ka Yun Yu were involved with performing the research and data analysis.

Chapter 3: Thomas Canam was involved with the design of the research, performing research, data analysis and manuscript preparation. Shawn Mansfield was involved with identification and design of the research program and manuscript preparation.

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CHAPTER ONE

Introduction

1.1 Plant invertases

1.1.1 Enzyme catalysis

Invertases, or β-fructofuranosidases (EC 3.2.1.26), are hydrolases that cleave the O-glycolytic bond of compounds possessing a terminal β-D-fructofuranoside residue. Sucrose, the smallest of the biologically active β-D-fructofuranoside compounds in plants, is the preferred substrate of enzymes classified as invertases (De Coninck et al. 2005). In this reaction, the glycolytic bridge of sucrose is cleaved releasing glucose and fructose monomers. Some plant invertases can also act on larger β-D-fructofuranosides, such as raffinose and stachyose, although weaker affinity for these compounds is typically observed (e.g. Hashizume et al. 2003, Nagaraj et al. 2005, Hsieh et al. 2006). In these reactions, there is evidence that the terminal β-D-fructofuranoside residue is released as fructose, leaving the remaining bonds between glucose and galactose residues unchanged (e.g. Cooper and Greenshields 1964, Konno et al. 1993, Nagaraj et al. 2005).

1.1.2 Subcellular locations

Plants possess a variety of invertases, defined by their subcellular locations: the cytosol, vacuole and cell wall. These distinctions are formed based on the amino acid sequences and enzymatic characteristics of the invertases (Tymowska-Lalanne and Kreis 1998). Cytosolic invertases are typically distinguished by their neutral to alkaline pH optima alone, which permits the enzymes to be active in the cytosolic space. In contrast, vacuolar and cell-wall invertases have acidic pH optima, matching the acidic nature of both the vacuole and cell-wall matrix, and therefore require further discrimination. The isoelectric point (pI) of these invertases serves as an additional classification parameter (Roitsch and Gonzalez 2004). Vacuolar invertases typically have acidic pI values, while cell-wall invertases have more alkaline pI values. Additionally, both types of invertases can be further distinguished at the amino acid level. For example, both vacuolar and cell-wall plant invertases have been shown to possess the WEC<u>P/V</u>D amino acid motif, with vacuolar invertases having a valine residue at the forth position in contrast to the proline residue found in cell-wall invertases (Goetz and Roitsch 1999, Roitsch and Gonzalez 2004).

1.1.3 Physiological functions

The presence of invertase isoforms in distinct subcellular locations suggests that these enzymes have a number of different roles with respect to plant physiology. The exact functions of each type of invertase (cytosolic, vacuolar or cell-wall) are not clearly defined, however, a number of potential roles have been suggested (Tymowska-Lalanne and Kreis 1998a). Broadly, it has been speculated that the cytosolic invertases serve to modulate sucrose and hexose levels in the cytosolic space and thereby act as a control point for general metabolism (e.g. glycolysis) and cell maintenance (e.g. respiration). Vacuolar invertases presumably play a role in cleaving sucrose stored in the vacuole, thereby releasing glucose and fructose into the vacuolar compartment for further storage, vacuolar metabolism or subsequent secretion into the cytosol. Cell-wall invertases, in contrast, have been speculated to be primarily involved in the regulation of the wound and stress response (e.g. Sturm and Chrispeels 1990) and phloem unloading in sink tissues (e.g. Eschrich 1980, Roitsch et al. 2003).

1.2 Phloem loading and sugar transport

There are two forms of phloem loading accepted in the current literature: apoplastic and symplastic loading. Both processes involve common cells in the same tissues performing a similar function, which is delivering sucrose and other metabolites from source tissues to sink tissues via the phloem. However, the types of phloem loading differ in two separate but not exclusive ways: (1) the path by which the sucrose and other metabolites are transferred from the mesophyll cells to the companion cell/sieve element complex in source tissues, and (2) the process by which sucrose and other metabolites accumulate against a concentration gradient.

1.2.1 Apoplastic loading

Photoassimilate in the form of sucrose is generated in the mesophyll cells of source tissues, which can be uploaded into the apoplastic space. In apoplastic loading species, companion cells (CCs) and sieve elements (SEs) are able to actively import the available sucrose directly from the apoplast (Oparka and Turgeon 1999). This active process of loading sucrose into the SEs of source tissues creates a concentration gradient between the source tissues and sink tissues, which indirectly drives the movement of sucrose to sink tissues where efflux occurs (Turgeon 2006). The sucrose

can then enter sink cells directly from the apoplast via sucrose transporters or indirectly through hexose transporters after hydrolysis by cell-wall invertase, although the specific processes surrounding post-phloem transport are not well understood (Fisher and Oparka 1996, Turgeon 2006).

1.2.2 Symplastic loading

In symplastic loading species, sucrose is transported from mesophyll cells, through bundle sheath cells and finally to companion cells via plasmodesmata (Turgeon 1996). As such, sucrose is transported from cell to cell directly through the symplast rather than the apoplast. In symplastic loading species, sucrose is delivered from bundle sheath cells to specialized companion cells known as intermediary cells (IC). Within the IC, sucrose is converted into raffinose family oligosaccharides (RFOs) through a series of enzymatic reactions. The resultant RFOs (e.g. raffinose and stachyose) are too large to effectively flow back to the bundle sheath cells and are trapped within the ICs. This process is known as polymer trapping (Turgeon 1996). According to this theory, the RFOs are then transported from the ICs to SEs and delivered from source to sink tissues via a concentration gradient similar to that described for apoplastic loading species. As with apoplastic loading species, the exact method of phloem unloading is unknown (Fisher and Oparka 1996, Turgeon 2006), although cell-wall invertases, which are typically able to hydrolyze raffinose and stachyose (e.g. Nagaraj et al. 2005), may also play a role in phloem unloading in symplastic loading species.

1.3 Cell-wall invertase gene expression

Cell-wall invertases were first proposed to be associated with the mechanisms surrounding phloem unloading by Eschrich (1980). In this model, invertases located in the cell-wall space cleave sucrose and other β-D-fructofuranosides at sink tissues that were transported from source tissues via the phloem. This mechanism is important to the growth and survival of higher plants and, as such, cell-wall invertases have been the focus of considerable study. The advent of gene sequencing and related technology considerably accelerated the understanding of plant invertases and their physiological roles by linking specific genes to specific invertase isoforms. After the first full-length cell-wall invertase gene was reported from carrot (Sturm and Chrispeels 1990), a number of studies examining the roles of cell-wall invertases from other species,

including monocots and dicots, were initiated in an effort to understand the roles these enzymes may play with respect to the overall physiology of plants. Generally, the results from these studies indicate that plants possess multiple cell-wall invertases that are differentially regulated and responsive to varying physiological conditions. For the sake of brevity, I will herein discuss the identification, expression analysis and the putative roles of cell-wall invertases from carrot and other dicots species including *Solanum tuberosum* (potato), *Lycopersicon esculentum* (tomato), *Nicotiana tabacum* (tobacco), *Chenopodium rubrum*, *Pisum sativum* (pea), *Vicia faba* (faba bean), *Beta vulgaris* (sugar beet), *Arabidopsis thaliana* and *Populus* spp. (poplar).

1.3.1 Carrot (*Daucus carota*)

Sturm and Chrispeels (1990) described the complete full-length cDNA sequence of the first cell-wall invertase gene (*Inv*Dc1*) from carrot. The authors were able to identify a specific gene product, deduce an amino acid sequence, predict an alkaline pI (9.9) and examine the expression patterns of the gene in a maturing carrot root. The results of that study showed that *Inv*Dc1* expression was undetectable in maturing carrot roots. However, despite the lack of expression in the maturing root, *Inv*Dc1* expression did increase in response to wounding and infection. Consequently, it was speculated that the "apoplastic β-fructosidase is probably a new and hitherto unrecognized pathogenesis-related protein" (Sturm and Chrispeels 1990). The authors further speculated that the increased invertase activity was necessary to mobilize carbohydrate reserves required for initiating the plant-wide defense response. Two additional studies of this gene showed that *Inv*Dc1* was expressed during early growth in the roots, petioles and leaf lamina (~2-8 weeks after germination) when the primary root was developing, thereby extending the role of this enzyme to include early growth and development (Ramloch-Lorenz et al. 1993, Sturm et al. 1995). Around the same time, two putative cell-wall invertase genes (*Inv*Dc2* and *Inv*Dc3*) were subsequently identified and reported from carrot (Lorenz et al. 1995). That study indicated that *Inv*Dc2* expression was limited to flower buds, while *Inv*Dc3* expression was observed exclusively in suspension-cultured cells. In sum, these studies showed that multiple cell-wall invertase genes existed in carrot with distinct expressional regulation.

1.3.2 Potato (*Solanum tuberosum*)

The first fully sequenced cell-wall invertase gene (*pCD111*) from potato was reported by Hedley et al. (1993). Like the *Inv*Dc1* (Sturm and Chrispeels 1990), the derived amino acid sequence contained the concensus sequences WECPD and NDPN. Expression analysis revealed the *pCD111* gene was being transcribed in both young and mature leaves, stem and tuber tissue (Hedley et al. 1993). A second cell-wall invertase gene from potato (*Inv*) was identified shortly after (Hedley et al. 1994) and was shown to be primarily expressed in source leaf and stem tissue (Hedley et al. 1994). Five years after the discovery of *pCD111* and *Inv*, two additional cell-wall invertase genes from potato (*invGE* and *invGF*) were reported (Maddison et al. 1999). This study investigated the expression of each gene by RT-PCR as well as by histochemical analysis, which involved linking their respective promoter regions to the reporter gene β-glucuronidase (*GUS*) and creating potato and tobacco transformants. The collective data revealed that *invGE* was expressed in both vegetative (lateral nodes, root and tuber) and reproductive tissue, while the expression of *invGF* was restricted to tissues involving the formation of pollen (Maddison et al. 1999). Due to the association with reproductive and meristematic tissue, the authors concluded that these invertase genes were involved in "diverse developmental processes" and speculated that "modulation of invertase activity might offer a means to regulate these developmental processes" (Maddison et al. 1999). Hedley et al. (2000) used a similar histochemical-based approach to examine the tissue-specificity of the previously reported *pCD111* gene (now referred to as *invCD111*), and the *Inv* gene (now referred to as *invCD141*). The promoters of both genes led to GUS activity in vascular tissue of transgenic potato. Specifically, the results suggested that *invCD111* was associated with the outer phloem of the stem, while *invCD141* was associated with the inner phloem (Hedley et al. 2000). Collectively, the results from these studies revealed that at least four cell-wall invertase genes existed in potato, each with unique expression patterns that suggested a variety of roles, such as photoassimilate transport and both vegetative and reproductive development.

1.3.3 Tomato (*Lycopersicon esculentum*)

Four cell-wall invertase genes from *Lycopersicon esculentum* (*Lin5*, *Lin6*, *Lin7* and *Lin8*) were identified using genomic PCR and RT-PCR techniques (Godt and Roitsch 1997).

Expression of two of these genes was associated with floral tissues, with highest expression observed in the gynoecia for *Lin5* and stamens/anthers for *Lin7*. In contrast, transcripts of *Lin6* were detected in seedling roots, flower buds and tumors, and this gene was also associated with growth elicitors and wounding (Godt and Roitsch 1997, Goetz et al. 2000). Supporting this finding, a *Lin6*-like gene (known as *Wiv-1*) was shown to be wound-inducible in tomato (Ohyama et al. 1998). The forth gene (*Lin8*) from the original study was not detected in any of the tissues investigated, leading the authors to speculate that this gene was a pseudogene lacking effective transcription (Godt and Roitsch 1997). However, a later study by Fridman and Zamir (2003) revealed that *Lin8* was expressed in both leaf and root tissue of tomato. That study also revealed further and more detailed expressional analysis of *Lin5* (petal, stamen, ovary and fruit) and *Lin7* (stamen and pollen). Further analysis of *Lin5* and *Lin7* using their respective promoters to drive *GUS* expression revealed that *Lin5* is expressed during fruit development, while *Lin7* is expressed in pollen grains and the tapetum (Proels et al. 2003). The *Lin5* promoter was also shown to be responsive to a variety of plant hormones such as auxins and gibberellins. It was later shown by Proels et al. (2006) that the *Lin7* promoter was also inducible by gibberellins. Collectively, the results of these experiments provided strong evidence for significant roles of cell-wall invertases in the reproductive cycle of tomato.

1.3.4 Tobacco (*Nicotiana tabacum*)

Unlike many of the cell-wall invertases identified, the first tobacco cell-wall invertase gene was identified (Greiner et al. 1995) after the enzyme it encodes was characterized (Weil and Rausch 1994). In that case, matching the pI value of the purified enzyme (9.5) with the predicted pI value of the gene provided the necessary evidence to link the gene with its putative product (Greiner et al. 1995). Expression data was not presented for this gene, however, a second and distinct tobacco cell-wall invertase gene (*Nin88*) was later investigated by Goetz et al. (2001). Linking the *Nin88* promoter region with *GUS* allowed the authors to investigate the expression pattern of *Nin88* in transgenic tobacco. Similar to some of the previously reported potato and tomato genes, the tobacco *Nin88* gene was determined to be anther-specific. Antisense suppression of *Nin88* clearly showed the importance of this cell-wall invertase to proper pollen development, as *Nin88* antisense tobacco resulted in male sterility (Goetz et al. 2001).

The authors speculated that the suppression of the cell-wall invertase decreased the sink strength of the developing anthers and subsequently limited the available carbohydrate necessary for development.

1.3.5 Red goosefoot (*Chenopodium rubrum*)

A cell-wall invertase gene (*CIN1*) was identified in *Chenopodium rubrum* by Roitsch et al. (1995). Whole-plant expression analysis suggested that this cell-wall invertase was associated with sink tissue as the root tissues contained the highest amount of transcript. Further evidence for the association of cell-wall invertase with sink tissues was provided when photoautotrophic suspension-culture cells of *C. rubrum* were switched to mixotrophic media. Under the heterotrophic conditions the level of *CIN1* transcript increased significantly, causing the authors to speculated that "sucrose hydrolysis further induces invertase expression in the sink cells (feed-forward regulation)" (Roitsch et al. 1995). Further investigation of *CIN1* came in a study focusing on the WECPD amino acid motif of plant cell-wall invertases (Goetz and Roitsch, 1999), where the effects of switching the proline residue of the motif with a valine residue (common to vacuolar invertases) using site-directed mutagenesis was investigated. Expression in *S. cerevisiae* indicated that the proline/valine residue affects the pH optima of cell-wall invertases, with the modified *CIN1* gene (WECVD) having a higher pH optimum in comparison to the native *CIN1* gene (WECPD). Other kinetic parameters such as K_m and V_{max} were unaffected by the mutation. The authors concluded that "the presence of a proline or valine residue in the WEC-P/V-D box of a cloned new invertase may be used as a reliable criterion for classification into the groups of extracellular or vacuolar invertases, respectively" (Goetz and Roitsch 1999).

1.3.6 Pea (*Pisum sativum*)

A cell-wall invertase from pea (*βfruct1*) was sequenced and reported by Zhang et al. (1996a). Expression of this gene, visualized by Northern blotting, was limited to root tissue in normal healthy pea plants. However, when leaf and stems were wounded, cell-wall invertase transcript abundance increased in the affected area. Tissues that were not subjected to wounding in the affected plants did not show significantly elevated cell-wall invertase transcript, providing evidence for a non-systemic and localized response by *βfruct1* to wounding (Zhang et al. 1996b). Further examination of *βfruct1* gene expression in detached leaves revealed sensitivity to abscisic acid and

jasmonic acid as both hormones induced cell-wall invertase expression in petiole feeding experiments and, to a lesser extent, spraying experiments (Zhang et al. 1996b). Transcript localization using *in situ* hybridization were consistent with the expression data, as the *in situ* hybridization experiments indicated that little to no transcript was present in unwounded stem and leaf tissue. However, examination of wounded stem and leaf tissue revealed transcript accumulation in the phloem tissue. The authors speculated that the association between phloem tissue in wounded pea plants and *βfruct1* expression was due either to "the maintenance of a steep sucrose concentration gradient between the source and sink regions of a plant or the provision of hexose, and thus energy, for the metabolism of companion cells" (Zhang et al. 1996b). It was later shown that the expression of this gene in detached leaves was enhanced by the presence of sucrose and glucose, which suggested that *PsInv-1* (*βfruct1*) was regulated by these sugars (Zhang et al. 1997).

1.3.7 Faba bean (*Vicia faba*)

Based on sequence homology with other known plant cell-wall invertases, two cell-wall invertases (*VfCWINV1* and *VfCWINV2*) were identified from faba beans (Weber et al. 1995). RT-PCR expression analysis of developing seeds linked *VfCWINV1* expression to developing anthers and seed coats approximately two weeks after flowering. *VfCWINV2* expression on the other hand was found in a variety of tissues including gynoecia, pods and seed coats, as well as root tissue of *V. faba* plants. Further analysis using *in situ* hybridization showed that *VfCWINV1* was expressed in the chalazal vein and thin-wall parenchyma in the seed coat. As a result of the association of *VfCWINV1* with the sieve-element end-point tissues, the authors speculated that *VfCWINV1* plays a role in establishing sink strength in developing seeds (Weber et al. 1995). *VfCWINV2* expression, by contrast, was localized to chlorenchyma and ground parenchyma, suggesting that the enzyme encoded by *VfCWINV2* plays a role in general metabolism and development of the seed.

1.3.8 Sugar beet (*Beta vulgaris*)

Due to the economic importance of tap roots of *B. vulgaris*, the expression of a number of sucrose-cleaving enzymes was investigated in this species (Godt and Roitsch 2006). Among the enzymes investigated were two putative cell-wall invertases, BIN35 and BIN46, based on the presence of the conserved amino acid motif WECPD. However,

both BIN35 and BIN46 have predicted pI values in the acidic range (5.7 and 4.7, respectively), which is in contrast to the alkaline pI values typical of most other plant cell-wall invertases (Tymowska-Lalanne and Kreis 1998). Additionally, the authors discuss the possibility that both enzymes may not be cell-wall invertases, as they share high sequence homology with known fructan-6-exohydrolases (FEHs) (Godt and Roitsch 2006). However, cell-wall invertase activity was correlated to *BIN35* expression in early stages of development of sugar beet roots, which provided evidence that this enzyme was indeed a cell-wall invertase. The authors suggested that the association between *BIN35* expression and early tap root development was evidence that this cell-wall invertase was primarily responsible for phloem unloading and establishing sink strength (Godt and Roitsch 2006). The *BIN35* gene was also shown to be expressed in mature leaves of *B. vulgaris* after mechanical wounding, which implies a second role for this cell-wall invertase in the wound response. Expression of *BIN46* was not detected in mechanically wounded leaves nor at any stage of development of the tap root (Godt and Roitsch 2006).

1.3.9 *Arabidopsis thaliana*

Atβfruct1 (Schwebel-Dugue et al. 1994) and *Atβfruct2* (Mercier and Gogarten 1995) were the first two cell-wall invertase genes identified in *Arabidopsis*. A comprehensive expression analysis of these two genes (Tymowska-Lalanne and Kreis 1998b) showed that *Atβfruct1* expression occurred in leaves of three week old plants and in leaves, stems, roots and flower buds of six week old plants. After eight weeks the plants had begun to form siliques. At this stage *Atβfruct1* was limited to leaf and stem tissue, while *Atβfruct2* expression was prominent and exclusive to the flowers. Since *Atβfruct2* was not detected in any of the vegetative tissue examined, the authors used a nonspecific cell-wall invertase probe to examine *Atβfruct1* expression using Northern blot analysis (Tymowska-Lalanne and Kreis 1998b). Using this method it was shown that total cell-wall invertase expression (presumably only that of *Atβfruct1*) was more prominent in the roots of plants that were grown under aeroponic conditions that favoured root growth, suggesting that cell-wall invertase plays a role in the development of sink organs such as the root (Tymowska-Lalanne and Kreis 1998b). It was also shown via Northern blot analysis that cell-wall invertase expression increased in the leaves of plants grown in the dark, suggesting that cell-wall invertase was involved with the switch from

photoautotrophic to heterotrophic conditions. Unfortunately, the conclusions based on the Northern blot analyses were put into question when four additional cell-wall invertases from *Arabidopsis* were described (Sherson et al. 2003). In the latter study, gene-specific RT-PCR analysis revealed that the previously examined *AtcwINV1* (*Atβfruct1*) was highly expressed in Arabidopsis seedlings, expanding leaves, flowers and seeds. As with the previous study by Tymowska-Lalanne and Kreis (1998b), *AtcwINV2* (*AtBfruct2*) expression was limited to the flower and seeds (Sherson et al. 2003). In contrast, *AtcwINV4* was weakly expressed in seedlings, highly expressed in flowers and to a lesser extent in seeds. The expression of *AtcwINV5* was weak and limited to flowers only. Expression analysis of *AtcwINV3* and *AtcwINV6* was also performed, however, the enzymes encoded by these genes were later functionally characterized as FEHs by De Coninck et al. (2005). For the sake of clarity, the expression profiles of *AtcwINV3* and *AtcwINV6* (both known FEHs) will not be discussed further. The expression profiles of the remaining cell-wall invertases (*AtcwINV1*, *AtcwINV2*, *AtcwINV4* and *AtcwINV5*) were also examined by Fridman and Zamir (2003). In that study, the expression patterns (assessed by RT-PCR) of *AtcwINV1*, *AtcwINV2* and *AtcwINV4* were essentially as described by Sherson et al. (2003), however, the expression of *AtcwINV5* differed. Instead of weak expression limited to the flowers, *AtcwINV5* expression was detected in root, stem and leaf tissue in addition to flowering tissues such as the silique and pod (Fridman and Zamir 2003).

1.3.10 Poplar (*Populus* spp.)

A study exploring the invertase gene family in *P. trichocarpa* identified 16 cytosolic invertases and eight acid invertases (Bocock et al. 2008). Of the eight acid invertases, five were reported as cell-wall invertases. Three cell wall invertases (*PtCIN1*, *PtCIN2* and *PtCIN3*) shared high sequence homology with other cell-wall invertases from *Arabidopsis*. *PtCIN4* and *PtCIN5*, although possessing the WECPD amino acid motif, shared higher amino acid sequence identities to known FEHs from *Arabidopsis* (*AtcwINV3* and *AtcwINV6*). This led the authors to speculate that "one or more of the five *PtCIN* genes described here may encode an FEH" (Bocock et al. 2008). Nevertheless, expression analysis was conducted on the five putative cell-wall invertase genes. Microarray data revealed that *PtCIN3* (a homolog to *PaxgINV1* described in Chapter four) was strongly expressed in mature and young leaves, while roots,

internodes and nodes had weaker expression. *PtCIN4* was weakly expressed in all the tissues examined by microarray (leaves, roots, internodes and nodes), and *PtCIN5* showed weak expression in leaves, roots and internodes only. The remaining two cell-wall invertases *PtCIN1* and *PtCIN2* were not detected in any of the vegetative tissues examined using microarray technology. Instead, both cell-wall invertases were detected in floral organs of mature field-grown female *P. deltoides*. Specifically, *PtCIN1* (a homolog to *PaxgINV2* described in Chapter four) was located only in post-zygotic floral tissue, while *PtCIN2* (a homolog to *PaxgINV3* in Chapter four) was located in catkins (Bocock et al. 2008).

1.4 Cellulose biosynthesis

1.4.1 Polymer structure

The cellulose polymer consists of repeating β-1,4-linked glucose residues successively oriented 180° to one another. As such, cellobiose is the actual repeating subunit, with cellulose formation occurring in n+2 fashion from the non-reducing end (Koyama et al. 1997). In higher plants, individual glucan chains are typically several thousand glucose residues in length. Microfibrils are formed when 36 of these glucan chains are linked together in parallel, resulting in a highly crystallized structure (Delmer and Haigler 2002).

1.4.2 Cellulose synthase complex

There is substantial evidence that the formation of cellulose in higher plants involves an organized enzymatic unit known as the cellulose synthase complex (CSC). The hexagonal complex, also known as a rosette, is composed of six subunits. Each of the six subunits presumably contains six catalytic sites, thereby facilitating the formation of a microfibril composed of 36 growing glucan chains (Delmer 1999, Doblin et al. 2002, Somerville 2006). Precise knowledge of cellulose biosynthesis has been limited due to difficulty isolating functional CSCs (Chapple and Carpita 1998). Equally as challenging has been the identification of the genes encoding the components of these complexes. A putative cellulose synthase gene was first discovered in the cellulosic bacterium *Acetobacter xylinum* (Saxena et al. 1990). The gene, now known as *AxCeSA*, is believed to encode a catalytic subunit of cellulose synthase, as the expressed enzyme is able to bind UDP-glucose, the putative substrate for the CSC (Pear et al. 1996).

Initially, homolog searches in plant gene databases revealed little similarities. A breakthrough came when hydrophobic cluster analysis of known UDP-specific glycosyltransferases revealed conserved regions surrounding aspartate residues and a conserved downstream QXXRW amino acid motif were identified (Saxena et al. 1995). Since that discovery several putative cellulose synthase genes and gene families have been discovered in plants including both cotton (Pear et al. 1996) and *Arabidopsis* (Taylor et al. 1999). Predicted tertiary peptide structures from these genes gave rise to several models regarding the assembly and molecular mechanics of the CSC (Brown et al. 1996, Delmer 1999, Brown and Saxena 2000, Doblin et al. 2002). Common to these models is an enzyme complex with distinct transmembrane helices and a globular cytosolic component, presumably the location of an active site involving UDP-glucose. Targeting the UDP-glucose active site to the cytosol led to the formation of additional models involving the enzymatic channelling of UDP-glucose to the CSC.

1.4.3 Cellulose biosynthetic pathway

There are several enzymes that may directly or indirectly affect the cytosolic pools of UDP-glucose, the immediate substrate for the CSC. Among these, the enzyme sucrose synthase (SuSy; EC 2.4.1.13; sucrose + UDP $\Leftrightarrow$ UDP-glucose + fructose) has received the most attention with respect to cellulose biosynthesis. Two forms of this enzyme have been identified; a soluble cytosolic form called S-SuSy and the cell-wall associated P-SuSy form (Amor et al. 1995). To date there is little evidence whether these two enzymes are distinct gene products or that they are subject to post-transcriptional modification (Delmer 1999). Of particular interest is the correlation between P-SuSy and cellulose in cells undergoing secondary cell wall synthesis. Patterns of P-SuSy in immunolocalization studies have been shown to mirror the pattern of cellulose microfibrils in cotton (Robinson 1996). This suggests that P-SuSy may be localized in close association or perhaps bound to the CSC. Two advantages of such an association have been suggested. The first is the ability to efficiently channel UDP-glucose directly to the CSC, which is not only energetically efficient, but also prevents the substrate from pooling and being consumed in other metabolic processes. Secondly, glycosyltransferases such as the CSC are known to be inhibited by UDP; P-SuSy would therefore effectively reduce the local concentration of free UDP

by converting the potential inhibitor into CSC substrate (Delmer and Amor 1995, Haigler et al. 2001).

A related enzyme correlated to cellulose biosynthesis is sucrose phosphate synthase (SPS; EC 2.4.1.14; UDP-glucose + fructose-6-phosphate ⇔ UDP + sucrose-6-phosphate). Studies on tracheary elements and fibres in cotton revealed up to 4-fold increases in SPS from primary to secondary wall deposition (Haigler et al. 2001). A similar association with fibre development was also observed in hybrid poplar overexpressing an SPS gene from *Arabidopsis* (Park et al. 2008). With respect to cellulose biosynthesis it has been suggested that SPS may prevent fructose, an inhibitor of SuSy activity, from pooling (Delmer and Haigler 2002). This theory critically depends on the rapid phosphorylation of fructose (SuSy product) to fructose-6-phosphate (SPS substrate). A tracer study of ^{14}C-glucose in cotton indicated that rapid phosphorylation of fructose may occur (Haigler et al. 2001), and it was shown that, after a short time period, surprisingly low amounts of the radioactive isotope were found in fructose, indicating rapid phosphorylation and anabolism of this metabolite. This supports the idea that, in cells undergoing substantial cellulose biosynthesis, SPS may play a role in recycling fructose-6-phosphate into sucrose-6-phosphate, which can then be converted into sucrose via sucrose-phosphate phosphatase (EC 3.1.3.24; sucrose-6-phosphate + H_2O ⇔ sucrose + phosphate). The sucrose supply may then be used by P-SuSy to fuel cellulose biosynthesis.

In addition to SuSy and SPS, the enzyme UDP-pyrophosphorylase (UGPase; EC 2.7.7.9; UTP + glucose-1-phosphate ⇔ UDP-glucose + PP$_i$) has also been associated with secondary wall synthesis. UDP-glucose has been shown to accumulate in cotton fibres undergoing secondary wall development (Carpita and Delmer 1981), and this increase is paralleled by an almost 2-fold increase in UGPase activity (Wafler and Meier 1994). In this case UGPase may be contributing to UDP-glucose pools for subsequent use by CSCs in cells undergoing substantial cellulose biosynthesis. An alternate model indicates that S-SuSy and SPS utilize cytosolic UDP-glucose as a substrate to produce sucrose and sucrose-6-phosphate, respectively, that can subsequently be used for cellulose biosynthesis (Haigler et al. 2001, Delmer and Haigler 2002). Experimentally, an increase in cellulose content was observed in hybrid poplar overexpressing UGPase

from *A. xylinum* (Coleman et al. 2007). The results of that study provided further evidence for the link between UGPase and cellulose biosynthesis and accumulation.

In contrast to SuSy, SPS and UGPase, there are several enzymes in developing cotton fibres that do not show increases in activity during secondary wall development. Of these enzymes, invertase is perhaps the most relevant to cellulose biosynthesis models, as this enzyme catalyzes the hydrolysis of sucrose, a key metabolite in the cellulose biosynthetic pathway. Wafler and Meier (1994) reported that total invertase activity gradually increased during cotton boll development before reaching a maximum 18 days post-anthesis. These data suggest that invertase may enable the concentration of hexose sugars to peak prior to the period of rapid consumption of UDP-glucose by the CSC. This increased hexose supply may help meet the substrate requirements of UGPase, which had a 2-fold increase in activity during secondary wall development (Wafler and Meier 1994). This trend concurs with current models of plant cellulose biosynthesis. In particular, it has been shown that cellulose biosynthesis is critically limited by sucrose supply rather than UDP-glucose directly (Pillonel et al. 1980), therefore unnecessary hydrolysis of sucrose by invertase during secondary wall development would be counterproductive. It should be noted that the minimum invertase activity recorded was nevertheless extremely high in comparison to the other enzymes examined (Wafler and Meier 1994). For example, invertase activity (>25 µkat mg^{-1} protein) was almost 1000-fold higher than that of UGPase (Wafler and Meier 1994). This implies that invertase plays a substantial role in hydrolyzing sucrose in cotton bolls regardless of the developmental period. Also, invertase is known to exist in several different isoforms in plants. The study by Wafler and Meier (1994) did not address the activity of the different isoforms separately, and therefore the exact roles and mechanisms of the distinct invertase isoforms remain unclear.

1.5 Yeast-derived plant invertases

Photosynthetic tissues are the primary source of fixed carbon in higher plants, with sucrose being the major translocated form of biologically active carbon produced by these tissues. Sucrose is distributed from source tissue to other regions in the plant, including non-photosynthetic sink tissues. In an effort to better understand this sink-source relationship, a yeast-derived invertase targeting the apoplast of plants was

created (von Schaewen et al. 1990). The authors hypothesized that the over-expression of an invertase in the apoplast would prevent sucrose export (phloem loading) to the remainder of the plant. They further hypothesized a concomitant inhibition of photosynthesis would result due to the surplus of carbohydrate in source cells. In addition to the yeast-derived apoplastic invertase, an invertase targeting the cytosol was later created to further explore not only sink-source interactions, but also the role of carbohydrate compartmentalization in plant development (Sonnewald et al. 1991). The *suc2* gene (invertase) of *Saccharomyces cerevisiae* was used as a basis for the genes targeting the different subcellular compartments. von Schaewen et al. (1990) provided two reasons for using the yeast invertase gene: the first was to avoid inhibition from endogenous plant invertase inhibitors, as the foreign invertase would not be recognized by plant systems. Secondly, the pH of different subcellular compartments within vascular plants was not known with certainty. The *S. cerevisiae* invertase was known to possess activity over a broad pH range, and therefore effectively addressed this issue.

1.5.1 Apoplastic invertase

The apoplastic invertase (*AI*) gene was generated by fusing the N-terminus of a vacuolar protein, proteinase inhibitor II (PI-II) from potato (Keil et al. 1986), to a portion of the *suc2* gene that was missing the native signal sequence (von Schaewen et al. 1990). To verify membrane specificity, the resulting gene was transcribed *in vitro* using an SP6 expression vector in the presence and absence of microsomes. The resulting proteins expressed in the presence of microsomes migrated slower through SDS gels than did the same proteins generated in the absence of microsomes. The additional molecular weight was attributed to a glycosylated (membrane-specific) version of the enzyme. An increase in gel mobility of the proteins after treatment with endoglycosidase H provided additional evidence for glycosylation. Furthermore, *Arabidopsis thaliana* protoplasts transformed with the chimeric gene secreted active enzyme in suspension culture, while only minor amounts of activity were detected in cell extracts of the protoplasts.

1.5.2 Cytosolic invertase

The *CI* gene was created by fusing the 5′-untranslated leader sequence of the PI-II gene to the coding region of the *suc2* gene (Sonnewald et al. 1991). Again, transgenic

protoplasts were used to generate functional enzyme. In contrast to protoplasts transformed with the *AI* gene, the CI transformants showed invertase activity in cell extracts only. In addition, SDS gel migration indicated CI was a non-glycosylated protein when compared to the migration pattern of AI. The protoplast-specific activity and lack of glycosylation were consistent with a functional cytosolic enzyme.

1.5.3 Transgenic plants expressing yeast-derived invertase genes

Following the creation of the chimeric invertase genes, transgenic research was initially conducted using *Arabidopsis* and tobacco (von Schaewen et al. 1990, Sonnewald et al. 1991), followed by several studies in potato. The different invertase isoforms under varying expressional control in a number of different plant species consequently generated a wide range of phenotypes. The following is a summary of key research conducted using tobacco and potato phenotypic backgrounds, as these two species have been studied extensively with respect to the yeast-derived plant invertases. For clarity, the research is organized based on the specific species involved.

1.5.3.1 Tobacco (*Nicotiana tabacum*)

Tobacco was one of two model plants used in the original paper describing the creation of the *AI* gene (von Schaewen et al. 1990). *Agrobacterium*-mediated transformation of tobacco with the AI gene under the regulatory control of the cauliflower mosaic virus *35S* (*35S*) promoter led to distinct phenotypic differences from wild-type tobacco. Tobacco overexpressing the AI gene exhibited reduced height, which was shown to be a function of reduced internodal distance not a reduction in leaf number. The leaves of these plants displayed bleached areas, which in some cases formed a mosaic pattern. Analysis of the bleached areas revealed an expected loss of chlorophyll, decreased photosynthesis, and an increase in respiration, starch, sucrose and hexose sugars. Collectively, the data implied the successful interference of source export to sink tissues. This reduction in sucrose export appeared to have led to a reduction in photosynthesis in source tissues, which the authors expected in tissues with limited sucrose exporting ability (von Schaewen et al. 1990).

The mechanism leading to decreased photosynthesis that was observed in bleached regions of tobacco overexpressing the AI gene was explored by Stitt et al. (1991). An AI tobacco transformant (A 41-10) from the original study by von Schaewen et al. (1990) was used to investigate the effect of AI on photosynthesis in tobacco.

