Functional characterization of flavonoid glycosyltransferases and an acid phosphatase from poplar (*Populus* spp.)

by

Vasko Veljanovski
HB.Sc., University of Toronto, 2002
M.Sc., Queen's University, 2005

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Supervisory Committee

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Abstract

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Plants have evolved a wide variety of physical and biochemical defense mechanisms to protect against herbivores and pathogens. When wounded, hybrid poplar (*Populus trichocarpa* X *P. deltoides*) upregulates a suite of defense-related genes, some of which encode anti-herbivore proteins. Among the most strongly insect- and wound-induced genes in poplar is an acid phosphatase gene (AP). APs are enzymes that function in hydrolyzing phosphate from P-monoesters and anhydrides and are involved in the remobilization of phosphate from these pools. However, APs may also play a role in the defense against insects by acting as anti-insect proteins. In poplar, AP mRNA induction occurs within 1.5 hours, which is similar to other known poplar defense genes. In the work described in this thesis, a 2 to 3-fold increase in the extractable AP activity was demonstrated in the leaves of saplings 4 days post wounding. These results suggest the poplar AP is part of the defense response against leaf-eating herbivores.

In another type of defense reaction, when hybrid poplar is infected by the pathogen *Melampsora medusae*, which causes poplar leaf rust, flavonoid pathway genes are induced. This induction leads to the accumulation of proanthocyanidins (PAs), compounds with antimicrobial activity. The expression of several flavonoid-specific glycosyltransferase (UGTs) genes were correlated with these PA genes, suggesting a role for them in PA biosynthesis. Therefore, the second objective of this thesis was to functionally analyze these UGT genes. UGTs are enzymes which catalyze glycosylation reactions, which is typically one of the last steps in the biosynthesis of plant phenolic compounds. Active recombinant proteins for two highly induced poplar UGTs (PtUGT1 and PtUGT2) were generated, and sequence analysis grouped these proteins with others

involved in the glycosylation of flavonols and anthocyanidins (UGT78 family), and not PA precursors as expected from microarray data. Enzymatic analysis of one of these proteins (PtUGT1) supports this phylogenetic grouping. By contrast, PtUGT2 does not use any known flavonoid substrates. To investigate the role of PtUGT1 *in planta*, transgenic poplars were produced that suppressed the expression of this gene using RNA interference. Phytochemical analysis of these knockdown plants were found to display decreased levels of PAs. Tissue survey analysis also implicates the PtUGT1 gene in PA biosynthesis since phytochemical analysis correlates with gene expression of PtUGT1 in the various tissues tested. Thus the data suggests that this UGT gene may be involved in PA biosynthesis.

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List of Abbreviations

4CL	4-coumarate CoA-ligase
ACT	Acyltransferase
ADP	Adenosine diphosphate
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
AP	Acid phosphatase
AUS	Auresidin synthase
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3' –indolyl phosphate
BLAST	Basic local alignment search tool
C4H	Cinnamate 4-hydroyxlase
cDNA	Complementary deoxyribonucleic acid
CHI	Chalcone isomerase
CHS	Chalcone synthase
CTAB	Cetyltrimethylammonium bromide
DAB	3,3’-diaminobenzidine tetrahydrochloride
DFR	Dihydroflavonol reductase
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
F3H	Flavanone 3β-hydroxylase
FLS	Flavonol synthase
FNS	Flavone synthase
GT	Glycosyltransferase
HCD	Hydroxylcinnamic acid derivative
His	Histidine
HPLC	High performance liquid chromatography
IFS	2-hydroxyisoflavone synthase

IPTG	Isopropyl-1-thio-B-D-galactoside
JGI	Joint Genome Institute
KTI	Kunitz type protease inhibitor
LAR	Leucoanthocyanidin reductase
LPI	Leaf plastochron index
MATE	Multidrug and toxic compound extrusion
MW	Molecular weight
NBT	Nitroblue tetrazolium chloride
OMT	O-methyltransferase
P	Phosphorus
PA	Proanthocyanidin
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PG	Phenolic glycoside
Pi	Phosphate
pNPP	p-nitrophenyl phosphate
PPi	Pyrophosphate
PPO	Polyphenol oxidase
PSPG	Plant secondary product glycosyltransferase
PVDF	Polyvinylidene difluoride
RNAi	RNA interference
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAIR	The Arabidopsis Information Resource
TCP	2,4,5-trichlorophenol
tt	Transparent testa
UDP	Uridine diphosphate
UGT	Uridine diphosphate glycosyltransferase
UV	Ultraviolet
VSP	Vegetative storage protein

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First off I want to begin by thanking my supervisor Dr. Peter Constabel for allowing me the opportunity to carry out this research. Additionally, I want to thank both the current and past members of my committee, Dr. Al Boraston, Dr. Abul Ekramoddoullah, Dr. Perry Howard and Dr. Robert Ingham for all the helpful input they had in this project. I would also like to thank all members in the Constabel lab. There have been many that have come through the lab! I especially want to thank Ms. Lan Tran for all her qPCR work. A special thanks goes out to Andrea Coulter, Natalie Prior, Alpha Wong, Nicole Dafoe, Catherine Franz and Eric Bol for all the good times we have had. And well, there are way too many other people to thank. So I will just say a big thank you to everyone else. You know who you are :)

Chapter 1: Introduction

1.1 Overview of plant defense strategies

In order to survive in their natural environments, plants must be able to respond to a wide range of stresses. These stresses can be either abiotic, such as heat, cold, drought, flooding, and UV radiation, or biotic, such as herbivore feeding and pathogen attack. Unlike animals, which are capable of moving away from their disturbances, plants are immobile: they are rooted and cannot move. Therefore, plants have evolved ways to overcome these stresses and actively defend themselves. Plant defenses can always be present in a plant (constitutive defenses) or they can be produced when needed (induced defenses).

As a first line of defense, plants have evolved pre-existing physical barriers. These barriers include thick cuticles, increased levels of cellulose and lignin in their cell wall, the formation of thick bark, and the production of thorns, spines, and/or trichomes along their leaves and stems. Such barriers help make the plant tougher, dangerous, and impermeable (Kessler and Baldwin, 2002). However, these physical barriers are not 100% efficient. When these defenses are not enough, plants are able to defend themselves by inducing a variety of defense mechanisms to prevent further damage. This induced defense response may occur locally at the site which is directly being damaged (termed a local defense), but it may also occur systemically, in tissues that are far away from the initial site of attack (termed a systemic response). Induced defenses include the production of proteins, enzymes and secondary compounds which can be anti-digestive,

anti-nutritive, or toxic (Howe and Jander, 2008; Walling 2000; Philippe and Bohlman, 2007; Mithöfer and Boland, 2012).

Protein-based defenses in plants

Plants contain many defense proteins, some of which can be strongly induced under herbivory, wounding and pathogen stress (Christopher et al., 2004; Major and Constabel, 2006; Ralph et al., 2006; Walling, 2000). The main mode of defense of these proteins is to decrease the nutritional content of the ingested plant tissue, or to act aggressively against the pests. Chitinases, lectins, proteinase inhibitors, peroxidases and polyphenol oxidases are examples of common plant defense proteins (Kessler and Baldwin, 2002).

Chitinases are enzymes that hydrolyze the chitin polymers which are found in insect exoskeletons and the cell walls of fungi (Sharma et al., 2011). They have been implicated in anti-insect defense by reducing the growth and development of insects. When Coloardo potato beetles were fed tomato leaves overexpressing a chitinase, growth was significantly reduced (Lawrence and Novak, 2006). It is hypothesized that the insecticidal activity of the chitinase is due to the degradation of the peritrophic membrane within the insect gut, but this has yet to be shown. Chitinases have also been shown to be able to inhibit the growth of a variety of fungi (Punja and Zhang, 1993; Sharma et al., 2011).

Similar to chitinases, lectins are both antifungal/antimicrobial and anti-insecticidal. They are a ubiquitous group of plant proteins that are able to recognize and bind to carbohydrate molecules (Vandenborre et al., 2011). Lectins have been shown to

be highly toxic to insect pests. Machuka et al. (1999) examined larval survival and development of a legume pod borer fed a diet of purified plant lectins, which were found to have a negative effect on growth. Lectins have also been implicated in pathogen resistance. Ma et al. (2010) showed that overexpression of a wheat lectin in tobacco plants increased its resistance to pathogen infection.

The largest group of characterized defense proteins are the Kunitz type protease inhibitors (KTI). KTIs in poplar have been studied extensively (Christopher et al., 2004; Haruta et al., 2001; Major and Constabel, 2006; Philippe and Bohlmann, 2007). They are encoded by a large gene family and are among the most strongly herbivore induced genes. KTIs function in plant defense by binding to digestive enzymes within the insect gut and thus inhibiting digestive activity (Howe and Jander, 2008). Decreases in growth and mortality of herbivores have been shown when KTIs are ingested by insects (McManus et al., 1999; Franco et al., 2004).

Polyphenol oxidases are another class of defense related proteins against pests and pathogens. Some have been found to be induced simultaneously with other anti-nutritive defense proteins, such as KTIs (Constabel et al., 1995; Constabel et al., 1996). PPOs also are encoded for by a large gene family and are similar to KTIs in that they are hypothesized to decrease protein digestibility. Quinones that are produced by PPOs in the herbivore gut are able to modify dietary proteins by cross-linking them and thus preventing their absorption and assimilation (Constabel and Barbehenn, 2008). This leads to decreased growth in herbivores. When larvae of forest tent caterpillar (*Malacosoma disstria*) were fed leaves from poplar that overexpressed a PPO gene, they

exhibited slower growth and higher mortality than caterpillars fed control leaves (Wang and Constabel, 2004).

An intriguing group of enzymes that has been found to be highly co-induced systemically with other defense proteins under wounding, herbivory and pathogen attack are acid phosphatases (APs) (Christopher et al., 2004). APs (E.C. 3.1.3.2; phosphoric-monoester phosphohydrolase) function in hydrolyzing phosphate (Pi) from P-monoesters and anhydrides by cleaving a covalent bond (Duff et al., 1994). Under Pi stress, APs are believed to be involved in the remobilization of Pi from P-monoester pools. The function of these enzymes in plant defense is unknown, but some have been found to be involved in defending plants during herbivory and disease. For example, an Arabidopsis AP was shown to inhibit the growth and to increase the mortality rates of insect larvae when ingested (Liu et al., 2005). Both coleopterans and dipterans were equally affected. Some APs also display peroxidase activity and it is hypothesized that APs may be involved in reactive oxygen species metabolism following pathogen attack (del Pozo et al., 1999). An induction of AP activity has been seen during disease responses of tomato and potato plants. In tomato, the transcript level of an AP gene was shown to be highly induced following bacterial infection of the plant (Stenzel et al., 2003). In a bacterial wilt-resistant potato, a purified AP was shown to display antimicrobial activity by inhibiting the growth of fungal colonies (Feng et al., 2003). Thus, APs seem to be associated with the plant defense response.

Phytochemical defenses in plants

In addition to proteins, plants are also able to produce a diverse arsenal of defensive secondary metabolites in response to biotic and abiotic stresses (Levin, 1976; Philippe and Bohlmann, 2007). Secondary metabolites are defined as small organic plant compounds that are not necessary for the normal day to day function of a plant but often have ecological functions (Croteau et al., 2000). Plants are able to synthesize a diverse variety of secondary metabolites including cyanogenic glycosides, terpenoids, glucosinolates, alkaloids, and phenolics.

Cyanogenic glycosides are present in many plant species (>2500) (Mithöfer and Boland, 2012). These toxic compounds are stored as inactive conjugates in the vacuole of plant cells. When cell damage occurs due to herbivore feeding, cellular structure is destroyed and leads to mixing of cellular contents allowing their activating glycosidases, which are located in the cytoplasm, to act on the cyanogenic glycosides and release toxic hydrogen cyanide (Mithöfer and Boland, 2012). Glucosinolates (sulphur containing glycosides) act in a manner similar to the cyanogenic glycosides. Their breakdown products, the isothiocyanates, are known to serve as direct defense compounds to insects and pathogens. They are also able to act as attractants for predators of feeding insects (Howe and Jander, 2008). Terpenoids are a diverse group of compounds that are derived from 5-carbon isoprene units which contribute to both direct and indirect defenses in plants. Some terpenoids are found in tree resin and can be quite toxic, and thus act as a direct repellent/deterrent to pests (Mumm and Hilker, 2006). Terpenoids are often volatile, and hence also play a role in indirect defenses in plants by attracting parasitoids of insect herbivores (Mithöfer and Boland, 2012). Another group of the intensely studied

group of defense compounds are the phenolics. This group of secondary metabolites consist of molecules based on an aromatic six carbon ring structure and can accumulate to high levels after a variety of biotic and abiotic stresses. The most studied natural phenolics are the flavonoids and their rapid induction under stress suggests their importance in a plant's inducible defense response (Aoki et al., 2000; Peters and Constabel, 2002). These are discussed in more detail below.

1.2 Flavonoids

Flavonoids are polyphenolic compounds found in all plant species. They form the largest group of plant phenols: over 9,000 different compounds have been identified to date (Martens and Mithofer, 2005; Taylor and Grotewold, 2005). Flavonoids have many functions that aid in plant survival, including UV protection, defense against pests and pathogens, colouration of flower and fruit pigmentation for attracting pollinators, regulating seed germination, acting as signaling molecules and contributing to plant structure (Dixon and Paiva, 1995; Koes et al., 1994; Kim et al., 2006a; Winkel-Shirley, 2002).