Supporting the earlier study, the pale areas of the leaves had increased levels of carbohydrates, decreased photosynthesis and an increase in respiration with respect to the wild-type. Examining the carbohydrate status of bleached areas revealed an increase in fructose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate. Other metabolite changes included a decrease in ribulose-phosphate and glycerate-3-phosphate, and an increase in triose phosphates and fructose-1,6-bisphosphate. Changes in key enzymes were also observed. In pale areas of tobacco leaves, ribulose-1,6-bisphosphate carboxylase/oxygenase (RubisCO) activity was two to three times lower, NADP-glyceraldehyde-3-phosphate dehydrogenase activity was reduced by as much as half, and fructose-1,6-bisphosphatase was also reduced. The above information indicates long-term inhibition of photosynthesis, particularly with respect to the Calvin cycle enzymes. The authors suggested that overexpression of the *AI* gene may lead to short-term inhibition of photosynthesis due to the sequestering of inorganic phosphate to the cytosol in the form of hexose-phosphates, which would limit the availability of inorganic phosphate in the chloroplasts for photosynthesis (Stitt et al. 1991).

The effects of *CI* gene overexpression, in addition to AI, were studied by Sonnewald et al. (1991). This study examined the role of compartmentalization, with respect to the foreign invertase genes, in tobacco. Interestingly, there were several similarities between AI and CI transformed plants. The height of transformed tobacco in tissue culture, as well as soil, was reduced in both types of transformants in comparison to wild-type tobacco. Both transformants also showed reduced leaf expansion, root development and seed setting. AI and CI transformants also exhibited late flowering, an indicator of prolonged life, which was attributed to an observed increase in the local sucrose supply (Sonnewald et al. 1991). In addition to sucrose, the concentration of hexoses also increased in pale leaf sections, where invertase activity was highest, in both types of transformants. Interestingly, CI transformants had higher levels of fructose than glucose, which was implicated in the curly-leaved phenotype observed in CI plants. The authors speculated that elevated fructose concentrations, attributed to increases in invertase activity, occurred on the upper side of the leaf where photosynthetic activity is maximal. These increased fructose concentrations may have led to an increase in water flux, which would trigger increased cell division and cell

expansion on the upper side of the leaf. This would consequently lead to differential growth between the upper and lower sides of the leaf, and ultimately result in leaf curling (Sonnewald et al. 1991).

In general, tobacco overexpressing the *CI* gene displayed a visible phenotype early in development and seemed to be more sensitive to invertase compared to plants overexpressing the *AI* gene (Sonnewald et al. 1991). The authors provided two explanations for these differences. The first explanation involves compartmentalization. Tobacco with CI have increased activity in the cytosol of targeted cells, therefore a significant strain is exerted on the plant as there is metabolic interference at the individual cell level. Apoplastic expression leads to metabolic interference between cells, and therefore represents a less significant stress at the individual cell level (Sonnewald et al. 1991). This theory led to the second explanation for the difference in sensitivity between AI and CI tobacco, which involves whole-plant development. AI tobacco showed signs of stress later in development as opposed to CI tobacco. It was speculated that AI tobacco showed signs of stress as the plant matured and sink-source relationships become increasingly important as growth and development progresses and the various tissues (e.g. leaves, roots) become spatially separated (Sonnewald et al. 1991).

Compartmentalization in tobacco overexpressing the *AI* and *CI* genes was again investigated by Heineke et al. (1994). This study focused on the compartmentalization of metabolites, specifically the hexoses liberated by invertase. It was found that over 97% of hexoses accumulated in the vacuoles of both types of transgenic tobacco cells. Additional evidence from this study indicated that hexoses in both wild-type and transgenic plants are sequestered to the vacuoles against a concentration gradient, which may be mediated by glucose and fructose transporters. This study also showed that AI and CI in tobacco did not affect photosynthesis directly by altering the osmotic balance of the cells, but instead had a more indirect, long-term inhibition of photosynthesis through the accumulation of photosynthetic product (Heineke et al. 1994).

In addition to the overexpression experiments, the *CI* gene was also studied under the regulatory control of the phloem-specific *rolC* promoter from *Agrobacterium rhizogenes* (Lerchl et al. 1995). This study was preceded by an earlier experiment

where overexpression of *E. coli* inorganic pyrophosphatase (PPase) led to a stunted-growth phenotype in tobacco, which was attributed to impaired sucrose export (Sonnewald 1992). It was hypothesized that sucrose export (phloem loading) in tobacco was dependent on inorganic pyrophosphate. This theory was tested by studying sucrose transport in the phloem of plants harbouring genes for both PPase and CI under the *rolC* promoter (Lerchl et al. 1995). It was shown that tobacco plants with *rolC*-directed *CI* gene expression had similar phenotypes with respect to growth when compared to wild-type tobacco. Unlike tobacco overexpressing the *CI* gene, the plants with tissue-specific expression had similar levels of sugar, starch and sucrose. Remarkably, tobacco with *rolC*-directed PPase gene expression in combination with *rolC*-directed *CI* gene expression displayed a similar phenotype to wild-type tobacco. This finding indicated that CI had restored the wild-type phenotype from the stunted phenotype observed with PPase gene expression alone. Collectively, the study by Lerchl et al. (1995) provided three critical pieces of information regarding the effects of yeast-derived invertases in plant systems: (1) the genes can be targeted to a specific tissue in addition to overexpression, (2) yeast-derived invertases can lead to dramatically different phenotypes in tobacco when under the regulatory control of different promoters, and (3) they can be used in combination with other foreign enzymes to generate unique phenotypes.

1.5.3.2 Potato (*Solanum tuberosum*)

In addition to tobacco, much of the transgenic research involving AI and CI (von Schaewen et al. 1990, Sonnewald et al. 1991) has focused on potato. Initial research involved the previously studied *35S::AI* construct (Heineke et al. 1992) to generate potato plants overexpressing the *AI* gene, which shared many phenotypic similarities to *35S::AI* tobacco plants. These similarities included reduced growth rates, higher glucose and fructose levels (4-8 fold increase), reduced rates of photosynthesis and increased respiration. However, despite these similarities other aspects of *AI* overexpression were unique to potato plants. For instance, potato expressing *AI* had a curly-leaf phenotype, which was limited to the CI transformants in tobacco (Sonnewald et al. 1991). Also, the AI potato plants showed little change in sucrose content when compared to wild-type plants, a sharp contrast to AI tobacco, which showed substantial increases in this metabolite. Additionally, unique to the overexpression of the *AI* gene

in potato was an accumulation of amino acids in leaf tissue. This accumulation was attributed to either a by-product of reduced amino acid export to sieve tubes or an increase in protein degradation (Heineke et al. 1992). Further analysis of individual amino acids revealed that the concentration of proline increased substantially. In one transformant, the proline concentration was 153 times higher than wild-type. Based on these findings the authors postulated that AI activity in potato results in osmotic conditions similar to those in plants experiencing water stress, as proline is a strong indicator of water stress in plants (Heineke et al. 1992). The presence of increased solutes such as malate, and an observed increase in osmotic pressure in the phloem sap further supported this theory.

To further study water-related stress in potato, the effects of *CI* gene expression in potato were examined (Bussis et al. 1997). As with tobacco and earlier studies in potato, reduced photosynthesis and stunted growth were observed with both invertase isogenes. However, unlike tobacco, overexpression of the *AI* and *CI* genes in potato led to substantial increases in proline, which concurs with previous data (Heineke et al. 1992). Aside from these similarities the two invertases also had several different effects on potato. Plants overexpressing *CI* were sensitive to light, whereas those expressing *AI* were not. In addition, AI plants showed early abscission, and displayed necrosis in older leaves. CI plants in comparison showed no signs of early necrosis or abscission. Also, CI potato plants had reduced fresh weight, indicating lower water content than AI plants. Along with this decrease in water content came a lack of turgidity in CI plants, which was so severe that stems had to be supported during growth (Bussis et al. 1997). In contrast, AI plants had no apparent change in fresh weight or water content, and maintained turgidity.

After initial overexpression studies showed a promising increase in hexoses, the tuber-specific patatin *B33* promoter was used in an effort to increase tuber yield of potato plants (Sonnewald et al. 1997). Once again, the *AI* and *CI* genes were employed in that sink-source study, however, unlike earlier investigations with potato, these experiments targeted a specific tissue. In both AI and CI potato the aerial portions of the plants were relatively unaffected and showed no visible phenotypic differences, which was taken as evidence that the promoter was effectively targeting potato tubers. Further analyses of the CI tubers showed a decrease in tuber fresh weight and

individual tuber size, although some CI plants showed an increase in tuber number. Along with these attributes, CI tubers also had an increase in glycolytic flux along with an increase in phosphorylated carbohydrates (Hajirezaei et al. 2000), and 96% less sucrose content than wild-type tubers (Sonnewald et al. 1997). A similar reduction in sucrose content (92%) was observed in tubers of AI plants, although these plants had opposite trends with respect to tuber size and number when compared to the CI plants. Specifically, AI tubers had reduced tuber number and increased yield with respect to fresh weight, although it should be noted that the dry weight, and therefore biomass, did not significantly differ from wild-type tubers. These findings effectively demonstrated three key points with respect to yeast-derived invertases: (1) that sink content can be manipulated by controlled expression of yeast-derived invertase genes, (2) that the site of yeast-derived invertase gene expression and consequently sucrose degradation and hexose accumulation is extremely important in determining phenotype, and (3) that the yeast-derived invertase genes under tissue-specific promoters can lead to drastic phenotypic differences in potato compared to those under constitutive control.

The ability to alter phenotype by tissue-specific expression of yeast-derived invertases in potato was further demonstrated using the phloem-specific *rolC* promoter with the *CI* gene (Hajirezaei et al. 2003). In contrast to CI overexpression in potato, plants with *rolC*-directed CI showed few visible phenotypic differences when compared to wild-type plants. Transformed and wild-type plants had similar size, growth rates, shoot branching patterns, leaf appearance, onset of flowering and tuber yield. The effectiveness of the phloem-specific promoter was addressed by measuring invertase activity in different tissues within tubers. The activity in the vascular tissue was ten-fold higher than the control, while activity was only slightly higher in the parenchyma. Furthermore, sucrose concentration decreased in the vascular tissue in transformed tubers, along with a concomitant increase in hexoses. In contrast, parenchyma cells in the same tubers showed little differences in either sucrose or hexose concentration (Hajirezaei et al. 2003). With respect to morphogenesis, this study revealed a reduction in sprouting of tubers expressing *CI* under the regulatory control of *rolC*. The authors hypothesized that sprout growth was dependent on sucrose supply translocated from the phloem, and that phloem-specific cytosolic invertase activity led to a reduction in the local sucrose concentration. It was further speculated that the increase in invertase

activity, and the concomitant increase in hexoses, led to the observed increase in respiration (Hajirezaei et al. 2003). An additional study of *rolC*-directed expression of *CI* in potato revealed an increase in the trisaccharide 6-kestose in leaf tissue, which later accumulated in the tubers of the transgenic potato (Zuther et al. 2004). Grafting experiments with transgenic scions on wild-type roots showed a similar accumulation of 6-kestose in the tuber tissue. This study showed that the expression of a yeast-derived invertase can cause the synthesis of unusual transport carbohydrates in potato, and that these carbohydrates can be transported via the phloem in this sucrose loading apoplastic species.

1.6 Promoters

The expression of specific genes in higher plants is regulated both developmentally and spatially by promoters. For this reason, a key aspect of transgenic research is the promoter associated with the gene or genes of interest. Constitutive expression has typically been achieved with the *35S* promoter (e.g. von Schaewen et al. 1990), while other promoters have been used for developmental, tissue-specific or cell-specific expression, such as the tuber-specific *B33* promoter (e.g. Sonnewald 1997). Similarly, a constitutive and a tissue-specific promoter were employed and described in this thesis. The following describes the creation and resulting tissue specificity of those two promoters.

1.6.1 Enhanced tandem cauliflower mosaic virus promoter (*2X35S*)

The creation of the tandem cauliflower mosaic virus 35S promoter (*t35S*) was originally described by Kay et al. (1987). To achieve this construct, a *35S* promoter fragment was excised from pUC13 as a *Cla*I-*Hin*dIII fragment, blunt-ended, and subcloned into pUC18 within the *Hin*cII site. The resulting plasmid, pCA1, was cut with *Hin*dIII and *Eco*RV, giving a new *35S* fragment, which was ligated into a second pCA1 that was cut with *Hin*dIII and *Pst*I (blunted). The resulting plasmid was labelled pCA2, which contained two *35S* promoters in tandem. These single and double *35S* promoters were taken as *Hin*dIII-*Bam*HI fragments from pCA1 and pCA2, respectively, and ligated into the binary plasmid pMON178 giving pCKR1 (*35S::NPTII*) and pCKR2 (*t35S::NPTII*). Transcript analysis of transgenic tobacco plants revealed a 10-fold increase of *NPTII* transcript level from the *35S* promoter, and a 100-fold increase for the *t35S* promoter,

when compared to expression resulting from the nopaline synthase (*NOS*) promoter. The results indicated that the tandem promoter (*t35S*) sequence had effectively increased expression by a factor of ten when compared to the single *35S* promoter (Kay et al. 1987).

Further experimentation on an enhanced version of the *t35S* promoter resulted in an even greater increase in gene expression (Datla et al. 1993). In this study the *t35S* promoter was excised from pCA2 (Kay et al. 1987) as a *Hin*dIII-*Bam*HI fragment and subcloned in pTZ18R, producing pBI-500. Subsequent site-directed mutagenesis created a *Bgl*II site at the 3′ end of the promoter (pBI-501). A synthetic alfalfa mosaic virus subgenomic RNA4 (AMV) untranslated leader sequence was then ligated as a *Bgl*II-*Xba*I fragment into pBI-501, which resulted in pBI-502. The newly constructed *t35S-AMV* (*2X35S*) promoter and *GUS* were subcloned from pBI-502 as a *Hin*dIII-*Eco*RI fragment into BIN19, creating the binary construct pBI-543. Protoplast suspensions of tobacco and white spruce (*Picea glauca*) were transformed with pBI-543 and pBI-542 (containing *t35S*) using electroporation. In both cell types, GUS activity increased almost ten-fold in *2X35S* cells when compared to *t35S* cells. These results, along with those of Kay et al. (1987), indicated that the tandem promoter in combination with the AMV sequence had a combined expression increase of approximately 100-fold in comparison to the *35S* promoter alone.

1.6.2 4-coumarate:CoA ligase promoter (*4CL*)

The 4-coumarate:CoA ligase gene promoter (*4CL*) originated from a study on gene expression of the 4CL enzyme, which is a key link between the phenylpropanoid biosynthetic pathway and committed branches of phenylpropanoid metabolism (Hauffe et al. 1991). To study gene expression of this enzyme, a *4CL* promoter from parsley (*Petroselinum crispum*) was isolated and its expressional control in tobacco was examined. The initial *4CL* promoter that was identified was 1530 bp in length, however, 5′ deletion experiments produced several other functional promoters including a 597 bp fragment, which was subsequently cloned with *GUS* into the binary construct BIN19 (Hauffe et al. 1991). *Agrobacterium*-transformed tobacco expressing the reporter gene were created, and were subsequently examined to determine the spatial and developmental expression patterns of the *4CL* promoter. Histochemical localization revealed a lack of GUS activity in young leaves and stems, and only minor activity in

primary xylem of developing leaf buds. Instead, most of the GUS activity was located in the secondary xylem of mature tobacco stems, specifically in ray parenchyma and other cells surrounding tracheary elements (Hauffe et al. 1991). Significant GUS activity was also present in the subapical cells of young lateral roots and elongated roots, and in root hairs and vascular tissue. Flowers and developing nectories also had vascular-specific GUS activity, although this activity decreased after pollination, which was followed by the presence of GUS activity in epidermal cells of the developing seed (Hauffe et al. 1991). Additional activity was also observed in the epidermal cells of mature stigmas, pollen grains and petal tissue. Together, these findings indicated that the 597 bp *4CL* promoter from parsley conferred tissue and cell specificity in tobacco. Specifically, the *4CL* promoter led to significant gene expression in vascular tissues and epidermal cells of the reproductive organs, and the parenchyma cells of developing and secondary xylem.

1.7 Model systems

The research described in this thesis employs three model biological systems: tobacco, poplar and *Pichia pastoris*. Specifically, chapters two and three of this thesis describe the use of tobacco (*Nicotiana tabacum* cv. Xanthi) and hybrid poplar (*Populus alba* x *grandidentata*) as phenotypic backgrounds to study the effects of yeast-derived invertase expression. Chapter four describes the expression of endogenous cell-wall invertases in hybrid poplar, which were subsequently heterologously expressed in the yeast *Pichia pastoris* (described in Chapter five). The following is a brief summary of each of the three model biological systems employed in this thesis.

1.7.1 Tobacco

Research with tobacco has primarily focused on either the effects of foreign enzymes within the native system (e.g. Sonnewald et al. 1991) or the effects of benign enzymes (e.g. GUS) in histochemical studies of gene promoters from other species such as parsley (Hauffe et al. 1991) and potato (Maddison et al. 1999). In general this species has become a common plant system for transformation-based studies, owing to the ability of tobacco to regenerate in tissue culture and the relative ease of transformation using traditional *Agrobacterium*-based methods. Additionally, tobacco has a relatively

short life cycle (2-3 months), produces well-defined flowers for reproduction studies and develops a substantial stem allowing for secondary cell wall analyses.

1.7.2 Poplar

The emergence of poplar as the model tree species was due to a variety of natural attributes including rapid growth, relative ease of tissue culture propagation and the ability to be transformed using traditional *Agrobacterium*-mediated techniques (Boerjan 2005, Jansson and Douglas 2007). In addition, the relatively small genome size of approximately 500 million bp (50 times smaller than *Pinus*) makes poplar especially suitable for genome and gene expression studies. Consequently, a substantial amount of tool building and resource development has been initiated including the landmark achievement of sequencing the complete genome of *P. trichocarpa* (Tuskan et al. 2006). Furthermore, over 350,000 expressed sequence tags (ESTs) from various physiological and environmental conditions are available (e.g. Sterky et al. 2004). In addition, microarrays representing thousands of poplar genes have been created allowing for thorough and detailed expression analysis (e.g. Ralph et al. 2006).

1.7.3 *Pichia pastoris*

P. pastoris is a methylotrophic yeast that is capable of efficiently metabolizing methanol as an alternative carbon source. It emerged as a suitable host for the production of heterologous proteins after an efficient transformation protocol was established by Cregg et al. (1985). A breakthrough in heterologous gene expression in *P. pastoris* occurred when the promoter for alcohol oxidase (*AOX1*), a key enzyme in the methanol metabolizing pathway, was identified as having the potential to drive high level expression of foreign genes (Cregg et al. 1993). Together, these findings led to the subsequent commercialization of *P. pastoris* GS115 along with expression vectors containing the *AOX1* promoter, which are available from Invitrogen (Carlsbad, CA). The suitability of *P. pastoris* as a eukaryotic expression system can be attributed to several factors including: (1) ease of growth when compared to insect and mammalian systems, (2) ability to properly translate and process eukaryotic gene products, (3) inducible expression via the *AOX1* promoter when cultures are grown in methanol-containing medium, (4) secretion of exogenous protein in the growth medium for easy purification, and (5) ability to grow cultures in fermentation reactors for continuous protein production (Cregg et al. 1993, Romanos 1995, Daly and Hearn 2005).

1.8 Goals and hypotheses

1.8.1 Expression of yeast-derived invertases in tobacco and hybrid poplar

The goal of these experiments was to investigate the effects of yeast-derived invertase genes (*AI* and *CI*) under the expressional control of either the constitutive *2X35S* promoter or the xylem-specific *4CL* promoter on *Nicotiana tabacum* (tobacco) and *Populus alba x grandidentata* (hybrid poplar). Analysis focused on growth rate, phenotype, biochemistry and chemistry of tissues undergoing secondary cell wall development. The hypothesis was that varied expression of yeast-derived invertase genes would alter the sink-source relationship during the growth and development of tobacco and hybrid poplar. In particular, the quantity of structural polymers in stem tissue would be affected by altering the amount of carbohydrate available to metabolic processes in the sink tissue, such as the cellulose biosynthesis pathway.

1.8.2 Cell-wall invertase gene identification and expression in hybrid poplar

The goal of this experiment was to identify the cell-wall invertase genes in hybrid poplar and examine the expression levels of these genes spatially and temporally during the first year of growth in an attempt to understand their physiological role(s) in cell wall development. The hypothesis was that multiple cell-wall invertase genes exist in hybrid poplar, each with varied expression levels both spatially and temporally. Furthermore, based on existing research on other plant species, it was anticipated that hybrid poplar contains at least one gene that is expressed in vegetative tissues during periods of growth, and at least one gene that is expressed solely or primarily in reproductive tissues.

1.8.3 Characterization of hybrid poplar cell-wall invertases

The goal of this experiment was to examine the enzymatic characteristics of the cell-wall invertases identified from hybrid poplar. Specifically, properties such as Km, pH and temperature optima, as well as substrate specificity were explored. The heterologous expression system *Pichia pastoris* was used to produce enzyme that could be directly correlated to a specific cell-wall invertase gene. The hypothesis was that each of the various cell-wall invertases would have unique properties and characteristics that would be related to their physiological role(s) in hybrid poplar growth and development.

1.9 References

Amor Y., C.H. Haigler, S. Johnson, M. Wainscott and D.P. Delmer. 1995. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. P. Natl. Acad. Sci. U.S.A. 92:9353-9357.

Bocock, P.N., A.M. Morse, C. Dervinis and J.M. Davis. 2008. Evolution and diversity of invertase genes in *Populus trichocarpa*. Planta. 227:565-576.

Boerjan W. 2005. Biotechnology and the domestication of forest trees. Curr. Opin. Biotech. 16:159-166.

Brown R.M. and I.M. Saxena. 2000. Cellulose biosynthesis: A model for understanding the assembly of biopolymers. Plant Physiol. Bioch. 38:57-67.

Brown R.M., I.M. Saxena and K. Kudlicka. 1996. Cellulose biosynthesis in higher plants. Trends Plant Sci. 1:149-156.

Bussis D., D. Heineke, U. Sonnewald, L. Willmitzer, K. Raschke and H.W. Heldt. 1997. Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol. Planta. 202:126-136.

Carpita N.C. and D.P. Delmer. 1981. Concentration and metabolic turnover of UDP-glucose in developing cotton fibers. J Biol. Chem. 256:308-315.

Chapple, C. and N. Carpita. 1998. Plant cell walls as targets for biotechnology. Curr. Opin. Plant Biol. 1:179-185.

Coleman, H.D., T. Canam, K.Y. Kang, D.D. Ellis and S.D. Mansfield. 2007. Over-expression of UDP-glucose pyrophosphorylase in hybrid poplar affects carbon allocation. J. Exp. Bot. 58:4257-4268.

Cooper R.A. and R.N. Greenshields. 1964. The partial purification and some properties of two sucrases of *Phaseolus vulgaris*. Biochem J. 92:357-364.

Cregg J.M., K.J. Barringer, A.Y. Hessler and K.R. Madden. 1985. Pichia pastoris as a host system for transformations. Mol. Cell Biol. 5:3376-3385.

Cregg J.M., T.S. Vedvick and W.C. Raschke. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio-Technol. 11:905-910.

Daly R. and M.T.W. Hearn. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. 18:119-138.

Datla R.S.S., F. Bekkaoui, J.K. Hammerlindl, G. Pilate, D.I. Dunstan and W.L. Crosby. 1993. Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. 94:139-149.

De Coninck B., K. Le Roy, I. Francis, S. Clerens, R. Vergauwen, A.M. Halliday, S.M. Smith, A. Van Laere and W. Van den Ende. 2005. *Arabidopsis* AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. Plant Cell Environ. 28:432-443.

Delmer D.P. and Y. Amor. 1995. Cellulose biosynthesis. Plant Cell. 7:987-1000.

Delmer D.P. 1999. Cellulose biosynthesis: Exciting times for a difficult field of study. Annu. Rev. Plant Phys. 50:245-276.

Delmer D.P. and C.H. Haigler. 2002. The regulation of metabolic flux to cellulose, a major sink for carbon in plants. Metab. Eng. 4:22-28.

Doblin, M.S., I. Kurek, D. Jacob-Wilk and D.P. Delmer. 2002. Cellulose biosynthesis in plants: from genes to rosettes. Plant Cell Physiol. 43:1407-1420.

Eschrich W. 1980. Free space invertase, its possible role in phloem unloading. Ber. Deut. Bot. Ges. 93:363-378.

Fisher D.B. and K.J. Oparka. 1996. Post-phloem transport: Principles and problems. J. Exp. Bot. 47:1141-1154.

Fridman E. and D. Zamir. 2003. Functional divergence of a syntenic invertase gene family in tomato, potato, and *Arabidopsis*. Plant Physiol. 131:603-609.

Godt D.E. and T. Roitsch. 1997. Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. Plant Physiol. 115:273-282.

Godt D. and T. Roitsch. 2006. The developmental and organ specific expression of sucrose cleaving enzymes in sugar beet suggests a transition between apoplasmic and symplasmic phloem unloading in the tap roots. Plant Physiol. Bioch. 44:656-665.

Goetz M. and T. Roitsch. 1999. The different pH optima and substrate specificities of extracellular and vacuolar invertases from plants are determined by a single amino-acid substitution. Plant J. 20:707-711.

Goetz M., D.E. Godt and T. Roitsch. 2000. Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role for steroid hormones in assimilate partitioning. Plant J. 22:515-522.

Goetz M., D.E. Godt, A. Guivarc'h, U. Kahmann, D. Chriqui and T. Roitsch. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. P. Natl. Acad. Sci. U.S.A. 98:6522-6527.

Greiner S., M. Weil, S. Krausgrill and T. Rausch. 1995. A tobacco cDNA coding for cell-wall invertase. Plant Physiol. 108:825-826.

Haigler C.H., M. Ivanova-Datcheva, P.S. Hogan, V.V. Salnikov, S. Hwang, K. Martin and D.P. Delmer. 2001. Carbon partitioning to cellulose synthesis. Plant Mol. Biol. 47:29-51.

Hajirezaei, M.R., Y. Takahata, R.N. Trethewey, L. Willmitzer and U. Sonnewald. 2000. Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. J. Exp. Bot. 51:439-445.

Hajirezaei M.R., F. Bornke, M. Peisker, Y. Takahata, J. Lerchl, A. Kirakosyan and U. Sonnewald. 2003. Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers (*Solanum tuberosum*). J. Exp. Bot. 54:477-488.

Hashizume H., K. Tanase, K. Shiratake, H. Mori and S. Yamaki. 2003. Purification and characterization of two soluble acid invertase isozymes from Japanese pear fruit. Phytochemistry. 63:125-129.

Hauffe K.D., U. Paszkowski, P. Schulze-Lefert, K. Hahlbrock, J.L. Dangl and C.J. Douglas. 1991. A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell. 3:435-443.

Hedley P.E., G.C. Machray, H.V. Davies, L. Burch and R. Waugh. 1993. cDNA cloning and expression of a potato (*Solanum tuberosum*) invertase. Plant Mol. Biol. 22:917-922.

Hedley P.E., G.C. Machray, H.V. Davies, L. Burch and R. Waugh. 1994. Potato (*Solanum tuberosum*) invertase-encoding cDNAs and their differential expression. Gene. 145:211-214.

Hedley P.E., A.L. Maddison, D. Davidson and G.C. Machray. 2000. Differential expression of invertase genes in internal and external phloem tissues of potato (*Solanum tuberosum* L.). J. Exp. Bot. 51:817-821.

Heineke D., U. Sonnewald, D. Bussis, G. Gunter, K. Leidreiter, I. Wilke, K. Raschke, L. Willmitzer and H.W. Heldt. 1992. Apoplastic expression of yeast-derived invertase in potato - effects on photosynthesis, leaf solute composition, water relations, and tuber composition. Plant Physiol. 100:301-308.

Heineke D., K. Wildenberger, U. Sonnewald, L. Willmitzer and H.W. Heldt. 1994. Accumulation of hexoses in leaf vacuoles - studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. Planta. 194:29-33.

Hsieh C.W., L.K. Liu, S.H. Yeh, C.F. Chen, H.I. Lin, H.Y. Sung and A.Y. Wang. 2006. Molecular cloning and functional identification of invertase isozymes from green bamboo *Bambusa oldhamii*. J. Agr. Food Chem. 54:3101-3107.

Jansson S. and C.J. Douglas. 2007. Populus: A model system for plant biology. Annu. Rev. Plant Biol. 58:435-458.

Kay R., A. Chan, M. Daly and J. McPherson. 1987. Duplication of CaMV-35S promoter sequences creates a strong enhancer for plant genes. Science. 236:1299-1302.

Keil M., J. Sanchezserrano, J. Schell and L. Willmitzer. 1986. Primary structure of a proteinase inhibitor-II gene from potato (*Solanum tuberosum*). Nucleic Acids Res. 14:5641-5650.

Konno Y., T. Vedvick, L. Fitzmaurice and T.E. Mirkov. 1993. Purification, characterization, and subcellular localization of soluble invertase from tomato fruit. J Plant Physiol. 141:385-392.

Koyama M., W. Helbert, T. Imai, J. Sugiyama and B. Henrissat. 1997. Parallel-up structure evidences the molecular directionality during biosynthesis of bacterial cellulose. P. Natl. Acad. Sci. U.S.A. 94:9091-9095.

Lerchl J., P. Geigenberger, M. Stitt and U. Sonnewald. 1995. Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell. 7:259-270.

Lorenz K., S. Lienhard and A. Sturm. 1995. Structural organization and differential expression of carrot beta-fructofuranosidase genes - identification of a gene coding for a flower bud-specific isozyme. Plant Mol. Biol. 28:189-194.

Maddison A.L., P.E. Hedley, R.C. Meyer, N. Aziz, D. Davidson and G.C. Machray. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol. Biol. 41:741-751.

Mercier R.W. and J.P. Gogarten. 1995. A 2nd cell-wall acid invertase gene in *Arabidopsis thaliana*. Plant Physiol. 107:659-660.

Nagaraj V.J., V. Galati, M. Luscher, T. Boller and A. Wiemken. 2005. Cloning and functional characterization of a cDNA encoding barley soluble acid invertase (HvINV1). Plant Sci. 168:249-258.

Ohyama A., S. Nishimura and M. Hirai. 1998. Cloning of cDNA for a cell wall-bound acid invertase from tomato (*Lycopersicon esculentum*) and expression of soluble and cell wall-bound invertases in plants and wounded leaves of *L. esculentum* and *L. peruvianum*. Genes Genet. Syst. 73:149-157.

Oparka K.J. and R. Turgeon. 1999. Sieve elements and companion cells - traffic control centers of the phloem. Plant Cell. 11:739-750.

Park, J.Y., T. Canam, K.Y. Kang, D.D. Ellis and S.D. Mansfield. 2008. Over-expression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. Transgenic Res. 17:181-192.

Pear J.R., Y. Kawagoe, W.E. Schreckengost, D.P. Delmer and D.M. Stalker. 1996. Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. P. Natl. Acad. Sci. U.S.A. 93:12637-12642.

Pillonel C., A.J. Buchala and H. Meier. 1980. Glucan synthesis by intact cotton fibers fed with different precursors at the stages of primary and secondary wall formation. Planta. 149:306-312.

Proels R.K., B. Hause, S. Berger and T. Roitsch. 2003. Novel mode of hormone induction of tandem tomato invertase genes in floral tissues. Plant Mol. Biol. 52:191-201.

Proels R.K., M.C. Gonzalez and T. Roitsch. 2006. Gibberellin-dependent induction of tomato extracellular invertase *Lin7* is required for pollen development. Funct. Plant Biol. 33:547-554.

Ralph, S., C. Oddy, D. Cooper et al. 2006. Genomics of hybrid poplar (*Populus trichocarpa x deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Mol. Ecol. 15:1275-1297.

Ramloch-Lorenz K., S. Knudsen and A. Sturm. 1993. Molecular characterization of the gene for carrot cell-wall beta-fructosidase. Plant J. 4:545-554.

Robinson D.G. 1996. SuSy ergo GluSy: New developments in the field of cellulose biosynthesis. Bot. Acta. 109:261-263.

Roitsch T., M. Bittner and D.E. Godt. 1995. Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol. 108:285-294.

Roitsch T., M.E. Balibrea, M. Hofmann, R. Proels and A.K. Sinha. 2003. Extracellular invertase: key metabolic enzyme and PR protein. J. Exp. Bot. 54:513-524.

Roitsch T. and M.C. Gonzalez. 2004. Function and regulation of plant invertases: sweet sensations. Trends Plant Sci. 9:606-613.

Romanos M. 1995. Advances in the use of *Pichia pastoris* for high-level gene expression. Curr. Opin. Biotech. 6:527-533.

Saxena I.M., F.C. Lin and R.M. Brown. 1990. Cloning and sequencing of the cellulose synthase catalytic subunit gene of *Acetobacter xylinum*. Plant Mol. Biol. 15:673-683.

Saxena I.M., B. Henrissat and R.M. Brown. 1995. Analysis of genes involved in cellulose biosynthesis - from sequence comparisons to mechanism of glycosyl transfer. Plant Physiol. 108:9-9.

Schwebel-Dugue N., N. El Mtili, M. Krivitzky, I. Jean-Jacques, J.H.H. Williams, M. Thomas, M. Kreis and A. Lecharny. 1994. *Arabidopsis* gene and cDNA-encoding cell-wall invertase. Plant Physiol. 104:809-810.

Sherson S.M., H.L. Alford, S.M. Forbes, G. Wallace and S.M. Smith. 2003. Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J. Exp. Bot. 54:525-531.

Somerville, C. 2006. Cellulose synthesis in higher plants. Annu. Rev. Cell. Dev. Bi. 22:53-78.

Sonnewald U., M. Brauer, A. von Schaewen, M. Stitt and L. Willmitzer. 1991. Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast - a powerful tool for studying sucrose metabolism and sink source interactions. Plant J. 1:95-106.

Sonnewald U. 1992. Expression of *Escherichia coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning. Plant J. 2:571-581.

Sonnewald U., M.R. Hajirezaei, J. Kossmann, A. Heyer, R.N. Trethewey and L. Willmitzer. 1997. Increased potato tuber size resulting from apoplastic expression of a yeast invertase. Nature Biotechnol. 15:794-797.

Sterky F., R.R. Bhalerao, P. Unneberg, B. Segerman, P. Nilsson, A.M. Brunner, L. Charbonnel-Campaa, J.J. Lindvall, K. Tandre, S.H. Strauss, B. Sundberg, P. Gustafsson, M. Uhlen, R.P. Bhalerao, O. Nilsson, G. Sandberg, J. Karlsson, J. Lundeberg and S. Jansson. 2004. A *Populus* EST resource for plant functional genomics. P. Natl. Acad. Sci. U.S.A. 101:13951-13956.

Stitt M., A. von Schaewen and L. Willmitzer. 1991. Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell-wall involves a decrease of the Calvin cycle enzymes and an increase of glycolytic enzymes. Planta. 183:40-50.

Sturm A. and M.J. Chrispeels. 1990. cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell. 2:1107-1119.

Sturm A., V. Sebkova, K. Lorenz, M. Hardegger, S. Lienhard and C. Unger. 1995. Development-specific and organ-specific expression of the genes for sucrose synthase and three isoenzymes of acid beta-fructofuranosidase in carrot. Planta. 195:601-610.

Taylor N.G., W.R. Scheible, S. Cutler, C.R. Somerville and S.R. Turner. 1999. The irregular xylem3 locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell. 11:769-779.

Turgeon R. 1996. Phloem loading and plasmodesmata. Trends Plant Sci. 1:418-423.

Turgeon R. 2006. Phloem loading: How leaves gain their independence. Bioscience. 56:15-24.

Tuskan G.A. et al. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science. 313:1596-1604.

Tymowska-Lalanne Z. and M. Kreis. 1998a. The plant invertases: Physiology, biochemistry and molecular biology. *In* Advances in Botanical Research. Vol 28., pp 71-117.

Tymowska-Lalanne Z. and M. Kreis. 1998b. Expression of the *Arabidopsis thaliana* invertase gene family. Planta. 207:259-265.

von Schaewen A., M. Stitt, R. Schmidt, U. Sonnewald and L. Willmitzer. 1990. Expression of a yeast-derived invertase in the cell-wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J. 9:3033-3044.

Wafler U. and H. Meier. 1994. Enzyme activities in developing cotton fibers. Plant Physiol. Bioch. 32:697-702.

Weber H., L. Borisjuk, U. Heim, P. Buchner and U. Wobus. 1995. Seed coat-associated invertases of fava bean control both unloading and storage functions: Cloning of cDNAs and cell type-specific expression. Plant Cell. 7:1835-1846.

Weil M. and T. Rausch. 1994. Acid invertase in *Nicotiana tabacum* crown-gall cells: Molecular properties of the cell-wall isoform. Planta. 193:430-437.

Zhang, L., N.S. Cohn and J.P. Mitchell. 1996a. A cDNA clone encoding a cell wall invertase from pea (Accession No. X85327). Plant Physiol. 110:1048.

Zhang L., N.S. Cohn and J.P. Mitchell. 1996b. Induction of a pea cell-wall invertase gene by wounding and its localized expression in phloem. Plant Physiol. 112:1111-1117.

Zhang L., N.S. Cohn and J.P. Mitchell. 1997. A pea cell-wall invertase gene (*PsInv-1*) with tissue-specific expression. Plant Physiol. Bioch. 35:751-760.

Zuther, E., M. Kwart, L. Willmitzer and A.G. Heyer. 2004. Expression of a yeast-derived invertase in companion cells results in long-distance transport of a trisaccharide in an apoplastic loader and influences sucrose transport. Planta. 218:759-766.

CHAPTER TWO

Varied growth, biomass and cellulose content in tobacco expressing yeast-derived invertases

2.1 Introduction

In woody plants, cellulose and lignin, the most abundant biopolymers on earth, make the largest contribution to the overall plant biomass. Genetic manipulation offers the ability to influence the production of these polymers to suit targeted commercial or industrial applications (Huntley et al. 2003, Li et al. 2003). Such manipulation can be achieved by affecting the pathways involved in the synthesis or degradation of these target polymers. In theory, polysaccharide production can be manipulated by altering substrate availability. For example, altering UDP-glucose or ADP-glucose availability in sink tissue should influence the accumulation of cellulose or starch, respectively. Since it is probable that UDP and ADP are recycled during the biosynthesis of both cellulose and starch, the availability of these nucleotide-sugars depends on the abundance of soluble carbohydrate. The influx of sucrose, and its subsequent breakdown to glucose and fructose, is critical to maintaining high rates of biosynthesis and concurrently manipulating the production of cellulose (Figure 2.1) and starch in sink cells and tissues.

In higher plants, sucrose synthase (EC 2.4.1.13) and invertase (EC 3.2.1.26) directly influence the abundance of sucrose available for metabolic processes (Roitsch and Gonzalez 2004). Altered expression of these enzymes has led to significant changes in the size and accumulation of biomass in sink tissues, such as potato tubers (Zrenner et al. 1995, Sonnewald et al. 1997) and carrot tap roots (Tang and Sturm 1999, Tang et al. 1999). The manipulation of invertase has largely involved the heterologous expression of yeast-derived invertase genes (von Schaewen et al. 1990, Sonnewald et al. 1991); one of the invertase genes encodes a cytosol-localized invertase (*CI*), while the other encodes an invertase that is targeted to the apoplast (apoplastic invertase, *AI*). These invertases have been expressed under the control of a number of promoters and studied in various plant species, including *Arabidopsis* (von Schaewen et al. 1990, Heyer et al. 2004), tobacco (Stitt et al. 1991, Sonnewald et al. 1991, Heineke et al. 1994, Lerchl et al. 1995), and potato (Heineke et al. 1992, Bussis et al. 1997, Sonnewald et al. 1997, Tauberger et al. 1999, Hajirezaei et al. 2003).

Generally, the heterologous expression of yeast invertase genes has led to a reduction in plant growth (Dickinson et al. 1991, Sonnewald et al. 1991, Bussis et al. 1997), inhibition of photosynthesis (Sonnewald et al. 1991, Heineke et al. 1992) and in

some cases a change in phenolic metabolites in leaf tissue (Herbers et al. 1996, Baumert et al. 2001). Nevertheless, invertase overexpression has been shown to alter yield in sink tissues. For example, the overexpression of the yeast-derived *AI* in potato, under the control of the tuber-specific patatin *B33* promoter, led to an increase in tuber size (Sonnewald et al. 1997). In this instance, tuber biomass (dry weight) was unaffected, suggesting that increased tuber size in the invertase-overexpressing plants was a function of increased water retention. Similarly, the overexpression of an *AI* transgene in *Arabidopsis* under the control of a meristem-specific promoter accelerated flowering and altered plant architecture, resulting in increased branching, increased silique numbers and a 30% increase in seed yield (Heyer et al. 2004). In contrast, the overexpression of *CI* under the control of the same promoter had an antagonistic effect on these same traits, and resulted in delayed flowering, decreased branching, and decreased seed yield (Heyer et al. 2004). Although invertase overexpression has been shown to affect plant stature, architecture, and reproductive potential, the relationship between invertase overexpression and the accumulation of vegetative biomass has not been explored.

In the present study, the effects of varied expression of yeast-derived *CI* and *AI* on parameters related to vegetative biomass in transgenic *Nicotiana tabacum* L. cv. Xanthi were examined. The *CI* and *AI* genes were expressed under the control of either a duplicated Cauliflower Mosaic Virus *35S* promoter (*2X35S*), or under the control of the *Petroselinum crispum* 4-coumarate:CoA ligase (*4CL*) promoter, which directs xylem-abundant transgene expression in vegetative tissues (Hauffe et al. 1991). The effects of these constructs on biomass, stem diameter and soluble and structural carbohydrate were examined in transgenic plants.

2.2 Materials and methods

2.2.1 Plasmid construction

The tandem *35S*-*35S* promoter (Kay et al. 1987) fused with an alfalfa mosaic virus untranslated leader sequence (*2X35S*; Datla et al. 1993), and the 4CL-1 promoter (*4CL*; Hauffe et al. 1991), were excised from pBI-426 and p99-G1-801, respectively, as *Hin*dIII-*Bam*HI fragments, and ligated into the multicloning site of pCAMBIA1390 (CAMBIA, Canberra, Australia); creating pSM-1 and pSM-2.

The yeast-derived apoplastic invertase (*AI*) and cytosolic invertase (*CI*) genes (von Schaewen et al. 1990, Sonnewald et al. 1991), each with an adjacent NOS terminator, were amplified from a pBINPLUS derivative using site-directed mutagenesis to create *Bgl*II sites at the 5′ termini. The amplicons were subsequently digested with *Bgl*II and *Eco*RI and ligated into the binary vectors pSM-1 and pSM-2, creating *Bam*HI/*Bgl*II fusion sites with the *2X35S* and *4CL* promoters. The binary vectors containing *2X35S::AI, 2X35S::CI*, *4CL::AI* and *4CL::CI* were confirmed by sequence analysis.

2.2.2 Tissue culture

Tobacco shoot cultures were maintained on MS media (Mirashige and Skoog 1962) solidified with agar (0.3% w/v) and phytagel (0.1% w/v), and supplemented with 3% sucrose (MS 3%). Shoot cultures were grown in GA-7 magenta vessels at a constant 25°C under 16 h days with an average photon flux density of 50 µmol m^{-2} s^{-1}. Plants were maintained by transferring nodes and apical regions at 4 week intervals.

2.2.3 Plant transformation

Agrobacterium tumefaciens EHA105 (Hood et al. 1993) was transformed with the binary plasmids using a traditional freeze-thaw method. Cultures were grown overnight at 225 rpm and 37°C in Lauria-Bertani media containing 50 µg mL^{-1} of kanamycin. An aliquot of the overnight suspension was transferred to liquid MS 3% supplemented with 100 µM acetosyringone and incubated at 225 rpm and 28°C for 2 days prior to the transformation.

Leaf discs were cut from the upper four leaves (> 2 cm) of tobacco shoot cultures 21 days after propagation using a number-4 cork borer. Leaf discs were added to 50 mL suspensions of liquid MS 3% containing EHA105 harbouring the plasmid of interest. Discs were incubated at 100 rpm and 28°C for approximately 30 min, then removed from the bacterial suspension, dried by blotting on sterile filter paper and placed abaxial side up on solid MS 3%-SIM (0.1 µM naphthalene acetic acid - NAA, 0.1 µM benzyladenine – BA, 0.1 µM thidiazuron) plates and supplemented with hygromycin (20 µg mL^{-1}). The MS 3%-SIM plates containing the leaf discs were placed in the dark at 25°C for 3 days, and subsequently transferred to fresh MS 3%-SIM plates containing carbenicillin (500 µg mL^{-1}), cefotaxime (250 µg mL^{-1}) and hygromycin (20 µg mL^{-1}). Healthy shoots (one per leaf disc) were excised and placed on solid MS

3% supplemented with 0.1 µM of BA, carbenicillin (500 µg mL^{-1}) and cefotaxime (250 µg mL^{-1}) for 3–4 weeks before being transferred to solid MS 3% with 0.1 µM of NAA for an additional 3–4 weeks. Regenerated shoots were confirmed as transformants by genomic PCR screening using the gene-specific oligonucleotides MP−7 (5′-gggccatgctacttccgatgatttgac-3′) and MP-8 (5′-agtagcaaaacgagaccagggaccagc-3′). The developing shoots were transferred in tissue culture to solid MS 3% with hygromycin (20 µg mL^{-1}) for root initiation, and then subsequently transferred to solid MS 3%.

2.2.4 Soluble carbohydrate quantification

Mature tissue from greenhouse-grown plants was lyophilized for 24 h prior to being ground with a mortar and pestle in liquid nitrogen. Approximately 50 mg of ground tissue was incubated for 16 h with 4 mL of methanol:chloroform:water (12:5:3) prior to centrifugation for 10 min at 6000 rpm and 4°C. The supernatant was collected and the pellet was twice washed and centrifuged as before with an additional 4 mL of methanol:chloroform:water. The supernatants were combined (12 mL) and phases were separated by adding 5 mL of water and centrifuging for 4 min at 4000 rpm and 4°C. An aliquot of the polar phase containing soluble sugars was vacuum centrifuged to dryness, resuspended with water and passed through a 0.45 µm filter. Soluble sugars along with fucose (internal control) were resolved with a CarboPac PA1 column and a DX-600 anion-exchange HPLC (Dionex, Sunnyvale, CA) using a 1 mL min^{-1} flowrate and an isocratic wash of 10% 1 M NaOH.

2.2.5 RNA isolation, cDNA synthesis and real-time RT-PCR

Approximately 0.5 g of fresh frozen tissue was ground in liquid nitrogen with a chilled mortar and pestle and total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A 10 µg aliquot of RNA was treated with TURBO DNase™ (Ambion, Austin, TX) to remove residual DNA. cDNA was generated from 1 µg of DNase-treated RNA using the Superscript II RT system with dT$_{16}$ oligonucleotides (Invitrogen).

Critical threshold (ct) values for *CI*, *AI* and *ACTIN-9* were quantified in triplicate with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and an Mx3000P Real-Time PCR System (Stratagene). The forward and reverse primers for RT-PCR analysis of the yeast-derived invertase isogenes were INV-RTF (5′-TATTGGGTCATGTTTATTTCTATC-3′) and INV-RTR (5′-TCCTTACCAAAATCTACCAC

TC-3′), respectively. Primers for *ACTIN-9* were as previously described (Volkov et al. 2003). Thermocycler conditions for all reactions were: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 30 s. Relative expression was determined according to Levy et al. (2004) using the following equation: $\Delta ct = 2^{-(ctCI \text{ or } AI - ctACTIN-9)}$.

2.2.6 Acid invertase activity

Total protein was isolated from the lower leaves of mature greenhouse-grown tobacco and was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Acid invertase was assayed according to a modified method of Pelleschi et al. (1997). Briefly, 100 mg of tissue was homogenized at 4.5 m s^{-1} for 45 s using a FastPrep® Instrument (Qbiogene, Carlsbad, CA) with Lysing Matrix D (Qbiogene) in 700 µL of an extraction buffer containing 50 mM HEPES-NaOH (pH 7.0), 10 mM $MgCl_2$, 1 mM Na_2EDTA, 2.6 mM DTT, 10% ethylene glycol and 0.02% Triton X-100. After centrifugation at 13000 rpm for 6 min at 4°C, desalting of the supernatant was performed using a 10 DG Desalting Column (Bio-Rad) pre-equilibrated with a stabilization buffer containing 50 mM HEPES-NaOH (pH 7.0), 10 mM $MgCl_2$, 1 mM Na_2EDTA and 2.6 mM DTT.

The assay consisted of 250 µL of desalted extract with 250 µL of 0.2 M sodium acetate (pH 4.8) and 100 µL of 0.6 M sucrose. The solution was incubated for 4 h at 30°C before termination by incubating at 95°C for 15 min. The glucose liberated from the invertase reaction was quantified using a CarboPac MA1 column and a DX-600 anion-exchange HPLC (Dionex) with an isocratic elution of 300 mM NaOH at a flow rate of 0.4 mL min^{-1}.

2.2.7 SDS-PAGE and Western blotting

Total protein was extracted from approximately 0.3 g of newly emerged tissue-cultured leaves in an extraction buffer (50 mM Tris–HCl pH 6.5, 1 mM $Na_2EDTA \cdot H_2O$, 100 mM NaCl and 0.1% Triton X-100) using a FastPrep® Instrument (Qbiogene) at 6.0 m s^{-1} for 40 s with Lysing Matrix D (Qbiogene). The sample was centrifuged for 2 min at 13000 rpm and the supernatant containing soluble proteins was collected. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Total protein (20 µg/well) was separated on a 10% polyacrylamide gel before being transferred to a 0.45 µm nitrocellulose membrane using a Trans-Blot SD cell (Bio-Rad). The membrane

was blocked and then treated with a 1:350 dilution of a custom anti-invertase antibody from rabbit (ImmuneChem Pharmaceutical Inc., Burnaby, BC Canada) using standard procedures. This was followed by incubation with a 1:10000 dilution of a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) before visualization using a coupled BCIP/NBT staining system (Sigma-Aldrich).

2.2.8 Greenhouse analysis

Plants were transferred from tissue culture conditions to 7.5 L pots in perennial soil (50% peat, 25% fine bark and 25% pumice; pH 6.0), acclimated for 5 days under 500 mL clear plastic cups (to initiate new growth), and were subsequently watered uniformly on flood tables in a greenhouse. Plants were grown under 18 h days and supplemented with overhead lighting when radiant flux density was less than 300 W m^{-2}. Height, stem diameter, fresh weight and biomass were recorded at maturity (onset of flower bud formation). Plant height was measured from the flowering apex to the base as early as possible after the flowering apex was visible. Stem diameter was measured below node 5 (node 0 was the first leaf with length > 7 cm). The leaves and stems (5 cm above soil level) were harvested, weighed immediately (fresh weight) and dried at 105°C for 48 h to determine biomass. Each primary transformant was represented by 8–14 clonally propagated plants.

2.2.9 Starch determination

Stem tissue (nodes 10–11) from mature tobacco was dried for 48 h and ground with a Wiley mill (30-mesh screen). Approximately 50 mg per sample was incubated for 24 h at −20°C with 4 mL of methanol:chloroform:water (M:C:W; 12:5:3). The samples were centrifuged for 10 min at 6000 rpm and 4°C, and the supernatant was discarded. The pellet was washed with an additional 8 mL of M:C:W, centrifuged for an additional 10 min at 6000 rpm and 4°C, and the supernatant was discarded. The pellet was dried at 55°C for 24 h before 5 mL of 4% H_2SO_4 was added. The solution was autoclaved at 121°C for 3.5 min, cooled and vortexed briefly before centrifuging for 10 min at 300 rpm and 4°C. The supernatant was analysed using HPLC and the quantity of starch was calculated as the quantity of glucose hydrolysed by the weak acid.

2.2.10 Structural carbohydrate and lignin determination

Acid soluble lignin, insoluble lignin and structural carbohydrates were determined using a modified Tappi method UM-250 (Cullis et al. 2004). Stems were harvested from

mature tobacco plants and dried at 105°C for 2 days prior to grinding nodes 5–9 with a Wiley mill using a 30-mesh screen. Extractives were removed through acetone extraction for 24 h using a soxhlet apparatus. The remaining tissue was dried for 24 h at 55°C before approximately 200 mg of tissue was taken for further analysis. The tissue was macerated at regular intervals in 3 mL of 72% H_2SO_4 for 2 h at 25°C. The acid solution was diluted to 4%, autoclaved at 121°C for 1 h, cooled and filtered through medium-coarse sintered glass crucibles. The retentate was rinsed with distilled water and dried at 105°C for 24 h to determine the amount of acid insoluble lignin. An aliquot of the filtrate was analysed at 205 nm using a UV spectrophotometer, and the amount of soluble lignin was determined using Beer's Law with an absorptivity constant of 110 L g^{-1} cm^{-1}.

The concentration of structural monosaccharides was determined by HPLC analysis of the filtrate. All samples were filtered through a 0.45 µm nylon filter prior to analysis. Glucose, xylose, mannose, galactose, arabinose and rhamnose were analysed using a DX-600 anion-exchange HPLC (Dionex) on a CarboPac PA1 column (Dionex). Sugar concentrations were determined using regression equations from calibration curves that were derived from external standards. In all cases fucose served as an internal standard. Cellulose was determined as the percentage of glucose per mass of dried tissue less the previously determined glucose percentage due to starch.

2.2.11 Statistical analysis

Data were analysed using unpaired two-tailed t-tests assuming unequal variances at the 95% confidence level.

2.3 Results

2.3.1 Transformations and tissue culture

Independent transformed lines were regenerated for each construct (*2X35S::AI*, *2X35S::C*I, *4CL::AI* and *4CL::CI*) using an *Agrobacterium*-mediated transformation protocol. Although we generated lines harbouring each of the constructs, the *4CL::CI* transformants had reduced growth rates (approximately 0.5 cm/month), and could not be efficiently propagated under tissue culture conditions; therefore, these transformants could not be examined further. Of the three *4CL::AI* transformed lines that could be recovered, two lines (*4CL::AI*-2 and -3) also had severely impaired growth (with and

without subdued lighting). Similarly, the propagation efficiency of *2X35S::CI*-4, *2X35S::CI*-5 and *2X35S::AI*-2 was decreased compared to wild-type. The remaining transformed lines (five lines of *2X35S::CI*, two lines of *2X35S::AI* and one line of *4CL::AI*) had similar growth rates relative to wild-type (WT) plants under standard growth conditions. Generally, there were no changes in the visible phenotype (i.e. leaf morphology, architecture, size) of the *2X35S::CI* and *2X35S::AI* transformants in tissue culture. The *4CL::CI*-1 line was the exception, which displayed reduced growth and substantial curling and chlorosis of the leaves at the base of the plant.

2.3.2 Transcript, activity and Western blot analysis

Transcript abundance of the invertase isogenes was examined in both leaf and stem tissue of mature greenhouse-grown plants using real-time RT-PCR (Figure 2.2). As expected with a heterologous transgene, all transformed lines had much higher transcript levels than the corresponding WT plants, which were similar to negative controls with no cDNA in the reaction. Relative to the other transgenic lines, *4CL::AI*-2 and *4CL::AI*-3 showed the highest transcript abundance in both upper and lower leaves (at nodes 3 and 15, respectively). The transcript level of *4CL::AI*-2 was almost 20-fold higher in the upper leaves and over 30-fold higher in the lower leaves than the highest *CI* transgenic. Furthermore, *4CL::AI*-2 had 3.5-fold (upper leaves) and 12-fold (lower leaves) greater transcript level than the next highest *4CL* expressor (*4CL::AI*-3). In contrast, the lines with the weakest transcript levels were *2X35S::CI*-1, *2X35S::CI*-5 and *2X35S::AI*-1.

In general, stem tissue had lower levels of the yeast-derived invertase transcripts (per µg total RNA) when compared to leaf tissue, however, the overall trend of expression was similar between lines: *4CL::AI*-2 and *4CL::AI*-3 had the highest expression levels, while *2X35S::CI*-1, *2X35S::CI*-5 and *2X35S::AI*-1 had the lowest. Interestingly, *2X35S*::CI-5 had no detectable transcripts in either the upper (internode 3) or lower (internode 15) stem tissue. This trend was also observed with young stem tissue of the same line in tissue culture (data not shown).

Western blots using an antibody raised against yeast invertase revealed a strong signal at approximately 65 kDa for *4CL::AI*-2 and *4CL::AI*-3 leaf protein extracts (Figure 2.3). These lines also had the highest transcript levels of all the transgenic plants examined. Protein that cross-reacted with the yeast invertase antibody was more

difficult to detect in the other transgenic lines, illustrating the lower levels of foreign invertase in these plants with respect to total protein level. In addition, activity assays of total acid invertase from the lower mature leaves of these relatively low-expressing lines generally did not show any statistical differences from WT activity, except *2X35S::AI*-2 (Table 2.1). The lines *4CL::AI*-2 and *4CL::AI*-3 had the highest levels of enzyme activity, which is consistent with the transcript and Western blot analyses of these same lines.

2.3.3 Soluble carbohydrate analysis

Total soluble carbohydrates (glucose, fructose and sucrose) of the transgenic tissues were quantified from upper and lower leaves (numbers 3 and 15, respectively) and upper and lower stems (internodes 3 and 15, respectively) of two separate sets of mature tobacco grown over two different growth periods in a greenhouse (Table 2.1). Sucrose, the predominant soluble carbohydrate in tobacco leaf tissue, exhibited the most variation in the upper leaves with five of the ten transformants having significantly reduced sucrose levels. One line (*2X35S::AI*-1) displayed an opposite trend, with a significant increase in sucrose within the upper leaf. The lower leaf showed no such increase in sucrose for this same line, however. Those lines exhibiting a reduction in upper leaf sucrose level (*2X35S::CI*-3, *2X35S::CI*-4, *2X35S::AI*-2, *4CL::AI*-1 and *4CL::AI*-3) also showed a significant decrease of the carbohydrate in the lower leaf with the exception of *4CL::AI*-1. In contrast, previous studies revealed an increase in sucrose levels in the leaves of transgenic tobacco (von Schaewen et al. 1990, Sonnewald et al. 1991). In both experiments, it was speculated that the accumulation of sucrose may be attributed to the direct inhibition of sucrose transport by the increased invertase activity.

Generally, stem tissue showed little or no change in sucrose levels, however, *2X35S::CI*-2, *2X35S::CI*-3 and *4CL::AI*-1 did show significant decreases in glucose and fructose concentration. Two additional lines, *2X35S::AI*-2 and *2X35S::CI*-3, also had reduced fructose levels, although these lines did not show a significant difference in the level of glucose. In general, there were less differences detected in the lower stem carbohydrate levels, with the exception of *4CL::AI*-2 and *4CL::AI*-3. Both of these lines had severely reduced sucrose levels ($P < 0.01$), with *4CL::AI*-2 also exhibiting severely reduced levels of glucose and fructose. It should be noted that the transgenic lines with the lowest abundance of yeast invertase transcript (*2X35S::CI*-1 and *2X35S::CI*-5)

showed no changes in carbohydrate level in any tissue examined. These two lines also had a phenotype that most closely resembled that of WT. No significant changes to either glucose or fructose in the upper leaves of most translines were evident, with the exception of *2X35S::AI*-1, which had an increase in both hexoses. The lower leaves generally had a slight reduction in the hexose levels, with a significant decrease in fructose level in the lower leaves of *2X35S::CI*-3 and *2X35S::CI*-4. In contrast, *4CL::AI*-2 showed a significant increase in fructose level, while the glucose level of this same transline was comparable to WT plants. These results contrast those of similar studies involving the overexpression of CI or AI in tobacco (von Schaewen et al. 1990, Sonnewald et al. 1991). In these experiments, lines expressing the transgenes accumulated hexoses in leaf tissue. Furthermore, these studies showed that in most cases fructose levels were higher or approximately equivalent to glucose levels, while in the present experiment, fructose levels were generally lower than glucose levels.

The hexose levels of the upper stems were also reduced with some lines showing a significant decrease in glucose (*2X35S::CI*-2, *2X35S::CI*-4 and *4CL::AI*-1) and fructose (*2X35S::CI*-2, *2X35S::CI*-3, *2X35S::CI*-4, *2X35S::AI*-2 and *4CL::AI*-1). The *2X35S::AI*-1 line showed an increasing level of glucose, although not statistically significant. Similar to the upper stems, the lower stems exhibited slightly reduced or unchanged levels of hexoses, with a significant reduction in fructose shown in *2X35S::CI*-3 and *4CL::AI*-1. The highest expressing line (*4CL::AI*-2) had a dramatic reduction in both glucose and fructose in the lower stems.

2.3.4 Phenotype of mature plants

In an attempt to normalize growth and biomass analyses, plastichron indices (leaf zero represented the first leaf greater than 7 cm in length from base to tip) were used to standardize developmental stages sampled from mature (onset of bud formation) transgenic and WT lines. Plant height, stem diameter (measured by caliper between nodes 5 and 6), whole-plant fresh weight and whole-plant biomass were subsequently evaluated (Figure 2.4). Fresh weight and biomass showed identical trends (data not shown). Four of the five *2X35S::CI* lines had a significant reduction in height (Figure 2.4a). In contrast, *2X35S::CI*-1 was 15% taller than the WT. The transgenic line *2X35S::CI*-4 demonstrated the greatest reduction in stature. Similar to four of the five *2X35S::CI* lines, the *2X35S::AI* and *4CL::AI* transgenic lines were also significantly

shorter than the corresponding WT plants. *2X35S::AI*-1, *2X35S::AI*-2, *4CL::AI*-2 and *4CL::AI*-3 were the shortest lines examined, having heights less than 80% of WT. In all invertase overexpressing lines, the difference in height was attributed to a change in internode length, rather than a change in the number of nodes per plant (data not shown). This phenotype was most dramatic in the *4CL::AI*-2 and *4CL::AI*-3 lines, where the internodes were essentially non-existent in some parts of the plant.

The diameter of one line (*2X35S::AI*-1) was 40% greater than that of the WT plants (Figures 2.4b, 2.5). Despite this increase in stem thickness, the dry weight of the total stem was not significantly different from the WT (data not shown), which is attributable to the corresponding decrease in stem length in this same transformed line. Stem diameter was also significantly reduced in *4CL::AI*-2 and *4CL::AI*-3, with a decrease of 12.1 and 17.2%, respectively.

Three of the transformed lines had significant increases in biomass (Figure 2.4c) compared to the WT; *2X35S::AI*-1 (44%), *2X35S::CI*-1 (20%), and *2X35S::CI*-5 (14%). The increased biomass of *2X35S::CI*-1 could be explained by the corresponding increase in height, unlike *2X35S::AI*-1, which had the most significant increase in biomass despite being 24% shorter than the WT. When considering both parameters, the biomass/height ratio of *2X35S::AI*-1 was 88% greater than the corresponding WT. Similar to *2X35S::AI*-1, the *2X35S::CI*-5 transformed line showed an increase in biomass despite a decrease in height, but to a lesser extent (biomass/height increased by 21%). In contrast to these transformed lines, the shortest plants (*2X35S::CI*-4, *2X35S::AI*-2, *4CL::AI*-2 and *4CL::AI*-3) had associated decreases in biomass.

2.3.5 Chemical analysis of stem tissue

Starch, structural carbohydrates, and lignin composition were determined from extractive-free dried stem tissue of mature tobacco to investigate the effects of the transgenes on carbohydrate-derived polymer production. The content of cellulose, starch, lignin (soluble and insoluble) and hemicellulose (arabinose, rhamnose, galactose, xylose and mannose) as a percentage of dry mass of both WT and transformed lines is shown in Table 2.3. Three different growth periods were used with three separate groups of plants to account for both differing growth and propagation rates in tissue culture and greenhouse availability.

The *2X35S::CI* lines generally showed very few significant differences from WT with respect to structural chemistry of the stem tissue. However, the lines expressing AI showed considerably more variation. *4CL::AI*-3 showed a severe reduction in cellulose content (28%), along with a decrease in starch and hemicellulose (specifically, xylose and mannose). *4CL::AI*-2 showed similar trends as *4CL::AI*-3, but to a lesser extent. In contrast, *2X35S::AI*-2 had a substantial increase in cellulose content (36%) along with all five of the primarily associated hemicellulose sugars (arabinose, rhamnose, galactose, xylose and mannose). Further evidence of altered stem tissue chemistry between *2X35S::AI*-2 and WT may be the visible difference between extractive-free tissue, with *2X35S::AI*-2 being noticeably lighter in colour than the WT tissue (Figure 2.6). Visible colour differences between extractive-free tissue from WT and other transformed lines were not observed, which is consistent with the comparable concentrations of cellulose among these plants.

2.4 Discussion

The effects of constitutive and xylem-localized heterologous expression of two yeast-derived invertase transgenes (*AI* and *CI*) under the control of a *2X35S* promoter or a parsley *4CL* promoter were investigated in tobacco. Several of the transgenic lines in this study exhibited such impaired growth that they could not be propagated beyond tissue culture. Given this result, the 'lower than normal' transformation efficiency in this study is attributed to a high incidence of detrimental phenotypes arising from the overexpression of the invertase transgenes.

The *4CL* promoter previously shown to direct the expression of a reporter gene to xylem vessels and tracheary elements in transgenic tobacco plants (Hauffe et al. 1991), was used to direct invertase expression in these same cells. With the exception of three *4CL::AI*-1 transformed lines, developing shoots from leaf discs transformed with either *4CL::CI* and *4CL::AI* showed signs of severe growth inhibition, particularly with respect to stem elongation. Two of the analysed transformants, *4CL::AI*-2 and *4CL::AI*-3, had very short internode distances in both tissue culture and mature greenhouse-grown plants. Similar trends were observed previously with tissue-cultured tobacco expressing yeast invertase (vacuolar, apoplastic and cytosolic) under the control of the Cauliflower Mosaic Virus *35S* (*35S*) promoter (Sonnewald et al. 1991). In addition to

severe dwarfing, *4CL::CI* transformants could not be effectively propagated in tissue culture. This is consistent with the results of Sonnewald et al. (1991) who found that overexpression of *CI* under the control of the *35S* promoter in tobacco resulted in plants that were generally more growth impaired than were plants overexpressing *AI*. The two *4CL::CI* transformants that did survive in this study had to be maintained under subdued lighting (average photon flux density of 2.1 µmol m^{-2} s^{-1}) on media supplemented with both auxin and cytokinin. Subdued lighting appears to minimize light stress on plants exhibiting growth inhibition by transgene expression, and was similarly required for the growth of potato expressing *CI* under the control of the companion cell-specific *rolC* promoter in tissue culture (Bussis et al. 1997). In the present study, two slow-growing *4CL::AI* transformants (2 and 3) also required subdued lighting in tissue culture to sustain growth. Aside from the reduced growth rates, three of the four slow-growing *4CL* transformants otherwise appeared healthy. One transformant, *4CL::CI-1*, was chlorotic, and displayed curling of the leaves at the base of the plant, which is consistent with a similar phenotype described for transgenic tobacco overexpressing yeast *CI* under the control of the *35S* promoter (Sonnewald et al. 1991).

In contrast to plants transformed with invertase under the control of *4CL*, plants expressing *CI* or *AI* under the control of *2X35S* generally displayed phenotypes comparable to WT plants in tissue culture, and could be propagated in vitro for further analyses. One explanation for this trend is that the tissues targeted by the *4CL* promoter in young tobacco are sensitive to increased cytosolic invertase levels. This finding suggests that the flux of soluble sugars in regions targeted by the *4CL* promoter, during the lignifying stages of cell wall development, is important for early growth; although it is not clear whether this is due to a direct effect, through the hydrolysis of sucrose, or an indirect effect, such as altered carbohydrate signalling or osmotic balance. It has been suggested that reduced rates of photosynthesis and growth may be due to altered osmotic conditions in potato plants overexpressing invertases (Heineke et al. 1992, Tauberger et al. 1999). However, a similar study in tobacco suggested that a reduction in photosynthetic capacity and growth was attributable to the accumulation of specific metabolites rather than to a disturbance in osmolarity (Heineke et al. 1994).

Real-time RT-PCR analyses of leaf and stem tissue from mature plants revealed similar transcript levels for both the *2X35S* and *4CL* promoters. This finding was unexpected for two reasons: (1) *2X35S* was previously found to drive reporter gene transcript levels higher than a single *35S* promoter in transgenic tobacco (Datla et al. 1993), and (2) the *4CL* promoter was found to direct low levels of reporter gene expression in the xylem of axillary buds and developing leaf veins of young tobacco (Hauffe et al. 1991). The transcript data from this study suggests that either the translines that could effectively be propagated represent "lower" expressors while active overexpression may be lethal, that the *2X35S* promoter does not lead to significant overexpression as expected, or the *4CL* promoter is directing higher levels of transcript than expected from previous studies. We suggest that the first explanation is more likely as the efficacy of positive invertase transformation selection was extremely low in comparison to control transformations.

With the *2X35S* promoter directing expression levels similar to the *4CL* promoter (based on real-time RT-PCR analyses), it is not surprising that similar levels of enzyme activity were detected between WT and transgenic lines. Strong phenotypes in both tissue culture (e.g. *4CL::CI*-1) and greenhouse conditions (e.g. *2X35S::AI*-1) suggest that modest levels of heterologous invertase expression may be sufficient to alter the WT phenotype. These findings are not unique to this study as significant differences in invertase activity were not detected in leaf mid ribs of *Solanum tuberosum* between a line expressing *CI* under the control of a companion cell-specific promoter (*rolC*) and WT plants (Zuther et al. 2004). Thus, it is plausible that the tissue-specific *4CL* promoter and the *2X35S* promoter lead to levels of heterologous invertase activity that are difficult to detect above background activity of native invertase, yet allow sufficient expression to generate phenotypes that differ from WT tobacco. Western blot analysis of total protein from leaves of transgenic tobacco revealed strongest signals for *4CL::AI-2* and *4CL::AI*-3, which directly corresponds to the transcript, enzyme activity and growth data (most retarded in overall growth), further suggesting a modest level of expression of the yeast-derived invertases in the other lines in this study. The sizes and pattern of the protein detected agree with previous Western blot analysis of yeast-derived AI (von Schaewen et al. 1990).

Significant differences in stem diameter, height and biomass were observed in select transformed lines relative to WT plants. The difference in height between all of the transformants was attributable to internode length rather than the number of nodes, which is consistent with previous findings when invertase transgenes were overexpressed under the control of the *35S* promoter (von Schaewen et al. 1990, Sonnewald et al. 1991). The height of some lines (*2X35S::CI*-2, *2X35S::CI*-4, *2X35S::AI*-1, *2X35S::AI*-2, *4CL::AI*-2 and *4CL::AI*-3) was less than 80% of the WT, which is similar to a phenotype of potato plants expressing either yeast-derived vacuolar, apoplastic and cytosolic invertase under the control of the *rolC* promoter (Bussis et al. 1997). Despite the reduction in plant height, two lines, *2X35S::CI*-5 and *2X35S::AI*-1, exhibited large increases in total biomass. In one of these lines (*2X35S::AI*-1), the biomass/height ratio was almost twofold higher than the WT. Such differences were visually obvious in the mature plants, as the stems of *2X35S::AI*-1 plants were 40% thicker than those of the corresponding WT plants (Figure 2.5). Interestingly, this transformant also had increased soluble sucrose levels ($P = 0.001$) in young leaves of mature greenhouse-grown plants. Such an anomaly may, in part, explain the drastically unique phenotype observed with *2X35S::AI*-1, as none of the other transformants displayed any increase in any of the three soluble carbohydrates in any tissue examined. Plants from the other *2X35S::AI* transformed line (*2X35S::AI*-2) did not demonstrate such dramatic increases in sucrose concentration in the leaves, nor did they show increases in stem diameter, biomass or biomass/height ratio.