Flavonoids are categorized according to their core chemical structure. They all contain the characteristic 15 carbon structure, which consists of two aromatic rings (ring A and B) linked by a heterocycle containing oxygen. The chalcones, flavones, flavonols, flavanones, dihydroflavonols, isoflavones, anthocyanins, and proanthocyanidins (PAs), are the major groups (Figure 1.1) (Winkel-Shirley, 2001). The flavonoid biosynthetic pathway (Figure 1.2) has been extensively characterized using both biochemical and molecular approaches (Dixon and Pavia, 1995), and all of the key enzymes involved in

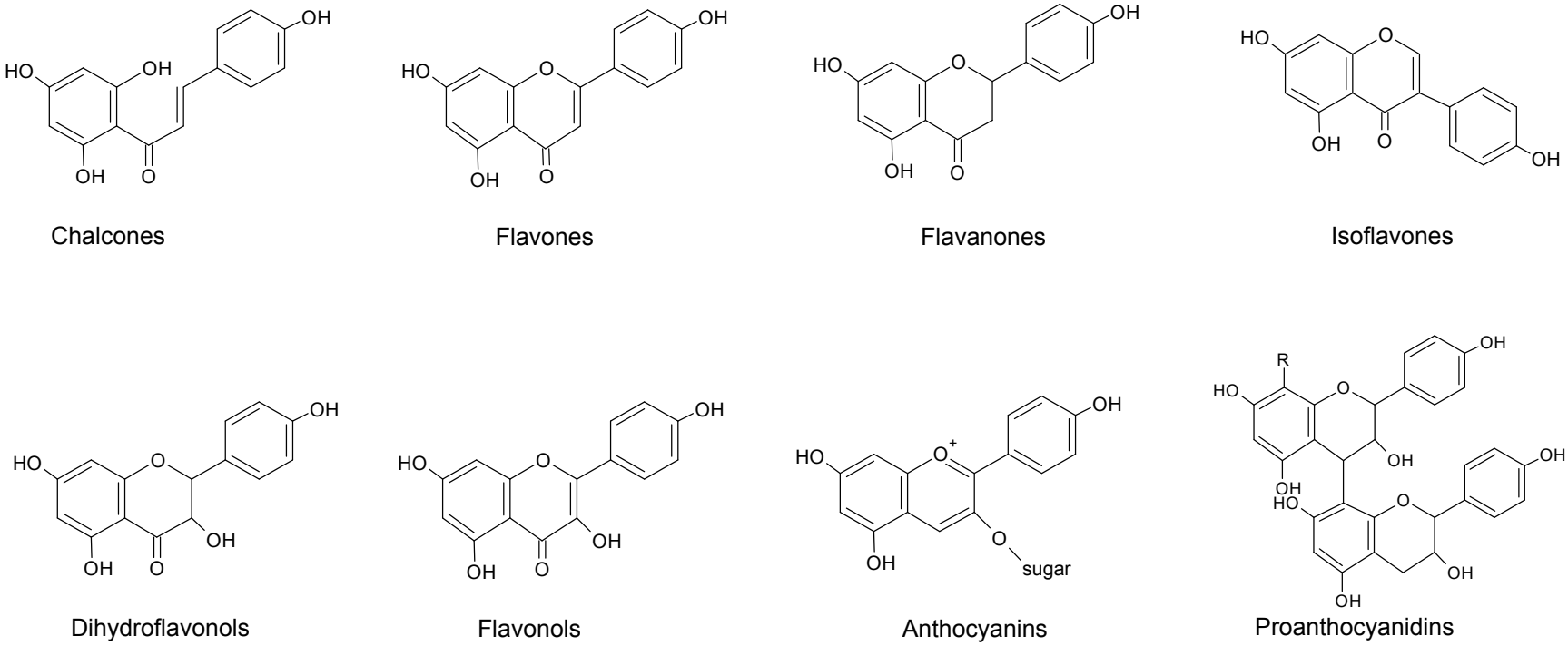


Figure 1.1: The major flavonoid groups.

Structures of the various flavonoid groups present in plants are shown.

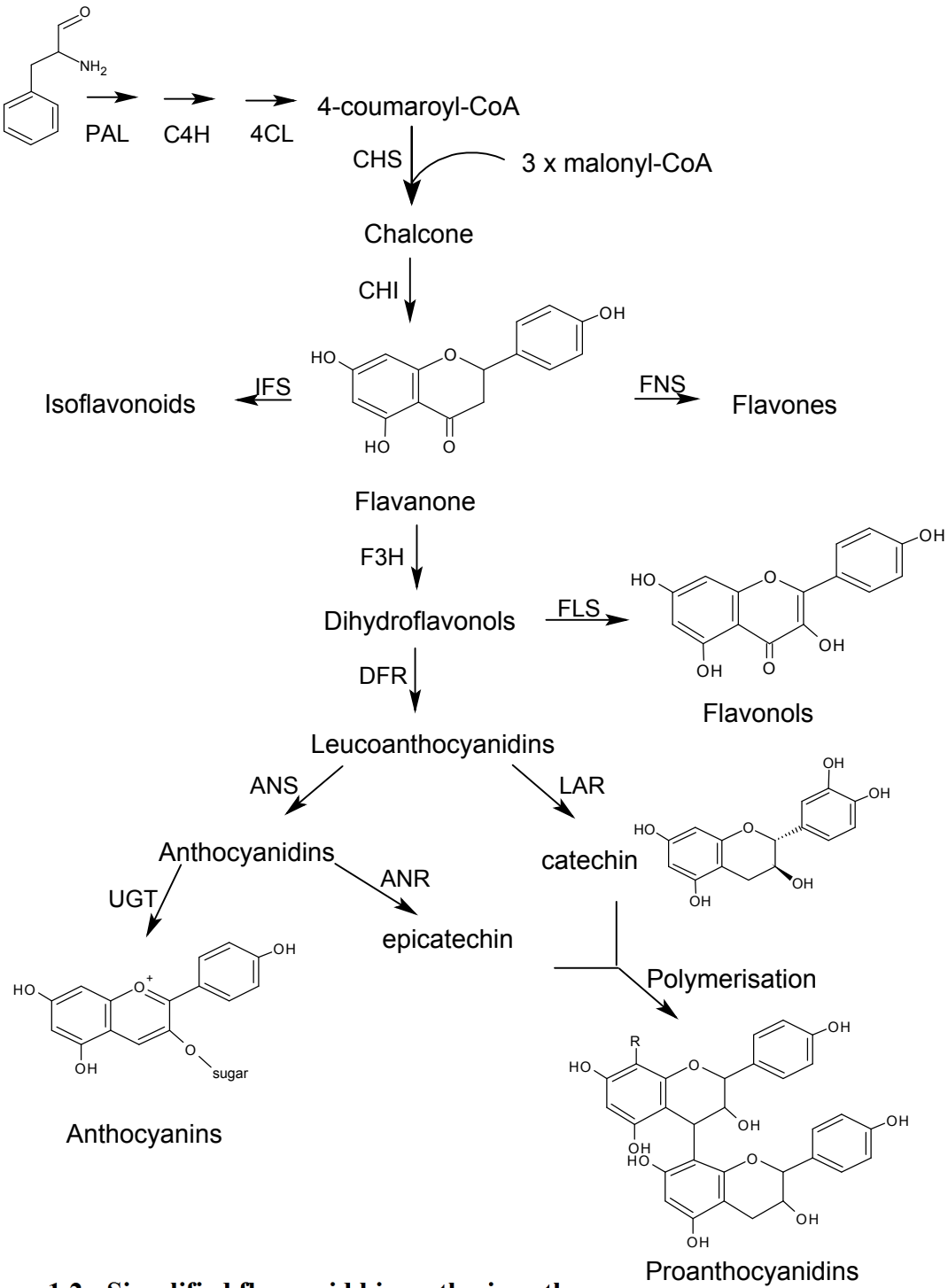


Figure 1.2: Simplified flavonoid biosynthesis pathway.
The main types of flavonoids are shown and the enzymes catalyzing key reactions are labelled. Enzyme abbreviation: PAL, phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase, 4CL: 4-coumaroyl-CoA ligase, CHS: chalcone synthase, CHI: chalcone isomerase, IFS: isoflavonoid synthase, FLS: flavonol synthase, F3H: flavanone-3β-hydroxylase, DFR: dihydroflavonol reductase, LAR: leucoanthocyanidin reductase, ANS: anthocyanidin synthase, ANR: anthocyanidin reductase, UGT: UDP glycosyltransferase.

synthesis have been identified. This was primarily accomplished by identifying and using mutants in a variety of plant species that were affected in flavonoid synthesis (Lepiniec et al., 2006). One of the advantages of studying flavonoid biosynthesis within Arabidopsis is all of the enzymes involved in synthesis are single copy genes, with the exception of FLS, which is encoded by six genes. Furthermore, mutations in any of the enzymes involved in the flavonoid pathway may lead to changes in the colour of the seed coat (testa) within Arabidopsis (Wiseman et al., 1998). Since the testa is affected, the mutant seeds are called transparent testa mutants or tt mutants (Buer et al., 2010). Over 22 tt mutants have been identified and characterized to date corresponding to all the major enzyme involved in flavonoid biosynthesis. For example, the tt4 mutant contains a mutation in the CHS gene, the first gene of flavonoid synthesis, and is unable to produce any flavonoids (Buer and Muday, 2004). The molecular analysis of tt mutants has greatly advanced the knowledge of flavonoid biosynthesis (Winkel-Shirley, 2001).

Flavonoid biosynthesis

The first committed step of the flavonoid pathway begins with the formation of naringenin chalcone, a 15 carbon compound (Figure 1.2). This intermediate compound is formed by the enzyme chalcone synthase (CHS). This enzyme catalyzes the condensation of three molecules of malonyl-CoA with a molecule of 4-coumaroyl-CoA. The malonyl-CoA is derived from the carboxylation of acetyl-CoA from fatty acid biosynthesis. The molecule of 4-coumaroyl-CoA is formed from phenylalanine by the actions of phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL). These steps constitute the general phenylpropanoid

pathway. Naringenin chalcone is either acted upon by aureusidin synthase (AUS) to produce the small class of flavonoids known as aurones, or it is isomerized by chalcone isomerase (CHI) and forms the 2-S-flavanones, with naringenin being the most common form (Croteau et al., 2000). From this point, the flavonoid pathway can branch into three different directions, leading either to the synthesis of flavones, isoflavones, or dihydroflavonols. If a double bond is formed between the C-2 and C-3 carbons of naringenin by the action of flavone synthase (FNS), this leads to the formation of the flavones. Isoflavones are formed by the action of 2-hydroxyisoflavonone synthase (IFS), which catalyzes the reaction that rearranges the B-ring from the C-2 position to the C-3 position of the C ring (Jung et al., 2000). Finally, the dihydroflavonols are produced from a C-3 hydroxylation by flavanone 3-hydroxylase (F3H) (Croteau et al., 2000). At this point, another branch point in the pathway leads to two groups of flavonoids: the flavonols and the flavan 3,4-diols (the leucoanthocyanidins) (Davies and Schwinn, 2006).

If the dihydroflavonols are acted upon by flavonol synthase (FLS), a double bond forms at the C-2 C-3 position. This gives rise to the flavonols, most commonly kaempferol, quercetin, or myricetin (Davies and Schwinn, 2006). Instead, if the dihydroflavonols are reduced at position 4 by the action of dihydroflavonol reductase (DFR), this leads to the flavan 3,4-diols, which are intermediates for the formation of both anthocyanidins and PAs. This is a key branch point, as these compounds have very different functions.

Leucoanthocyanidins can be converted to anthocyanidins by a reduction reaction carried out by anthocyanidin synthase (ANS). These molecules can then be converted to anthocyanins through the action of glycosyltransferases (GTs) that attach sugar residues

onto the molecules. Glycosylation helps stabilize the anthocyanins (Winkel, 2006). To produce PAs, which are polymers synthesized from flavan-3-ol molecules, there are two routes which in most plants act concurrently. One route involves the formation of 2,3-trans-flavan-3-ols (catechins). These are formed from leucoanthocyanidins that have had their 4-hydroxyl group removed by leucoanthocyanidin reductase (LAR). The other route involves the formation of 2,3-cis-flavan-3-ols (epicatechins). These are formed by the enzymatic action of anthocyanidin reductase (ANR) on anthocyanidins (Davies and Schwinn, 2006). These two flavano-3-ols are polymerized to PAs which accumulate in the vacuole, though the details of how this occurs are unknown.

Methylations, acylations, and glycosylations can occur throughout every branch point in flavonoid biosynthesis. These reactions occur via O-methyltransferases (OMTs), acyltransferases (ACTs) and GTs respectively (Winkel, 2006). These final decorations lead to the large variety of flavonoid metabolites seen in plants. GTs form the main subject of this thesis and are described in detail in following sections.