The soluble carbohydrate and transcript data may partially explain the observed changes in morphology of the *2X35S::CI* transformed lines as well. For instance, the *2X35S::CI*-1 line was significantly taller than the WT, and had relatively lower transcript levels of *CI* in both leaves and stem compared to the other transformed lines. Similarly, the mature plant height of the other transformed line with low transgene transcript abundance (*2X35S::CI*-5) was very similar to the WT. In addition, both *2X35S::CI*-1 and *2X35S::CI*-5 showed no significant changes to glucose, fructose or sucrose content in any of the tissues examined from mature greenhouse-grown plants. By comparison, the shortest line (*2X35S::CI*-4) had the highest abundance of *CI* transcript in leaf tissue, and also had significant reductions in soluble carbohydrates in upper and lower leaves and upper stem tissue. The shortest *4CL::AI* lines (-2 and -3) also had very high leaf

transcript levels and showed substantially reduced levels of hexoses and sucrose in the lower stem tissue. An inverse relationship between foreign invertase expression in leaf tissue and height was also observed in transgenic tomato (*Lycopersicon esculentum*) expressing an *AI* under the control of the *35S* promoter, where growth inhibition was most severe in transformants with the highest level of *AI* transcript (Dickinson et al. 1991). Leaf morphology was also visibly different in the transgenic tomato lines expressing *AI* relative to WT plants. Similarly, in the current study *2X35S::AI*-1 leaves were darker and narrower relative to those of the WT; however, none of the other transformed lines, including the other *2X35S::AI* transformant, had visible differences in leaf morphology. The notable increase in fructose and sucrose concentration in the young leaves of *2X35S::AI*-1 may, in part, explain this observed phenotype, as the altered leaf morphology was more prominent in younger leaves.

In addition to changes in properties such as height, biomass and stem diameter, significant changes to the structural chemistry of the stem tissue from mature greenhouse-grown lines were also detected. *2X35S::AI*-2 showed a dramatic increase in cellulose content. The concomitant increase in hemicellulose sugars (important for the cellulose framework) is further evidence for an increase in cellulose content. Additionally, the colour of the extractive-free stem tissue from *2X35S::AI*-2 was visibly lighter than that of the other transformed lines and WT. It is highly likely that the observed colour difference of the extractive-free tissue is due to the increase in cellulose, as the percentage of both soluble and insoluble lignin was unchanged. In contrast, stems tissue from *4CL::AI*-3 and *4CL::AI*-2 showed opposing results, as a significant decrease in cellulose content and the associated hemicellulose sugars, xylose and mannose, was observed.

Transgenic lines expressing *CI* generally showed no significant changes to structural chemistry of stem tissue, while lines expressing *AI* showed a greater number of differences compared to WT with respect to cellulose, hemicellulose, lignin and starch content. This corresponds well to existing theory, as an apoplastic invertase should have the greatest effect on cell-wall (structural) chemistry, with CI having less of a direct impact on cell-wall composition. However, despite the apparent lack of effect of CI on cell-wall chemistry in this experiment, it is clear that the expression of a foreign

CI, and indeed an AI, can alter the general growth characteristics of tobacco, along with the soluble carbohydrate status of the leaf and stem tissue.

In summary, we have shown that it is possible to significantly alter biomass, stem diameter and structural chemistry through the expression of apoplastic and cytosolic yeast-derived invertases in tobacco using either the *2X35S* or *4CL* promoter. In some cases significant increases in biomass and cellulose content were achieved. Such increases in biomass have rarely been reported (Heyer et al. 2004), and to our knowledge such changes in structural carbohydrate are unique to this study. These findings are encouraging for crop improvement programs aimed at increasing biomass or cellulose production. Specifically increasing biomass and stem diameter, while concurrently suppressing height, could generate desirable traits, especially in trees, which may have widespread commercial and industrial applications (Campbell et al. 2003). Similarly, an increase in cellulose content and/or changes to tissue colour in tree species could have implications for the pulp and paper industry.
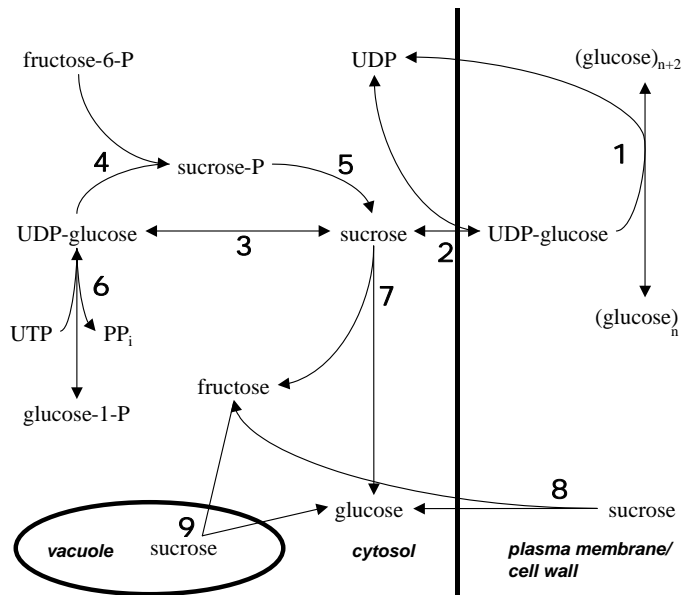
Figure 2.1. Model of the interactions between the following major enzymes in the putative cellulose biosynthetic pathway: (1) cellulose synthase complex, (2) particulate sucrose synthase, (3) soluble sucrose synthase, (4) sucrose phosphate synthase, (5) sucrose phosphate phosphatase, (6) UDP-glucose pyrophosphorylase, (7) cytosolic invertase, (8) cell-wall (apoplastic) invertase and (9) vacuolar invertase. Adapted from Haigler et al. (2001) and Delmer and Haigler (2002).
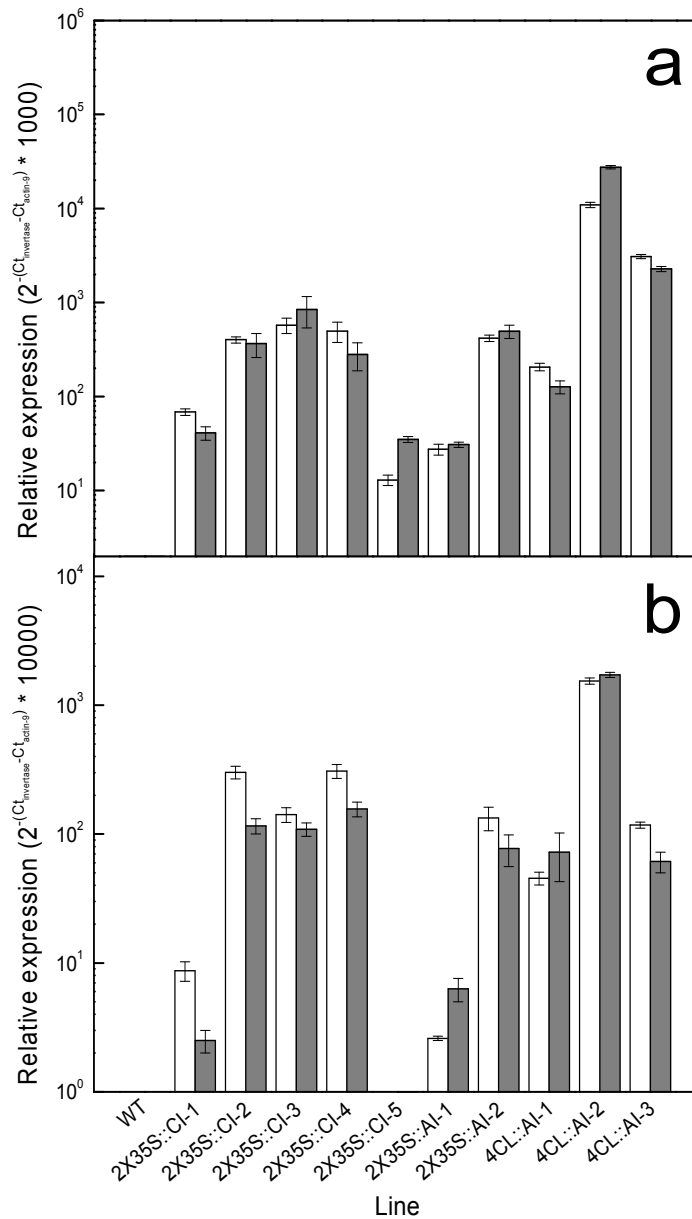
Figure 2.2. Real-time RT-PCR analysis of wild-type (WT) and transgenic tobacco depicting the number of transcripts of either *CI* or *AI* in upper (open bars) and lower (closed bars) leaf tissue (a) and upper (open bars) and lower (closed bars) stem tissue (b). Data are expressed as the difference between *CI* or *AI* and *ACTIN-9* critical threshold (ct) values. Error bars represent the standard deviation (n=3).
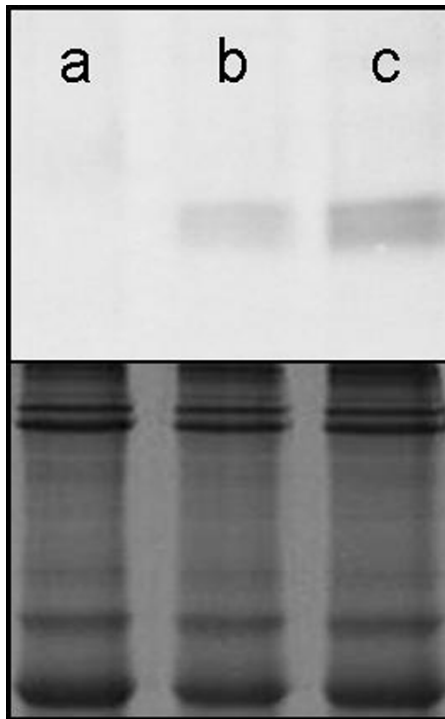
Figure 2.3. Western blot (top) of total protein extracted from wild-type (a), *4CL::AI*-2 (b) and *4CL::AI*-3 (c) tobacco leaves. The corresponding segment of a 10% polyacrylamide gel visualized using Coomassie R-250 stain (bottom) serves as a loading control. Protein was bound to a nitrocellulose membrane and AI (~65 kDa) was detected using an NBT/BCIP system.
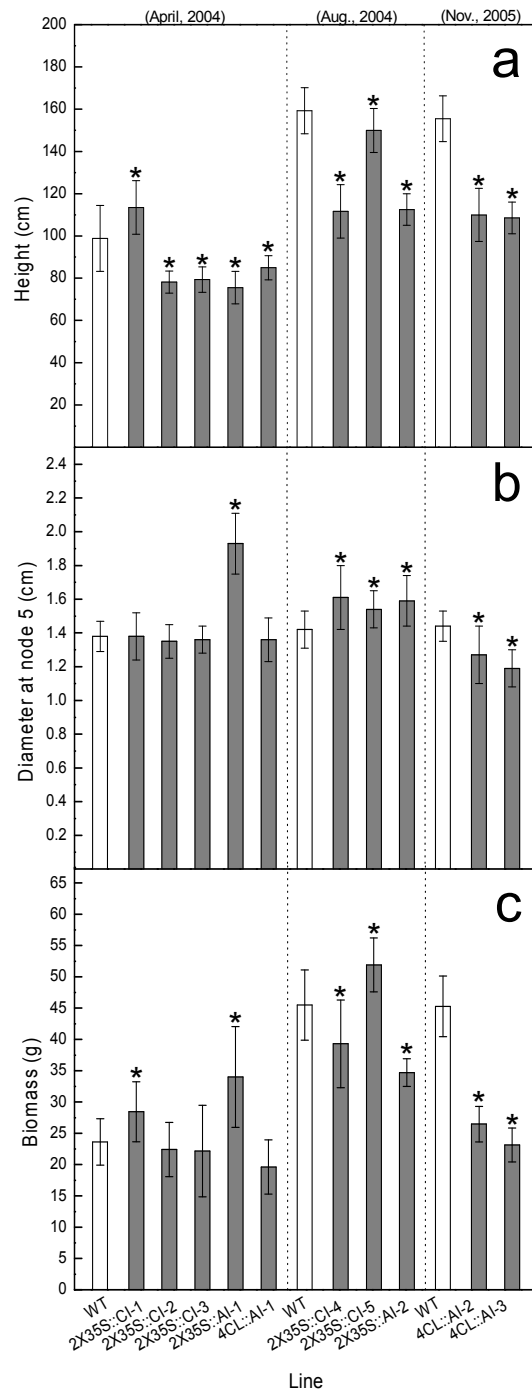
Figure 2.4. Height (a), diameter at node 5 (b) and biomass (c) of three groups of transgenic (closed bars) and wild-type (WT; open bars) tobacco at maturity for three separate growth periods. Error bars represent the standard deviation (n=8-14). Asterisks represent statistical significance at the 95% confidence level.
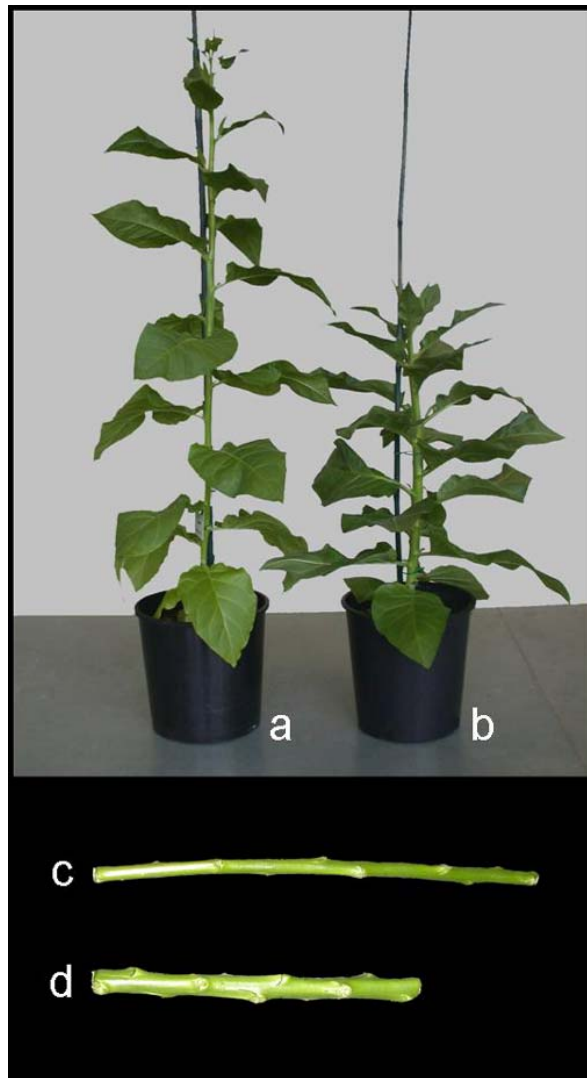
Figure 2.5. Wild-type (a) and *2X35S::AI*-1 (b) tobacco plants at maturity, and the corresponding wild-type (c) and *2X35S::AI*-1 (d) stems between nodes five and fifteen (right to left).
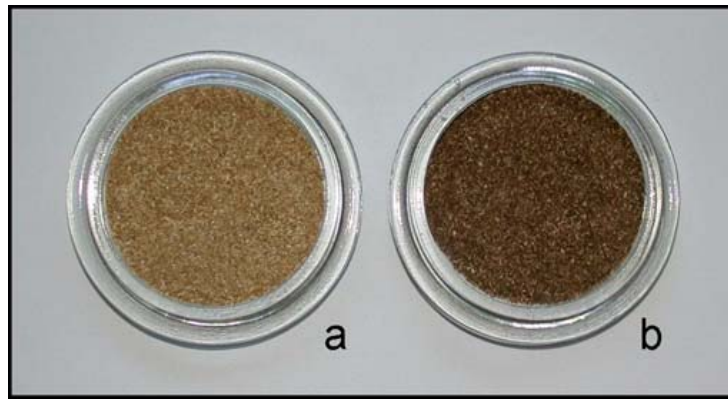
Figure 2.6. Dried, extractive-free mature stem tissue of *2X35S::Al*-2 (a) and wild-type (b) tobacco after grinding to pass a 30 μm mesh.

Table 2.1. Acid invertase activity from the lower leaf of mature greenhouse-grown transgenic and wild-type (WT) tobacco. Values represent the mean and standard deviation of three biological replicates. Units are quantity of glucose (mg) liberated by invertase per total protein (mg) added to the assay. Bold values represent a significant difference from the corresponding WT at the 95% confidence level.

Growth period	Line	Lower leaf
August, 2004	WT	5.5 ± 1.4
	2X35S::CI-1	4.2 ± 0.8
	2X35S::CI-2	6.0 ± 2.7
	2X35S::CI-3	4.7 ± 1.1
	2X35S::CI-4	3.7 ± 1.5
	2X35S::CI-5	4.2 ± 0.2
	2X35S::AI-1	6.7 ± 1.0
	2X35S::AI-2	**24.2 ± 2.4**
	4CL::AI-1	5.1 ± 1.0
November, 2004	WT	6.2 ± 1.9
	4CL::AI-2	**108.5 ± 12.5**
	4CL::AI-3	**21.4 ± 3.7**

Table 2.2. Glucose (glc), fructose (fru) and sucrose (suc) levels in different tissues of mature greenhouse-grown transgenic and wild-type (WT) tobacco. Units are mass (µg) of carbohydrate per mass (mg) of lyophilized tissue. Bold values represent a significant difference from the corresponding WT at the 95% confidence level (n=3).

Growth period	Line	Upper leaf			Lower leaf			Upper stem			Lower stem		
		Glc	fru	suc	glc	fru	suc	Glc	fru	Suc	glc	fru	suc
August, 2004	WT	2.0±0.1	0.4±0.1	7.9±0.2	3.9±1.3	2.2±0.5	6.4±0.7	27.5±3.8	18.6±2.4	8.2±0.9	18.7±1.6	14.1±0.6	10.5±2.0
	2X35S::Cl-1	2.0±0.4	0.6±0.2	6.5±0.7	2.8±0.6	1.4±0.6	4.9±0.1	19.5±4.3	15.8±4.6	7.0±2.1	18.0±2.8	11.8±2.0	8.0±1.4
	2X35S::Cl-2	1.7±0.2	0.4±0.1	5.8±1.4	2.9±0.7	0.8±0.6	4.9±0.5	**16.6±1.5**	**7.8±0.5**	8.1±1.5	17.7±2.9	10.0±1.6	8.2±0.8
	2X35S::Cl-3	2.0±0.1	0.6±0.2	**6.9±0.3**	2.0±0.3	**0.6±0.4**	**4.4±0.2**	22.5±2.3	**11.5±1.9**	6.8±1.3	12.5±3.9	**5.3±1.6**	8.0±3.4
	2X35S::Cl-4	1.8±0.4	0.5±0.3	**6.1±0.6**	1.6±0.1	**0.2±0.0**	**3.9±0.0**	**13.1±1.4**	**3.2±0.3**	7.3±1.6	16.3±3.3	9.1±3.2	8.4±2.0
	2X35S::Cl-5	2.3±0.6	0.8±0.5	7.8±0.2	4.4±1.7	2.4±1.2	6.3±0.6	28.6±4.1	18.8±1.9	7.9±0.9	22.6±1.6	16.6±1.5	9.0±1.0
	2X35S::Al-1	6.6±2.4	**2.0±0.5**	**10.4±0.3**	2.8±0.3	0.8±0.5	5.6±1.4	37.2±6.7	20.6±6.6	6.5±0.5	16.4±2.9	8.7±2.3	5.5±2.2
	2X35S::Al-2	1.9±0.2	0.9±0.5	**5.5±0.9**	2.7±0.9	1.3±0.4	**3.9±0.4**	18.8±2.9	**9.2±2.8**	4.9±2.4	20.2±1.0	10.4±2.8	7.4±0.3
	4CL::Al-1	1.5±0.3	0.2±0.1	**5.8±0.7**	1.9±0.4	0.6±0.4	4.8±1.0	**17.8±3.1**	**6.4±3.6**	8.0±0.6	16.4±0.5	**9.2±1.5**	9.0±0.2
November, 2004	WT	5.8±4.7	3.4±1.8	26.2±3.6	12.7±3.9	12.9±2.3	23.1±0.1	99.5±6.4	67.2±7.5	22.6±9.3	66.3±5.9	48.5±5.6	39.1±3.5
	4CL::Al-2	9.1±0.4	9.7±4.0	21.4±2.1	12.2±2.7	**23.4±1.5**	12.2±4.6	60.8±18.1	43.4±12.4	0.7±0.6	**8.8±5.2**	**10.0±4.4**	**0.3±0.3**
	4CL::Al-3	10.9±6.2	9.6±4.8	**14.6±1.1**	9.2±4.1	9.4±1.5	**9.3±3.9**	80.1±12.0	56.2±10.4	12.4±1.9	52.0±13.8	44.4±10.0	**15.3±3.5**

Table 2.3. Dry mass percentage of structural carbohydrates, lignin and starch of stem tissue from mature greenhouse-grown transgenic and wild-type (WT) tobacco. Values represent the mean and standard deviation (n=3). Bold values represent a statistical difference at the 95% confidence level.

Growth period	Line	Cellulose	Hemicellulose[a]	Lignin[b]	Starch
April, 2004	WT	30.9 ± 3.5	17.1 ± 1.3	21.0 ± 1.2	0.65 ± 0.07
	2X35S::CI-1	28.5 ± 4.3	15.5 ± 1.6	18.6 ± 0.7	0.59 ± 0.01
	2X35S::CI-2	32.7 ± 1.4	18.3 ± 0.5	21.7 ± 0.9	0.74 ± 0.06
	2X35S::CI-3	32.2 ± 0.7	17.8 ± 0.2	23.0 ± 1.3	0.66 ± 0.05
	2X35S::AI-1	24.0 ± 1.2	**12.7 ± 0.5**	19.0 ± 1.7	0.81 ± 0.34
	4CL::AI-1	31.4 ± 4.0	17.3 ± 1.7	20.3 ± 0.9	0.66 ± 0.03
August, 2004	WT	26.0 ± 1.5	14.2 ± 0.7	18.6 ± 1.5	0.53 ± 0.05
	2X35S::CI-4	29.2 ± 1.2	15.4 ± 0.7	19.7 ± 1.8	0.52 ± 0.03
	2X35S::CI-5	30.3 ± 2.3	**16.9 ± 1.1**	19.8 ± 0.8	0.51 ± 0.04
	2X35S::AI-2	**34.9 ± 1.4**	**18.2 ± 0.7**	18.6 ± 1.0	0.51 ± 0.07
November, 2005	WT	27.0 ± 0.8	14.0 ± 0.4	20.5 ± 1.0	1.34 ± 0.11
	4CL::AI-2	24.8 ± 2.3	**11.5 ± 0.8**	19.7 ± 1.2	**0.80 ± 0.07**
	4CL::AI-3	**19.4 ± 0.6**	**9.2 ± 0.8**	**15.4 ± 2.5**	**0.67 ± 0.06**

[a]Represents total arabinose, rhamnose, galactose, xylose and mannose monomers detected.
[b]Represents soluble and insoluble lignin.

2.5 References

Baumert A., H.P. Mock, J. Schmidt, K. Herbers, U. Sonnewald and D. Strack. 2001. Patterns of phenylpropanoids in non-inoculated and potato virus Y-inoculated leaves of transgenic tobacco plants expressing yeast-derived invertase. Phytochemistry. 56:535-541.

Bussis D., D. Heineke, U. Sonnewald, L. Willmitzer, K. Raschke and H.W. Heldt. 1997. Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol. Planta. 202:126-136.

Campbell M.M., A.M. Brunner, H.M. Jones and S.H. Strauss. 2003. Forestry's fertile crescent: The application of biotechnology to forest trees. Plant Biotech. J. 1:141-154.

Cullis I.F., J.N. Saddler and S.D. Mansfield. 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnol. Bioeng. 85:413-421.

Datla R.S.S., F. Bekkaoui, J.K. Hammerlindl, G. Pilate, D.I. Dunstan and W.L. Crosby. 1993. Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. 94:139-149.

Delmer D.P. and C.H. Haigler. 2002. The regulation of metabolic flux to cellulose, a major sink for carbon in plants. Metab. Eng. 4:22-28.

Dickinson C.D., T. Altabella and M.J. Chrispeels. 1991. Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. Plant Physiol. 95:420-425.

Haigler C.H., M. Ivanova-Datcheva, P.S. Hogan, V.V. Salnikov, S. Hwang, K. Martin and D.P. Delmer. 2001. Carbon partitioning to cellulose synthesis. Plant Mol. Biol. 47:29-51.

Hajirezaei M.R., F. Bornke, M. Peisker, Y. Takahata, J. Lerchl, A. Kirakosyan and U. Sonnewald. 2003. Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers (*Solanum tuberosum*). J. Exp. Bot. 54:477-488.

Hauffe K.D., U. Paszkowski, P. Schulze-Lefert, K. Hahlbrock, J.L. Dangl and C.J. Douglas. 1991. A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell. 3:435-443.

Heineke D., U. Sonnewald, D. Bussis, G. Gunter, K. Leidreiter, I. Wilke, K. Raschke, L. Willmitzer and H.W. Heldt. 1992. Apoplastic expression of yeast-derived invertase in potato - effects on photosynthesis, leaf solute composition, water relations, and tuber composition. Plant Physiol. 100:301-308.

Heineke D., K. Wildenberger, U. Sonnewald, L. Willmitzer and H.W. Heldt. 1994. Accumulation of hexoses in leaf vacuoles - studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. Planta. 194:29-33.

Herbers K., P. Meuwly, W.B. Frommer, J.P. Metraux and U. Sonnewald. 1996. Systemic acquired resistance mediated by the ectopic expression of invertase: Possible hexose sensing in the secretory pathway. Plant Cell. 8:793-803.

Heyer A.G., M. Raap, B. Schroeer, B. Marty and L. Willmitzer. 2004. Cell wall invertase expression at the apical meristem alters floral, architectural, and reproductive traits in *Arabidopsis thaliana*. Plant J. 39:161-169.

Hood E.E., S.B. Gelvin, L.S. Melchers and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.

Huntley S.K., D. Ellis, M. Gilbert, C. Chapple and S.D. Mansfield. 2003. Significant increases in pulping efficiency in C4H-F5H-transformed poplars: Improved chemical savings and reduced environmental toxins. J. Agr. Food Chem. 51:6178-6183.

Kay R., A. Chan, M. Daly and J. McPherson. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science. 236:1299-1302.

Lerchl J., P. Geigenberger, M. Stitt and U. Sonnewald. 1995. Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell. 7:259-270.

Levy M., O. Edelbaum and I. Sela. 2004. Tobacco mosaic virus regulates the expression of its own resistance gene *N*. Plant Physiol. 135:2392-2397.

Li L., Y.H. Zhou, X.F. Cheng, J.Y. Sun, J.M. Marita, J. Ralph and V.L. Chiang. 2003. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. P. Natl. Acad. Sci. U.S.A. 100:4939-4944.

Mirashige T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Pelleschi S., J.P. Rocher and J.L. Prioul. 1997. Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell Environ. 20:493-503.

Roitsch T. and M.C. Gonzalez. 2004. Function and regulation of plant invertases: Sweet sensations. Trends Plant Sci. 9:606-613.

Sonnewald U., M. Brauer, A. von Schaewen, M. Stitt and L. Willmitzer. 1991. Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast - a powerful tool for studying sucrose metabolism and sink source interactions. Plant J. 1:95-106.

Sonnewald U., M.R. Hajirezaei, J. Kossmann, A. Heyer, R.N. Trethewey and L. Willmitzer. 1997. Increased potato tuber size resulting from apoplastic expression of a yeast invertase. Nature Biotechnol. 15:794-797.

Stitt M., A. von Schaewen and L. Willmitzer. 1991. Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell-wall involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes. Planta. 183:40-50.

Tang G.Q. and A. Sturm. 1999. Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. Plant Mol. Biol. 41:465-479.

Tang G.Q., M. Luscher and A. Sturm. 1999. Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell. 11:177-189.

Tauberger E., S. Hoffmann-Benning, H. Fleischer-Notter, L. Willmitzer and J. Fisahn. 1999. Impact of invertase overexpression on cell size, starch granule formation and cell wall properties during tuber development in potatoes with modified carbon allocation patterns. J. Exp. Bot. 50:477-486.

Volkov R.A., Panchuk, II and F. Schoffl. 2003. Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. J. Exp. Bot. 54:2343-2349.

von Schaewen A., M. Stitt, R. Schmidt, U. Sonnewald and L. Willmitzer. 1990. Expression of a yeast-derived invertase in the cell-wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J. 9:3033-3044.

Zrenner R., M. Salanoubat, L. Willmitzer and U. Sonnewald. 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L). Plant J. 7:97-107.

Zuther E., M. Kwart, L. Willmitzer and A.G. Heyer. 2004. Expression of a yeast-derived invertase in companion cells results in long-distance transport of a trisaccharide in an apoplastic loader and influences sucrose transport. Planta. 218:759-766.

CHAPTER THREE

Expression of yeast-derived invertase genes in hybrid poplar

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3.1 Introduction

Plants possess a large and diverse group of enzymes known as invertases (EC 3.2.1.26) that irreversibly hydrolyze sucrose releasing glucose and fructose. Plant invertases have been classified as either vacuolar, cytosolic or apoplastic in nature depending on their subcellular location, pH optima and/or isoelectric point (Tymowska-Lalanne and Kreis 1998). The multiplicity of invertases in key subcellular locations highlights the importance of sucrose concentration, and by extension glucose and fructose levels, to plant metabolism.

Sucrose is the primary transport sugar in most higher plants, and as such, is the primary carbon source for growth, maintenance and respiration. Invertases mediate the transition of sucrose, as a transport carbohydrate from source tissues, to glucose and fructose moieties in sink tissues, which then participate in a wide range of primary (e.g. glycolysis) and secondary (e.g. cellulose biosynthesis) metabolic pathways. This pivotal role in sink-source interaction has been the subject of considerable study, particularly with respect to transgenic research. The work of von Schaewen et al. (1990) represents one of the first transgenic approaches investigating the potential for invertases to affect sink-source metabolism. The authors employed a modified yeast invertase gene (*suc2*) that was directed to the apoplast and cytosol of both *Nicotiana tabacum* (tobacco) and *Arabidopsis thaliana* using the cauliflower mosaic virus 35S promoter. The presence of these exogenous invertases led to altered growth rates and quantifiable changes in carbohydrate metabolism. This study not only illustrated the ability for exogenous invertases to affect plant phenotype, but also served as a catalyst for subsequent research involving the modified yeast invertases in plants such as *Solanum tuberosum* (Heineke et al. 1991, Heineke et al. 1992, Bussis et al. 1997, Sonnewald et al. 1997, Trethewey et al. 1998, Tauberger et al. 1999, Trethewey et al. 1999a, Trethewey et al. 1999b, Hajirezaei et al. 2000, Deryabin et al. 2003, Zuther et al. 2004, Deryabin et al. 2005, Hajirezaei et al. 2003), *Nicotiana tabacum* (Stitt et al. 1991, Sonnewald et al. 1991, Heineke et al. 1994, Lerchl et al. 1995, Fukushima et al. 2001, Tomlinson et al. 2004, Canam et al. 2006), *Arabidopsis thaliana* (Heyer et al. 2004) and *Vicia narbonensis* (Weber et al. 1998, Neubohn et al. 2000). Collectively, these efforts have shown that the expression of the foreign invertases leads to impaired growth, inhibited photosynthetic capacity, increased respiration, altered reproductive structure

and capacity (Goetz et al. 2001, Heyer et al. 2004) and increased heterotrophic behaviour of leaf tissue (Heineke et al. 1994). However, not all of the yeast-derived invertase studies have resulted in detriment to the host plant. For example, the use of the tuber-specific *B33* promoter with a yeast-derived apoplastic invertase in potato appeared to have no effect on aerial phenotypes, while resulting in a significant increase in tuber size (Sonnewald et al. 1997, Tauberger et al. 1999).

Despite the extensive volume of research investigating the effects of yeast-derived invertases on herbaceous plant phenotypes, the effects of overexpressing these enzymes in tree species has not been explored. The primary components of wood, cellulose and lignin, are the most abundant biopolymers on earth. As such, trees represent one of the largest repositories of carbon, and are therefore excellent models to study sink-source interactions. Previous research has demonstrated that it is possible to significantly alter the lignin content of hybrid poplar using transgenic approaches (Huntley et al. 2003). Furthermore, we have previously shown that overexpressing yeast-derived invertases altered the proportion of cellulose and lignin in tobacco stems, with one transline manifesting a 36% increase in cellulose content (Canam et al. 2006).

In the present study we explored the effects of overexpressing yeast-derived invertases targeting the cytosol and apoplast under the expressional control of a tandemly-duplicated 35S promoter (*2X35S*) and the xylem-specific 4-coumarate CoA:ligase (*4CL*) promoter in hybrid poplar (*Populus alba* x *grandidentata*). The phenotype and biochemistry of the translines were assessed, including the cellulose and lignin composition of the wood.

3.2 Materials and methods

3.2.1 Plasmid construction

Cytosolic (*CI*) and apoplastic (*AI*) yeast-derived invertase genes, described by von Schaewen et al. (1990), were excised along with their corresponding NOS terminators from pBI-121 with *Bam*HI and *Eco*RI, and subsequently cloned into pCAMBIA1390 containing either the *2X35S* promoter (Datla et al. 1993) or the *4CL* promoter (Hauffe et al. 1991) as described previously (Canam et al. 2006).

3.2.2 Plant transformations

Agrobacterium tumefaciens strain EHA105 was transformed with each of the vectors containing the yeast-derived invertase genes (*2X35S::AI*, *2X35S::CI*, *4CL::AI* and *4CL::CI*). *Agrobacterium*-mediated transformation was then used to create hybrid poplar transformants using the method described previously by Canam et al. (2006), except woody plant medium (WPM; McCown and Lloyd 1981) served as the basis for all transformation media rather than Mirashige and Skoog (MS) medium.

3.2.3 Plant growth

Explants from the transformation events were cultured on WPM supplemented with 0.01 µM naphthalene acetic acid (NAA) to promote root formation and were maintained under 16 h days with an average photon flux density of 50 µmol m^{-2}s^{-1}. The hybrid poplar were propagated at four week intervals before being transferred (12 individuals per transgenic line) to soil in 2L pots in a greenhouse in Vancouver, British Columbia, Canada (49° North, 123° West) during March 2007. The plants were randomly placed on a flood table under supplemental lighting with 16 h days and watered daily with fertilized water.

3.2.4 Growth parameters and harvest

After three months of growth, the height of each hybrid poplar was measured from one inch above the soil to the apex of the plant. The diameter of the stem was also recorded using digital calipers at plastichron index (PI) five, where PI=0 corresponded to the first leaf greater than seven centimetres in length from the apex of the tree. Three plants from each transline were then destructively harvested and the resulting tissues (leaves, phloem, cambium, xylem and roots) collected and placed in liquid nitrogen on site prior to being stored at -80°C. An additional segment of xylem (phloem and cambium were removed) between PI=5 and =15 was collected for structural chemical analysis.

3.2.5 Structural chemistry analysis

Xylem tissue was ground to pass a 40-mesh screen using a Wiley Mill, treated with acetone for 24 h using a Soxhlet apparatus and dried for 24 h at 105°C. Approximately 200 mg of dried extractive-free tissue was treated with 72% sulphuric acid for 2 h and then autoclaved at 121°C for 90 min according to a modified Tappi method UM-250 (Cullis et al. 2004). The solution was then filtered through a medium coarseness

crucible. The retentate was dried at 105°C and the mass representing insoluble lignin was recorded. An aliquot of the filtrate was analyzed for soluble lignin using a spectrophotometer and Beer's Law as previously described (Canam et al. 2006). A portion of the remaining filtrate was analyzed for monosaccharides representing hydrolyzed cellulose and hemicellulose using a Dionex 600 HPLC (Dionex, Sunnyvale, CA) with a PA-1 CarboPac column (Dionex) and electrochemical detector. Fucose served as an internal control. The percentages of cellulose and hemicellulose were calculated as previously described (Canam et al. 2006).

3.2.6 Starch analysis

Approximately 50 mg of dried extractive-free xylem tissue was incubated with 4% sulphuric acid and autoclaved at 121°C for 3.5 min. After cooling, the samples were centrifuged at 200 rpm for 5 min. An aliquot of the resulting supernatant was analyzed using an HPLC as described for the structural chemistry analysis. Glucose was quantified, which represented starch that was hydrolyzed by the weak acid treatment. Fucose again served as an internal control.

3.2.7 Soluble sugar analysis

Frozen tissues were ground using a mortar and pestle with liquid nitrogen prior to lyophilizing for 24 h. Samples were then weighed and treated with 4 mL of methanol:chloroform:water (M:C:W; 12:5:3) and incubated at 4°C for 24 h. The solutions were centrifuged at 4000 rpm for 10 min and the supernatant was collected. The pellet was twice washed with an additional 4 mL of M:C:W, centrifuged as before and the supernatants were combined. A 5 mL aliquot of water was added to supernatant (12 mL total) and the mixture was centrifuged at 4000 rpm for 5 min. An aliquot (2 mL) of the upper phase was collected and spun to dryness using a vacuum centrifuge. The pellet containing soluble carbohydrate was resuspended in 0.5 mL of water and analyzed for sucrose using an HPLC as described above with a CarboPac MA-1 column (Dionex). Fucose served as an internal control.

3.2.8 SDS-PAGE and Western blot analysis

Approximately 0.5 mg of frozen tissue was ground using a mortar and pestle with liquid nitrogen and then mixed with 1 mL of extraction buffer (50 mM Tris-HCl pH 6.5, 1 mM $Na_2EDTA \cdot 2H_2O$, 100 mM NaCl, 1% Triton X-100). The suspension was centrifuged at 13000 rpm for 5 min and an aliquot of the supernatant was assayed to determine

protein concentration using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). An aliquot of the remaining supernatant was mixed with a 2X reducing buffer (100 mM Tris pH 6.5, 4% SDS, 20% glycerol, 1 mM DTT, 0.01% bromophenol blue) and heated at 100°C for 5 min. 10 µg of protein per sample was separated using 10% SDS-PAGE at 120 V for 1 h. Gels were either visualized with Coomassie blue or transferred to nitrocellulose (0.25 µm) using a semi-dry transfer cell (Bio-Rad) at 10 V for 30 min. Nitrocellulose membranes containing the sample proteins were blocked with 3% milk powder in Tris-buffered saline (TBS+3%) prior to being exposed to a 1:200 dilution (in TBS+3%) of a custom antibody from rabbit (ImmuneChem Pharmaceutical Inc., Burnaby, BC Canada) raised against a commercially available *Saccharomyces cerevisiae* yeast invertase (Sigma, St. Louis, MO). The membranes were then exposed to a 1:10000 dilution of a mouse anti-rabbit alkaline phosphate conjugate (Sigma) in TBS+3% and subsequently visualized using a coupled NBT/BCIP alkaline phosphatase assay.

3.2.9 Invertase activity

Total protein was extracted and invertase activity assayed according to a modified method of Peleschi et al. (1999) that was previously demonstrated to be effective in assaying the yeast-derived invertases (Canam et al. 2006). Briefly, 0.5 g of frozen leaf tissue was ground using a mortar and pestle with liquid nitrogen prior to the addition of 2 mL of an extraction buffer (50 mM HEPES pH 7.0, 10 mM $MgCl_2 \cdot 6H_2O$, 1 mM $Na_2EDTA \cdot 2H_2O$, 2.6 mM DTT). The suspension was centrifuged at 13000 rpm for 5 min and an aliquot of the supernatant was assayed to determine protein concentration using the Bio-Rad Protein Assay (Bio-Rad). The remaining supernatant was desalted using Econo-Pac 10 DG desalting columns (Bio-Rad) that were pre-equilibrated with extraction buffer. A 250 µL aliquot of the eluant was added to 250 µL of 200 mM sodium acetate (pH 4.8) and 100 µL of 600 mM sucrose. The reaction was incubated at 30°C for 3 h and was terminated by incubating at 100°C for 5 min. The reaction mixture was analyzed for glucose concentration using a Dionex 600 HPLC with a CarboPac MA-1 column (Dionex). Specific activity was calculated as the quantity of glucose liberated (µg) per quantity of total protein added to the assay (µg).

3.2.10 RNA extraction and cDNA synthesis

RNA was extracted from approximately 0.5 g of frozen ground tissue as per Kolosova et al. (2004). An aliquot representing 10 µg of RNA was treated using TURBO DNase™ (Ambion, Austin, TX) and requantified. One µg of DNase-free RNA was used to generate cDNA using poly(T)$_{16}$ oligonucleotides and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA).

3.2.11 Quantitative RT-PCR

Quantitative RT-PCR reactions contained 1 µL of cDNA, 1 µL each of forward and reverse primers (5 pmol), 9.5 µL of water and 12.5 µL of 2X Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). For detection of the cytosolic and apoplastic yeast-derived invertase transcripts the forward primer was 5′-GTCCCTGGTCTCGTTTTGC-3′ and reverse primer was 5′-GGTCATGTTCACAGATCCTAG-3′. For detection of transcription initiation factor 5A (TIF-5A) transcript, which represented a housekeeping gene (Ralph et al. 2006), the forward primer was 5′-GACGGTATTTTAGCTATGGAATTG-3′ and reverse primer was 5′-CTGATAACACAAGTTCCCTGC-3′. The reaction conditions were as follows: 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 40 s. Data was collected with an Mx3000P Real-Time PCR System (Stratagene) and analyzed using the supplied MxPro software. Relative expression of the yeast-derived invertase genes was calculated as previously described ($2^{-[CTINV-CTTIF5A]}$), using TIF-5A transcript abundance for normalization (Canam et al. 2006 and the references therein).

3.2.12 Statistical analysis

Data were analysed using unpaired two-tailed t-tests assuming unequal variances at the 95% confidence level.

3.3 Results

3.3.1 Plant transformation

Three hybrid poplar translines containing *2X35S::CI* and one transline containing *4CL::AI* constructs were created using *Agrobacterium*-mediated transformation and confirmed by genomic DNA screening. These transformants represent the outcome of several transformation events, highlighting the difficulty in generating viable transformants harbouring these yeast-derived invertase genes. Poor transformation

efficiency with the yeast-derived invertases under the *2X35S* and *4CL* promoters was also seen in a parallel study using tobacco (Canam et al. 2006). As with that study, the low transformation efficiency may be attributed to the inherent risk of overexpressing an enzyme central to primary metabolism, which could compromise the viability of young transformants.

3.3.2 Growth and phenotype

Hybrid poplar trees were grown in soil in a greenhouse for three months to assess the effects of foreign invertase expression on tree phenotype. No differences in growth rate were observed during development, and upon termination of the growth trial there were no statistical differences in either height or diameter of the transformants when compared to wild-type trees (Figure 3.1). Additionally, there was no visible change in the number of leaves or nodes, nor any visible difference in leaf size, shape or colour between the translines and wild-type. Furthermore, there were no visible below-ground phenotypes, as the structure and architecture of the roots appeared normal.

3.3.3 Transgene expression

In combination with phenotype assessment, the expression of the foreign invertases in select tissues of the three-month old greenhouse-grown translines was explored using real-time RT-PCR (Figure 3.2). Transcripts were clearly detected in all tissues in all four transgenic lines, with the exception of the cambium in *2X35S::CI*-2. The overall expression pattern of *2X35S::CI*-2 closely resembled that of *2X35S::CI*-1. However, the expression pattern of the third *2X35S::CI* transline differed, showing much higher expression in the vascular tissues (cambium, phloem and xylem) and in the roots (Figure 3.2). Highest expression of the *2X35S::CI* translines was observed in the upper leaf tissue, which was a trait not shared by the *4CL::AI-1* transline. Instead, the cambium and xylem of *4CL::AI-1* had the highest expression, which agrees with the data collected in GUS-expressing tobacco where the *4CL* promoter was found to be largely xylem-specific (Hauffe et al. 1991). However, in the present study it is clear that the *4CL* promoter does confer expression, and relatively equally, in a range of tissues in hybrid poplar including leaves, phloem and roots.

3.3.4 SDS-PAGE, Western blots and enzyme activity

Total protein was extracted from upper (PI=5) and lower (PI=15) leaves, cambium, phloem, xylem and root tissue of the three-month old greenhouse-grown hybrid poplar

and examined using SDS-PAGE. There were no observable differences between proteins from the transgenic lines and WT when Coomassie-stained gels were examined (data not shown). To examine the presence of the foreign invertases the proteins were transferred to nitrocellulose for immunodetection. The membranes containing proteins from a number of tissues were treated with a custom antibody raised against *S. cerevisiae* yeast invertase (Canam et al. 2006) and visualized using an alkaline phosphatase-conjugated secondary antibody with a NBT/BCIP coupled assay (Figure 3.3). The *4CL::AI*-1 line had a distinct band approximately 80 kDa in size in the leaf, phloem, xylem and root tissue, however, a doublet appeared in approximately the same size range in the cambium samples. No bands were detected in the mature protein size range in any of the three *2X35S::CI* translines nor WT. However, faint bands were detected at approximately 5 kDa in leaf samples of *2X35S::CI* lines (Figure 3.4). These bands did not appear in the WT nor in the *4CL::AI*-1 transline. Enzyme activity analysis of leaf tissues revealed an increasing trend between the translines and WT with *4CL::AI*-1 having significantly more activity (Table 3.1).

3.3.5 Structural carbohydrate

The composition of the three-month old greenhouse-grown hybrid poplar xylem was analyzed to explore the effects of transgene expression on starch and the structural cell wall components cellulose, hemicellulose and lignin (Table 3.2). The typical proportions of these four polymers in hybrid poplar xylem was determined in WT (47% cellulose, 22% hemicellulose, 20% lignin and 1% starch) and no statistical difference was determined between WT and the *2X35S::CI* translines. However, the *4CL::AI*-1 transline had statistically higher lignin content (22%) and significantly lower starch content (0.8%), although the proportion of cellulose and hemicellulose did not differ significantly from WT. The cellulose content did, however, show an increasing trend amongst translines despite a relatively unchanged hemicellulose content.

3.3.6 Soluble sugar analysis

The level of sucrose in the upper and lower leaf, cambium, phloem and root tissues was examined (Table 3.3). The concentration of sucrose, as expected, was highly variable between the three individual plants per line that were examined, which is reflected in the high standard deviations. The data indicate no significant changes to the sucrose level

in any of the tissues examined between WT and *2X35S::CI*-1,*2X35S::CI*-2 and *4CL::AI*-1. However, the *2X35S::CI*-3 transline showed a significant decrease of sucrose in the roots and cambium.

3.4 Discussion

The goal of the present study was to explore the effects of yeast-derived invertase expression in a perennial tree species. Hybrid poplar (*P. alba* x *grandidentata*) was chosen for its ability to be tissue cultured, the relative ease of transformation compared to other tree species and the emerging role of *Populus* as a model tree system (e.g. Tuskan et al. 2006). *Agrobacterium*-mediated transformation was used to transform hybrid poplar with yeast-derived apoplastic invertase (AI) and cytosolic invertase (CI) under the expressional control of either the *2X35S* (Datla et al. 1993) or the xylem-specific *4CL* (Hauffe et al. 1991). The output of these transformations was poor, and despite repeated attempts using an established hybrid poplar transformation protocol (e.g. Coleman et al. 2006) only four lines harbouring a yeast-derived invertase transgene were established: three *2X35S::CI* and one *4CL::AI*. Similar difficulty in establishing viable transformants was also observed in tobacco using the same promoter and gene constructs (Canam et al. 2006), but not with constructs harbouring GUS in place of the yeast-derived invertases. As such, it is speculated that the presence of a foreign invertase is affecting primary metabolism and compromising the ability of transformed callus tissue to survive and establish a healthy rooting plant. To that end, the viable transformants generated in this study may actually be weakly expressing the transgenes or represent plants that have effectively inactivated the foreign invertase.

After preliminary tests to verify the presence of the transgene transcripts of the four transformed hybrid poplar lines in tissue culture, the plants were transferred to a greenhouse and allowed to develop in soil for three months to assess visible phenotype, as well as to generate tissue for chemical and biochemical analyses. All of the transgenic lines survived out-planting and appeared to develop at the same rate as wild-type trees throughout the growth period. Three months after transfer to the greenhouse, the growth statistics (height and diameter) of all translines and WT were measured. There was no significant difference in phenotype between the translines

and WT trees, including no apparent change to the number of nodes nor leaf size, shape or colour. Root structure also appeared to be unchanged. A lack of visible phenotype was similarly observed for the aerial portions of potato expressing AI or CI under the tuber-specific *B33* promoter, although significant changes to below-ground phenotype were observed (Sonnewald et al. 1997, Tauberger et al. 1999). Due to the obvious lack of phenotype manifested by the transgenic lines in this study, no further whole-plant phenotype analysis was performed. Instead, the focus shifted to the biochemistry of the greenhouse-grown plants, and more specifically, whether the transgenes had continued to be expressed beyond the tissue culture stage.

Upper and lower leaves, phloem, cambium, xylem and root tissue were collected from three representative plants of each transline for RNA analysis. Real-time RT-PCR revealed that all tissues continued to express the transgenes in all tissues examined, with the exception of the cambial tissue of *2X35S::CI*-2. As expected, WT did not show any amplification with the transgene-specific primers. It is interesting that although *4CL* was previously shown to be primarily xylem-specific in tobacco (Hauffe et al. 1991), the promoter seems to confer relatively equal expression throughout various tissues in hybrid poplar. Although, it should be noted that the vascular tissues (cambium, xylem and phloem) had the highest expression in the *4CL::AI*-1 transline. Taken together, the data from the real-time analysis of the three-month old greenhouse-grown plants indicate that transgene expression had continued beyond the tissue culture stage and was ongoing at the time of harvest.

Parallel with validation of transcript abundance of the transgenes in the hybrid poplar translines, we examined the presence of mature protein in the same tissues. Western blots were employed in an attempt to visualize translated AI and CI using a custom rabbit antibody developed against a *S. cerevisiae* invertase that had previously been show to be effective against the yeast-derived invertases (Canam et al. 2006). A clear band was detected in proteins extracted from *4CL::AI*-1, although the apparent molecular weight of the protein appeared to be approximately 80 kDa in size, which is higher than the predicted 65kDa size that was observed with Western blots in *4CL::AI* tobacco (Canam et al. 2006). The consistency of this size range in all the tissues examined, and lack of banding in wild-type tissues, suggests that this 80 kDa band is indeed AI. We hypothesize that this discrepancy in size may be the result of

glycosylation of the AI protein, as was observed with *in vitro* and *in vivo* translation of AI in *Arabidopsis* (von Schaewen et al. 1990). The channels through which secretion-bound proteins travel may be different in hybrid poplar compared to tobacco, and therefore could result in such a difference in apparent molecular weight of AI between the two plant systems. In *Arabidodpsis*, the glycosylation of the yeast invertase targeted to the apoplast was taken as evidence for effective endoplasmic reticulum/golgi apparatus channeling on route to protoplast secretion (von Schaewen et al. 1990). Additionally, it is possible that whatever modifications occurred to the protein (i.e. glycosylation) may have seriously compromised the activity of the enzyme or rendered it completely inactive, which may explain the lack of phenotype of the *4CL::AI*-1 transline.   In contrast to the *4CL::AI*-1 immunodetection results, the *2X35S::CI* translines showed no such banding profile, despite transgene amplification. However, a single faint band in each of the *2X35S::CI* translines was detected at approximately 5 kDa in the upper leaf sample (Figure 3.4), which represents the highest expressing tissue as determined by real-time analysis. Similar bands were not detected in WT or *4CL::AI*-1 protein extractions. Therefore, we hypothesize that the cytosolic yeast-derived invertase may be degraded either before or after reaching its mature form. This would explain the lack of mature protein being visualized using immunodetection and the lack of phenotype in the translines expressing CI. Despite the strong evidence for partial to complete inactivation of AI and CI, this study did provide evidence suggesting at least minor exogenous invertase activity may been present in the transgenic lines. All translines had increased invertase activity, although only 4CL:AI-1 had a statistically significant increase. In addition, the *2X35S::CI*-3 transline also had significantly lower sucrose levels in the roots and cambial tissue. However, none of the changes to the sucrose levels observed in this study were dramatic; as compared to those previously observed for two *4CL::AI* translines in tobacco (Canam et al. 2006), *B33*-driven *AI* and *CI* in potato tubers (Sonnewald et al. 1997), *35S::AI* in potato leaves (Heineke et al. 1992), and seeds of *Vicia narbonensis* expressing *AI* (Weber et al. 1998). With respect to structural chemistry the *4CL::AI*-1 transline also had a significant increase in lignin content of the stem when compared to WT, along with a concomitant decrease in starch content. A decrease in starch, albeit much more dramatic, was also observed in the cotyledons of *Vicia narbonensis* expressing *AI*, where it was concluded that starch

accumulation may be directly related to sucrose concentration (Weber et al. 1998). The differences in structural carbohydrate polymer content in *4CL::AI*-1 compared to WT, despite being minor, represent the best evidence in the present study for a transgenic effect incurred by a yeast-derived invertase in hybrid poplar. We therefore speculate it is possible that minor AI activity in the cell wall of hybrid poplar may directly or indirectly affect the processes related to starch and lignin biosynthesis. The evidence of a mature protein by immunodetection in the *4CL::AI*-1 line may lend support to this theory by verifying the existence of the enzyme, albeit putatively post-translationally modified. Furthermore, the trend for increasing cellulose content coupled with an increase in apparent enzyme activity of leaf tissue observed in the translines may be taken as additional evidence for at least minor levels of foreign invertase activity. To that end, changes to structural carbohydrate polymers conferred by the expression of yeast-derived invertases were also observed in tobacco expressing the same promoter/gene combination (Canam et al. 2006).

In summary, we explored the effects of yeast-derived *AI* and *CI* under the expressional control of either the overexpressing *2X35S* promoter or the tissue-specific *4CL* promoter in hybrid poplar. Greenhouse growth experiments revealed no visible phenotype, including no significant difference in overall height or diameter of the stem. Further biochemical analysis revealed that the translines were expressing transcript, however, the translated product appeared to be compromised. Specifically, the evidence gathered in this experiment suggested that CI expressed in hybrid poplar was being degraded, while AI was post-transcriptionally modified, which may have negatively affected enzyme activity. Despite these scenarios, further evidence from this experiment suggested that the presence of AI increased the lignin and decreased the starch content of the xylem tissue in one transline of hybrid poplar, while a slight increase in cellulose content was observed for all translines. Collectively, the data from this experiment suggests that the yeast-derived invertases do not confer the same dramatic biochemical and phenotypic changes in hybrid poplar as those observed in herbaceous plants such as tobacco, which provides supporting evidence for the fundamental differences in sink-source interaction between apoplastic-loading tobacco and poplar, a putative symplastic loader (Turgeon and Medville 2004). However, changes to the structural chemistry (e.g. cellulose and lignin) were observed despite

only minor increases in invertase activity. This study therefore underscores the potential to alter the composition of structural carbohydrate in trees, such as hybrid poplar, using enzymes involved in carbohydrate metabolism, and adds to the growing body of research in this field (e.g. Coleman et al. 2006). Finally, although it appears that foreign invertases confer only limited phenotypic changes in hybrid poplar, this group of enzymes nevertheless may prove to be useful in future transgenic approaches with respect to wood modification, perhaps by misregulating (e.g. RNAi technology or overexpressing) endogenous invertases.
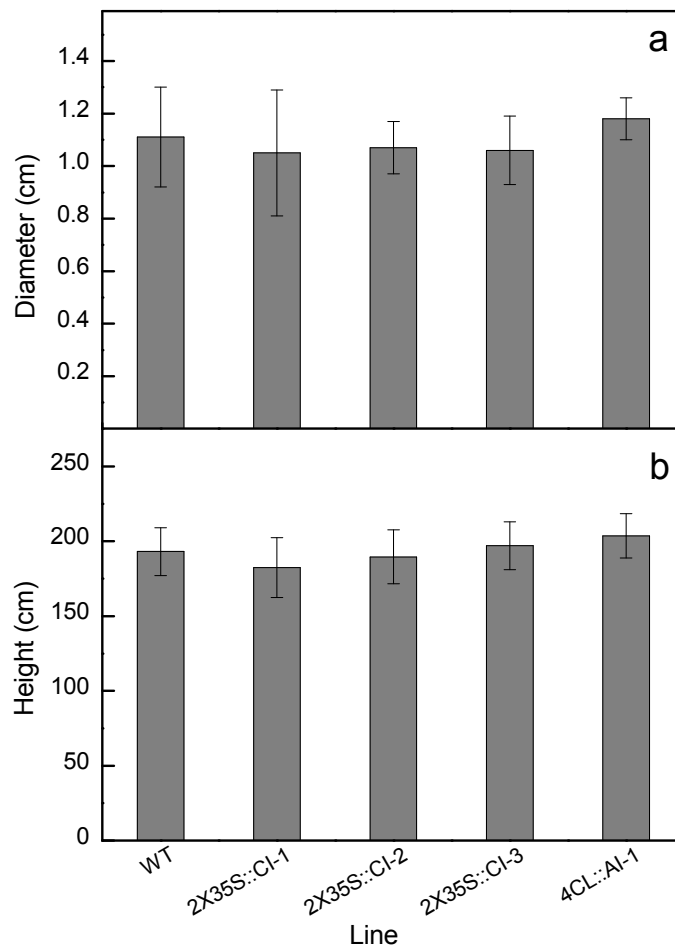
Figure 3.1. Diameter at PI=5 (a) and height from the base of the stem to the apex (b) of three-month old greenhouse-grown hybrid poplar. Bars represent the mean and standard deviation (n=12).
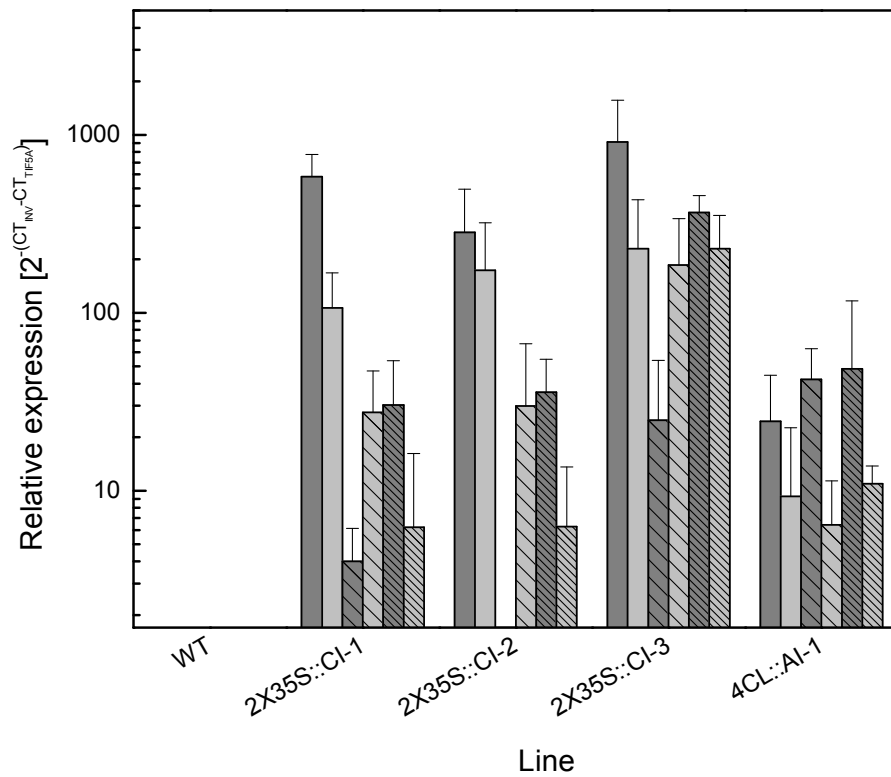
Figure 3.2. Transcript abundance of yeast-derived invertase (INV) in tissues of three-month old greenhouse-grown hybrid poplar calculated as expression relative to transcription initiation factor 5A (TIF5A; a housekeeping gene) using real-time RT-PCR. Bars represent the mean relative expression with the standard deviation (n=3) for upper leaves (PI=5) (dark gray, solid), lower leaves (PI=15) (light gray, solid), cambium (dark gray, sparse stripe), phloem (light gray, sparse stripe), xylem (dark gray, dense stripe) and roots (light gray, dense stripe).
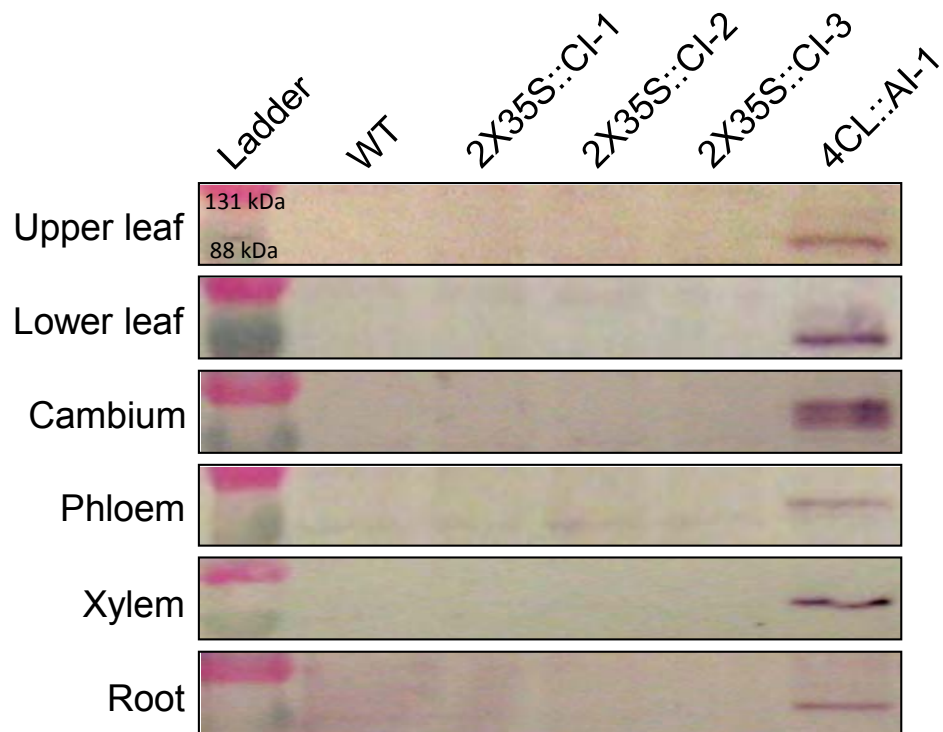
Figure 3.3. Western blots of total proteins (10 µg per lane) extracted from selected tissues of three-month old greenhouse-grown hybrid poplar using a custom rabbit antibody raised against *S. cerevisiae* invertase. Bands were visualized using a coupled BCIP/NBT system. Highlighted sections represent proteins between approximately 131 kDa and 88 kDa as estimated using kaleidoscope pre-stained standards (Bio-Rad).
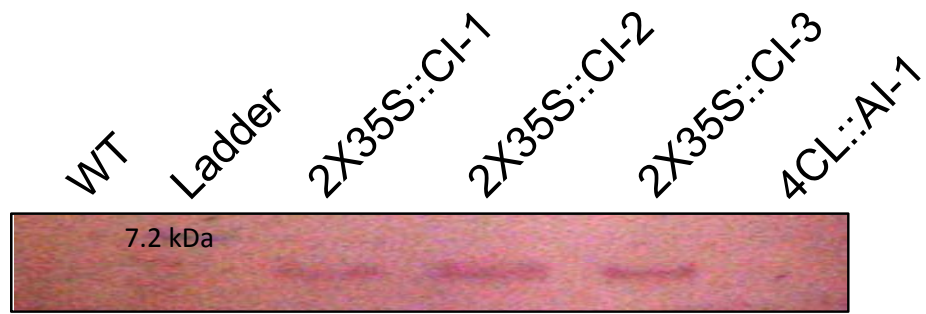
Figure 3.4. Western blot of total proteins (10 µg per lane) extracted from the upper leaves of three-month old greenhouse-grown hybrid poplar using a custom rabbit antibody raised against *S. cerevisiae* invertase. Highlighted section represents proteins/polypeptides less than approximately 10 kDa as estimated using kaleidoscope pre-stained standards (Bio-Rad).

Table 3.1. Invertase activity from the upper leaf tissue (PI=5) of three-month old poplar. Values represent the mean and standard deviation with bold values corresponding to a statistical difference from wild-type (WT) at the 95% level (n=3).

Line	ng glucose μg^{-1} protein min^{-1}
WT	4.3 ± 2.0
2X35S::CI-1	8.3 ± 0.4
2X35S::CI-2	8.7 ± 1.6
2X35S::CI-3	10.4 ± 2.9
4CL::AI-1	**16.8 ± 1.6**

Table 3.2. Chemical analysis of xylem from three-month old hybrid poplar. Values are the percentage of each cell wall polymer in dried extractive-free tissue with bold values representing a statistical difference from wild-type (WT) at the 95% level (n=3).

Line	Cellulose %	Hemicellulose[a] %	Lignin[b] %	Starch %
WT	47.1 ± 1.5	22.4 ± 0.2	19.7 ± 0.6	1.0 ± 0.1
2X35S::CI-1	48.2 ± 0.2	22.7 ± 0.8	20.3 ± 1.0	1.0 ± 0.1
2X35S::CI-2	48.6 ± 2.0	22.4 ± 0.5	21.0 ± 1.5	1.0 ± 0.2
2X35S::CI-3	49.8 ± 2.2	21.9 ± 1.1	20.2 ± 2.0	1.1 ± 0.3
4CL::AI-1	48.0 ± 0.8	22.5 ± 1.1	**22.3 ± 0.8**	**0.8 ± 0.1**

[a]Represents the sum of arabinose, xylose, mannose, rhamnose and galactose
[b]Represents the sum of soluble and insoluble lignin

Table 3.3. The concentration of sucrose in select tissues of three-month old greenhouse-grown hybrid poplar. Values are mean and standard deviation and units are quantity of carbohydrate (mg) per mass of dried tissue (g). Bold values indicate a significant difference from wild-type (WT) at the 95% level (n=3).

Line	Upper leaves	Lower leaves	Cambium	Phloem	Roots
WT	30.3 ± 12.9	22.8 ± 10.0	11.8 ± 1.5	16.7 ± 1.5	8.5 ± 1.7
2X35S::Cl-1	25.8 ± 6.1	17.7 ± 4.2	9.4 ± 4.1	15.7 ± 0.4	5.6 ± 0.9
2X35S::Cl-2	17.9 ± 8.0	19.1 ± 10.8	7.7 ± 2.1	23.9 ± 13.0	6.0 ± 2.5
2X35S::Cl-3	11.0 ± 4.8	18.2 ± 12.0	**5.4 ± 2.6**	15.9 ± 4.1	**3.3 ± 1.6**
4CL::Al-1	21.8 ± 3.4	32.6 ± 17.9	8.5 ± 2.3	25.4 ± 8.5	7.2 ± 2.2

3.5 References

Bussis, D., D. Heineke, U. Sonnewald, L. Willmitzer, K. Raschke and H.W. Heldt. 1997. Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol. Planta. 202:126-136.

Canam, T., J.Y. Park, K.Y. Yu, M.M. Campbell, D.D. Ellis and S.D. Mansfield. 2006. Varied growth, biomass and cellulose content in tobacco expressing yeast-derived invertases. Planta. 224:1315-1327.

Coleman, H.D., D.D. Ellis, M. Gilbert and S.D. Mansfield. 2006. Up-regulation of sucrose synthase and UDP-glucose pyrophosphorylase impacts plant growth and metabolism. Plant Biotech. J. 4:87-101.

Cullis, I.F., J.N. Saddler and S.D. Mansfield. 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. Biotechnol. Bioeng. 85:413-421.

Datla, R.S.S., F. Bekkaoui, J.K. Hammerlindl, G. Pilate, D.I. Dunstan and W.L. Crosby. 1993. Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. 94:139-149.

Deryabin, A.N., I.M. Dubinina, E.A. Burakhanova, N.V. Astakhova, E.P. Sabel'nikova and T.I. Trunova. 2005. Influence of yeast-derived invertase gene expression in potato plants on membrane lipid peroxidation at low temperature. J. Therm. Biol. 30:73-77.

Deryabin, A.N., T.I. Trunova, I.M. Dubinina, E.A. Burakhanova, E.P. Sabel'nikova, E.M. Krylova and G.A. Romanov. 2003. Chilling tolerance of potato plants transformed with a yeast-derived invertase gene under the control of the *B33* patatin promoter. Russ. J. Plant. Physiol. 50:449-454.

Fukushima, E., Y. Arata, T. Endo, U. Sonnewald and F. Sato. 2001. Improved salt tolerance of transgenic tobacco expressing apoplastic yeast-derived invertase. Plant Cell Physiol. 42:245-249.

Goetz, M., D.E. Godt, A. Guivarc'h, U. Kahmann, D. Chriqui and T. Roitsch. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. P. Natl. Acad. Sci. U.S.A. 98:6522-6527.

Hajirezaei, M.R., F. Bornke, M. Peisker, Y. Takahata, J. Lerchl, A. Kirakosyan and U. Sonnewald. 2003. Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers (*Solanum tuberosum*). J. Exp. Bot. 54:477-488.

Hajirezaei, M.R., Y. Takahata, R.N. Trethewey, L. Willmitzer and U. Sonnewald. 2000. Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. J. Exp. Bot. 51:439-445.

Hauffe, K.D., U. Paszkowski, P. Schulze-Lefert, K. Hahlbrock, J.L. Dangl and C.J. Douglas. 1991. A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell. 3:435-443.

Heineke, D., G. Gunter, U. Sonnewald, L. Willmitzer and H.W. Heldt. 1991. Metabolic responses of potato leaves to an expression of yeast invertase in the apoplast. Biol. Chem. H.-S. 372:674-674.

Heineke, D., U. Sonnewald, D. Bussis, G. Gunter, K. Leidreiter, I. Wilke, K. Raschke, L. Willmitzer and H.W. Heldt. 1992. Apoplastic expression of yeast-derived invertase in potato - effects on photosynthesis, leaf solute composition, water relations, and tuber composition. Plant Physiol. 100:301-308.

Heineke, D., K. Wildenberger, U. Sonnewald, L. Willmitzer and H.W. Heldt. 1994. Accumulation of hexoses in leaf vacuoles - studies with transgenic tobacco plants

expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. Planta. 194:29-33.

Heyer, A.G., M. Raap, B. Schroeer, B. Marty and L. Willmitzer. 2004. Cell wall invertase expression at the apical meristem alters floral, architectural, and reproductive traits in *Arabidopsis thaliana*. Plant J. 39:161-169.

Huntley, S.K., D. Ellis, M. Gilbert, C. Chapple and S.D. Mansfield. 2003. Significant increases in pulping efficiency in C4H-F5H-transformed poplars: Improved chemical savings and reduced environmental toxins. J. Agr. Food Chem. 51:6178-6183.

Kolosova, N., B. Miller, S. Ralph, B.E. Ellis, C. Douglas, K. Ritland and J. Bohlmann. 2004. Isolation of high-quality RNA from gymnosperm and angiosperm trees. Biotechniques. 36:821-824.

Lerchl, J., P. Geigenberger, M. Stitt and U. Sonnewald. 1995. Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell. 7:259-270.

McCown, B.H. and G. Lloyd. 1981. Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant species. Hortscience. 16:453-453.

Neubohn, B., S. Gubatz, U. Wobus and H. Weber. 2000. Sugar levels altered by ectopic expression of a yeast-derived invertase affect cellular differentiation of developing cotyledons of *Vicia narbonensis* L. Planta. 211:325-334.