Localization of flavonoid enzymes – the concept of metabolons

Enzymes involved in flavonoid biosynthesis have long been thought to be soluble enzymes within the cytosol of plant cells, yet many are found to be localized to specific areas within the cell, mainly the ER membrane. More than 30 years ago, Helen Stafford proposed the idea that enzymes involved in a biosynthetic sequence were not free floating throughout the cytoplasm but were actually organized into a multi-enzyme complex, or metabolon (Stafford, 1974). In this metabolon configuration, the enzymes involved in a pathway are physically organized into a chain-like assembly, such that a direct transfer of

the product from one enzyme to the next is facilitated (Hrazdina and Jensen, 1992; Sweetlove and Fernie, 2005; Winkel-Shirley, 2001; Winkel, 2004). The idea of the channelling of intermediates between enzymes is appealing for many reasons. First, there would be an increase in the rate of product output because of the direct transfer of intermediates from one enzyme to the next. Therefore, the reaction rate would not be dependent on the diffusion rates of products and the randomness of encountering the next enzyme. Second, there would be less competition between competing pathways or branch points within pathways because each could have there own metabolon. Third, metabolons would not allow highly unstable and toxic intermediates to diffuse freely within a cell and would help protect the breakdown of unstable intermediates. Fourth, there could be the prevention of inhibitory compounds from attaching to enzymes and decreasing their activity. Fifth, there is the potential to have fine metabolic control of the pathways and allow for quick changes to occur (Achnine et al., 2004; Jorgensen et al., 2005; Kutchan, 2005; Stafford, 1981; Winkel-Shirley, 2001). However, studying these complexes is rather difficult because they are believed to be held together by relatively weak interactions and most likely dissociate during enzyme extraction. Therefore, no complexes have ever been purified intact (Stafford, 1974, Stafford 1981, Winkel-Shirley, 1999). Nevertheless, there is indirect overwhelming evidence that a multienzyme complex exists that leads to the formation of flavonoids.

The first piece of evidence for the organization of flavonoid metabolons was in 1984 when Wagner and Hrazdina used sucrose gradient centrifugation and were able to show that CHS, CHI and a GT were associated with both the rough ER and the smooth ER in *Hippeastrum* petals. Later, more enzymes involved in flavonoid biosynthesis were

found to be localized to the ER membrane (Hrazdina et al., 1987). These 'soluble' enzymes were hypothesized to all be bound to the ER under *in vivo* conditions. To confirm these results, antibodies for CHS were used to immunologically detect the enzyme on ER membranes (Hrazdina et al., 1987). Furthermore in 1999, Burbulis and Winkel reported protein-protein interactions between enzymes within the flavonoid pathway, adding additional evidence to support the idea of metabolons. Using yeast two-hybrid experiments, they were able to show that the CHS, F3H, and CHI enzymes were able to interact with each other. All this data has lead to the model of flavonoid biosynthesis in which all the flavonoid enzymes are organized onto the ER membrane in a linear fashion, but since CHS is able to interact with F3H, it may not in fact be linear, but more of a globular arrangement (Jorgensen et al., 2005; Winkel-Shirley, 1999). All the evidence to date points to the formation of a flavonoid metabolon in which flavonoids are made at the ER membrane.

The diverse function of flavonoids

Flavonoids exhibit a diverse range of biological functions (Treutter, 2006). One of the well known roles of flavonoids is their function as visual cues for animals. Anthocyanins, the most familiar group of flavonoids, are responsible for the red and purple colours seen in flower petals and fruits (Koes et al., 1994). These flashy colours have a role in attracting pollinators to the plant. Flavonoids are also involved in the formation of symbiotic relationships between plants and microbes. Isoflavonoids are released by leguminous plants and induce *nod* genes within surrounding rhizobia bacteria, initiating an interaction (Buer et al., 2010; Koes et al., 1994; Winkel-Shirley,

1996; Taylor and Grotewold, 2005). Another important role of flavonoids is their involvement in polar auxin transport. Flavonols, such as quercetin and kaempferol, can compete with an auxin efflux inhibitor for transporters, and thus can negatively regulate polar auxin transport. In mutated plants without the ability to produce flavonols, developmental abnormalities are seen which can be rescued by the addition of missing flavonols. Thus, flavonoids are important for the formation of polar auxin gradients within plants (Buer et al., 2010; Taylor and Grotewold, 2005).

Many flavonoids are rapidly produced in response to stress conditions such as pest damage, intense UV radiation, drought, low nutrient levels, low temperatures, and pathogen attack (Dixon and Paiva, 1995; Marais et al., 2005, Izaguirre et al., 2007). UV protection is one of the most significant roles of flavonoids. Flavonoids have the ability to absorb light over a wide range of the light spectrum including the UV(Winkel-Shirley, 1996; Stafford, 1991). Flavonoids in the epidermal layers of rye plants have been shown to increase under UV light and thus protect the underlying internal tissues from UV damage (Reuber et al., 1996). Often, during intense visible light conditions, an increase in the levels of anthocyanins and PAs is observed. Anthocyanins have been implicated as 'sunscreens' for plants by absorbing excessive visible light. They could function as a light screen for the photosynthetic apparatus in order to avoid the production of reactive oxygen species (Middleton and Teramura, 1993; Koes et al., 1994; Winkel-Shirley, 2001).

Some flavonoids have also been seen to be upregulated during herbivore and pathogen attack (Treutter, 2006; Miranda et al., 2007). They have been found to increase around the site of plant damage to concentrations that are thought to be toxic (Dixon and

Paiva, 1995). For example, the isoflavone luteone is induced following infection by fungal pathogens and has been shown to display antifungal activity against pathogens (Harborne et al., 1976; Tahara et al., 1984). Another group of inducible flavonoids that are anti-nutritive to herbivores and potentially toxic to pathogenic fungi are the PAs, also called the condensed tannins (Levin, 1976, Bauce el al., 2005). These are central to this thesis and are described in greater detail.

Proanthocyanidins

Proanthocyanidins are a class of large molecular weight polyphenolic compounds that are synthesized as polymers of flavan-3-ols (Vogt, 2010). PAs are well known for their ability to strongly bind to and precipitate proteins, and thus can decrease protein digestion in mammalian herbivores (Barbehenn and Constabel, 2011; Ayres et al., 1997). This is due to the many hydroxyl groups which interact with proteins and form strong complexes which lead to the precipitation of the proteins. By binding the proteins, PAs are thought to be able to inhibit digestion of the proteins by herbivores. PAs can also exhibit toxic effects to herbivores. It is believed that ingested PAs can lead to the production of reactive oxygen species which can cause direct damage to herbivore tissues leading to decreased growth (Barbehenn and Constabel, 2011). In some insect herbivores fed high levels of PAs, growth rates were significantly lower than larvae fed low PA diets (Bauce et al., 2005). Examination of insect digestive tracts found lesions had formed in their midgut epithelium, which are believed to be due to the ROS molecules formed from ingestion of PAs (Barbehenn and Constabel, 2011).

PAs may also be toxic to pathogenic fungi. In chestnuts, high PA levels were found to allow resistance to chestnut blight fungus. Similarly, in strawberry and apricot, PAs have been believed to be involved in resistance to a wilt fungus (Levin, 1976). In bilberry plants infected with *Botrytis cinerea*, total soluble PAs were increased within 24 hours of infection (Koskimaki et al., 2009). Furthermore, in a study done on poplar leaf tissue that was infected with the pathogenic leaf rust, *Melampsora medusae*, PAs were found to accumulate after infection. Microarray analysis of the transcripts from this experiment showed that 6 days after infection with the fungus, all the flavonoid biosynthetic genes encoding enzymes needed for the synthesis of PAs were highly upregulated, leading to the de novo synthesis of PAs (Miranda et al., 2007). Thus PAs appear to be induced for defense. In addition to herbivory and pathogen infection, PAs have also been found to accumulate to high levels under UV light stress and nutrient limitation conditions in some woody plant species, including poplar, oak, and birch (Peters and Constabel, 2002; Schultz and Baldwin, 1982; Bryant et al., 1993; Mellway and Constabel, 2009; Hemming and Lindroth, 1999).

PAs are primarily sequestered within the vacuoles of plant cells (Stafford, 2000; Barbehenn and Constabel, 2011). For example, PAs have been found in the vacuole of epidermal and mesophyll cells from the leaves of beech trees, from persimmon fruit leaves, and from grapes leaves and skins (Bussotti et al., 1998; Gagne et al., 2006; Ikegami et al., 2007). In order for PAs to be sequestered within the vacuole, the involvement of transporters is required (Bussotti et al., 1998). The major transporters that transport PAs are the proton dependent transporters and the multidrug and toxic compound extrusion (MATE) transporters. The pH of the central vacuole of plants is

known to be mildly acidic, estimated to have a pH between 5 and 6. The action of two proton pumps, the V-ATPase and the V-PPase, are responsible for this. These pumps translocate H^+ ions into the vacuole by using the energy released from the breakdown of ATP to ADP + Pi or PPi to two molecules of Pi (Maeshima, 2000; Rea and Sanders, 1987; Roytrakul and Verpoorte, 2007). It has been speculated that this pH gradient between the cytosol and the vacuole is able to provide the energy needed to allow the movement of substances into the vacuole. Using the Arabidopsis *tt12* mutants, a vacuolar flavonoid/H^+ antiporter was found to be active in the seed coats of PA accumulating cells. When this protein was characterized in yeast, it was shown to be an antiporter, moving cyanidin-3-O-glucoside and H^+ ions in opposite directions (Marinova et al., 2007a). Molecular cloning of the TT12 gene and sequencing led to the finding that it encoded a 507 amino acid protein, which contained 12 putative transmembrane segments. BLAST analysis of the protein identified it as being related to the MATE family of carrier transporters found in prokaryotes and eukaryotes (Debeaujon et al.., 2001). The transporter was localized to the tonoplast membrane and was found to transport glycosylated flavonoids across the membrane (Marinova et al., 2007b). More recently, a Medicago MATE transporter similar to Arabidopsis tt12 was identified which could preferentially transport epicatechin 3'-O-glucoside, precursors for PA synthesis (Zhao and Dixon, 2009).

1.3 Glycosylation and glycosyltransferases

Glycosylation is one of the last steps involved in the biosynthesis of many plant defense compounds including phenolics, glucosinolates, salicylates, anthocyanins, and

flavonoids (Vogt and Jones, 2000, Bowles et al., 2006; Jones and Vogt, 2001). Glycosylation involves the addition of glycan subunits to target molecules. The glycan units may influence the stability, the biological activity, and the solubility of metabolites (Ko et al., 2006). Furthermore, it is hypothesized that glycosylation may be crucial for the targeting of molecules to specific compartments in a cell (Rayon et al., 1998). Glycosylation reactions are carried out by a large group of enzymes known as GTs. These enzymes are found in all living organisms, including plants, animals, and bacteria (Ross et al., 2001; Keegstra and Raikhhel, 2001). GTs transfer sugar residues to a variety of acceptor molecules. GTs have been classified based on their amino acid sequence and structural similarities, and this has led to the identification of 94 distinct families (denoted as GTx where x is the family number) (Hu and Walker, 2002; Paquette et al., 2003; Cantarel et al., 2008). This number is continually increasing as new GT genes are being identified and biochemically characterized (for example, in 2003, there were 54 families indentified; in 2006, this number had increased to 78, and this has increased to the 94 seen today) (Paquette et al., 2003; Breton et al., 2006; Hansen et al., 2009; Yonekura-Sakakibara and Hanada, 2011) . These various families have been organized into a database that is freely available on the internet. The CAZy (Carbohydrate-Active Enzyme) database (http://www.cazy.org/) is a comprehensive resource that has specialized in organizing the enzymes used in the building and breakdown of carbohydrates and glycol-conjugates (Cantarel et al., 2008; Park et al., 2010). The data is manually curated and is constantly updated providing an invaluable resource for CAZymes. The GTs represent approximately 40% of the enzymes present on the CAZy website (Cantarel et al., 2008).

GT family 1 has been found to be the largest family in the plant kingdom. This family transfers sugar residues from uridine-diphosphate activated monosaccarides to low molecular weight substates (Vogt and Jones, 2000), and are often referred to as uridine diphosphate glycosyltransferases (UGTs) (Bowles et al., 2006). This family is further defined by the presence of a consensus sequence, a PSPG box (Plant Secondary Product Glycosyltransferase motif) located near the C-terminal end of the protein (Coutinho et al., 2003; Lorenc-Kukula et al., 2004; Offen et al., 2006; Hughes and Hughes, 1994). This box consists of a short 44 amino acid fragment that is involved in the binding of the enzyme to the UDP moiety of the sugar nucleotide (Lorenc-Kukula et al., 2004). Using this PSPG box consensus sequence as a search tool, 117 putative UGT genes were identified in the *Arabidopsis thaliana* genome. There appear to be 187 and 202 UGT genes in *Medicago truncatula* and in rice, respectively (Gachon et al., 2005; Kim et al., 2006b; Ko et al., 2006; Yonekura-Sakakibara and Hanada, 2011). Thus, family 1 UGTs contain a large number of genes. This abundance has led to a novel nomenclature strategy, recommended by the UGT Nomenclature Committee (Mackenzie at al., 1997). UGTs are named based on amino acid sequence identity. The genes are named as follows: UGT to denote they are UDP-dependent glycosyltransferases, a number from 1-200 to denote the family the gene belongs to, a letter (A-Z) to denote the subfamily, and finally a number to signify the individual gene. For family number, values of 1-50 are used for animal UGTs, 51-70 for yeast, 71-100 for plants, and 101-200 for bacteria. Grouping of the UGTs within a family denotes greater then 40% amino acid similarity; 60% or more is seen in the subfamilies (Campbell et al., 1997; Coutinho et al., 2003; Yonekura-Sakaibara and Hanada, 2011; Ross et al., 2001). Phylogenetic analysis of

UGTs can also be used to divide them into 14 distinct groups (Group A – Group N) based on bootstrap values. This was first performed by Li et al. (2001) when they examined the UGTs from Arabidopsis.