Pelleschi, S., J.P. Rocher and J.L. Prioul. 1997. Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell Environ. 20:493-503.

Ralph, S., C. Oddy, D. Cooper et al. 2006. Genomics of hybrid poplar (*Populus trichocarpa x deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Mol. Ecol. 15:1275-1297.

Sonnewald, U., M. Brauer, A. von Schaewen, M. Stitt and L. Willmitzer. 1991. Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast - a powerful tool for studying sucrose metabolism and sink source interactions. Plant J. 1:95-106.

Sonnewald, U., M.R. Hajirezaei, J. Kossmann, A. Heyer, R.N. Trethewey and L. Willmitzer. 1997. Increased potato tuber size resulting from apoplastic expression of a yeast invertase. Nature Biotechnol. 15:794-797.

Stitt, M., A. von Schaewen and L. Willmitzer. 1991. Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell-wall involves a decrease of the Calvin cycle enzymes and an increase of glycolytic enzymes. Planta. 183:40-50.

Tauberger, E., S. Hoffmann-Benning, H. Fleischer-Notter, L. Willmitzer and J. Fisahn. 1999. Impact of invertase overexpression on cell size, starch granule formation and cell wall properties during tuber development in potatoes with modified carbon allocation patterns. J. Exp. Bot. 50:477-486.

Tomlinson, K.L., S. McHugh, H. Labbe, J.L. Grainger, L.E. James, K.M. Pomeroy, J.W. Mullin, S.S. Miller, D.T. Dennis and B.L.A. Miki. 2004. Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: analysis of tobacco seed development and effects of overexpressing apoplastic invertase. J. Exp. Bot. 55:2291-2303.

Trethewey, R.N., P. Geigenberger, K. Riedel, M.R. Hajirezaei, U. Sonnewald, M. Stitt, J.W. Riesmeier and L. Willmitzer. 1998. Combined expression of glucokinase and

invertase in potato tubers leads to a dramatic reduction in starch accumulation and a stimulation of glycolysis. Plant J. 15:109-118.

Trethewey, R.N., P. Geigenberger, A. Hennig, H. Fleischer-Notter, B. Muller-Rober and L. Willmitzer. 1999a. Induction of the activity of glycolytic enzymes correlates with enhanced hydrolysis of sucrose in the cytosol of transgenic potato tubers. Plant Cell Environ. 22:71-79.

Trethewey, R.N., J.W. Riesmeier, L. Willmitzer, M. Stitt and P. Geigenberger. 1999b. Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. Planta. 208:227-238.

Turgeon, R. and R. Medville. 2004. Phloem loading. A reevaluation of the relationship between plasmodesmatal frequencies and loading strategies. Plant Physiol. 136: 3795-3803.

Tuskan, G.A., S. DiFazio, S. Jansson et al. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science. 313:1596-1604.

Tymowska-Lalanne, Z. and M. Kreis. 1998. The plant invertases: Physiology, biochemistry and molecular biology. *In* Advances in Botanical Research. Vol 28., pp. 71-117.

von Schaewen, A., M. Stitt, R. Schmidt, U. Sonnewald and L. Willmitzer. 1990. Expression of a yeast-derived invertase in the cell-wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J. 9:3033-3044.

Weber, H., U. Heim, S. Golombek, L. Borisjuk, R. Manteuffel and U. Wobus. 1998. Expression of a yeast-derived invertase in developing cotyledons of *Vicia narbonensis* alters the carbohydrate state and affects storage functions. Plant J. 16:163-172.

Zuther, E., M. Kwart, L. Willmitzer and A.G. Heyer. 2004. Expression of a yeast-derived invertase in companion cells results in long-distance transport of a trisaccharide in an apoplastic loader and influences sucrose transport. Planta. 218:759-766.

CHAPTER FOUR

Spatial and temporal expression profiling of cell-wall invertase genes during early development in hybrid poplar

4.1 Introduction

Invertases (EC 3.2.1.26) represent a diverse family of sucrose-cleaving enzymes that has been divided into distinct subclasses based on three subcellular locations: the cytosol, the vacuole and the cell wall. Cytosolic invertases have neutral or alkaline pH optima, while the vacuolar and cell-wall invertases have lower pH optima, presumably to match the acidic nature of their subcellular location (Tymowska-Lalanne and Kreis 1998a). Vacuolar and cell-wall invertases can be further differentiated based on the charge of the mature protein, with vacuolar and cell-wall invertases having acidic and basic isoelectric points, respectively (Tymowska-Lalanne and Kreis 1998a). Additionally, these two latter subclasses can be differentiated at the sequence level as both invertases share a conserved amino acid active-site motif (WEC<u>P/V</u>D), with vacuolar invertases having a valine at the forth residue in contrast to the proline residue that is common to cell-wall invertases (Goetz and Roitsch 1999, Maddison et al. 1999).

Despite their differences, all plant invertases share the common ability to irreversibly hydrolyze sucrose, a major transport sugar in terrestrial plants. Since this class of enzyme influences such a central component to plant growth and survival, considerable efforts have gone into understanding the physiological roles of these key proteins. For example, the cell wall invertases have been proposed to play a fundamental role in creating and maintaining sink strength (Eschrich 1980). This model describes the movement of sucrose from photosynthetic source tissue to heterotrophic sink tissue via the phloem, at which point the disaccharide is released into the cell-wall space. Cell-wall invertase subsequently hydrolyzes the sucrose in the apoplasm releasing glucose and fructose, which are then transferred into the surrounding cells via hexose transporters. This catalytic breakdown of sucrose in the cell-wall space creates a concentration gradient, thereby maintaining the flow of sucrose from source to sink tissues (Eschrich 1980).

As a result of their critical role in influencing sink strength, cell-wall invertases have been investigated in a number of commercial crop species where sink tissues are of central importance, including carrot (*Daucus carota* L.; Sturm and Chrispeels 1990, Ramloch-Lorenz et al. 1993, Lorenz et al. 1995, Sturm et al. 1995), potato (*Solanum tuberosum* L.; Hedley et al. 1993, Hedley et al. 1994, Maddison et al. 1999), maize (*Zea mays* L.; Miller and Chourey 1992, Cheng et al. 1996, Xu et al. 1996, Cheng and

Chourey 1999, Cheng et al. 1999, Taliercio et al. 1999, Kim et al. 2000), peas (*Pisum sativum* L.; Zhang et al. 1996), tobacco (*Nicotiana tabacum* L.; Weil and Rausch 1994, Greiner et al. 1995, Goetz et al. 2001), faba beans (*Vicia faba* L.; Weber et al. 1995), tomato (*Lycopersicon esculentum* L.; Godt and Roitsch 1997, Ohyama et al. 1998, Proels et al. 2003, Proels et al. 2006), barley (*Hordeum vulgare* L.; Weschke et al. 2003), sugar beet (*Beta vulgaris* L.; Godt and Roitsch 2006), rice (*Oryza sativa* L.; Cho et al. 2005), and wheat (*Triticum aestivum* L.; Koonjul et al. 2005). Additionally, cell-wall invertases have been extensively investigated in *Arabidopsis thaliana* L. Heynh. (Schwebel-Dugue et al. 1994, Mercier and Gogarten 1995, Tymowska-Lalanne and Kreis 1998b, Sherson et al. 2003, Mitsuhashi et al. 2004, De Coninck et al. 2005) and *Chenopodium rubrum* L. (Roitsch et al. 1995, Goetz and Roitsch 1999). Collectively, these and other studies have led to a number of proposed roles for cell-wall invertases in addition to the classical phloem unloading hypothesis, including involvement in carbohydrate signalling, wound response, pollen formation, and creation of osmotic potential for tissue expansion (for reviews see Sturm 1999, Roitsch et al. 2003, Koch 2004, Roitsch and Gonzalez 2004). However, despite the plethora of research on these enzymes, the identity and expression pattern of cell-wall invertases in tree species has only recently been evaluated (Bocock et al. 2008). Cellulose and lignin, the two major constituents of wood, are the most abundant biopolymers on earth and consequently represent enormous carbon sinks. As such, the enzymes and overlying mechanisms that govern carbon allocation to sink tissues in tree species are of great interest both economically and socio-politically.

The primary objectives of the present experiment were to: (1) identify cell-wall invertase genes in hybrid poplar (*Populus alba x grandidentata*), (2) determine the spatial and temporal expression patterns of these genes in vegetative tissues, and (3) suggest possible roles for the respective enzymes in development.

4.2 Materials and methods
4.2.1 Gene isolation and sequence analysis
The 3′ end of *PaxgINV1* was amplified from hybrid poplar cDNA using the FirstChoice[®] RLM RACE Kit (Ambion, Austin, TX) with a gene-specific primer (5′-CCTTTTGCTGGATTTGTTGATGT-3′) that was created using the homologous

sequence from the *P. trichocarpa* genome (Tuskan et al. 2006; genome.jgi-psf.org/Poptr1_1). The 5′ end of *PaxgINV1* was amplified from hybrid poplar genomic DNA using a forward primer located upstream of the predicted start codon (5′-CTTTTCAGACTCTAACAACAAC-3′) and a reverse primer located within the expected coding region (5′-CCGTTGATCCAGTTCTTAGG-3′). The 5′ and 3′ amplicons were cloned into the pCR®-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. The resulting sequence was used to create forward (5′-ATGGATAAGCTATTAGGGACGG-3′) and reverse (5′-TTAGCTCTTTACAGGGACATTCA-3′) primers designed to amplify the coding region of *PaxgINV1* from hybrid poplar cDNA.

The 5′ end of *PaxgINV2* was amplified using a forward primer located upstream of the predicted coding sequence (5′-AGCTCTTTCGATATTATTCATTG-3′) with a reverse primer located in the coding sequence (5′-TATGGACTTGGTTCACGTTCT-3′). The 3′ end of *PaxgINV2* was amplified using a forward primer in the coding sequence (5′-CCATCTCATCTAGGGTTTATCC-3′) and a reverse primer downstream of the predicted stop codon. The amplicons were cloned as above and the resulting sequences were used to design forward (5′-ATGATGGTTATGCCACACACTC-3′) and reverse (5′-TCAACTCTTTATAGGAACATTCATG-3′) primers to amplify the coding region of *PaxgINV2* from hybrid poplar cDNA.

The entire genomic region of *PaxgINV3* including introns was amplified from hybrid poplar genomic DNA with a forward primer designed upstream of the predicted start codon (5′-AAGCACGTAAACTCTCATTCT-3′) and a reverse primer designed downstream of the predicted stop codon (5′-GATCTAGGTTGTCTTAGTAAATAC-3′). The resulting amplicon was ligated into a cloning vector as above and sequenced using universal M13 forward and reverse primers along with three gene-specific forward primers (5′-GTTTTCAATGGCATGACATGAG-3′, 5′-TTCCGTGATCCGACCACTGC-3′, 5′-GCTTGGTGGAATTTATTGTTAAC-3′) that were designed from the *P. trichocarpa* homologue. The coding region of *PaxgINV3* was deduced from the genomic sequence using the coding region of *PaxgINV2* as a guide.

4.2.2 Phylogenetic analysis

Complete and partial plant cell-wall invertase amino acid sequences derived from cDNA were obtained from GenBank and entered along with the predicted amino acid

sequences of *PaxgINV1*, *PaxgINV2* and *PaxgINV3* into a ClustalW program available online (http://align.genome.jp). The data output was represented as a dendrogram.

4.2.3 Plant growth and tissue collection

Hybrid poplar trees were propagated in tissue culture on semi-solid WPM medium (McCown and Lloyd 1981) supplemented with 0.01 µM of naphthalene acetic acid. The trees were allowed to develop with 16 h days at 25°C under an average photon flux density of 50 µmol m^{-2}s^{-1}. After three weeks of growth in GA-7 Magenta$^{®}$ vessels (Sigma-Aldrich, St. Louis, MO) approximately 50 plants were transferred to soil in 4 L pots. The trees were permitted to grow in a greenhouse for one year (July 2006 to June 2007) under ambient temperatures which mimicked those of the outside local environment (Vancouver, British Columbia, Canada: 49° North, 123° West). Three trees were destructively harvested together at approximately 30-day intervals throughout the growth trial, between noon and 2 p.m. each month. Tissues were separated and immediately frozen in liquid nitrogen on site prior to being stored at –80°C.

4.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 1 g of ground hybrid poplar tissue using a method specific for tree species (Kolosova et al. 2004). The resulting RNA (10 µg) was then treated using the Turbo DNA-*free*^{TM} kit (Ambion, Austin, TX) to ensure complete removal of contaminating DNA template. An aliquot (1 µg) of treated RNA was used to generate cDNA using SuperScript^{TM}II Reverse Transcriptase (Invitrogen) in a total volume of 12 µL.

4.2.5 Semi-quantitative RT-PCR

Each PCR consisted of 2mM dNTP, 10 pmol each of a forward and reverse primer, 2.5 U of Taq DNA Polymerase with ThermoPol Buffer (New England Biolabs, Ipswich, MA) and 1 µL of cDNA in a total reaction volume of 20 µL. The *PaxgINV1* and *PaxgINV2* primer pairs were designed in regions having limited consensus between the two genes, and were as follows: PaxgINV1F (5′-GGGACGGCTTTGTTAAAGTTC-3′), PaxgINV1R (5′-TTCATGTCTTGTTGCACAGA-3′), PaxgINV2F (5′-TATGGGACAAAATGTGAAA-3′) and PaxgINV2R (5′-TGCTTATCTTTCTGAGGATC-3′). PCR was performed using a PTC-100 thermocycler (MJ Research, Waltham, MA) under the following conditions: 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. An aliquot (10 µL) of each PCR product was

separated on a 2% agarose gel in TAE buffer. Gels were visualized with ethidium bromide under ultraviolet light and images were captured with an AlphaImager™ 2200 (Alpha Innotech, San Leandro, CA).

4.3 Results

4.3.1 Gene isolation and sequence analysis

Three putative cell-wall invertase genes (Table 4.1) were identified from the fully sequenced *Populus trichocarpa* (Torr. & Gray) genome (Tuskan et al. 2006) and associated online database (http://genome.jgi-psf.org/Poptr1_1). The homologues of these genes from hybrid poplar were identified from genomic DNA, and their corresponding transcripts from cDNA were isolated.   Two genes (*PaxgINV1* and *PaxgINV2*) were readily detectable from cDNA corresponding to a variety of hybrid poplar tissues. These genes were subsequently cloned and their putative coding regions sequenced. Transcript of the third cell-wall invertase gene (*PaxgINV3*) was not detectable in any vegetative tissues, nor in male and female flower tissues. Due to the absence of detectable transcript, the coding region of *PaxgINV3* was deduced by sequencing the entire genomic region of *PaxgINV3*, aligning the resultant sequence with the coding region of *PaxgINV2* and removing the predicted intron regions *in silico*. The deduced amino acid sequences of the three putative cell-wall invertases were aligned to determine regions of consensus (Figure 4.1). All three sequences had the predicted β-fructosidase (NDPN) and cell-wall invertase active site (WECPD) amino acid motifs common to plant cell-wall invertases (Maddison et al. 1999). Additionally, all three genes had predicted isoelectric points in the expected neutral to basic pH range (*PaxgINV1*: 8.62, *PaxgINV2*: 8.98, *PaxgINV3*: 7.27).

4.3.2 Phylogenetic analysis

The deduced amino acid sequences of the three hybrid poplar cell-wall invertase genes along with the cDNA-derived amino acid sequences of a number of other plant cell-wall invertases identified from GenBank were subjected to ClustalW analysis.   A clear dichotomy was apparent between monocot and dicot cell-wall invertases, and for the sake of brevity, only the dendrogram for the dicotyledonous species is shown (Figure 4.2).   As expected, members of the Solanaceae family (tobacco, tomato and potato) grouped together, including the close grouping of the tomato *Lin5* and *Lin7* genes (Godt

and Roitsch 1997) with their respective homologues, *invGE* and *invGF*, from potato (Maddison et al. 1999). The carrot cell-wall invertases on the other hand grouped together as an independent clade, as did the three hybrid poplar cell-wall invertase sequences. The amino acid sequence of the *VfCWINV1* gene from *Vicia faba* (Weber et al. 1995) was shown to be the most similar sequence to the three hybrid poplar genes, while the cell-wall invertase gene from *Carica papaya* (AF420223; unpublished) had the highest similarity to the hybrid poplar genes when the same ClustalW analysis was performed using the corresponding nucleotide sequences (dendrogram not shown).

4.3.3 Spatial and temporal expression profiles during a complete year of growth

In an attempt to investigate the expression patterns of the cell-wall invertase genes from hybrid poplar, an experiment was conducted to examine the spatial and temporal transcript abundance of *PaxgINV1* and *PaxgINV2* in a complement of vegetative tissues in hybrid poplar. *PaxgINV3* was not investigated in this manner due to the lack of expression in any vegetative tissues examined at various developmental periods. Tissue cultured trees were transferred to soil and permitted to grow in a greenhouse for a full year at ambient outside temperatures, and thereby mimicked the local annual seasonal environment (Vancouver, British Columbia, Canada), which allowed natural physiological processes to occur (e.g. dormancy). Trees were destructively harvested monthly throughout the growth cycle (July 2006 to June 2007), and the tissues were subsequently analyzed for *PaxgINV1* and *PaxgINV2* expression using semi-quantitative RT-PCR.

4.3.3.1 Expression profile of *PaxgINV1*

The expression of *PaxgINV1* was limited to the dormancy and post-dormancy stages of development (Figure 4.3a), and overall had the weakest expression of the two cell-wall specific genes examined. No transcript was detectable during the early stages of development from 30 to 120 days after transfer (DAT) to the greenhouse when the most dramatic aerial and root growth occurred. *PaxgINV1* expression appeared to be most prevalent in the apex, young leaf, phloem and root tissues in December, after buds had developed, temperatures had dropped considerably and active growth had effectively stopped. Additionally, isolated periods of expression were apparent during the dormant months (no significant change in growth). For example, expression was detected in the

fine roots in January and the apex and buds in February (Figure 4.4). However, no transcript was detected during March and April (during bud break). In May, expression was again detectable in the apex, phloem and fine roots as temperatures increased and branch development initiated. When growth had fully resumed in June, the expression of *PaxgINV1* was weak and limited to the apex of the tree and leaves in the branch tissues when many of the auxillary branches were being formed (Figure 4.5). Expression was not detected in wood (xylem) or cambial tissue of the stem or branches at any stage of development.

4.3.4.2 Expression profile of *PaxgINV2*

In comparison to *PaxgINV1* the expression of *PaxgINV2* was very strong (Figure 4.3b), especially during the early stages of growth (60-120 DAT) when *PaxgINV1* expression was not detectable. The expression of *PaxgINV2* during this period was ubiquitous, with relatively equal transcript abundance in all tissues examined. There was a marked decrease in expression in December after bud set and temperatures lowered. The expression of *PaxgINV2* during December was similar to *PaxgINV1*, except *PaxgINV2* was still weakly expressed in the middle stem wood (Figure 4.3b). *PaxgINV2* continued to be weakly expressed throughout the dormant months in all tissues examined until March when there was a significant spike in expression in the phloem, which coincided with the initiation of bud break. This elevated level of expression was similar to the high levels observed in the buds in March and the emerging shoots in April (Figure 4.4). The level of *PaxgINV2* generally increased in most tissues from April to June, with the highest expression being in the lower stem in June, when rapid growth in girth was occurring. The expression of *PaxgINV2* in the branches from June (Figure 4.5) was highest in the apex, petiole and leaf tissues with very weak expression in the phloem and wood tissue.

4.4 Discussion

4.4.1 Three cell-wall invertase genes exist in hybrid poplar

The recently sequenced *Populus trichocarpa* genome (Tuskan et al. 2006) has quickly proven to be an invaluable tool in the field of tree genetics and genomics. In this study, the online JGI database of the *P. trichocarpa* genome was used to expedite the identification of the three putative cell-wall invertases. The homologues for these

genes were subsequently identified and sequenced from hybrid poplar (*P. alba x grandidentata*). The deduced amino acid sequences of *PaxgINV1*, *PaxgINV2* and *PaxgINV3* possessed conserved β-fructosidase (NDPN) and cell-wall invertase (WECPD) motifs, providing strong evidence for the identification of these three genes as cell-wall invertases (Goetz and Roitsch 1999, Maddison et al. 1999). In addition, the predicted isoelectric points of the deduced peptide sequences are in the neutral to basic pH range, which has been identified as a common characteristic of cell-wall invertases.

Homologs to these three genes were also recently identified as cell-wall invertases in *P. trichocarpa* (*PtCIN1*, *PtCIN2* and *PtCIN3*; Bocock et al. 2008), along with two additional putative cell-wall invertases (*PtCIN4* and *PtCIN5*), although the latter two genes appear to be more closely related to fructan exohydrolases, such as *AtcwINV6*, and were not examined further in the present study. Caution was exercised due to the re-classification of putative cell-wall invertase genes (*AtcwINV3* and *AtcwINV6*), containing NDPN and WECPD motifs, as fructan exohydrolases in *Arabidopsis* by De Coninck et al. (2005). Furthermore, preliminary expression analysis of *PaxgINV1* and *PaxgINV2* cDNA in *Pichia pastoris* indicates that the mature proteins are able to effectively hydrolyze sucrose (data not shown). Taken together, along with the close sequence similarity between *PaxgINV2* and *PaxgINV3*, we speculate that only three cell-wall invertases (*PaxgINV1*, *PaxgINV2* and *PaxgINV3*) exist in hybrid poplar.

4.4.2 The evolutionary relationship of cell-wall invertases

An amino acid sequence-derived dendrogram of several putative plant cell-wall invertases, including the three hybrid poplar genes described in this study, was created using ClustalW. There was a distinct separation of the cell-wall invertases representing monocotyledonous species (including rice, maize and barley) and those representing dicotyledonous species. Similar phylogenetic analyses have also shown divisions between cell-wall invertases of the two broad plant classifications (Hirose et al. 2002, Cho et al. 2005, De Coninck et al. 2005, Van Riet et al. 2006).

Within the dicotyledonous species there was very high homology between three members of the Solanaceae family (tobacco, tomato and potato). This has been well documented, particularly with respect to the cell-wall invertases *invGE* and *invGF* from potato and their respective tomato homologues, *Lin5* and *Lin7* (Godt and Roitsch 1997, Maddison et al. 1999, Proels et al. 2003). These homologues not only share similar

sequences, but are similarly regulated. For example, *invGE* and *Lin5* are expressed in both vegetative and reproductive tissues, while *invGF* and *Lin7* are expressed exclusively in reproductive tissues (Godt and Roitsch 1997, Maddison et al. 1999). Additionally, these homologue pairs have been shown to exist in tandem repeat within their respective genomes, separated by 1.7 kb in potato (Maddison et al. 1999) and 1.5 kb in tomato (Proels et al. 2003). These phenomena can be explained in part by the close genetic relationship between tomato and potato (Tanksley et al. 1992). However, the tandem duplication of cell-wall invertase genes is not unique to Solanaceae. Analysis of the *P. trichocarpa* genome revealed that the *PaxgINV2* and *PaxgINV3* genes in poplar exist in a tandem repeat with 6.8 kb separating the two genes. However, *PaxgINV2* and *PaxgINV3* are more similar to each other than to the orthologous *invGE*/*invGF* and *Lin5*/*Lin7* gene pairs (Figure 4.2). This may be evidence for parallel evolution of the tandem cell-wall invertases between the distantly related Solanaceae and Salicaceae families as it appears that the tandem duplication event occurred after the species diverged from the common ancestor. The possibility of parallel evolution with respect to cell-wall invertases has been discussed previously (Fridman and Zamir 2003) when a similar trend was identified between cell-wall invertases of *Arabidopsis* and tomato, which are predicted to have diverged approximately 112 million years ago (Ku et al. 2000). Interestingly, the phylogenetic tree constructed in this study shows a species-specific grouping of cell-wall invertases in carrot as well. This further supports the suggestion of parallel evolution of cell-wall invertase gene duplication, although this is highly speculative and would at minimum require information regarding the genomic organization of *Inv*Dc2* and *Inv*Dc3*.

4.4.3 *PaxgINV1* expression is associated with dormancy in hybrid poplar

The use of a perennial dicot in this study enabled the unique monitoring of cell-wall invertase gene expression throughout three distinct developmental stages: (1) early growth, (2) dormancy and (3) post-dormancy. Semi-quantitative RT-PCR analyses revealed that *PaxgINV1* expression was undetectable during the early stages of growth, as defined by days post planting, and was instead first detectable in a variety of tissues (apex, leaf, phloem, root and fine roots) in December when temperatures had decreased, most of the leaves had senesced, and growth had effectively ceased. After a spike in expression of *PaxgINV1* in December, the level of transcript decreased until

107

no expression was detected at the end of dormancy in March. Similarly, no expression was detected after bud break in April. This period of undetectable expression was followed by an increase in transcript in the apex, phloem and fine roots in May when lateral branches and leaves were forming and whole-plant growth was initiating. The expression decreased again in June, and was limited to only the apex of the tree and leaves from the newly formed branches. The lack of expression of *PaxgINV1* both temporally and spatially during times of rapid young growth after transplantation (September to November) and bud break (March and April), in combination with the presence of transcript in young source leaves, suggests that the enzyme encoded by *PaxgINV1* does not play a role in sink-source metabolism. The lack of association with sink tissue is not novel as Sturm et al. (1995) have previously shown the absence of cell-wall invertase transcript in the developing tap roots of carrots. As such, alternative roles for cell-wall invertase have been suggested, including roles in the generation and/or maintenance of osmotic pressure, as well as a roles in regulating/influencing genes associated with cell wall carbohydrate biosynthesis (for reviews see Sturm 1999, Roitsch et al. 2003, Koch 2004, Roitsch and Gonzalez 2004). Further investigation is necessary to determine the exact role this cell-wall invertase plays in hybrid poplar, however, we suggest that *PaxgINV1* plays either a direct or indirect role in processes relating to dormancy. This conclusion is in contrast to an extracellular invertase from tobacco, which was shown to delay senescence in the presence of cytokinins (Lara et al. 2004). Nevertheless, both this study and that from tobacco emphasize the association of cell-wall invertase with the molecular processes surrounding senescence and more generally, the seasonal reallocation of carbohydrate.

4.4.4 *PaxgINV2* expression is associated with active growth in hybrid poplar

Expression profiling of *PaxgINV2* showed very high levels of transcript abundance in all tissues evaluated during early stages of growth. This coincided with periods of rapid vertical and horizontal growth, suggesting that these developmental stages are reliant on the PaxgINV2 enzyme to provide energy and carbon skeletons to the rapidly expanding tissues. The dramatic decrease in expression in December, when growth had effectively stopped, further provides evidence for PaxgINV2's role in phloem-unloading, as was proposed by Eschrich (1980), who hypothesized that cleavage of sucrose by cell-wall invertase in sink tissues created a concentration gradient with the

source tissues. This proposed role has been corroborated in subsequent studies with several plants species. For example, in *C. rubrum* temporal and spatial expression of a root-specific invertase (*CIN1*) was documented, which implied that cell-wall invertase functioned to provide heterotrophic tissue with monomeric carbohydrates for respiration and/or carbon skeletons for polymer biosynthesis (Roitsch et al. 1995). Additionally, the expression of a cell-wall invertase gene in *Arabidopsis*, *Atβfruct1*, was shown to be highest during aeroponic conditions when root growth was stimulated (Tymowska-Lalanne and Kreis 1998b).

Although the transcript abundance of *PaxgINV2* decreased markedly from December to March, which coincided with dormancy, expression during this period was still weakly quantifiable in most tissues. With the subsequent onset of bud break, the transcript level of *PaxgINV2* increased noticeably in the phloem tissue and buds. Transcript induction at this time likely represented a need to reallocate carbon reserves from the phloem tissue to newly forming tissues, which require significant carbohydrate moieties, as has been demonstrated previously in Norway spruce (Egger et al. 1996). We therefore suggest that *PaxgINV2* may be involved in the mobilization of carbohydrate in the phloem tissue to support the heterotrophic processes surrounding bud break in hybrid poplar.

Finally, in June a notable spike in *PaxgINV2* transcript abundance was prevalent in the lower xylem tissue, which represents the most mature tissue being evaluated. This effectively represents stem tissue undergoing girth growth and initiation of latewood formation in the xylem in the second year. An earlier study in Scots pine evaluating the transition between earlywood and latewood showed that the major sucrose-cleaving enzyme involved in mature xylem formation was cell-wall invertase, which suggested that the majority of the carbohydrate required for growth in maturing xylem arrived via an apoplastic route (Uggla et al. 2001). Given the conclusions from that study and the evidence presented here, we suggest that PaxgINV2 has a major role in providing developing woody tissue with the energy and carbon skeletons required for rapid rates of cellulose and lignin biosynthesis in hybrid poplar.

4.4.5 *PaxgINV3* expression is tightly regulated or non-existent in hybrid poplar

Three cell-wall invertase genes were confirmed at the genomic level in hybrid poplar. Transcripts for two of these genes, *PaxgINV1* and *PaxgINV2*, were subsequently identified in a variety of vegetative tissues of hybrid poplar during the primary year of growth. Transcript for the third gene, *PaxgINV3*, was not detectable at any time in any tissues examined in this study. However, such limited expression of a cell-wall invertase is not unique. For example, the *Arabidopsis Atβfruct2* gene was shown to be expressed exclusively in the floral tissue (Tymowska-Lalanne and Kreis 1998b). Similarly, transcripts of the *invGF/Lin7* gene group in Solanaceae were detected exclusively in anthers and pollen of reproductive tissue (Godt and Roitsch 1997, Maddison et al. 1999). These findings, along with the discovery of pollen-specific elements associated with the promoter of the downstream tandemly repeated cell-wall invertase in *Arabidopsis*, led to the hypothesis that the floral-specific expression of the 3′ gene of tandemly arranged cell-wall invertase pairs may be a common feature in plant species (Proels et al. 2003). The current data from this experiment do not refute this hypothesis, however it should be noted that *PaxgINV3* is located upstream of the vegetatively-expressed *PaxgINV2* in *P. trichocarpa*, not downstream as would be predicted. Additionally, the expression of *PaxgINV3* was not investigated in the reproductive tissue of hybrid poplar in this experiment due to the time frame required for poplar to develop reproductive tissues (7-10 years). However, a recent study by Bocock et al. (2008) found that the homolog to *PaxgINV3* (*PtCIN2*) was weakly expressed solely in the female catkin tissue of field-grown *P. deltoides*, lending support to the theory of limited floral-specific expression of *PaxgINV3.*

Alternatively, *PaxgINV3* may represent a silent gene, although sequence analysis of this gene at the genomic level did not reveal premature stop sequences nor any other sequence abnormality that would infer improper post-transcriptional processing. The tomato cell-wall invertase gene (*Lin8*) was previously shown to have undetectable transcript as well (Godt and Roitsch 1997), although transcript of this gene was later reported in root and leaf tissue in a separate experiment (Proels et al. 2003). Interestingly, *Lin8* is located over 5 kb upstream in a tandem repeat with *Lin6* (Proels et al. 2003), which was shown to be expressed in a number of sink tissues during active growth (Godt and Roitsch 1997). These genomic and functional similarities show that

the tandem duplication of *PaxgINV3/PaxgINV2* closely resembles the *Lin8/Lin6* tandem duplication in tomato, although this relationship may not have an evolutionary basis.

In summary, we have identified three putative cell-wall invertase genes from hybrid poplar using the recently sequenced *P. trichocarpa* genome (Tuskan et al. 2006) as a guide. The hybrid poplar cell-wall invertase genes share more sequence homology to each other than to other plant cell-wall invertase genes, which lends support to the theory that cell-wall invertase genes have evolved variable regulation and function independently in a number of different species (Fridman and Zamir 2003). Such varied regulation was evident in hybrid poplar as each cell-wall invertase gene in this study showed very unique expression patterns both temporally and spatially. *PaxgINV1* expression appeared to be involved with processes related to dormancy, while *PaxgINV2* expression was correlated with actively growing tissues. *PaxgINV3* expression was not detected in any tissue in this experiment and may represent a floral-specific gene, which seems to be a common feature among dicotyledonous species (Lorenz et al. 1995, Weber et al. 1995, Godt and Roitsch 1997, Tymowska-Lalanne and Kreis 1998b, Maddison et al. 1999), including *P. deltoides* (Bocock et al. 2008). Further investigations involving the reproductive tissue of hybrid poplar, as well as examining the properties of the enzymes encoded by the three cell-wall invertases, may provide additional information with respect to the roles cell-wall invertases play in hybrid poplar development.

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                   10        20        30        40        50        60        70        80        90       100
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  MDKLLGTALLKFLPVLPLFALLFVLSNNGVEASHKIYLRYQSLSVDKVKQIHRTGYHFQPPKNWINDPNGPLYYKGLYHLFYQYNPKGAVWGNIVWAHSV
PaxgINV2  MM-----VMPHTLPVLALFALLFVLSNNGAEASHKIYSRYQNLSVENVNQVHRTGYHFQPPRHWINDPNAPMYYKGLYHLFYQYNPKGAVWGNIVWAHSV
PaxgINV3  MM-----AMPHTLPVLALFALLFVLSNNGAEASHKIYPRLQTLSVENVNQVHRTGYHFQPLRNWINDPNAPMYYKGLYHLFYQYNPKGAVWGNIVWAHSV
          *      **** *********** ******* * * ***  * * *********  ****** * **************************

                  110       120       130       140       150       160       170       180       190       200
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  SKDLINWESLEPAIYPSKWFDNYGCWSGSATILPNGEPVIFYTGIVDGNNRQIQNYAVPANSSDPYLREWVKPDDNPIVYPDPSVNASAFRDPTTAWRVG
PaxgINV2  SKDLINWESLEPAIYPSKWFDYYGCWSGSATVLPNGEPVILYTGIVDKNNSQIQNYAVPANLSDPYLREWVKPDDNPIVNPDANVNGSAFRDPTTAWWAD
PaxgINV3  SKDLINWESLEPAIYPSKWFDSYGCWSGSATILPNGEPVILYTGIADKNNSQIQNYAVPANLSDPYLREWVKPDDNPILNPDVSVNGSAFRDPTTAWWAD
          ******************** ********* ******* **** * ** ********** ****************  **  ** *********

                  210       220       230       240       250       260       270       280       290       300
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  GHWRILIGSKKRDRGIAYLYRSLDFKKWFKAKHPLHSVQGTGMWECPDFFPVSLSGEEGLDTSVGGSNVRHVLKVSLDLTRYEYYTIGTYDEKKDRYYPD
PaxgINV2  GHWRILIGSRRKQRGVAYLYRSKDFKKWVKAKHPLHSVQGTGMWECPDFFPVSLSGKNGLDPSVMGQNVKHVLKVSLDMTRYEYYTMGTYNKKKDKYFPD
PaxgINV3  GHWRILIGSRRNHVGVAYLYRSRDLKKWAKTKHPLHSVQGTGMWECPDFFPVSSFGENGLDPSVNGQNVKHALKVSLDLTRYEYYTLGTYDNKKEKYFPD
          *********    * ****** * *** *  ********************** *  *** ** * ** * ****** ******* ***  **  * **

                  310       320       330       340       350       360       370       380       390       400
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  EALVDGWAGLRYDYGNFYASKTFFDPSKNRRILWGWANESDSVQQDMNKGWAGIQLIPRRVWLDPSGKQLLQWPVAELEKLRSHNVQLRNQKLYQGYHVE
PaxgINV2  EGLVDGWAGLRLDYGNFYASKTFFDPSTNRRVLWGWANESDDPQKDKDKGWAGIQLIPRKVWLDPSGKQLLQWPVAELEKLRGHNVQLRNQKLNQGNHVE
PaxgINV3  VGLVDGWAGLRLDYGNFYASKTLFDPSTNRRILWGWANESDVVQQDTNKGWAGIQLIPRKVWLDPSGEQLLQWPVVELEKLRGHNVQLSNQKLYQGDHAQ
           ********* ********** ****  *** ********* * *  *********** ******* ******* ****** ***** **** ** *

                  410       420       430       440       450       460       470       480       490       500
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  VKGITAAQADVDVTFSFPSLDKAEPFDPKWAKLDALDVCAQKGSKAQGGLGPFGLLTLASEKLEEFTPVFFRVFKAA-DKHKVLLCSDARSSSLGEGLYK
PaxgINV2  VKVITAAQADVDVTFSFPSLDKAEPFDPKWAKLDALDVCDQKGSKDPGGLGPFGLLTLASKNLEEFTPVFFRVFKAAADKHKVLLCSDARSSSLGKGLYK
PaxgINV3  VKGITVAQADVDVTFSFPSLDKAEPFDPKWAKLDALDVCAQKGSKAPGGLGPFGLLTLASKNLEEFTPVFFKVFKAA-DKHKVLLCSDARSSSLGKGLYK
          ** ** ********************************** *****  ************** ***** ***** ***************** ****

                  510       520       530       540       550       560       570       580       590       600
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
PaxgINV1  PPFAGFVDVDLTDKKLTLRSLIDHSVVESFGAGGRTVITSRVYPIIAVFEKAHLFVFNNGSETVTVESLDAWSMKMPVMNVPVKS
PaxgINV2  PSFAGFVDVDLTGKKLSLRSLIDHSVVESFGVGGRIAISSRVYPTIAVSEKAHLYVFNNGSETITVENLNAWSMNTPVMNVPIKS
PaxgINV3  PSFAGFVDVDLTDKKLSLRSLIDHSVIEGFGAGGRIAISSRVYPTIAVSEKAHLYVFNNGSETITVENLNAWSMNLPVMDVPIKDRVGENPRNE
          * ********** *** ********* * ** ***  * ***** *** ***** ******** *** * ****  *** ** *
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Figure 4.1. Deduced amino acid sequence alignment of three cell-wall invertases from hybrid poplar. Putative β-fructosidase (NDPN) and cell-wall invertase active site (WECPD) motifs are underscored and bold. Asterisks signify consensus among the three sequences.
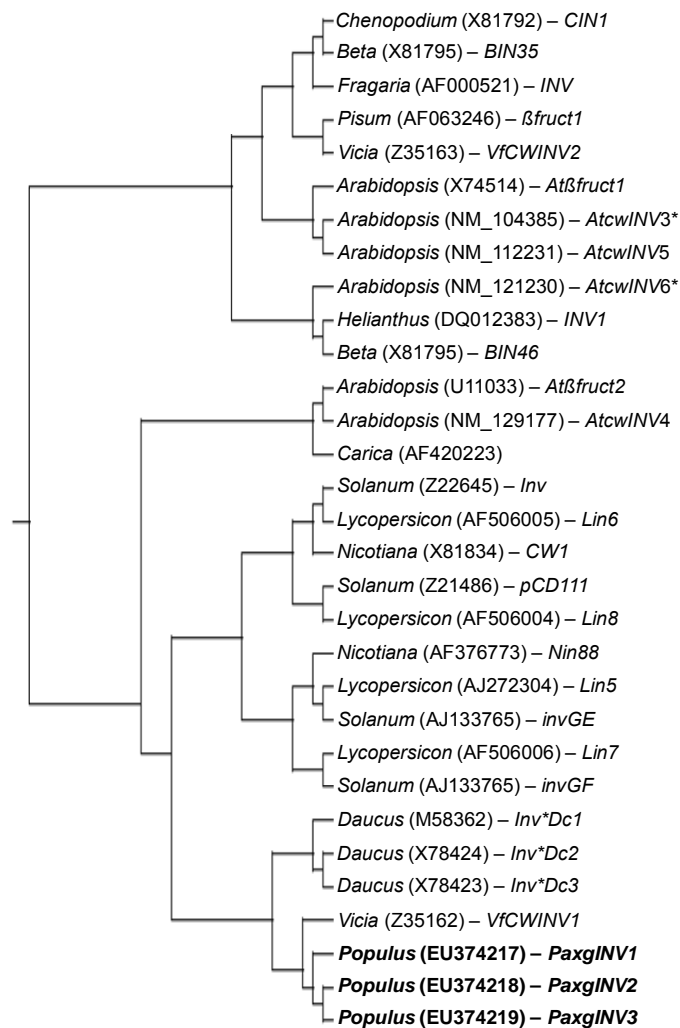
Figure 4.2. A dendrogram constructed with ClustalW using the cDNA-derived amino acid sequences of confirmed and putative cell-wall invertases from dicotyledonous plants. GenBank accession numbers are provided in parentheses and, where possible, gene names from the literature are cited: *Inv*Dc1* (Sturm and Chrispeels 1990); *pCD111* (Hedley et al. 1993); *Inv* (Hedley et al. 1994); *Atβfruct1* (Schwebel-Dugue et al. 1994); *CIN1* (Roitsch et al. 1995); *Atβfruct2* (Mercier and Gogarten 1995); *Inv*Dc2* and *Inv*Dc3* (Lorenz et al. 1995); *VfCWINV1* and *VfCWINV2* (Weber et al. 1995); *CW1* (Greiner et al. 1995); *βfruct1* (Zhang et al. 1996); *invGE* and *invGF* (Maddison et al. 1999); *Nin88* (Goetz et al. 2001); *Lin5-8* (Godt and Roitsch 1997); *AtcwINV3-6* (De Coninck et al. 2005); *BIN35* and *BIN46* (Godt and Roitsch 2006); *INV*, *INV1* and the *Carica* sequences to date are unpublished. The cell-wall invertases from this study are in bold. *These enzymes were shown to have no sucrose-cleaving ability and have been functionally characterized as fructan exohydrolases (De Coninck et al. 2005).
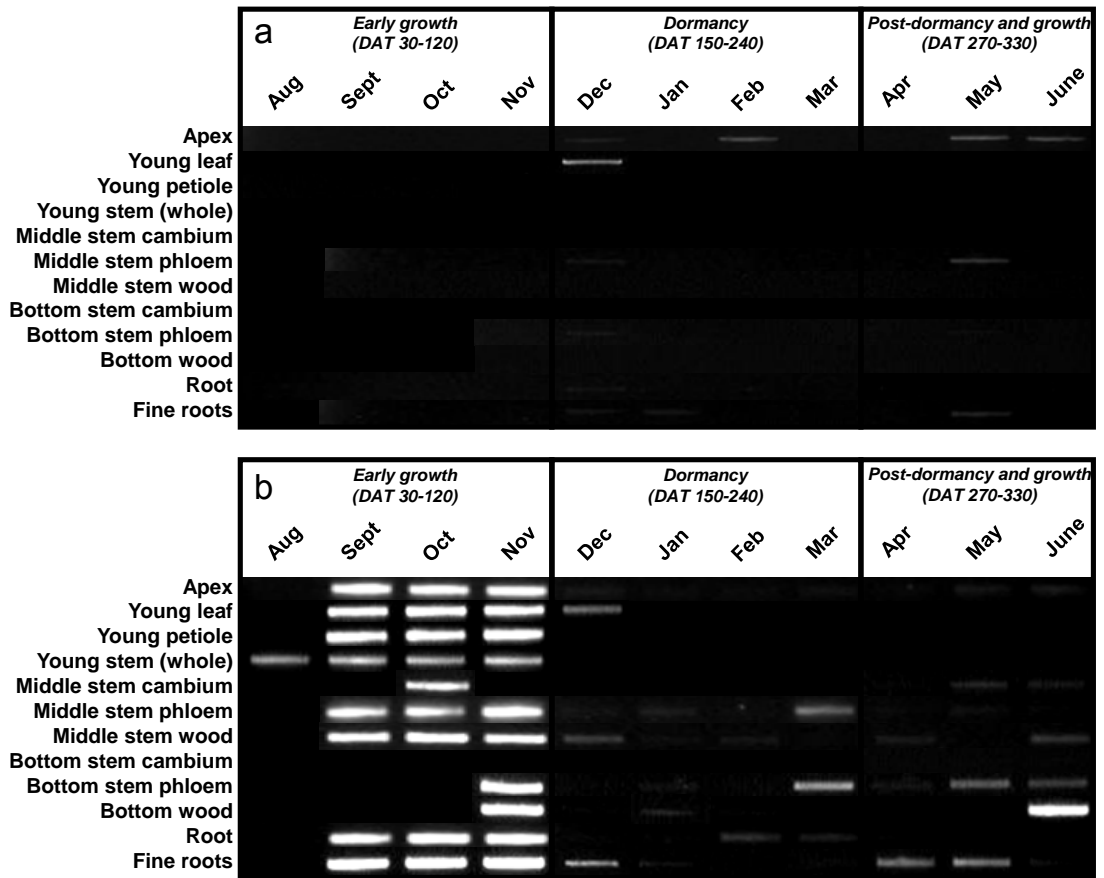
Figure 4.3. Expression profiles of *PaxgINV1* (a) and *PaxgINV2* (b) in several hybrid poplar tissues 30 to 330 days after transfer (DAT) from tissue culture to soil. Plants were grown in a greenhouse under ambient temperatures mirroring the outside environment, permitting dormancy to proceed as normal. cDNA was generated from equal quantities of RNA and all lanes are loaded with equal volumes of PCR product.
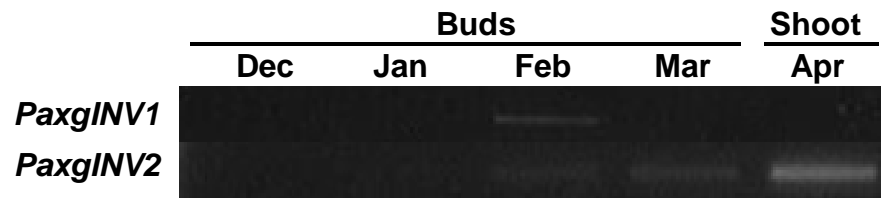
Figure 4.4. Expression pattern of *PaxgINV1* and *PaxgINV2* in buds during dormancy, along with an emerging shoot at the end of dormancy from hybrid poplar. cDNA was generated from equal quantities of RNA and all lanes were loaded with equal volumes of PCR product.
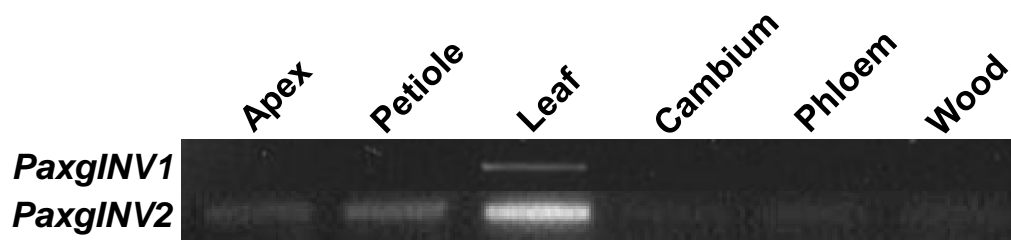
Figure 4.5. Expression pattern of *PaxgINV1* and *PaxgINV2* from newly formed branch tissues of plants 330 days after transfer from tissue culture to soil (June 2007). cDNA was generated using equal quantities of RNA and all lanes were loaded with equal volumes of PCR product.

Table 4.1. GenBank accession numbers of three putative cell-wall invertases from hybrid poplar and the location of their homologs within the *P. trichocarpa* genome database.

Gene	Accession no.	*P. trichocarpa* database homolog	
		Scaffold	Position
PaxgINV1	EU374217	LG_VI	13892977-13897519
PaxgINV2	EU374218	LG_XVI	5725761-5729453
PaxgINV3	EU374219	LG_XVI	5736234-5739915

4.5 References

Bocock, P. N., A. M. Morse, C. Dervinis and J. M. Davis. 2008. Evolution and diversity of invertase genes in *Populus trichocarpa*. Planta. 227:565-576.

Cheng, W. H., E. W. Taliercio and P. S. Chourey. 1996. The Miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell. 8:971-983.

Cheng, W. H. and P. S. Chourey. 1999. Genetic evidence that invertase-mediated release of hexoses is critical for appropriate carbon partitioning and normal seed development in maize. Theor. Appl. Genet. 98:485-495.

Cheng, W. H., E. W. Taliercio and P. S. Chourey. 1999. Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3 ' untranslated region in a cell suspension culture of maize. P. Natl. Acad. Sci. U.S.A. 96:10512-10517.

Cho, J. I., S. K. Lee, S. H. Ko, H. K. Kim, S. H. Jun, Y. H. Lee, S. H. Bhoo, K. W. Lee, G. H. An, T. R. Hahn and J. S. Jeon. 2005. Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa* L.). Plant Cell Rep. 24:225-236.

De Coninck, B., K. Le Roy, I. Francis, S. Clerens, R. Vergauwen, A. M. Halliday, S. M. Smith, A. Van Laere and W. Van den Ende. 2005. Arabidopsis AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. Plant Cell Environ. 28:432-443.

Egger, B., W. Einig, A. Schlereth, T. Wallenda, E. Magel, A. Loewe and R. Hampp. 1996. Carbohydrate metabolism in one- and two-year-old spruce needles, and stem carbohydrates from three months before until three months after bud break. Physiol. Plantarum. 96:91-100.

Eschrich, W. 1980. Free space invertase, its possible role in phloem unloading. Ber. Deut. Bot. Ges. 93:363-378.

Fridman, E. and D. Zamir. 2003. Functional divergence of a syntenic invertase gene family in tomato, potato, and Arabidopsis. Plant Physiol. 131:603-609.

Godt, D. E. and T. Roitsch. 1997. Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. Plant Physiol. 115:273-282.

Godt, D. and T. Roitsch. 2006. The developmental and organ specific expression of sucrose cleaving enzymes in sugar beet suggests a transition between apoplasmic and symplasmic phloem unloading in the tap roots. Plant Physiol. Bioch. 44:656-665.

Goetz, M. and T. Roitsch. 1999. The different pH optima and substrate specificities of extracellular and vacuolar invertases from plants are determined by a single amino-acid substitution. Plant J. 20:707-711.

Goetz, M., D. E. Godt, A. Guivarc'h, U. Kahmann, D. Chriqui and T. Roitsch. 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. P. Natl. Acad. Sci. U.S.A. 98:6522-6527.

Greiner, S., M. Weil, S. Krausgrill and T. Rausch. 1995. A tobacco cDNA coding for cell-wall invertase. Plant Physiol. 108:825-826.

Hedley, P. E., G. C. Machray, H. V. Davies, L. Burch and R. Waugh. 1993. cDNA cloning and expression of a potato (*Solanum tuberosum*) invertase. Plant Mol. Biol. 22:917-922.

Hedley, P. E., G. C. Machray, H. V. Davies, L. Burch and R. Waugh. 1994. Potato (*Solanum tuberosum*) invertase-encoding cDNAs and their differential expression. Gene. 145:211-214.

Hirose, T., M. Takano and T. Terao. 2002. Cell wall invertase in developing rice caryopsis: Molecular cloning of *OsCIN1* and analysis of its expression in relation to its role in grain filling. Plant Cell Physiol. 43:452-459.

Kim, J. Y., A. Mahe, S. Guy, J. Brangeon, O. Roche, P. S. Chourey and J. L. Prioul. 2000. Characterization of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. Gene. 245:89-102.

Koch, K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr. Opin. Plant Biol. 7:235-246.

Kolosova, N., B. Miller, S. Ralph, B. E. Ellis, C. Douglas, K. Ritland and J. Bohlmann. 2004. Isolation of high-quality RNA from gymnosperm and angiosperm trees. Biotechniques. 36:821-824.

Koonjul, P. K., J. S. Minhas, C. Nunes, I. S. Sheoran and H. S. Saini. 2005. Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat. J. Exp. Bot. 56:179-190.

Ku, H. M., T. Vision, J. P. Liu and S. D. Tanksley. 2000. Comparing sequenced segments of the tomato and Arabidopsis genomes: Large-scale duplication followed by selective gene loss creates a network of synteny. P. Natl. Acad. Sci. U.S.A. 97:9121-9126.

Lara, M. E. B., M. C. G. Garcia, T. Fatima, R. Ehness, T. K. Lee, R. Proels, W. Tanner and T. Roitsch. 2004. Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. Plant Cell. 16:1276-1287.

Lorenz, K., S. Lienhard and A. Sturm. 1995. Structural organization and differential expression of carrot beta-fructofuranosidase genes - identification of a gene coding for a flower bud-specific isozyme. Plant Mol. Biol. 28:189-194.

Maddison, A. L., P. E. Hedley, R. C. Meyer, N. Aziz, D. Davidson and G. C. Machray. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol. Biol. 41:741-751.

McCown, B. H. and G. Lloyd. 1981. Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant species. Hortscience. 16:453-453.

Mercier, R. W. and J. P. Gogarten. 1995. A 2nd cell-wall acid invertase gene in *Arabidopsis thaliana*. Plant Physiol. 107:659-660.

Miller, M. E. and P. S. Chourey. 1992. The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell. 4:297-305.

Mitsuhashi, W., S. Sasaki, A. Kanazawa, Y. Y. Yang, Y. Kamiya and T. Toyomasu. 2004. Differential expression of acid invertase genes during seed germination in Arabidopsis thaliana. Biosci. Biotech. Bioch. 68:602-608.

Ohyama, A., S. Nishimura and M. Hirai. 1998. Cloning of cDNA for a cell wall-bound acid invertase from tomato (*Lycopersicon esculentum*) and expression of soluble and cell wall-bound invertases in plants and wounded leaves of *L. esculentum* and *L. peruvianum*. Genes Genet. Syst. 73:149-157.

Proels, R. K., B. Hause, S. Berger and T. Roitsch. 2003. Novel mode of hormone induction of tandem tomato invertase genes in floral tissues. Plant Mol. Biol. 52:191-201.

Proels, R. K., M. C. Gonzalez and T. Roitsch. 2006. Gibberellin-dependent induction of tomato extracellular invertase *Lin7* is required for pollen development. Funct. Plant Biol. 33:547-554.

Ramloch-Lorenz, K., S. Knudsen and A. Sturm. 1993. Molecular characterization of the gene for carrot cell-wall beta-fructosidase. Plant J. 4:545-554.

Roitsch, T., M. Bittner and D. E. Godt. 1995. Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol. 108:285-294.

Roitsch, T., M. E. Balibrea, M. Hofmann, R. Proels and A. K. Sinha. 2003. Extracellular invertase: key metabolic enzyme and PR protein. J. Exp. Bot. 54:513-524.

Roitsch, T. and M. C. Gonzalez. 2004. Function and regulation of plant invertases: sweet sensations. Trends Plant Sci. 9:606-613.

Schwebel-Dugue, N., N. El Mtili, M. Krivitzky, I. Jean-Jacques, J. H. H. Williams, M. Thomas, M. Kreis and A. Lecharny. 1994. *Arabidopsis* gene and cDNA-encoding cell-wall invertase. Plant Physiol. 104:809-810.

Sherson, S. M., H. L. Alford, S. M. Forbes, G. Wallace and S. M. Smith. 2003. Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J. Exp. Bot. 54:525-531.

Sturm, A. and M. J. Chrispeels. 1990. cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell. 2:1107-1119.

Sturm, A., V. Sebkova, K. Lorenz, M. Hardegger, S. Lienhard and C. Unger. 1995. Development-specific and organ-specific expression of the genes for sucrose synthase and three isoenzymes of acid beta-fructofuranosidase in carrot. Planta. 195:601-610.

Sturm, A. 1999. Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol. 121:1-7.

Taliercio, E. W., J. Y. Kim, A. Mahe, S. Shanker, J. Choi, W. H. Cheng, J. L. Prioul and P. S. Chourey. 1999. Isolation, characterization and expression analyses of two cell wall invertase genes in maize. J. Plant Physiol. 155:197-204.

Tanksley, S. D., M. W. Ganal, J. P. Prince, M. C. Devicente, M. W. Bonierbale, P. Broun, T. M. Fulton, J. J. Giovannoni, S. Grandillo, G. B. Martin, R. Messeguer, J. C. Miller, L. Miller, A. H. Paterson, O. Pineda, M. S. Roder, R. A. Wing, W. Wu and N. D. Young. 1992. High-density molecular linkage maps of the tomato and potato genomes. Genetics. 132:1141-1160.

Tuskan, G.A., S. DiFazio, S. Jansson et al. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science. 313:1596-1604.

Tymowska-Lalanne, Z. and M. Kreis. 1998a. The plant invertases: Physiology, biochemistry and molecular biology. *In* Advances in Botanical Research. Vol 28., pp. 71-117.

Tymowska-Lalanne, Z. and M. Kreis. 1998b. Expression of the *Arabidopsis thaliana* invertase gene family. Planta. 207:259-265.

Uggla, C., E. Magel, T. Moritz and B. Sundberg. 2001. Function and dynamics of auxin and carbohydrates during earlywood/latewood transition in Scots pine. Plant Physiol. 125:2029-2039.

Van Riet, L., V. Nagaraj, W. Van den Ende, S. Clerens, A. Wiemken and A. Van Laere. 2006. Purification, cloning and functional characterization of a fructan 6-exohydrolase from wheat (*Triticum aestivum* L.). J. Exp. Bot. 57:213-223.

Weber, H., L. Borisjuk, U. Heim, P. Buchner and U. Wobus. 1995. Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. Plant Cell. 7:1835-1846.

Weil, M. and T. Rausch. 1994. Acid invertase in *Nicotiana tabacum* crown-gall cells: Molecular properties of the cell-wall isoform. Planta. 193:430-437.

Weschke, W., R. Panitz, S. Gubatz, Q. Wang, R. Radchuk, H. Weber and U. Wobus. 2003. The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J. 33:395-411.

Xu, J., W. T. Avigne, D. R. McCarty and K. E. Koch. 1996. A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: Evidence from a maize invertase gene family. Plant Cell. 8:1209-1220.

Zhang, L., N. S. Cohn and J. P. Mitchell. 1996. Induction of a pea cell-wall invertase gene by wounding and its localized expression in phloem. Plant Physiol. 112:1111-1117.

CHAPTER FIVE

Heterologous expression and functional characterization of two hybrid poplar cell-wall invertases

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5.1 Introduction

A number of plant invertases have been enzymatically characterized using traditional extraction and purification methods, such as ammonium sulphate precipitation and various forms of ionic exchange chromatography (e.g. Miller and Ranwala 1994, Tang et al. 1996). However, these classical purification procedures are often laborious and generally lead to low yield relative to the initial quantities of enzyme. An additional complication that frequently arises when attempting to purify plant enzymes from native tissue is the presence of multiple isoforms. For example, sixteen cytosolic invertases, three vacuolar and at least three cell-wall invertases have been identified in poplar (Bocock et al. 2008, Canam et al. 2008). To circumvent the difficulty in purifying distinct invertases to complete homogeneity from native plant tissues, heterologous expression of invertase genes has been shown to be effective in *Escherichia coli* (Gallagher and Pollock 1998), *Saccharomyces cerevisiae* (Goetz and Roitsch 1999) and *Pichia pastoris* (Fu et al. 2003a, Fu et al. 2003b, Huang et al. 2003, De Coninck et al. 2005, Nagaraj et al. 2005, Wang et al. 2005, Hsieh et al. 2006).

Heterologous protein expression has become an increasingly popular method for the production of large quantities of a target protein. Yeast systems, for example, are particularly advantageous due to the relative ease of transformation and simple growth requirements when compared with insect or mammalian systems. Additionally, yeast contain all the necessary protein translation and processing machinery required to properly synthesize proteins originating from complex eukaryotic organisms, such as poplar (for review see Boer et al. 2007). The yeast *Pichia pastoris*, first described in detail as a host system by Cregg et al. (1985), has emerged as a key heterologous protein expression system stemming from the ability of *P. pastoris* to metabolize methanol as an alternate carbon source. When grown in a medium supplemented with methanol, a series of enzymes, notably alcohol oxidase (AOX), are synthesized enabling the conversion of methanol into an energy source (for review see Daly et al. 2005). This has facilitated the use of the inducible alcohol oxidase promoter (*AOX1*) to drive the expression of heterologously recombinant proteins when the culture is switched to a methanol-containing medium.

The first heterologous expression of a plant invertase in *Pichia pastoris* was described by Huang et al. (2003). The vacuolar invertase gene *Ibβfruct1* was isolated

from *Ipomoea batatas* L. (sweet potato) and cloned into a *P. pastoris* expression vector utilizing the *AOX1* promoter. Under methylotrophic conditions the vacuolar invertase was successfully expressed, easily purified and was shown to be functionally active (Huang et al. 2003). Two additional vacuolar invertases from *I. batatas* (*Ibβfruct2* and *Ibβfruct3*) were later expressed using a similar expression system (Wang et al. 2005). These two studies not only confirmed that it is possible to express high quantities of a recombinant plant invertase with *P. pastoris*, but also provided valuable information on the biochemical nature of each enzyme through functional characterization studies. Additionally, it was shown that the vacuolar invertases expressed in *P. pastoris* had similar properties to those isolated directly from *I. batatas* tissues, thereby validating the heterologous expression system as a comparable method for functionally characterizing plant invertases. The strong functional agreement between enzymes heterologously expressed and those isolated from native tissues was further demonstrated with invertases isolated from *Bambusa oldhamii* (Munro) (Hsieh et al. 2006).

Two studies describing the expression of a vacuolar invertase from *Hordeum vulgare* L. (Nagaraj et al. 2005) and putative cell-wall invertases from *Arabidopsis thaliana* L. (Heynh.) (De Coninck et al. 2005) highlighted the ability for heterologously expressed invertases to be used to investigate substrate specificity. Specifically, it was shown that the vacuolar *H. vulgare* invertase (HvINV1) did not have fructosyltransferase activity despite sharing high homology with known fructosyltransferases (Nagarej et al. 2005). Similarly, the putative *Arabidopsis* cell-wall invertases, AtcwINV3 and AtcwINV6, were shown to be fructanexohydrolases, with neither enzyme having the ability to cleave sucrose despite possessing known cell-wall invertase amino acid motifs (De Coninck et al. 2005). The conclusions drawn from these studies were undoubtedly enhanced by the certainty of the gene-enzyme relationship unique to heterologous expression systems, such as *P. pastoris*.

The goal of the current study was to heterologously express and characterize two hybrid poplar (*Populus alba* x *grandidentata*) cell-wall invertases (PaxgINV1 and PaxgINV2) using the *P. pastoris* expression system. The genes encoding these enzymes were previously shown to be differentially expressed, both spatially and temporally, in poplar vegetative tissues (Canam et al. 2008). Specifically, PaxgINV1 appeared to be involved with processes relating to dormancy, while PaxgINV2

appeared to be primarily involved in tissues undergoing rapid growth and expansion. The results described herein clearly show that both enzymes exhibit unique characteristics and properties, which provides further evidence for their unique physiological functions with respect to the growth and development of hybrid poplar.

5.2 Materials and methods

5.2.1 Gene identification and cloning

Putative cell-wall invertase genes were initially identified from the online *Populus trichocarpa* genomic database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home .html), and their homologs from hybrid poplar, PaxgINV1 (EU374217) and PaxgINV2 (EU374218), were isolated from leaf cDNA as previously described (Canam et al. 2008). Primers were used to introduce *Sna*BI and *Avr*II restriction sites at the 5′ and 3′ ends, respectively, of *PaxgINV1* and *PaxgINV2*, allowing each gene to be subcloned as a *Sna*BI-*Avr*II fragment into pPIC9K (Invitrogen, Carlsbad, CA) creating pPIC9K+PaxgINV1 and pPIC9K+PaxgINV2.

5.2.2 *Pichia pastoris* transformation

Electrocompetent *P. pastoris* GS115 cells (Invitrogen) were prepared as per the manufacturer's instructions, and electroporated with approximately 10 µg of *Sal*I-linearized plasmid DNA (pPIC9K, pPIC9K+PaxgINV1 and pPIC9K+PaxgINV2) using an Electroporator 2510 (Eppendorf). Electroporated cells were plated on MM medium (1.34% yeast nitrogen base without amino acids, 0.00004% biotin and 0.5% methanol) and incubated at 28°C for 48 h. Colonies were screened using genomic PCR with AOX primers as described by the *Pichia* Expression Kit (version M) instructions (Invitrogen).

5.2.3 Cell growth and induction

P. pastoris GS115 colonies were grown at 28°C and 265 rpm for 16 h in 25 mL of BMGY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.00004% biotin and 1% glycerol), pelleted by centrifugation at 1500 rpm for 5 min and resuspended in 100 mL of BMMY induction medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.00004 % biotin and 0.5% methanol). Cells were supplemented with 0.5% methanol every 24 h and allowed to grow for five days at 28°C and 265 rpm.

5.2.4 Protein purification

After five days of methanol-induced growth, the *P. pastoris* GS115 cell suspensions were centrifuged at 10000 rpm for 10 min. The resulting supernatant was concentrated approximately 25-fold using an Amicon® Ultra-15 (10 000 MWCO) centrifugal filter device (Millipore, Billerica, MA) at 5000 *g*. Approximately 3 mL of concentrated supernatant was desalted into a 4 mL volume with either 50 mM sodium acetate at pH 4.5 (PaxgINV1) or 50 mM MES at pH 6.0 (PaxgINV2) using an EconoPac 10 DG column (Bio-Rad, Hercules, CA) pre-equilibrated with the appropriate buffer.

5.2.5 Enzyme reactions

For the determination of pH optima, reactions containing 40 mM of sodium acetate (pH 3.5-7) and 40 mM sodium citrate (pH 3.5-7) with 10 mM sucrose were incubated at 30°C for 1 h followed by inactivation at 85°C for 5 min. For the determination of temperature optima, the reactions consisted of 40 mM sodium acetate (pH 4.5) or 40 mM MES (pH 6.0) with 10 mM sucrose and were incubated at 5-70°C for 1 h, at the pre-established pH optima, prior to inactivation at 85°C for 5 min. Kinetic assays were performed in 40 mM sodium acetate (pH 4.5) or 40 mM MES (pH 6.0) with 0-20 mM of substrate and were incubated at 30°C for 1-4 h followed by 85°C for 5 min. Substrate specificity reactions comprised of 40 mM sodium acetate (pH 4.5) or 40 mM MES (pH 6.0) with 10 mM substrate (sucrose, raffinose, stachyose, verbascose) and were incubated at 30°C for 4 h followed by 85°C for 5 min. Metal cation reactions were conducted in 40 mM sodium acetate (pH 4.5) or 40 mM MES (pH 6.0) with 10 mM sucrose and 5 mM of either $LiCl$, KCl, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $FeCl_2$, $ZnCl_2$, $HgCl_2$ or $CuCl_2$.

5.2.6 Activity analysis

All enzyme reactions, except those with metal cations, were quantified as the amount of hexose sugar liberated by the invertase reaction using a colorimetric neocuproine assay for reducing sugars (Chaplin and Kennedy 1986). Enzyme activity was directly correlated to the absorbance at 450 nm measured using a spectrophotometer. For the metal cation reactions, the concentration of glucose released was quantified by HPLC using a DX-600 equipped with an MA-1 CarboPac column and electrochemical detector (Dionex, Sunnyvale, CA). Carbohydrates were eluted at 0.4 mL min^{-1} using an isocratic eluant of 300 mM sodium hydroxide.

5.2.7 Protein models and prediction tools

Theoretical molecular weight and pI values were generated from predicted amino acid sequences with the Compute MW/pI tool from ExPASy (http://us.expasy.org/tools/pi_tool.html). Three-dimensional models of PaxgINV1 and PaxgINV2 were generated by 3D-JIGSAW ver. 2.0 (Bates et al. 2001) and visualized using RasMol ver. 2.6 (Sayle and Milner-White 1995). K_m values were generated using the Michaelis-Menten nonlinear regression model in Graphpad Prism 5.00 (Graphpad Software, San Diego, CA).

5.3 Results

5.3.1 Structure analysis and predicted properties

The two hybrid poplar cell-wall invertases (PaxgINV1 and PaxgINV2) possess the conserved WECPD and NDPN amino acid motifs that are common amongst plant cell-wall invertases (Goetz and Roitsch 1999, Maddison et al. 1999). Three dimensional modeling of both enzymes using 3D-JIGSAW (Bates et al. 2001) coupled with visualization using RasMol (Sayle and Milner-White 1995) indicated that these amino acid segments exist opposite to one another on the inside of a channel of the mature protein (Figure 5.1). In addition to the conserved amino acid motifs, both enzymes had theoretical pI values in the alkaline range (8.6 for PaxgINV1 and 9.0 for PaxgINV2) and molecular masses of approximately 66 kDa.

5.3.2 Stability, pH and temperature optima

Purified PaxgINV1 from *P. pastoris* was stable for several months in 50 mM sodium acetate (pH 4.5) at 4°C, while PaxgINV1 was only stable for several weeks at the same temperature in 50 mM MES (pH 6.0). PaxgINV1 was active over a broad pH range (3.5-7), yet had a distinct optimum at approximately pH 4.8 (Figure 5.2). In contrast, PaxgINV2 had a distinct pH optimum at approximately pH 5.6 (Figure 5.2), and showed significant loss of activity below pH 4.8. Both enzymes were active over a broad temperature range (5-70°C) with maximal activities at 45°C and 40°C for PaxgINV1 and for PaxgINV2, respectively (Figure 5.3).

5.3.3 Substrate specificity and affinity

Both PaxgINV1 and PaxgINV2 were able to effectively hydrolyze the glycolytic bond between glucose and fructose in sucrose, raffinose, stachyose and verbascose.

However, PaxgINV2 was much more effective at catalyzing the degradation of the larger oligosaccharides than PaxgINV1 (Table 5.1). HPLC analysis indicated that PaxgINV1 and PaxgINV2 released only fructose and not galactose from the raffinose family of oligosaccharides (Figure 5.4), thus confirming that PaxgINV1 and PaxgINV2 are β-fructofuranosidases. Additionally, K_m values were calculated for sucrose, raffinose and stachyose. PaxgINV1 had K_m values for sucrose and raffinose in the 1-2 mM range and a K_m of 5.0 mM for stachyose. In contrast, PaxgINV2 had K_m values in the 1-2 mM range for all three substrates (Table 5.2). PaxgINV1 and PaxgINV2 were also able to hydrolyze the fructose residue from verbascose, but to a lesser extent (data not shown).

5.3.4 Metal cation sensitivity

The sucrolytic activity of PaxgINV1 and PaxgINV2 was examined in the presence of several metal cations. The activity of PaxgINV1 was inhibited slightly by most metals with the exception of Fe^{2+} (Table 5.3), with the strongest inhibition occurring in the presence of Hg^{2+} (71%) and Cu^{2+} (75%). In contrast, PaxgINV2 exhibited increased activity in the presence of Li^+ (117%) and K^+ (113%), but was severely inhibited by Fe^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} (Table 5.3).

5.4 Discussion

Two cell-wall invertase genes (*PaxgINV1* and *PaxgINV2*) were previously shown to display markedly different expression patterns throughout the growing season in a variety of vegetative tissues of hybrid poplar (Canam et al. 2008). Specifically, *PaxgINV1* was shown to be predominately expressed in tissues at the onset and emergence of dormancy, while *PaxgINV2* was highly expressed in tissues undergoing rapid growth and expansion. In addition, *PaxgINV2* was highly expressed in the phloem prior to and during bud break (Canam et al. 2008). The aim of this study was to heterologously express the enzymes encoded by these genes, and to explore their properties and characteristics in an effort to elucidate the physiological roles these enzymes may play in growth and development.