With the current availability of many plant genomes, genome-wide sequence analysis is a very useful tool in order to find candidate UGTs. Classification of these genes into the various families mentioned can help to identify potential substrates of the UGTs. However, it can be difficult to predict the function and substrate specificity of a putative UGT based solely on primary sequence homology with known and characterized UGTs. There are many examples of closely related sequences having very different catalytic activity, and diverse sequences having similar activities (Vogt and Jones, 2000; Breton et al., 2006; Osmani et al., 2009; Wang and Hou, 2009). For example, two UGTs identified in *Dorotheanthus bellidiformis* which showed below 20% sequence similarity, were able to glycosylate similar acceptor molecules (Vogt 2002). Similarly, when UGT sequences from enzymes able to glycosylate cytokinins were compared from Arabidopsis and maize, low sequence similarity was found even though they both worked on the same substrate (Hou et al., 2004). Thus, the relationship between primary sequence homology and UGT function is complex. Sometimes the prediction of substrates based on phylogenetic clades is accurate, while other times it is not. Hence, one needs to characterize UGT genes in order to truly determine their function.

Family 1 UGT: Flavonoid-specific UGTs

Flavonoid UGTs have been identified and characterized from many plant species. As mentioned earlier, they are ‘soluble’ enzymes in the cytosol of plant cells, though they

may be associated with other enzymes in a metabolon. Most of the data on flavonoid UGTs is from characterized recombinant enzymes produced in yeast or bacteria, though some have been purified to homogeneity from plant extracts (Lorenc-Kukula et al., 2004).

3-O glycosyltransferases

UGTs involved in the attachment of UDP-sugars to the 3-O-position of flavonols and anthocyanidins are the most frequently studied enzymes and many have been characterized (Lorenc-Kukula et al., 2004; Vogt, 2000). For example, recently an anthocyanidin 3-O-glycosyltransferases (3-UGT) was cloned from Concord grape (*Vitis labrusca*) (Hall et al., 2011). It was able to glycosylate anthocyanidins including malvidin, peonidin, delphinidin, and cyanidin, the preferred substrate. An anthocyanidin 3-UGT was also recently identified in the flesh of red kiwifruit (Montefiori et al., 2011). Recombinant protein of this UGT was able to add galactose onto the 3-OH position of cyanidin *in vitro* leading to the formation of cyanidin-O-galactoside. An interesting finding in kiwifruit is that the cyanidin-O-galactoside could be further glycosylated by another UGT. This enzyme was able to add a xylose to yield cyanidin 3-O-xylo(1-2)-galactoside which is the most prominent anthocyanin in kiwifruit (Montefiori et al., 2011). Several enzymes with activities toward flavonols have recently been cloned and characterized. In grapevine (*Vitis vinifera*), two UGTs (Vv GT5 and Vv GT6) were identified and cloned and shown to be specific for flavonol substrates. VvGT5 was only able to use UDP-glucuronic acid as the sugar donor, and was only able to glycosylate flavonols. It is thus defined as a UDP-glucuronic acid:flavonol–3-O-glucuronosyltransferase. VvGT6 was similar in it ability to only glycosylate flavonols,

but it used UDP-galactose and not UDP-glucose as its sugar donor (Ono et al., 2010). Similarly in strawberry, two UGTs were cloned and biochemical analysis identified them as primarily involved in the glucosylation of flavonols, but they were also able to attach UDP-glucose onto some flavanones *in vitro* (Griesser et al., 2008).

7-O glycosyltransferases

Glycosylation of the A and B rings of flavonoids has also been found to occur in plants. Glycosylation of the A ring most often occurs at position 7 by 7-O-glycosyltransferases (7-UGTs) and many such enzymes have been identified in plants. In Arabidopsis, a 7-UGT was identified, cloned and shown to be active with many flavonoids: flavonols, flavones, and flavanones (Kim et al., 2006b). In contrast, a yellow onion (*Allium cepa*) UGT was only able to use a very narrow range of flavonoids as substrates. It added a glucose residue to the C-7 position of a flavonol (isoquercitrin) and to an isoflavone (genistein) (Kramer et al., 2003). In rice, a UGT could only transfer glucose onto the 7-hydroxl group of isoflavones (genistein and daidzein) (Ko et al., 2008). Yet in all cases, the enzymes showed regiospecificity by modifying the 7-OH group of the A-ring.

5-O glycosyltransferases

The last major group of UGTs are those that glycosylate the B ring at position 5; hence they are called 5-UGTs. The best known 5-UGTs are those which act on 3-glycosylated anthocyanins. These modifications are important for the synthesis of stable anthocyanin compounds. Furthermore, glycosylation of anthocyanins contributes to the colour variations seen in pigmentation (Lorenc-Kukula et al., 2004). Anthocyanin 5-UGTs have been identified and characterized in many species including *Iris hollandica*

(Imayama et al., 2004), *Petunia hybrida* (Yamazaki et al., 2002) *Perilla frutescenes* var. *crispa* (Yamazaki and Saito, 2006), *Gentiana triflora* (Nakatsuka et al., 2008), and *Solanum tuberosum* (Lorenc-Kukula et al., 2005). All 5-UGT enzymes were able to catalyse glycosylation of the 5-moiety of anthocyanidin-3-glycosides.

UGTs and plant stress

UGTs respond to a variety of plant stresses. For example, the induction of flavonoid biosynthetic pathway enzymes have been found to occur in grape plants as well as in poplar plants under increased light. UGT transcripts were also rapidly induced under this stress (Sparvoli et al., 1994; Mellway and Constabel, 2009). A similar finding has also been seen in tomato plants; UGTs were upregulated after wounding or pathogen infection (O'Donnell et al., 1998; Truesdale et al., 1996). Furthermore, UGTs may also have a role in aiding in a plant's response to pathogen attack. In tobacco plants, when a UGT gene involved in the production of the hydroxycoumarin scopoletin had decreased expression in transgenic plants, the resulting plant contained lower levels of scopolin (scopoletin-glycoside) and had a reduced ability to defend itself against infection by tobacco mosaic virus (Chong et al., 2002). When this gene was overexpressed in tobacco plants, it had enhanced resistance to virus attack (Matros and Mock, 2004). In Arabidopsis, T-DNA mutants of the UGT73B3 and UGT73B5 genes decreased the plants resistance to *P. syringae* pv *tomato-AvrRpm1* infection, implicating the UGT genes in being necessary for defense (Langlois-Meurinne et al., 2005). Also, microarray studies on poplar leaf tissue infected with rust fungus found the accumulation of UGT transcripts

after 48 hours (Miranda et al., 2007). Thus, UGTs may have an important part in a plants' response to biotic and abiotic stresses.

1.4 Poplar as model tree

The genus *Populus*, which includes the trees commonly called poplars, aspens and cottonwoods, refers to a group of approximately 30 species that can be found throughout the Northern Hemisphere (Stettler et al., 1996). They are widespread across North America, especially in the boreal forest of Canada. *Populus* is considered to be the model woody plant for genomics since its entire genome has been sequenced and there is a large collection of genomic resources available (including cDNA clones, expressed sequence tags and numerous microarray data sets) (Tuskan et al., 2006). Poplar (*Populus trichocarpa*) was the third plant, and first woody plant to be sequenced. It was chosen due to it relatively small genome size (450Mb), its fast growth and short life cycle, its ability to be easily transformed by *Agrobacterium*-mediated transformation, and its ease of propagation (Meilan and Ma, 2006; Ridge et al., 1986; Wullschleger et al., 2002; Taylor, 2002; Jansson and Douglas, 2007). All these factors make it an excellent model species.

Poplars are also an important component of many ecosystems. A majority of poplars in North America are naturally found in riparian ecosystems which help shape the growth of these environments. Trembling aspen (*P. tremuloides*) is a dominant species in aspen parklands and the southern boreal forest. Many are planted to help serve as windbreaks and to prevent erosion (Rood et al., 2003). Furthermore, poplars have been known to be used in phytoremediation, for carbon sequestration and nutrient cycling by

scientists (Brunner et al., 2004; Haycock and Pinay, 1993). They are also a habitat for a variety of wildlife and can provide food for animals (Stettler et al., 1996). Not only are poplars important ecologically, but they are also important economically. They are a major crop for woodlot owners which use the timber for the production of pulp and paper and other wood products (Rood et al., 2003). A recent thrust of poplar research is on wood formation in order to produce poplars with improved digestibility of cellulose for use as biofuels (Sannigrahi et al., 2010).

As trees, poplars must be able to cope with a large variety of pests, pathogens, and other stresses over their lifespan. Thus, poplars must have evolved a diverse set of defense systems to help deal with them. Poplars accumulate a broad array of phenolic and phenylpropanoid compounds of relevance in defense. The three major classes are: salicylate based phenolic glycosides (PGs), PAs, and hydroxycinnamic acids and their derivatives (HCDs) (Constabel and Lindroth, 2010). The genus *Populus* contains more then 20 structurally different salicin based PGs which include salicin, salicortin, tremuloidin and tremulacin. They are a large class of compounds that are exclusive to the Salicaceae family and can comprise up to 30% of the dry weight of poplar leaves (Tsai et al., 2006). Many PGs are considered to be effective anti-herbivore compounds due to their toxicity to insects (Lindroth and Hwang, 1996; Lindroth et al., 1988). The PAs are a second major class of defensive phytochemicals in poplar, which are found in many woody plants and were discussed earlier. Lastly, the HCDs and derivatives are a third prominent class of secondary metabolites in poplar. These molecules are produced by hydroxylations and O-methylations of cinnamate (Constabel and Lindroth, 2010). HCDs include molecules such as chlorogenic acid and caffeic acid.

1.5 Objectives

Previous transcript profiling experiments identified a strongly wound-inducible AP gene. The upregulation of this gene suggested that it was an important component in poplar defense against herbivores and wounding. The first objective of this thesis was to characterize this wound- and herbivory- induced AP in poplar and examine its gene expression, protein expression and enzymatic activity (Chapter 2). The second objective of this thesis was to functionally analyze and characterize two flavonoid specific UGTs, called PtUGT1 and PtUGT2. These were implicated in the poplar defense response and in PA biosynthesis. In pathogen stressed poplar, as well as in transgenic poplars overexpressing a MYB transcription factor leading to the production of high levels of PAs, these UGTs were also found to be highly upregulated (Miranda et al., 2007; Mellway, 2009). The strong co- expression pattern of the UGTs with genes required for the production of PAs suggested their involvement in the synthesis of PAs for plant defense. These two genes were cloned and the gene products were biochemically characterized to determine substrates (Chapter 3). PtUGT1 showed the most interesting enzyme activity and was chosen for further study of its *in planta* function. This was achieved by producing poplars with RNAi-suppressed PtUGT1 expression and biochemically characterizing these plants (Chapter 4).

Chapter 2: Induction of acid phosphatase transcripts, protein and enzymatic activity by simulated herbivory of hybrid poplar

(Veljanovski V, Major IT, Patton JJ, Bol E, Louvet S, Hawkins BJ, Constabel CP (2010) Phytochemistry 71: 619-626)

Seven authors contributed to the work completed in this manuscript. As first author, I contributed the experiments pertaining to the enzymatic activity of the AP (Table 2.1, Figure 2.4, 2.5). I also aided in the production of Figure 2.1 with IT Major, who also produced Figure 2.2. J Patton performed experiments leading to the findings presented in Figure 2.3. I helped supervise E Bol who performed the immunoblots in Figure 2.6 from tissue obtained in previous experiments from S Louvet.

2.1 Abstract

Herbivory and wounding upregulate a large suite of defense genes in hybrid poplar leaves. A strongly wound- and herbivore-induced gene with high similarity to Arabidopsis vegetative storage proteins (VSPs) and acid phosphatase (AP) was identified among genes strongly expressed during the poplar herbivore defense response. Phylogenetic analysis showed that the putative poplar acid phosphatase (PtdAP1) gene is part of an eight-member AP gene family in poplar, and is most closely related to a functionally characterized soybean nodule AP. Unlike the other poplar APs, PtdAP1 is expressed in a variety of tissues, as observed in an analysis of EST data. Following wounding, the gene shows an expression profile similar to other known poplar defense genes such as protease inhibitors, chitinase, and polyphenol oxidase. Significantly, we show for the first time that subsequent to the wound-induction of PtdAP1 transcripts, AP protein and activity increase in extracts of leaves and other tissues. Although its mechanism of action is as yet unknown, these results suggest in hybrid poplar PtdAP1 is likely a component of the defense response against leaf-eating herbivores.