5.4.1 Predicted structure

PaxgINV1 and PaxgINV2 have amino acid sequence characteristics common to plant cell-wall invertases, including the predicted cell-wall invertase specific WECPD and β-

fructosidase NDPN amino acid motifs (Goetz and Roitsch 1999, Maddison et al. 1999). To investigate the location of these amino acid sites on each of the mature proteins, the deduced amino acid sequences of PaxgINV1 and PaxgINV2 were analyzed using 3D-JIGSAW (Bates et al. 2001) and visualized graphically in RasMol (Sayle and Milner-White 1995). Using these tools, a distinct channel was visualized in the predicted three-dimensional models of both PaxgINV1 and PaxgINV2. Interestingly, the NDPN and WECPD amino acid segments are both located on the inside of this channel directly opposite one another (Figure 5.1). Due to the conserved nature of these motifs in cell-wall invertases and the importance of the WECPD site to substrate specificity in dicots (Goetz and Roitsch 1999), it is likely that this channel is the catalytic centre of the two cell-wall invertases of hybrid poplar. However, it should be noted this conclusion is based on predictive models only and as such, this channel, although probable, is not known to exist with certainty.

5.4.2 Heterologous expression in *Pichia pastoris*

P. pastoris was chosen as a heterologous expression system primarily due to the relative ease of growth and the inherent lack of invertase activity in this microbe (Tschopp et al. 1987). In addition, *P. pastoris* can effectively secrete protein into the culture medium allowing simple and rapid enzyme purification. In the present study, we established *P. pastoris* cultures (strain GS115) that secreted functional PaxgINV1 and PaxgINV2 that were subsequently partially purified using simple protein concentration and desalting techniques. As expected, no β-fructofuranosidase activity was found in the growth medium of cultures transformed with empty vector (data not shown).

5.4.3 pH and temperature optima

The pH optima of plant cell-wall invertases has been well documented and is typically in the pH 3-5 range (e.g. Masuda and Sugawara 1980, Doelhart et al. 1987, Hsieh et al. 2006). The pH optimum of PaxgINV1 was approximately 4.5-5.0, which is in close agreement with cell-wall invertases from *Oryza sativa* L. (Charng et al. 1994), *H. vulgare* (Karuppiah et al. 1989), *Zea mays* L. (Doelhart et al. 1987), *Triticum aestivum* L. (Krishnan et al. 1985) and *Picea abies* L. (Karst) (Salzer and Hager 1993). PaxgINV2, however, exhibited a pH maximum of pH 5.5-6.0. This represents one of the highest reported pH optima for a plant cell-wall invertase, although several acid invertases (some with unknown subcellular locations) have been characterized with pH

optima in the 5-6 range (e.g. Unger et al. 1992, Van den Ende and Van Laere 1993, Tang et al. 1996). The temperature optima exhibited by PaxgINV1 and PaxgINV2 (45 and 40°C, respectively) are also similar to acid invertases previously studied from other plant species such as sweet potato (Huang et al. 2003), *Arabidopsis* (Tang et al. 1996) and *Carica papaya* L. (Chan and Kwok 1976).

5.4.4 Substrate specificity and affinity

Acid invertases from plants are typically able to hydrolyze not only sucrose, but also the glucose-fructose bond of other β-fructofuranosides such as raffinose and stachyose. However, experimental evidence suggests that vacuolar invertases are less efficient than cell-wall invertase at catalyzing these reactions (e.g. Goetz and Roitsch 1999, Hashizume et al. 2003). Accordingly, the hybrid poplar cell-wall invertases reported herein can efficiently use both raffinose and stachyose, as well as verbascose as substrates. The activity of PaxgINV1 is roughly correlated to the size of the β-fructofuranoside, with the enzyme having 52% activity with raffinose, 33% with stachyose and 19% with verbascose when compared to the activity of sucrose. By comparison, PaxgINV2 is much more effective at hydrolyzing these substrates, displaying nearly identical activities among raffinose and sucrose. Remarkably, the PaxgINV2 activities towards stachyose and verbascose are 92% and 85%, respectively. To our knowledge, this represents the highest level of raffinose, stachyose and verbascose catalysis relative to sucrose for any plant cell-wall invertase, although extremely high raffinose activity (92%) was also observed with a *B. oldhamii* cell-wall invertase (Hsieh et al. 2006).

Further evidence for the high affinity of the hybrid poplar cell-wall invertases PaxgINV1 and PaxgINV2 for a variety of β-fructofuranosides is the relatively low K_m values for these substrates. Sucrose K_m values for plant invertases (alkaline, neutral and acid) are typically in the 1-100 mM range (e.g. Charng et al. 1994). The K_m values for PaxgINV1 and PaxgINV2 towards sucrose are 1.7 and 1.6 mM, respectively. This high affinity for sucrose by poplar invertases concurs with previously reported plant cell wall invertases, such as 0.4 mM in *B. oldhamii* (Hsieh et al. 2006), 0.7 mM in *Urtica dioica* L. (Fahrendorf et al. 1990), 1.2 mM in *Beta vulgaris* L. (Masuda and Sugawara 1980) and 1.7 mM in *T. aestivum* (Krishnan et al. 1985). The K_m values for raffinose and stachyose are less frequently reported, yet are typically higher than the K_m values

for sucrose. For instance, with *U. dioica,* Fahrendorf et al. (1990) reported a K_m of 3.6 mM for raffinose, while Pressey and Avants (1980) demonstrated stachyose K_m values of 14 mM and 25 mM for two acid invertases from *Avena sativa* L. The raffinose K_m values for PaxgINV1 (1.8 mM) and PaxgINV2 (1.7 mM) appear to be among the lowest reported K_m values for raffinose, along with 1.6 mM reported for an acid invertase from *Lilium longiflorum* (Thunb.) (Miller and Ranwala 1994). Similarly, stachyose K_m values of 5.0 (PaxgINV1) and 1.9 (PaxgINV2) are also the lowest to our knowledge, and may represent the first stachyose K_m value reported for a plant cell-wall invertase. However, it should be stated that it is unclear whether the acid invertases examined by Pressey and Avants (1980) and Isla et al. (1988) were localized to the vacuole or the cell-wall.

5.4.5 Metal cation sensitivity

Enzyme activities are frequently affected by the presence of ions, which may have physiological relevance, such as K^+ and Ca^{2+} ions (e.g. phloem and xylem flux), or may underscore the importance of certain amino groups to enzyme activity (e.g. Hg^{2+} binding of cysteine). To that end, the effects of various metal cations on the sucrolytic activity of PaxgINV1 and PaxgINV2 were explored. With the exception of Fe^{2+} (an activator), PaxgINV1 showed a mild reduction in activity in the presence of all cations tested such as K^+, Mg^{2+} and Ca^{2+}, and to a greater extent Mn^{2+} and Cu^{2+}. Of the metal ions tested, PaxgINV1 was most sensitive to Hg^{2+}, although the enzyme still retained 71% of its activity. This finding is in contrast to a number of similar studies that have shown Hg^{2+} as a strong inhibitor of acid invertase activity (e.g. Pressey and Avants 1980, Hsieh et al. 2006). In fact, a number of studies have reported complete inhibition of acid invertase activity by the presence of Hg^{2+} (e.g. Krishnan et al. 1985, Karuppiah et al. 1989, Asthir et al. 1998). Similar to those studies, a severe inhibitory effect was observed with PaxgINV2, which showed only 5% activity in the presence of Hg^{2+}. PaxgINV2 was similarly inhibited by Cu^{2+} (3.5%) and Zn^{2+} (5.3%). The sensitivity of invertases to these three heavy metals (Cu^{2+}, Zn^{2+} and Hg^{2+}) has previously been observed in other studies (e.g. Masuda et al. 1987, Ranwala et al. 1991, Lin and Sung 1993), and it has been suggested that they inhibit activity by interfering with protein thiol groups that are crucial for effective catalysis (Karuppiah et al. 1989). Cysteine, for example, contains a thiol side chain, and as such is vulnerable to the reactivity of Cu^{2+}, Hg^{2+} and Zn^{2+} ions (Belcastro et al. 2005). To that end, the putative active site for

vacuolar (WECVD) and cell-wall (WECPD) invertases contains a cysteine residue, which may provide the mechanistic means for inactivation of acid invertases by this group of cations.

5.4.6 Conclusions

We have cloned and heterologously expressed two cell-wall invertases from hybrid poplar (PaxgINV1 and PaxgINV2) with the methylotrophic yeast *P. pastoris*. Both enzymes have characteristics of cell-wall invertases such as alkaline pI, acidic pH optima and the ability to hydrolyze fructose from sucrose and other fructofuranosides such as raffinose, stachyose and verbascose. However, despite these similarities, the invertases respond differently to the presence of metal cations and have different substrate affinities. For example, PaxgINV2 has a much higher affinity for stachyose than PaxgINV1, and can utilize raffinose and stachyose as substrates almost as effectively as sucrose.

The ability for PaxgINV1 to hydrolyze the raffinose family of oligosaccharides (RFOs), coupled with the expression of this enzyme in various tissues immediately following senescence (Canam et al. 2008), suggests that PaxgINV1 may be hydrolyzing RFOs to provide energy for residual cellular activity when photosynthesis has ceased and sucrose levels are low. Supporting this theory was an observed increase in RFOs in *Populus tremuloides* Michx. during early winter (Cox and Stushnoff 2001), which correlates with the increased expression of PaxgINV1 observed during this same time (Canam et al. 2008).

Previous experimental evidence showed that the expression of PaxgINV2 was highest in tissues undergoing rapid growth and expansion (i.e. sink tissue) and in phloem tissue during bud break (Canam et al. 2008). It was therefore proposed that PaxgINV2 played a role in phloem unloading and the seasonal reallocation of carbohydrate. The ability for PaxgINV2 to effectively utilize transport carbohydrates such as sucrose and the RFOs supports such a role in phloem unloading. Furthermore, *Populus* has been categorized as a type 1 species with respect to plasmodesmatal frequency, suggesting that poplar utilizes RFOs to transport carbohydrate from sink to source tissue (Turgeon and Medville 2004). Additionally, raffinose and stachyose have been shown to increase during dormancy in *P. tremuloides* (reaching a maximum in mid-winter) before decreasing in twigs and buds prior to bud break (Cox and Stushnoff

2001). This concurs with the expression of PaxgINV2, which was shown to be weakly expressed throughout dormancy and strongly expressed in phloem and buds prior to bud break (Canam et al. 2008), providing additional support to the hypothesis that PaxgINV2 plays a role in the processes surrounding bud break.

In summary, the variation in spatial and temporal expression and functional characterization data between PaxgINV1 and PaxgINV2 indicates that these two invertase enzymes have distinct physiological roles in hybrid poplar. Specifically, PaxgINV1 may be involved in providing the energy currency required for cellular activity during the early stages of dormancy, while PaxgINV2 may be involved in phloem unloading and the seasonal reallocation of carbohydrate prior to and during bud break.
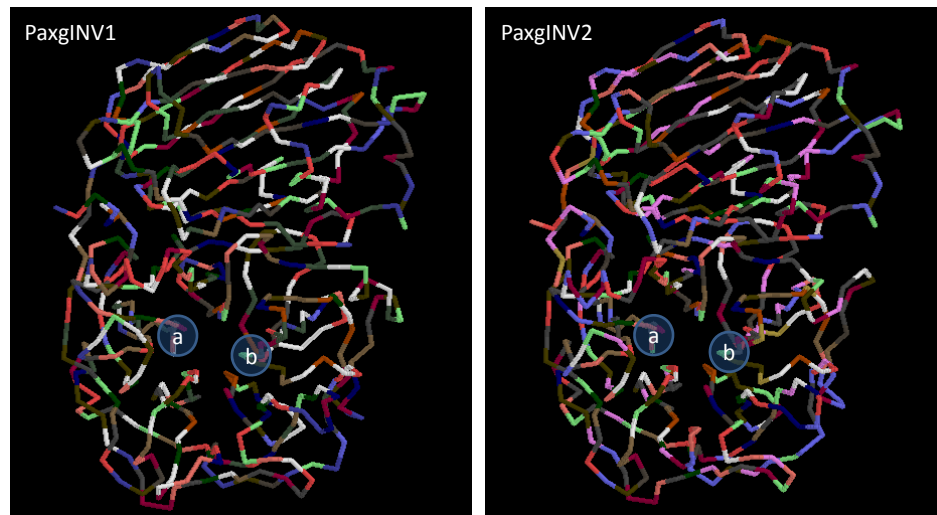
Figure 5.1. Three dimensional model of PaxgINV1 and PaxgINV2 generated using 3D-JIGSAW (Bates et al. 2001) and visualized using RasMol (Sayle and Milner-White 1995). The predicted positions of the β-fructosidase amino acid motif NDPN (a) and cell-wall invertase active site motif WECPD (b) are shown.
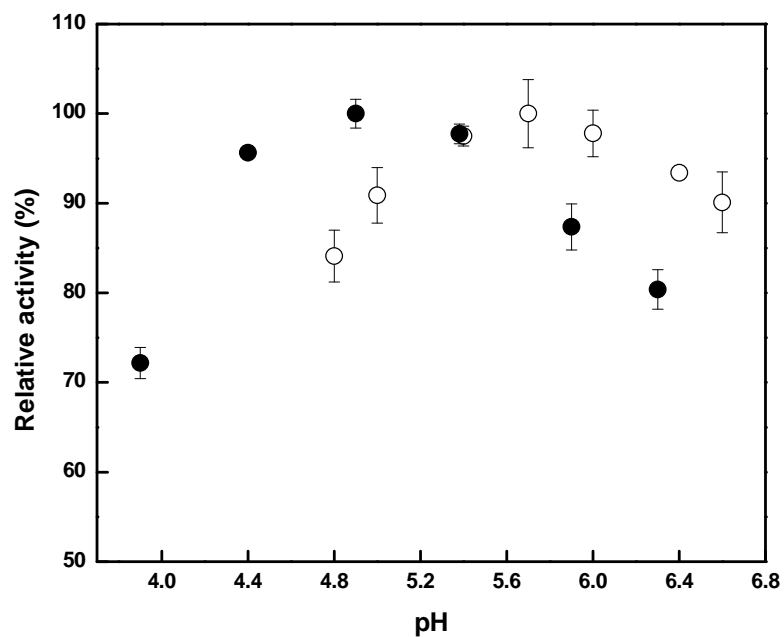
Figure 5.2. The activity of PaxgINV1 (closed circles) and PaxgINV2 (open circles) in response to pH. Assay conditions included 10 mM sucrose in either 40 mM sodium acetate (PaxgINV1) or 40 mM sodium citrate (PaxgINV2) at 30°C. Error bars represent the standard deviation (n=3).
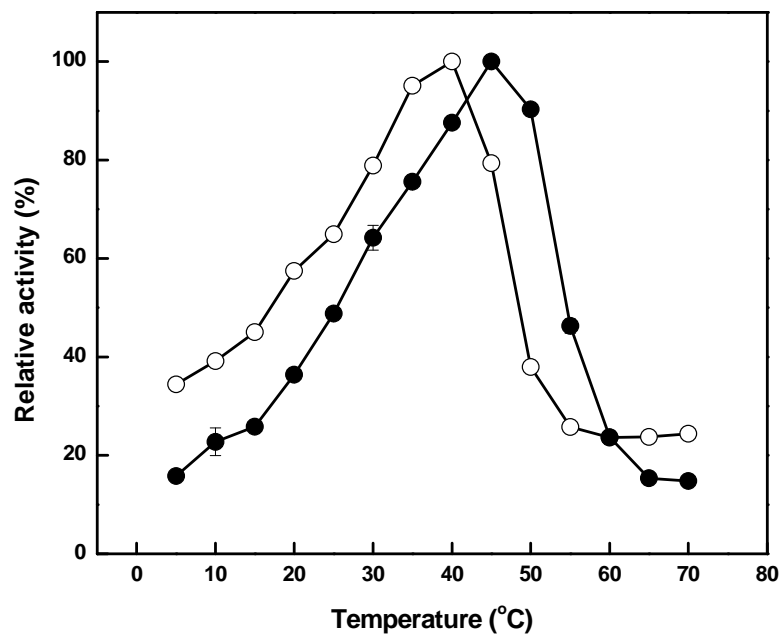
Figure 5.3. Activity of PaxgINV1 (closed circles) and PaxgINV2 (open circles) at various temperatures. Assay conditions included 10 mM sucrose in either 40 mM sodium acetate at pH 4.5 (PaxgINV1) or 40 mM MES at pH 6.0 (PaxgINV2). Error bars represent the standard deviation (n=3).
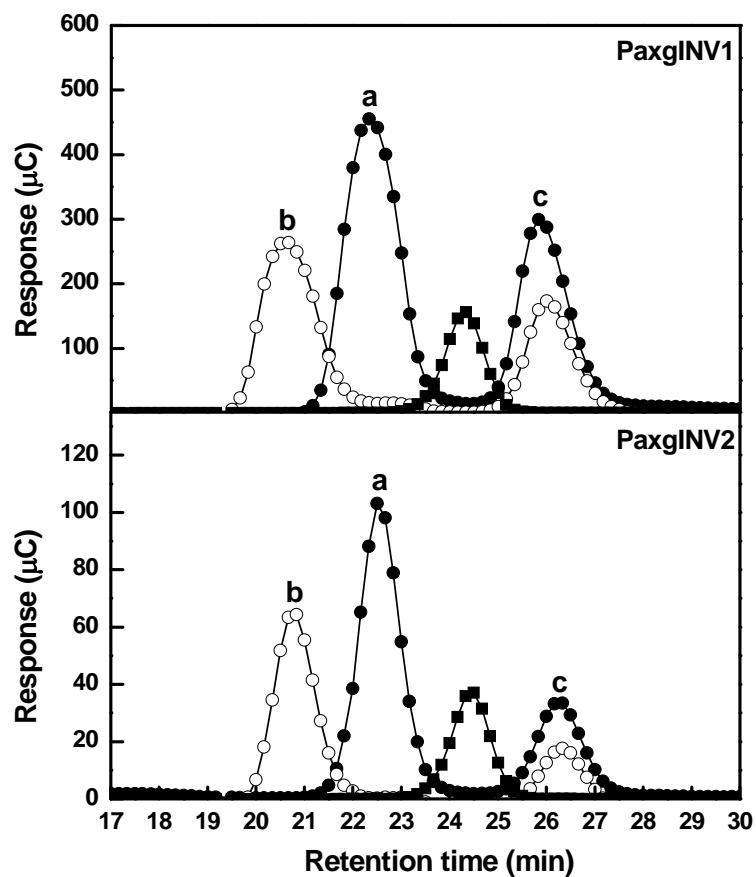
Figure 5.4. HPLC chromatograms showing the breakdown products of raffinose (closed circles) and stachyose (open circles) by PaxgINV1 and PaxgINV2 along with a galactose standard (closed squares). Breakdown products are melibiose (a), melibiose+galactose (b) and fructose (c).

Table 5.1. Percent activity (relative to sucrose) of PaxgINV1 and PaxgINV2 on 10 mM of substrate. Values are the mean and standard deviation (n=3).

Substrate	PaxgINV1	PaxgINV2
sucrose	100.0 ± 0.6	100.0 ± 0.6
raffinose	51.9 ± 0.1	100.2 ± 0.7
stachyose	32.8 ± 0.3	91.7 ± 0.3

Table 5.2. K_m values (mM) of PaxgINV1 and PaxgINV2 for sucrose, raffinose and stachyose. Values are the mean and standard deviation (n=3).

Substrate	PaxgINV1	PaxgINV2
sucrose	1.7 ± 0.1	1.6 ± 0.1
raffinose	1.8 ± 0.1	1.7 ± 0.1
stachyose	5.0 ± 0.4	1.9 ± 0.2

Table 5.3. Percent activity of PaxgINV1 and PaxgINV2 on 10 mM sucrose in the presence of metal cations (5 mM). Values represent the mean and standard deviation (n=3)**.**

Cation	PaxgINV1	PaxgINV2
none	100.0 ± 7.6	100.0 ± 3.7
Li^+	82.4 ± 9.5	117.3 ± 4.7
K^+	86.3 ± 8.9	113.7 ± 3.3
Mg^{2+}	88.5 ± 10.7	91.8 ± 4.1
Mn^{2+}	79.7 ± 9.9	86.7 ± 3.1
Co^{2+}	87.6 ± 6.3	74.4 ± 2.0
Ca^{2+}	89.5 ± 11.9	73.1 ± 6.3
Fe^{2+}	109.1 ± 4.4	15.6 ± 2.5
Zn^{2+}	92.5 ± 4.0	5.3 ± 0.3
Hg^{2+}	71.0 ± 3.7	5.0 ± 1.5
Cu^{2+}	74.5 ± 4.3	3.5 ± 0.5

5.5 References

Asthir, B., A. Kaur and A.S. Basra. 1998. Cultivar variation in heat stability and kinetic properties of soluble invertase in wheat grains. Acta Physiol. Plant. 20:339-345.

Bates, P.A., L.A. Kelley, R.M. MacCallum and M.J.E. Sternberg. 2001. Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins. 45:39-46.

Belcastro, M., T. Marino, N. Russo and M. Toscano. 2005. Interaction of cysteine with Cu^{2+} and Group IIb (Zn^{2+}, Cd^{2+}, Hg^{2+}) metal cations: a theoretical study. J. Mass Spectrom. 40:300-306.

Bocock, P.N., A.M. Morse, C. Dervinis and J.M. Davis. 2008. Evolution and diversity of invertase genes in *Populus trichocarpa*. Planta. 227:565-576.

Boer, E., G. Steinborn, G. Kunze and G. Gellissen. 2007. Yeast expression platforms. Appl. Microbiol. Biot. 77:513-523.

Canam, T., S.W.Y Mak and S.D. Mansfield. 2008. Spatial and temporal expression profiling of cell-wall invertase genes during early development in hybrid poplar. Tree Physiol. 28:1059-1067.

Chan, H.T. and S.C.M. Kwok. 1976. Isolation and characterization of a β-fructofuranosidase from papaya. J. Food Sci. 41:320-323.

Chaplin, M.F. and J.F. Kennedy 1986. Carbohydrate analysis: a practical approach. *In* Practical Approach Series Eds. D. Rickwood and B.D. Hames. IRL Press Limited, Oxford, England.

Charng, Y.Y., R.H. Juang, J.C. Su and H.Y. Sung. 1994. Partial purification and characterization of invertase isozymes from rice grains (*Oryza sativa*). Biochem. Mol. Biol. Int. 33:607-615.

Cox, S.E. and C. Stushnoff. 2001. Temperature-related shifts in soluble carbohydrate content during dormancy and cold acclimation in *Populus tremuloides.* Can. J. Forest Res. 31:730-737.

Cregg, J.M., K.J. Barringer, A.Y. Hessler and K.R. Madden. 1985. *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 5:3376-3385.

Daly, R. and M.T.W. Hearn. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. 18:119-138.

De Coninck, B., K. Le Roy, I. Francis, S. Clerens, R. Vergauwen, A.M. Halliday, S.M. Smith, A. Van Laere and W. Van den Ende. 2005. Arabidopsis AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. Plant Cell Environ. 28:432-443.

Doehlert, D.C. and F.C. Felker. 1987. Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernels. Physiol. Plantarum. 70:51-57.

Fahrendorf, T. and E. Beck. 1990. Cytosolic and cell-wall-bound acid invertases from leaves of *Urtica dioica* L.: a comparison. Planta. 180:237-244.

Fu, R.H., Y.L. Wang and H.Y. Sung. 2003a. Cloning, characterization and functional expression of a new β-D-fructofuranosidase (*Osβfruct2*) cDNA from *Oryza sativa*. Biotechnol. Lett. 25:455-459.

Fu, R.H., A.Y. Wang, Y.C. Wang and H.Y. Sung. 2003b. A cDNA encoding vacuolar type β-D-fructofuranosidase (*Osβfruct3*) of rice and its expression in *Pichia pastoris*. Biotechnol. Lett. 25:1525-1530.

Gallagher, J.A. and C.J. Pollock. 1998. Isolation and characterization of a cDNA clone from *Lolium temulentum* L. encoding for a sucrose hydrolytic enzyme which shows alkaline/neutral invertase activity. J. Exp. Bot. 49:789-795.

Goetz, M. and T. Roitsch. 1999. The different pH optima and substrate specificities of extracellular and vacuolar invertases from plants are determined by a single amino-acid substitution. Plant J. 20:707-711.

Hashizume, H., K. Tanase, K. Shiratake, H. Mori and S. Yamaki. 2003. Purification and characterization of two soluble acid invertase isozymes from Japanese pear fruit. Phytochemistry. 63:125-129.

Hsieh, C.W., L.K. Liu, S.H. Yeh, C.F. Chen, H.I. Lin, H.Y. Sung and A.Y. Wang. 2006. Molecular cloning and functional identification of invertase isozymes from green bamboo *Bambusa oldhamii*. J. Agr. Food Chem. 54:3101-3107.

Huang, W.C., A.Y. Wang, L.T. Wang and H.Y. Sung. 2003. Expression and characterization of sweet potato invertase in *Pichia pastoris*. J. Agr. Food Chem. 51:1494-1499.

Isla, M.I., M.A. Vattuone, M.I. Gutierrez and A.R. Sampietro. 1988. Acid invertase from *Tropaeolum* leaves. Phytochemistry. 27:1993-1998.

Karuppiah, N., B. Vadlamudi and P.B. Kaufman. 1989. Purification and characterization of soluble (cytosolic) and bound (cell-wall) isoforms of invertases in barley (*Hordeum vulgare*) elongating stem tissue. Plant Physiol. 91:993-998.

Krishnan, H.B., J.T. Blanchette and T.W. Okita. 1985. Wheat invertases: Characterization of cell wall-bound and soluble forms. Plant Physiol. 78:241-245.

Lin, S.S. and H.Y. Sung. 1993. Partial purification and characterization of soluble acid invertases from rice (*Oryza sativa*) leaves. Biochem. Mol. Biol. Int. 31:945-953.

Maddison, A.L., P.E. Hedley, R.C. Meyer, N. Aziz, D. Davidson and G.C. Machray. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. Plant Mol. Biol. 41:741-751.

Masuda, H. and S. Sugawara. 1980. Purification and some properties of cell wall-bound invertases from sugar beet seedlings and aged slices of mature roots. Plant Physiol. 66:93-96.

Masuda, H., T. Takahashi and S. Sugawara. 1987. The occurrence and properties of alkaline invertase in mature roots of sugar beets. Agr. Biol. Chem. Tokyo. 51:2309-2314.

Miller, W.B. and A.P. Ranwala. 1994. Characterization and localization of three soluble invertase forms from *Lilium longiflorum* flower buds. Physiol. Plantarum. 92:247-253.

Nagaraj, V.J., V. Galati, M. Luscher, T. Boller and A. Wiemken. 2005. Cloning and functional characterization of a cDNA encoding barley soluble acid invertase (HvINV1). Plant Sci. 168:249-258.

Pressey, R. and J.K. Avants. 1980. Invertases in oat seedlings: Separation, properties, and changes in activities in seedling segments. Plant Physiol. 65:136-140.

Ranwala, A.P., S.S. Iwanami and H. Masuda. 1991. Acid and neutral invertases in the mesocarp of developing muskmelon (*Cucumis melo* L. cv Prince) fruit. Plant Physiol. 96:881-886.

Salzer, P. and A. Hager. 1993. Characterization of wall-bound invertase isoforms of *Picea abies* cells and regulation by ectomycorrhizal fungi. Physiol. Plantarum. 88:52-59.

Sayle, R.A. and E.J. Milner-White. 1995. Rasmol: biomolecular graphics for all. Trends Biochem. Sci. 20:374-376.

Tang, X.W., H.P. Ruffner, J.D. Scholes and S.A. Rolfe. 1996. Purification and characterisation of soluble invertases from leaves of *Arabidopsis thaliana*. Planta. 198:17-23.

Tschopp, J.F., G. Sverlow, R. Kosson, W. Craig and L. Grinna. 1987. High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. Bio-Technol. 5:1305-1308.

Turgeon, R. and R. Medville. 2004. Phloem loading. A reevaluation of the relationship between plasmodesmatal frequencies and loading strategies. Plant Physiol. 136:3795-3803.

Unger, C., J. Hofsteenge and A. Sturm. 1992. Purification and characterization of a soluble β-fructofuranosidase from *Daucus carota*. Eur. J. Biochem. 204:915-921.

Van den Ende, W. and A. Van Laere. 1993. Purification and properties of an invertase with sucrose:sucrose fructosyltransferase (SST) activity from the roots of *Cichorium intybus* L. New Phytol. 123:31-37.

Wang, L.T., A.Y. Wang, C.W. Hsieh, C.Y. Chen and H.Y. Sung. 2005. Vacuolar invertases in sweet potato: Molecular cloning, characterization, and analysis of gene expression. J. Agr. Food Chem. 53:3672-3678.

CHAPTER SIX

Summary and future research

6.1 Thesis summary

Plants expressing yeast-derived invertases have previously been shown to alter phenotype, with many species displaying impaired growth and a general interference with sink-source metabolism. Although the existing research examined general sink strength, the effects on specific sink polymers was not thoroughly investigated. Our goal was to study the effects of these invertases not only on general plant phenotype, but also on specific sink polymers such as cellulose and lignin, two of the most abundant biopolymers on earth. The results of our efforts (Chapter two) show that the yeast-derived invertases disrupted sink-source metabolism in tobacco, as had previously been shown. However, we were able to extend the findings to show for the first time that the sink polymers cellulose and lignin had been influenced in stem tissue. Specifically, some transgenic lines showed significant increases in cellulose and lignin content, while others showed significant decreases, and in each case it appeared to be dependent on the level of expression and location. When the same invertases were expressed for the first time in a perennial tree species (Chapter three), the phenotypes were far less dramatic. However, marginal increases in cellulose content were observed. The precise reasons for this are not known with certainty, but these results may highlight the importance of the putative fundamental difference in phloem transport between herbaceous plants (apoplastic loaders), and woody plants, such as poplar (a symplastic loader). Additionally, we provided evidence for post-translational modification of the yeast-derived invertases in hybrid poplar, such as enzyme degradation and glycosylation, which was not observed with tobacco expressing these same genes. This highlights putative differences in foreign protein recognition between the herbaceous plants and woody plants, such as poplar. Overall, the efficacy of the yeast-derived invertases at providing a strong phenotype in hybrid poplar was poor, thus suggesting that biochemical techniques such as mis-regulation of endogenous invertases may be a more suitable alternative for future studies involving sink regulation in tree species.

The genes of a number of endogenous invertases have been discovered and reported for a variety of herbaceous plants including *Arabidopsis*, tomato and potato. However, the genes encoding cell-wall invertases in *Populus* had not adequately been explored. To that end, we identified three cell-wall invertases in hybrid poplar that

represented the first family of invertase genes to be fully sequenced and profiled for a tree species (Chapter four). Interestingly, the three hybrid poplar genes were more similar to each other than to their respective homologs from other plant species, suggesting that the duplication events had occurred after divergence from a common ancestor. This finding lends support to the existing theory of parallel evolution of multiple invertase isozymes in dicotyledonous species. Furthermore, the expression of these genes varied both spatially and temporally, which agreed with the expression data of a number of herbaceous plant species. For instance, hybrid poplar appears to have cell-wall invertases involved in vegetative growth and a different cell-wall invertase specifically involved in reproductive processes, which is similar to cell-wall invertase expression patterns seen with members of the Solanaceae family.

To expand on the cell-wall invertase gene discovery and expression analysis described in Chapter four, we functionally characterized two of the genes involved in vegetative biochemical processes using a heterologous expression system (Chapter five). This represented the first heterologous expression of invertases from a tree species. The expression of two poplar invertases in *P. pastoris* facilitated the functional characterization of each of the two cell-wall invertase genes involved in vegetative growth and development. We were not only able to conclude that the genes discovered in Chapter four were indeed cell-wall invertases, but that each of the two invertases had distinct functional properties. For instance, PaxgINV2 was able to very effectively hydrolyze the fructose residue from the raffinose family of oligosaccharides, while PaxgINV1 was less efficient at catalyzing these same reactions. Furthermore, both invertases responded differently to the presence of various biologically relevant cations and had different pH and temperature optima. Collectively, the data suggests potential roles for the two vegetatively expressed cell-wall invertases, with PaxgINV2 being closely linked to the processes surrounding phloem unloading and the seasonal reallocation of carbohydrate, while PaxgINV1 appears to be associated with processes surrounding dormancy. In summary, the research described represents the first time invertase genes have been completely sequenced and characterized from a tree species.

6.2 Future research

The research described in this thesis has expanded the existing knowledge base related to the effects of yeast-derived invertases on sink-source interaction and the expression of cell-wall invertase genes in plant species. However, there are three key research projects that could immediately add to our understanding of the role invertases play with respect to plant growth and development.

6.2.1 Heterologous expression of yeast-derived plant invertases

Chapters two and three of this thesis collectively showed that the yeast-derived invertases confer different phenotypes between tobacco and hybrid poplar. This suggests, as previously discussed, that herbaceous plants, such as tobacco, may transport carbohydrate in a fundamentally different manner than hybrid poplar. Existing phloem transport studies support this theory as members of the Solanaceae family have been identified as apoplastic loaders, while members of the Salicaceae family are believed to be primarily symplastic in nature. The yeast-derived plant invertases may not be capable of efficiently hydrolyzing the raffinose family of oligosaccharides. A weak affinity for raffinose and stachyose would explain why those enzymes are relatively ineffective at conferring a strong phenotype on hybrid poplar, as the tree would be able to circumvent the strain of unnecessary sucrose hydrolysis by transporting soluble carbohydrates such as raffinose and stachyose. Heterologous expression of the yeast-derived invertases in *P. pastoris*, similar to that described in Chapter five, would allow the functional characterization of the foreign invertases. With that in mind, *P. pastoris* colonies transformed with both apoplastic and cytosolic yeast invertase have already been created and have been shown to express the invertases via Western blots with the yeast invertase antibody described in Chapters two and three.

6.2.2 Antisense suppression of endogenous invertase in hybrid poplar

It was previously shown in Chapter three that yeast-derived invertases did not confer a strong phenotype on hybrid poplar. We hypothesized that the foreign invertases were for the most part ineffective at properly interfering with sink-source processes in hybrid poplar. We also suggested that the mis-regulation of endogenous invertases may be a more promising method to effectively alter the sink metabolism in tree species. Chapter four detailed the identification of three cell-wall invertase genes in hybrid poplar, and

along with the data from Chapter five we suggested that one of those genes (*PaxgINV2*) was involved in phloem unloading and soluble carbohydrate transport. If this putative role is correct, down-regulating *PaxgINV2* should interfere with basic sink metabolism and may provide a strong phenotype to compare to the wild-type condition. RNA interference (RNAi) and antisense suppression are two common techniques of suppressing gene regulation in plants. We have previously attempted to create RNAi hybrid poplar against the cell-wall invertases, and were unsuccessful at generating viable explants. We speculated that the suppression of cell-wall invertases manifested by RNAi was so extreme that little to no sucrose was being hydrolyzed to provide the hexoses necessary for vital processes such as glycolysis and respiration. Antisense suppression is generally less severe than RNAi; typically generating a range of suppression from weak to strong. To that end, we recently generated hybrid poplar expressing an antisense transcript for *PaxgINV2*, the cell-wall invertase believed to be involved in phloem unloading. The phenotype manifested along with subsequent biochemical analysis may provide clues pertaining to both the role of that cell-wall invertase in hybrid poplar.

6.2.3 Functional characterization of PaxgINV3

Chapter four described the identification of three cell-wall invertases from hybrid poplar. Two of these genes (*PaxgINV2* and *PaxgINV3*) were shown to be spatially and temporally regulated in vegetative tissues, and were functionally characterized (Chapter five). In contrast, expression of the third gene (*PaxgINV3*) was not detected in any of the vegetative tissues. As a result, we hypothesized that this gene was involved in reproductive tissue, as floral-specific cell-wall invertases had been shown to exist in other dicot species, including *PtCIN2* from female catkins of *P. deltoides*. As such, a comprehensive examination of mRNA from the reproductive tissues from mature hybrid poplar should reveal *PaxgINV3* transcript. Subsequent heterologous expression of PaxgINV3, similar to that described in Chapter five, would allow functional characterization of this enzyme. The information gathered from the proposed study would not only allow comparisons to be made between the previously characterized cell-wall invertases from hybrid poplar, but would also, to the best of our knowledge, represent the first enzymatic characterization of a floral-specific cell-wall invertase from a dicot species.