2.2 Introduction

Phosphorus (P) is a key element for plant metabolism and is often a limiting nutrient in crop plants (Vance et al., 2003). Phosphatases (EC 3.1.3.2) hydrolyse the phosphate anions (Pi) from orthophosphate monoesters, and are thus important enzymes of plant metabolism at many levels (Duff et al., 1994). Phosphatases are traditionally classified as either acidic or basic depending on their pH optima. Alkaline phosphatases typically show absolute specificity and take only single substrates, while the acid phosphatases (APs) usually have broad substrate preferences and can accept a variety of phosphorylated substrates (Duff et al., 1994). This low substrate specificity of APs makes it difficult to assign physiological roles to specific enzymes based on substrate preferences. For example, a purified AP from potato tubers was able to release Pi from a wide variety of substrates, such as small molecules and model substrates including phosphoenol pyruvate, pyrophosphate, p-nitrophenyl phosphate (pNPP), ATP, as well as phosphotyrosine and several phosphorylated potato proteins (Gellatly et al., 1994).

The expression of AP genes is often associated with Pi limitation, and active AP enzymes are commonly secreted into the rhizosphere in response to Pi starvation (Tadano et al., 1993; Vance et al., 2003). Therefore, a major role of the extracellular root APs appears to be in Pi acquisition, i.e., facilitating the release of Pi from various organic compounds in the soil (Fernandez and Ascencio, 1994; Vance et al., 2003). Extracellular secretion of AP into the soil from roots has been investigated in many species, and is considered an indicator of P stress. Intracellular APs, which may be either vacuolar or cytoplasmic, are also induced by Pi starvation, sometimes in aerial plant organs (Duff et al., 1991; Baldwin et al., 2001; Tian et al., 2003; Veljanovski et al., 2006). Such leaf APs

may contribute to plant nutrition by mobilizing internal stores of Pi from senescing tissues and cells. However, Yan et al. (2001) found no correlation of induced intracellular APs with improved nutrition, and thus the significance of these APs for Pi metabolism is still unclear.

In some plants, proteins with strong similarity to APs have been found to be vegetative storage proteins (VSPs), defined by their temporal patterns of synthesis and degradation that reflect the tissue-specific nitrogen supply in vegetative tissues. For example, soybean VSPa and VSPb accumulate dramatically in stems and other vegetative tissues of soybean plants following removal of seed pods. Their function is presumed to be temporary nitrogen storage in vegetative tissues when the plant is deprived of its normal nitrogen sink (Staswick et al., 1994). These VSPs have significant sequence similarity to APs, yet the gene product demonstrated only low levels of AP enzymatic activity (Leelapon et al., 2004). This confirms that their primary role is for nitrogen/amino acid storage, rather than Pi metabolism. Synthesis of the soybean VSPs can be stimulated by the wound and defense signal methyl jasmonate (Franceschi and Grimes, 1991), an observation that first provided a link of AP-like proteins to defense responses.

In Arabidopsis, two genes (AtVSP1 and AtVSP2) with similarity to the soybean VSPs and APs are strongly induced by herbivory, wounding, and jasmonate (Berger et al., 1995, Berger et al., 2002). These expression patterns are most consistent with a function in direct defense rather than in temporary nitrogen storage. The Arabidopsis VSP genes are regulated via the JA signaling defense pathway, and mutants without the capacity to synthesize JA do not accumulate VSPs (Berger et al., 1996). The link of VSPs

to plant defense was strengthened via the analysis of Arabidopsis mutants, in which VSP expression is correlated with defense capacity against lepidopteran herbivores (discussed by Liu et al., 2005). Significantly, recombinant AtVSP2 protein demonstrated anti-insect activity in bioassays with coleopteran pests as well as a dipteran species (Liu et al., 2005). AtVSP2 with a mutated active site lost its inhibitory activity against the test insects, thus linking AP activity to its biological effects. The mechanism of action against pest insects is not known, however.

In hybrid poplar as in many other species, wounding and leaf damage by herbivory can trigger dramatic changes to the leaf transcriptome, affecting the expression of hundreds to thousands of genes (Ralph et al., 2006; Major and Constabel, 2006). Many of the most strongly upregulated genes typically encode proteins with demonstrated activity against leaf-eating insects. Among these, Kunitz protease inhibitor transcripts are very prominent in terms of number of genes and proportion of transcripts (Christopher et al., 2004); these were subsequently shown to be active against mammalian and insect proteases (Major and Constabel, 2008). Other induced genes encode chitinases, polyphenol oxidase, and peroxidases, all enzymes for which anti-insect activity has been experimentally demonstrated (reviewed in Constabel and Lindroth, 2010). In addition, wounded leaves of some poplar species can lead to enhanced levels of proanthocyanidins (Peters and Constabel, 2002), which some studies have linked to pest resistance. Therefore, poplar leaves can clearly respond actively to herbivore attack with an active mobilization of defenses targeted to insect pests.

During previous transcript profiling and genomics studies of the hybrid poplar defense response, we identified a strongly wound inducible AP gene among the top ten

most abundant transcripts in a systemically wounded leaf library (Christopher et al., 2004; Major and Constabel, 2006). The strong upregulation of this gene in several, independent differential screening and herbivory experiments suggested that the gene product should be an important component of poplar defense against leaf-eating herbivores. Despite this strong co-expression, as well as independent work showing direct anti-insect effects of Arabidopsis AP-like AtVSP2, to date there are no reports for any plant species of either a wound- or herbivore-inducible AP enzyme activity. Here, we directly address this question in hybrid poplar, and further characterize AP induction at the transcript, protein, and enzyme activity levels. Our results demonstrate that wounding induces elevated AP activity in leaves and other tissues of hybrid poplar, supporting a defense role for this AP gene.

2.3 Methods

Plant material and treatments

Poplar hybrid H11–11 (*Populus trichocarpa* X *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research Program, was propagated from greenwood cuttings and grown in the Bev Glover Greenhouse of the University of Victoria as described (Major and Constabel, 2006). For wound treatment and herbivory simulation, plants were wounded along the margins to simulate herbivory. Leaves were numbered from apex basipetally using the leaf plastochron index (LPI) (Larson and Isebrands, 1971). Systemic induction by mechanical damage was performed by wounding six fully expanded leaves with pliers (LPI 9–14) directly below the designated systemic leaves (LPI 3–8). Mechanical wounding of leaf margins with pliers

was repeated two more times at 1.5 h intervals. Each leaf tissue set consisted of three adjacent leaves. Stem tissues were sampled by collecting the outer tissues (all tissue layers except the wood) from stem sections corresponding to leaf LPIs. All samples were frozen in liquid N_2 and stored at -80 ^{o}C until analysis. For nutrient manipulations, plants were grown in the greenhouse 15 cm-diameter pots containing a peat mix (Sunshine Mix #4, Sungro, Seba Beach, AB, Canada). Plants were watered every other day with 200 ml of a nutrient solution. Five nutrient treatments varied the proportions of N and Pi in solution. Treatments 1, 2, and 3 varied the amount of N ('low', 0.72 mM N; 'medium', 3.6 mM N, and 'high', 18 mM N), respectively, with a 'medium' concentration of Pi (0.32 mM). Treatments 4 and 5 had a 'low' (0.032 mM) and high (1.6 mM) concentration of Pi with the 'medium' concentration of N. All treatments had 5 mM K and 4 mM Ca in solution. N was supplied as NH_4NO_3 in treatment 1 and a mix of NH_4NO_3 and $Ca(NO_3)_2$ in the other treatments. Pi was supplied as KH_2PO_4. K was supplied as a mix of KH_2PO_4 and K_2SO_4, depending on Pi treatment. Ca^{2+} was supplied as a mix of $Ca(NO_3)_2$ and $CaCl_2$, depending on N treatment. Micronutrients were added to all treatments as 0.03 g L^{-1} chelated micronutrient mix (Plant Products Co. Ltd., Brampton ON, Canada).

Sequence analysis and digital northerns

AP genes were retrieved using keyword and BLAST searches from the P. trichocarpa genome database version 1.1 hosted at the Joint Genome Institutes (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The gene models and completeness of coding sequences were confirmed by further comparisons and BLAST searches against the TAIR and NCBI nr databases, and corrected as necessary. Sequence alignment and phylogenies were constructed using MEGA4 (Tamura et al., 2007). For

digital northern analyses, ESTs were retrieved from BLAST queries of the NCBI EST database for each AP. The retrieved ESTs were assembled into AP genes using ContigExpress in Vector NTI software and again manually verified to ensure they matched the AP transcript IDs from the poplar genome. ESTs that were sequenced multiple times were combined for the EST counts. Each gene was represented by at least four ESTs. EST counts per library were enumerated and converted to a heat map of all the libraries for ease of presentation

RNA extraction and northern analysis

RNA was extracted from hybrid poplar leaves as previously described (Haruta et al., 2001). For Northern blot analysis, total RNA (7 ug) per lane was separated on a 1% (w/v) agarose–formaldehyde gel in MOPS buffer (pH 7) (0.4 M MOPS, 100 mM NaOAc-$3H_2O$, 10 mM Na_2EDTA) and transferred by capillary blotting onto Zeta-Probe membranes (Bio-Rad, Hercules, CA) using standard protocols (Sambrook et al., 1989). RNA was fixed to a Zeta-Probe membrane (Bio-Rad) with UV using a GS Gene Linker UV chamber (Bio-Rad). Prehybridization was performed for 2 h at 42 ^{o}C in 5X NaCl/sodium phosphate/EDTA (SSPE), 50% (v/v) $HCONH_2$, 5X Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulphate, and 100 mg mL^{-1} denatured salmon sperm DNA. DNA probes were obtained by random priming (T7 Quickprime kit, Pharmacia Biotech, Piscataway, NJ) and hybridization carried out for 16–18 h. The membranes were washed twice with 5X SSPE and 1% (w/v) SDS for 15 min at room temperature, twice with 1X SSPE/1% (w/v) SDS at 65 ^{o}C for 30 min, and once with 0.1X SSPE/1% (w/v) SDS at 65 ^{o}C for 30 min. Hybridization blots were autoradiographed using a Phosphorimager system (Molecular Dynamics, Sunnyvale, CA).

Protein extraction, enzyme assays, and western blotting

Hybrid poplar tissue was powdered under liquid N_2 using a mortar and pestle, and protein was extracted (1:4, w/v) in ice-cold 50 mM $NaOA_2$ (pH 5.6), 1 mM EDTA, 1 mM DTT, and 5 mM thiourea with a pinch of sand. Extracts were centrifuged at 16,000g for 20 min at 4 ^{o}C, and the resulting supernatant was assayed for AP activity and total protein using the Bradford method using BSA as a standard.

Phosphatase activity was determined by measuring the inorganic Pi released from substrates (Drueckes et al., 1995). Acid washed 96 well microtitre plates were used for all assays, which consisted of 80 mM $NaOA_2$ (pH 5.6), 10 mM $MgCl_2$, 6 mM pNPP or ATP as the substrate, and protein extract. Assays were initiated by addition of substrate and were allowed to progress for 9 min. The reaction was terminated by the addition of developing reagent (125 ul). This reagent was prepared daily and consisted of 4 volumes freshly made 10% (w/v) ascorbic acid to 1 volume of 10 mM ammonium molybdate in 15 mM Zn-acetate (pH 5.0) solution. After addition of developing reagent, the samples were incubated for 30 min at 37 ^{o}C and the A630 read using a Sunrise microplate spectrophotometer (Tecan). To calculate activities, a standard curve in the range of 1–133 nmol Pi was constructed.

Denaturing SDS/PAGE (10% acrylamide) was performed using a Bio-Rad Mini-Protean II apparatus. Prior to running of the SDS–PAGE, samples were diluted in SDS sample buffer and incubated at 100 ^{o}C for 3 min. Gels were run at a constant voltage of 200 V. Immunoblotting was performed by transferring the proteins from SDS gels to PVDF membrane by electroblotting overnight at 30 V. Membranes were stained with Ponceau S to confirm even loading and transfers. Antibody incubations and washes were

carried out using standard procedures with antisera dilutions of 1:10,000 (PPO, Win4), or 1:5000 (soy VSP). Immunocomplexes were visualized using an alkaline phosphatase-conjugated secondary antibody (Bio-Rad) and developed colorimetrically with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

2.4 Results

Phylogenetic analysis of a wound-induced poplar AP gene

Previous transcriptomic analyses had led to the identification of a strongly wound- and herbivore-inducible gene, corresponding to the JGI protein ID705836, with high sequence similarity to acid phosphatases (Christopher et al., 2004; Major and Constabel, 2006). To confirm this annotation and to identify additional genes belonging to this family in poplar, we searched the genome databases. In total, eight putative poplar AP genes were identified and verified with available ESTs in GenBank at NCBI. The sequences were compared to the ten known Arabidopsis APs (Liu et al., 2005) in a phylogenetic analysis. We also included AP genes in GenBank from other species with functional data that could confirm AP activity of the corresponding gene product. In the resulting phylogeny, the poplar and Arabidopsis AP gene families showed a similarly wide distribution within the tree (Figure 2.1). The new poplar AP gene grouped most closely with GmACP, a soybean gene encoding a root nodule-specific protein with demonstrated AP activity and broad substrate specificity (Penheiter et al., 1997, Penheiter et al., 1998; Leelapon et al., 2004). Two additional genes in the group encode the soybean VSPs (VSPa and VSPb), proteins with AP activity (DeWald et al., 1992). Arabidopsis AtVSP1 and AtVSP2 genes also belong to this clade, and recent evidence indicates that AtVSP-2 has both AP and anti-insect activity. Since the new poplar gene clusters closely

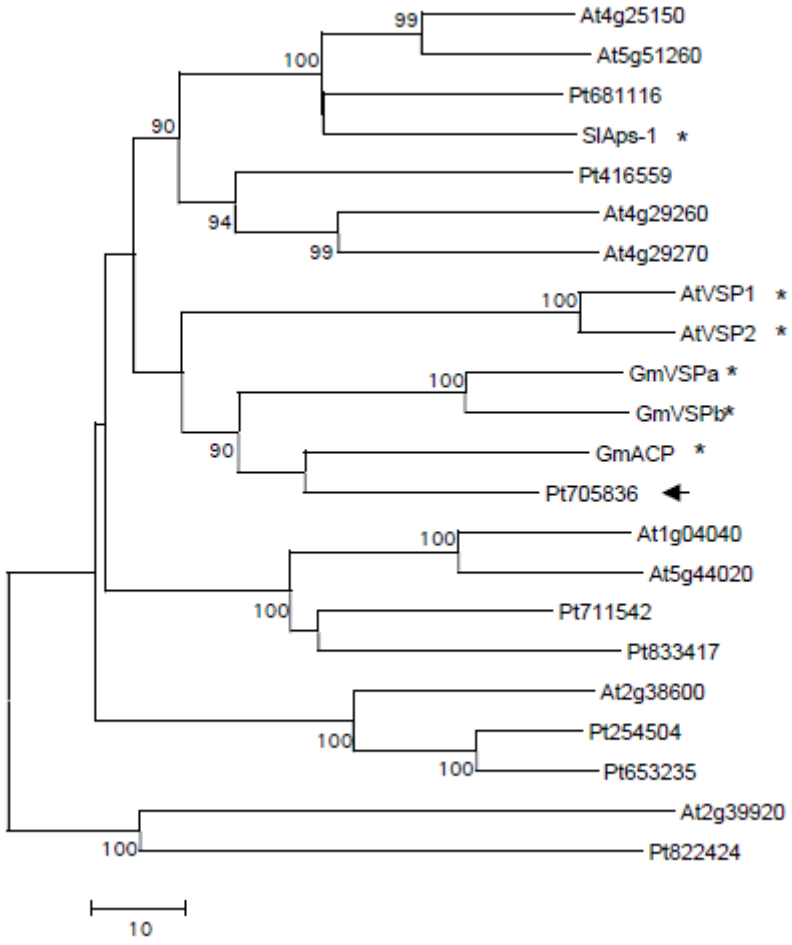


Figure 2.1: Phylogeny of Populus acid phosphatases (APs) constructed by neighbor-joining of protein distance.

AP sequences were retrieved from the P. trichocarpa genome. Numbers at branches represent bootstrap support from 2000 replicates (only values >80% are shown for clarity). Poplar APs are annotated with the prefix ‘‘Pt” and the protein ID from the P. trichocarpa v1.1 genome sequence (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). Arabidopsis APs and four functionally characterized plant APs that have sequence similarity are shown for comparison. Arabidopsis APs are indicated by AGI accessions, except AtVSP1 (At5g24780) and AtVSP2 (At5g24770). APs from other plants include SlAps-1 (Solanum lycopersicon Aps-1; NCBI accession P27061), GmVSPa (Glycine max VSPa; P15490), GmVSPb (G. max VSPb; P10743), and GmACP (G. max ACP; CAA11075). Scale bar represents the number of amino acid differences. Stars indicate genes shown to encode functional AP proteins, the arrow indicates the wound-induced gene PtdAP1.

with genes whose products have clear enzymatic activity and in some cases are linked to plant defense, it is very likely that the poplar gene also encodes a functional AP with potential to impact insect performance. We therefore named this gene PtdAP1 and undertook experiments to investigate its expression in more detail.

Constitutive and induced expression of the PtdAP1 gene

To obtain more information on the expression of this candidate poplar defense gene relative to the other poplar APs, we performed a 'digital northern' analysis using the abundance of ESTs deposited in the NCBI GenBank data base in different Populus libraries, with an approach demonstrated by Sterky et al. (2004). BLAST searches were used to identify Populus AP ESTs, which were then manually matched with the gene models identified in the JGI P. trichocarpa genome database. We compared the total numbers of ESTs found in libraries derived from different tissues, including tissues treated with one of several abiotic or biotic stress treatments. These data were used to generate a heat map to represent abundance of transcripts, roughly approximating gene expression, within that tissue (Figure 2.2). While this analysis does not easily permit direct comparisons among tissues due to differences in library size, it does allow for a semi-quantitative comparison of expression of poplar AP genes within any one tissue, i.e. within a library or set of libraries. Overall inspection of the heat map showed that PtdAP1 was represented by the largest number of ESTs, and was expressed in the greatest number of tissue types. Thus this gene showed more abundant transcripts than any other poplar AP in libraries derived from shoot tissues including leaf, stem, wood and bark, and roots. Most of the other AP genes were more restricted in expression and ESTs were detected only in a few tissue types and with typically lower inferred expression levels compared to

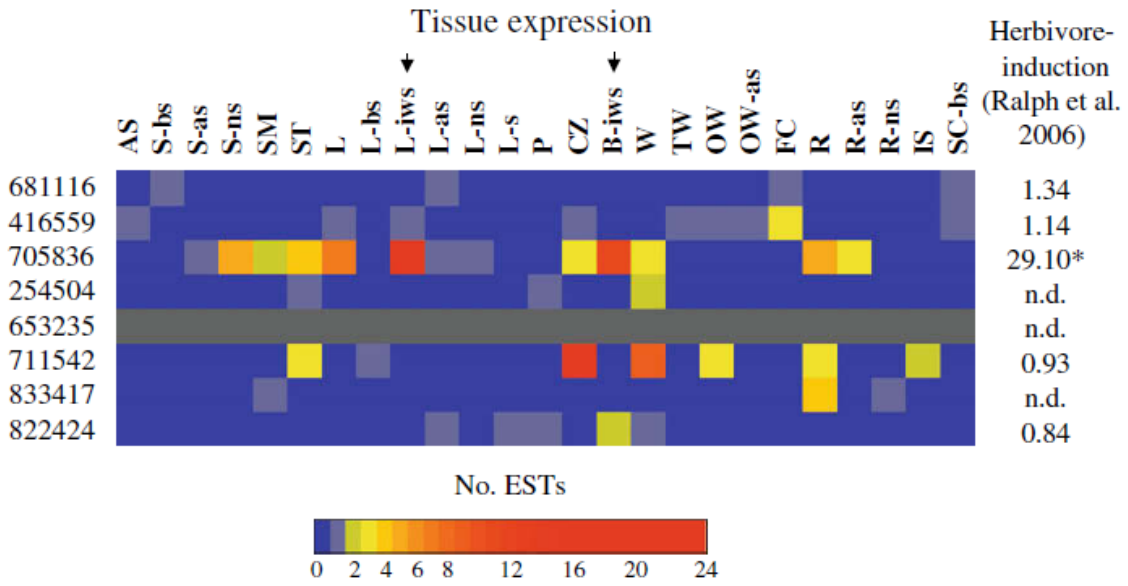


Figure 2.2: Digital northern analysis of expression data for poplar acid phosphatase (AP) transcripts.

ESTs were retrieved from BLAST queries of the NCBI EST database for each AP. The retrieved ESTs were assembled with APs (ContigExpress, Vector NTI) and manually verified correspond to the AP transcript IDs from the poplar genome. No ESTs corresponded to AP 653235. The number of ESTs corresponding to each AP (JGI protein ID) are shown. Tissue abbreviations: AS, apical shoot; S-bs, shoot – biotic stress; S-as, shoot – abiotic stress; S-ns, shoot – nutrient stress; SM, shoot meristem; ST, stem; L, leaves; L-bs, leaves – biotic stress; L-iws, leaves – insect/wound stress; L-as, leaves – abiotic stress; L-ns, leaves – nutrient stress; L-s, leaves – senescence; P, petioles; CZ, cambial zone; B-iws, bark – insect/wound stress; W, wood; TW, tension wood; OW, opposite wood; OW-as, opposite wood – abiotic stress; FC, female catkins; R, roots; R-as, roots – abiotic stress; R-ns, roots – nutrient stress; IS, imbibed seeds; SC-bs – suspension cells -biotically stressed. Biotic stresses include treatment with SA, BTH, MJ, chitosan, Pollacia radiosa elicitor, and infection with Marssonia pathogen; insect/wound stresses include mechanical wounding and herbivory by forest tent caterpillar (Malacosoma disstria) or poplar borer (Cryptorhynchus lapathi); abiotic stresses include dehydration, salt, chilling, heat, and treatment with ABA, H_2O_2, and ozone; nutrient stresses include low and high ammonium treatments and N starvation. Arrows indicate insect or wound stress treatments.

PtdAP1 (Figure 2.2). An exception to this pattern was Pt711452, which showed strong representation in the cambial zone and wood libraries, as well as root, and stem, but little expression elsewhere. Of all poplar APs, PtdAP1 showed by far the highest EST representation in the real or simulated herbivory libraries, from both leaf and bark tissues (see arrows, Figure 2.2). The low EST counts for most other AP genes in these libraries make their comparison only semi-quantitative, but the much greater abundance of PtdAP1 ESTs is most consistent with a role of this gene in induced defense. None of the other AP genes were as abundant as PtdAP1 in the herbivore-induced tissue libraries, suggesting that herbivore and biotic stress induction is not a general response of APs. The specificity of herbivore-inducibility of PtdAP1 could be further confirmed by an analysis of publicly available microarray data from Ralph et al. (2006). In this experiment, PtdAP1 was upregulated almost 30-fold by caterpillar feeding on hybrid poplar leaves, while none of the other APs on the 15,500 element cDNA array showed significant upregulation (Figure 2.2). This corroborates the digital northern analysis and our conclusion that PtdAP1 is regulated as an active herbivore defense gene.

To validate the wound-induction of PtdAP1 and obtain insight into the kinetics of induction, we carried out northern analysis and time course experiments. Poplar saplings were wounded with pliers to mimic herbivory, and leaves harvested for RNA analysis. Leaves from different positions on the saplings were harvested to determine if leaf age is important in the responsiveness. Our previous work had demonstrated that under our growth conditions, mechanical wounding with pliers induces a strong defense response in hybrid poplar leaves (Major and Constabel, 2006). These experiments demonstrate that in leaves of different ages, the gene is upregulated following wounding (Figure 2.3A). AP

transcripts began to visibly accumulate in the first 6 h following wounding, and their levels increased up to 36 h. There was no clear effect of leaf age on AP inducibility, as these genes were induced similarly regardless of position on the plant. The PtdAP1 mRNA profile parallels that seen for the enzyme polyphenol oxidase (Figure 2.3A), a marker of the poplar defense response (Constabel et al., 2000).

A hallmark of induced herbivore defense in poplar is its systemic expression, so that damage in one part of the plant also triggers defense gene activation in distant leaves (Davis et al., 1991; Constabel and Ryan, 1998; Constabel et al., 2000). In an analysis of upper unwounded leaves on plants with lower leaves that had been wounded, we found that expression of PtdAP1 in these 'systemically-wounded' leaves was also rapidly induced, again paralleling PPO gene expression (Figure 2.3B). As noted previously, leaf age or position did not affect the strength of the induction significantly. Thus, these experiments confirmed the induced AP expression by wounding, with kinetics and a systemic pattern of expression that is tightly associated with the defense response.

Wound-induced increase in AP protein and activity

If PtdAP1 is to have a role in pest defense of poplar as suggested from the phylogenetic and northern analyses, AP activity is expected to increase in leaves following wound treatments. To test this hypothesis, crude protein extracts were prepared from leaves and stem tissues and assayed for AP activity. Using pNPP, a commonly used assay substrate which functions with many phosphatases (Duff et al., 1994), we detected strong AP activity in several tissue extracts. Furthermore, in wounded leaf time course experiments, the activity in leaves was found to be clearly induced within two days, with

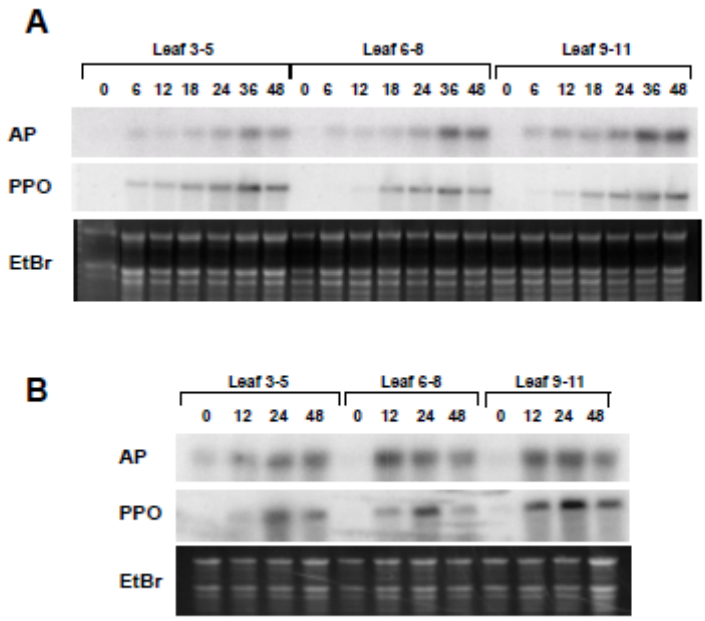


Figure 2.3: Northern analysis of wound-induced PtdAP1 gene expression.
Leaves of different LPI ranges were wounded along margins with pliers and harvested at the times indicated (h) for RNA extraction and northern analysis. Blots were probed with the PtdAP1 (AP) and PtdPPO (PPO) cDNAs. EtBr stained gel is shown as a loading control. (A) Leaves directly wounded by pliers on leaf margins. (B) Unwounded (systemic) leaves on wounded plants.

an induction of at least two-fold, and remained high until day 5 (Figure 2.4A). Greater levels of AP activity were detected in young compared to mature leaves, when normalized for total soluble protein. The kinetics of induction of AP activity and its subsequent decrease are similar to those observed for other inducible defense enzymes in hybrid poplar leaves, such as PPO and Kunitz TI (Constabel et al., 2000; Haruta et al., 2001). In stems, we also observed induction by wounding and with similar kinetics, but the overall specific activity was higher than in leaf extracts (Figure 2.4B).

To confirm that the induced AP activity is not due to enzyme activation but to an increase in protein levels, we used western blots to examine changes in levels of AP protein following wounding. We obtained an antibody raised against the soybean VSP-b (GmVSP-b; Figure 2.1) and tested it in western blots with diverse poplar protein extracts (Figure 2.5A). The antibody detected a single, wound inducible protein of the expected size for PtdAP1 (32 kDa) in all tissues tested. The close match to the predicted size and the wound-responsiveness of the immunoreactive band suggests it represents the PtdAP1 gene product. Following wound treatment, the intensity of the AP band increased an estimated 2–3-fold depending on the tissues. This is consistent with assays of AP in similar extracts, which demonstrated a wound-induction of AP activity by 2–3-fold relative to the controls (Figure 2.5B). Overall, our western analysis confirms that the induction of PtdAP1 mRNA is followed by greater AP protein levels that match changes in enzyme activity in extracts.

When ATP was provided as the phosphatase substrate in extracts of various tissues, the induction profile and pattern was very similar to that for pNPP (Figure 2.5B). This suggests that the same enzyme is able to use both substrates, although this will have

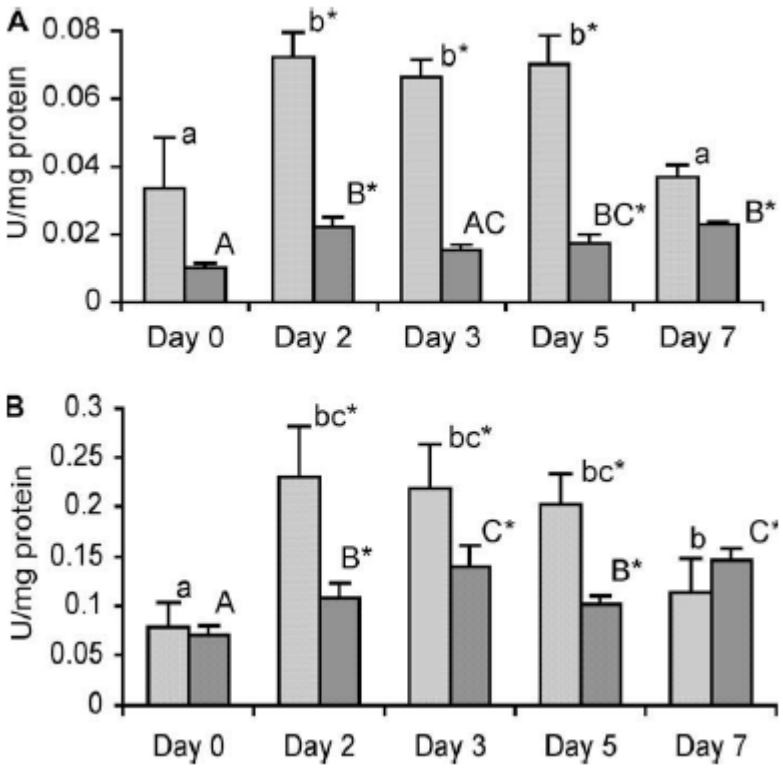


Figure 2.4: Analysis of AP enzyme activity in wounded poplar extracts.

Time course experiments were harvested on the days indicated and crude extracts assayed for AP activity using pNPP as substrate. Light bars indicate young tissues (LPI 3–5), dark bars indicate mature tissues (LPI 9–11). Both leaf (A) and stem (B) extracts were assayed. Bars represent means (+/- SE, n = 3). Points labelled by different letters indicate significant differences ($P < 0.05$) between means, with large and small case indicating values for young and mature tissues, respectively. Time points marked by asterisks indicate those means significantly different from the corresponding day 0 mean.

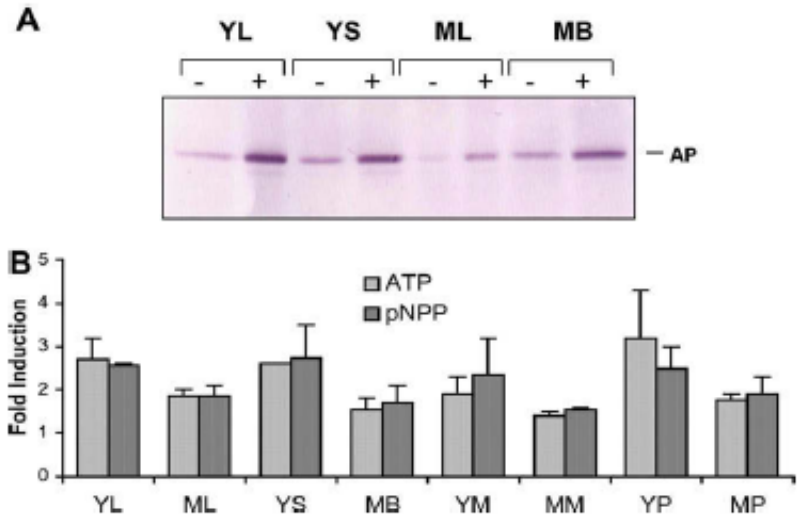


Figure 2.5: Wound-inducibility of AP protein and activity in various poplar tissues.

(A)Western blot analysis of wounded poplar plants using an antibody directed against a soybean AP (Gm VSPb) to visualize poplar AP protein. Samples were either wounded (+) or left as unwounded controls (-). Membranes were stained with Ponceau S to confirm even loading and transfers. (B) Fold-induction of AP activity in a variety of wounded poplar tissues, using both ATP and pNPP as assay substrates. YL, young leaf; ML, mature leaf; YS, young stem; MB, bark from medium stem; YM, young midvein; MM, mature midvein; YP, young petiole; MP, mature petiole. Mature and young is defined as in legend to Figure 4. Bars indicate means (+/- SE).

to be tested with either pure native or pure recombinant AP protein. In addition, we also tested a number of ions and other metabolites with our assays for their ability to activate or inhibit AP, as APs are often stimulated by the presence of divalent metal ions (Duff et al., 1994). Although most ions had a negligible effect on the AP activity in crude extracts, it was enhanced by both Ca^{2+} and Mg^{2+} (Table 1). Vanadate but not tartrate was able to inhibit AP activity effectively, consistent with many APs (Duff et al., 1994).

Impact of nutrient availability on AP protein levels

Since APs are often described in the context of P stress and nutrition, we tested if reduced or modified Pi levels in the nutrient solution would induce accumulation of AP protein. Plants were grown in peat-based planting mix, and watered every other day by hand with nutrient solutions containing limiting (low) and sufficient (med) Pi levels, both at an intermediate level of nitrogen (N). As previous studies have demonstrated a stimulatory effect of N on defense gene activation (Lou and Baldwin, 2004), we also included a high N (at medium Pi) treatment. Plants that had been grown under these nutrient regimes for at least 4 weeks were wounded, so that the nutrient limitations on wound-responsiveness could also be assessed. In addition to AP, we also monitored PPO and Win4 proteins by western blot as positive controls. The latter is an inducible protein of unknown biochemical activity with similarity to seasonally regulated vegetative storage proteins (Davis et al., 1993). All three proteins showed parallel patterns of expression under the different nutrient treatments tested (Figure 2.6) suggesting that AP follows an expression profile more similar to defense proteins rather than a nutrition-related protein. At the sufficient (med) N levels, only the low Pi treatment gave detectable expression of these proteins; while this may superficially suggest that AP is

Table 2.1
Effect of various divalent metal cations and metabolites on AP activity in crude leaf extracts of poplar. AP activity is expressed as % of control assays.

Cation/metabolite	$CaCl_2$	$CoCl_2$	KCl	$MgCl_2$	$MnCl_2$	NaCl	EDTA	Tartrate	Vanadate
Relative activity	121	94	86	132	90	80	91	100	51

[a] Shown as means of three independent replicates, all with variation <10%.

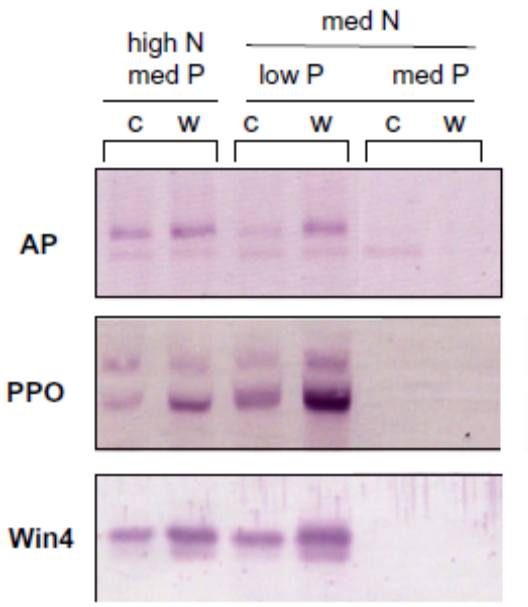


Figure 2.6: Western blot analysis of poplar defense proteins extracted from poplar leaves of plants grown under different nitrogen (N) and phosphate (P) levels as described in Section 2.4.
AP, acid phosphatase; PPO, polyphenol oxidase; Win4, poplar VSP; c, control; w, wounded leaves. Membranes were stained with Ponceau S to confirm even loading and transfers.

regulated by low Pi levels, the simultaneous presence of PPO or Win4 proteins also suggests that these nutrient treatments are affecting the leaves in other unknown ways. Therefore, we speculate that other proteins not directly connected to plant nutrition are also perturbed. However, Bradford tests indicate that these samples all contain normal levels of soluble protein (data not shown). Leaf extracts from plants grown under the same Pi (med) levels but at higher N levels showed similar protein patterns for all lanes. Thus, it appeared that it was the ratio of Pi:N rather than their absolute levels that was important. Furthermore, wounding induced the expression of AP, PPO, and Win4 in the high N/med Pi and med N/low Pi treatments relative to unwounded control leaves, as expected for herbivore defense-related proteins. N levels per se did not appear to be a key parameter for wound-induction, as inducible accumulation of all three gene products was similar at both nitrogen levels. From this experiment, we tentatively conclude that AP expression may not be specifically affected by Pi level; rather, accumulation of this protein follows PPO and Win4 defense proteins, and is thus more likely to be involved in herbivore defense. However, more experimentation will be required to clearly dissect the interaction of N and P with poplar defense genes.

2.5 Discussion

Defense-related expression of PtdAP1

The identification of PtdAP1 in several EST and genomics projects focused on herbivore defense in poplar motivated us to study this gene in more detail. We characterized its inducible expression on real and digital northern blots, and demonstrated wound-induced AP activity in poplar tissues that correlated with AP protein on western

blots. This is significant, since changes in transcript abundance do not always lead to changes in protein levels or enzymatic activity.

While we have not yet directly demonstrated that the PtdAP1 gene product has AP activity, in a phylogenetic analysis most of the APs genes with confirmed enzyme function cluster to the same clade as PtdAP1. This strongly suggests that PtdAP1 is likely to encode a functional enzyme. By contrast, the other members of the AP gene family are not highly expressed in herbivore damaged tissues, and generally have an expression profile based on EST abundance and microarray data that is very different from PtdAP1. The fact that PtdAP1 is distinct in its expression suggests it has a physiological role that is different from the other members of the AP gene family. Since the expression of PtdAP1 closely parallels the expression of other genes involved in protein-based defenses such as PPO, trypsin inhibitors and chitinases (Figure 2.3, and Patton and Constabel, unpublished data), we believe that PtdAP1 most likely also plays a defense-related role in poplar.

Earlier work had demonstrated changes in transcript levels of putative AP genes in response to herbivore stress and wounding. Our results are the first to demonstrate a wound-induced leaf AP activity, a key step towards establishing an anti-herbivore function for this enzyme. Our data also correlates the induction of AP activity with an increase in AP protein, as visualized by western analysis. Although these experiments do not prove that either the induced leaf AP activity or the visualized AP protein band is due to the induced PtdAP1 gene expression, this is the strongest candidate, given that none of the other poplar AP genes are wound-induced. We note that AP activity was induced only 2–3-fold above background in crude extracts, which contrasts with a more dramatic

induction at the transcript level. However, this modest induction is consistent with other defense genes and gene products in poplar for example, the wound-induction of trypsin inhibitors (Haruta et al., 2001; Major and Constabel, 2008).

2.6 Concluding remarks: potential functions of AP in plant defense

APs have been reported in the context of inducible defenses in only a few species, and thus their potential defensive function is unclear. The elegant work by Liu et al. (2005) demonstrating insecticidal activities against larvae of two coleopteran pest insects, as well as the dipteran Drosophila melanogaster, provides good evidence that AP can have strong biological activity. However, there are few clues as to the mechanism of action. Plant anti-insect proteins typically have one of two general modes of action: (i) destroying nutrients, i.e., acting as anti-nutrients, or (ii) direct damage to the digestive system (Zhu-Salzman et al., 2008). Trypsin inhibitors and oxidative enzymes such as either polyphenol oxidase or arginase typically act as anti-nutrients; they make dietary proteins unavailable or destroy essential amino acids, though via distinct mechanisms (Zhu-Salzman et al., 2008). Other proteins can directly attack the midgut or its peritrophic membrane, for example cysteine proteases which degrade proteins from the peritrophic membrane (Pechan et al., 2002; Mohan et al., 2006) and the anti-insect chitinases which may damage glycoproteins of the peritrophic membrane (Lawrence and Novak, 2006). Pi is a key nutrient for leaf-eating insects, potentially limiting growth, yet Pi metabolism in insect and the insect gut are poorly understood (Woods et al., 2002). In Manduca sexta, a lepidopteran, limited evidence points to either a ‘phosphate cycle’ or exchange of organic Pi between hemolymph and midgut. Phosphate metabolism is clearly

important for pest insects, and thus it is very likely that a defensive AP targets an aspect of herbivore phosphate metabolism (Zhu-Salzman et al., 2008). Interestingly, our molecular analysis of the induced herbivore defense response had also identified an apyrase (nucleoside phosphohydrolases) as a candidate herbivore defense gene (Major and Constabel, 2006). The possible involvement of other plant defense proteins targeting Pi metabolism of pests provides additional motivation to investigate the interplay of phosphate metabolism in insects in the context of plant defense.

Chapter 3: Molecular cloning and biochemical characterization of two UGT genes from Poplar

(manuscript in preparation for Phytochemistry)

3.1 Introduction

Flavonoids are polyphenolic compounds that are found in all higher plant species. They form the largest group of plant phenols that includes more than 9000 different compounds (Buer et al., 2010; Taylor and Grotewold, 2005). Flavonoids are classified by their chemical structure and include flavonols, flavones, flavanones, isoflavones, and anthocyanidins (Winkel-Shirley, 2001). Many of these compounds have various functions including UV protection, pathogen defense, and colouration of flowers (Kim et al., 2006a, Dixon and Paiva, 1995, Kimura et al., 2003). Flavonoids may contribute to induced defense responses in some plants against environmental stresses such as temperature stress, high light, UV light, pathogen attack and wounding (Treutter, 2006). Many but not all flavonoid compounds are stabilized by glycosylation.

Glycosylation is often the final step in the biosynthesis of secondary plant metabolites (Vogt, 2000; Vogt and Jones, 2000). Glycosylation involves the addition of glycan subunits to target molecules, and is important for stability, biological activity, and solubility of the metabolites (Bowles et al., 2006; Jones and Vogt, 2001). Furthermore, it is hypothesized that glycosylation may be crucial for the transport of molecules to specific compartments in a cell, such as the vacuole or cell wall (Bowles et al., 2006). Three major classes of transporters that transport flavonoids are the proton dependent transporters, the ATP-binding cassette transporters, and the multidrug and toxic compound extrusion (MATE) transporters (Bussotti et al., 1998). Recently, two MATE

transporters which transport anthocyanins and the proanthocyanidin (PA) precursor epicatechin 3'-O-glucoside into the vacuole of Medicago have been identified (Zhao and Dixon, 2009; Zhao et al., 2011). The sugars are essential for the transport of these molecules into the vacuole.

Sugars are transferred to secondary products by a class of enzymes known as glycosyltransferases (GTs). GTs are ubiquitous in all living organisms; they are found in plants, animals, fungi and bacteria (Hu and Walker, 2002; Paquette et al., 2003). GTs are categorized into numbered families based on protein sequences similarities. Currently 94 GT families are defined, as found on the CAZY website (http://www.cazy.org/GlycosylTransferases.html) (Cantarel et al., 2008). In plants, GT family 1 which contains the glycosyltransferases that utilize UDP-activated sugars as donor molecules is by far the largest (Yonekura-Sakakibar and Hanada, 2011, Vogt and Jones, 2000; Lim et al., 2003). These GTs are specifically called uridine diphosphate glycosyltransferases (UGTs) (Bowles et al., 2006). UGTs contain a consensus sequence, a PSPG box (Plant Secondary Product Glycosyltransferase motif), which is located at the C-terminal end of the protein (Li et al., 2001; Paquette et al., 2003). This motif consists of a short 44-amino acid fragment, and is highly conserved in all flavonoid-specific UGTs (Lorenc-Kukula et al., 2004; Gachon et al., 2005). It is involved in the binding of the UDP moiety of the sugar molecule to the enzyme (Offen et al., 2006, Ross et al., 2001). Using the PSPG box sequence as a search tool, more than 117 UGT genes were identified in the *Arabidopsis thaliana* genome, 187 genes in *Medicago truncatula*, and 202 in rice (Gachon et al., 2005; Kim et al., 2006a; Ko et al., 2006). Thus in plants,

UGTs exist as large multi-gene families (Li et al., 2001; Bowles, 2002; Lim et al., 2003). The availability of the poplar genome can facilitate a genome-wide search of UGT genes.

Previous microarray data in the Constabel lab had identified two UGTs which were highly upregulated in poplar leaves after infection with the fungal pathogen *Melampsora medusae* together with several other genes of PA synthesis (Miranda et al., 2007). Furthermore, in transgenic poplar plants overexpressing a MYB transcription factor (PtMYB134) that controls PA synthesis in poplar, the expression of one of these UGT genes was increased 45-fold (Mellway, 2009). This upregulation of the UGT genes correlated closely with the upregulation of all other known genes involved in the synthesis of PAs, suggesting that these UGTs might also play a role in PA biosynthesis. The use of microarray data and transcriptome co-expression analysis to identify UGT genes involved in flavonoid biosynthesis has been highly successful previously (Yonekura-Sakakibara et al., 2007; Kusano et al., 2011; Toghe et al., 2005). For example, in transgenic Arabidopsis plants expressing a different MYB transcription factor, PAP1 which regulates anthocyanin production, microarray data revealed the induction of many flavonoid genes, including two novel UGTs which were then found to perform previously uncharacterized steps in anthocyanin biosynthesis (Toghe et al., 2005). Therefore, co-expression can be a powerful tool for gene discovery in poplar.

To date over 150 flavonoid UGTs have been cloned and biochemically characterized for a variety of plant species (Vogt and Jones, 2000; Ross et al., 2001; Bowles, 2002; Lim and Bowles, 2004; Bowles et al., 2006). Here we report the first cloning and characterization of UGTs from poplar. Substrate specificity and kinetic analysis suggest putative roles for one of these UGTs in the modification of flavonols and

anthocyanidins but not in flavan-3-ols as hypothesized. In addition, these UGTs were placed in a phylogenetic context by carrying out a phylogeny of the poplar UGT family 1 sequences.

3.2 Experimental

Materials/Chemicals

All chemicals were obtained from Sigma (www.sigmaaldrich.com; Oakville, Canada) unless otherwise stated. Cyanidin and pelargonidin were obtained from Dr. Stefan Martens (Centro Ricerca e Innovazione, Department of Food Quality and Nutrition, Istituto Agrario San Michele all'Adige–IASMA, Italy). Petunidin was obtained from Jocelyn Ozga (Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada). UDP-glucose and UDP-galactose were purchased from Calbiochem (San Diego, California).

Cloning of the PtUGT1 and PtUGT2 genes from Populus

Primers designed to be complementary to full length cDNA clones of PtUGT1 and PtUGT2 genes were used to amplify DNA from a hybrid poplar cDNA library (Constabel et al., 2000). The primers contained BamHI and HindIII restriction enzyme sites to facilitate for cloning into protein expression vectors. PtUGT1 primers (PtGT1F – 5' GGGGGATCCATGTCAAGCTGCACAACAC 3'; PtGT1R – 5' GGGCTCGAGTTAAAGCTTCACCGATGTGATCTTTTC 3') and PtUGT2 primers (PtGT2F – 5' GGGGGATCCATGTCAGAAGCCAGAAAT 3'; PtGT2R – 5' GGGAAGCTTCTTGGCGACCACCTCTAA 3') yielded PCR products of 1452 bp and 1371 bp respectively. Proofreading Pfx DNA polymerase (Invitrogen) was used to

amplify the DNA. The products were then purified and digested with the restriction enzymes. The sequences were cloned into pET21a and pQE30 overexpression vectors. The final constructs were verified by DNA sequencing.

Production and purification of recombinant PtUGT1 and PtUGT2

Overexpression constructs containing the PtUGT1 and PtUGT2 coding regions were transformed into various *Escherichia coli* strains and the recombinant protein expression was assessed. A variety of conditions were tested to optimize the yield of expressed soluble protein. This included testing different *E. coli* strains with and without chaperone proteins to help in the folding of recombinant protein, using different overexpression vectors with different tags (N-terminal His-tag, C-terminal His-tag, GST-Tag), growing cultures at various temperatures (18^{o}C-37^{o}C), varying cell lysis procedures (sonication, French press, cell wall extraction reagents), and using a range of isopropyl-1-thio-B-D-galactoside (IPTG) concentrations to induce expression (0.1 mM – 2 mM). For each set of conditions, soluble protein was extracted and assayed by SDS-PAGE and western blots using a His-tag antibody. Expression of PtUGT1 and PtUGT2 in pET21a and pQE30 vectors in M15 *E. coli* cells, induction with 0.2 mM IPTG, and growth at 22^{o}C for 18 hours, yielded the most active soluble protein. These conditions were thus used for all subsequent recombinant enzyme experiments. For large scale experiments, a 25 ml culture of LB medium containing 50 ug ml $^{-1}$ ampicillin and 25 ug ml $^{-1}$ kanamycin was inoculated with the bacterial clone and grown overnight at 37 ^{o}C. These starter cultures were used to induce 500 ml cultures the following day. These 500 ml cultures were grown at 37 ^{o}C to an OD_{600} of ~0.4, the temperature was reduced to 22 ^{o}C, and IPTG was added to a final concentration of 0.2 mM. After 18 hours, the cells were

harvested by centrifugation at 4000g for 25 minutes, and pellets were stored at -80 ^{o}C until further use.

For protein purification, frozen 125 ml *E. coli* cell pellets were resuspended in 30 ml of resuspension buffer (50 mM NaH_2PO_3, 300 mM NaCl). Resuspended pellets were lysed using a French press, and the resulting solution was centrifuged at 4000g for 25 minutes at 4^{o}C. The supernatant was incubated with Ni-NTA resin (Promega) for at least 60 minutes at 4^{o}C with gentle mixing. The His-tagged PtUGT protein was eluted following a batch protocol (as described in the QIAexpressionist manual) using 250 mM imidazole in the elution buffer. Protein concentration was determined using the Bradford method with BSA as a standard. Denaturing SDS-PAGE was performed using a Bio-Rad Mini-Protean II (Bio-Rad) apparatus as described in Veljanovski et al. (2010). Immunoblotting was performed by transferring proteins from an SDS gel to PVDF membrane by electroblotting for 90 minutes at 100 volts. For His-tag detection, blots were incubated with a 1:3000 antiserum dilution of anti-His antibody (GE Healthcare Life Science, Quebec). The antigenic polypeptides were detected using a horseradish peroxidase tagged secondary antibody (Bio-Rad) and blots were developed colourimetrically with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma).

UGT enzyme assays and analysis of reaction products with high performance liquid chromatography (HPLC)

The standard reaction mixture for the UDP-glycoslytransferase enzyme assay consisted of 5 µg of affinity purified recombinant protein, 100 µM acceptor substrate, 5 mM UDP-sugar in 50 mM KPi (pH 7.4) in a reaction volume of 200 µl. Reactions were

incubated for 30 minutes at 30 ^{o}C. Reactions were stopped by the addition of 50 µl of 100% glacial acetic acid (for flavonols) or 150 µl of 5% HCl (for anthocyanins). To determine the pH optimum, the buffer systems consisted of Na-acetate (pH4.0-5.5), MES (pH5.5 – pH6.6), MOPS (pH6.5-7.9), Tris-HCl (pH7.4-9.0), or CAPS (pH9.5-11.0).

Prior to HPLC, UGT reaction products were partially purified using StrataX 33-μm solid-phase extraction columns, according to the manufacturer's instructions (Phenomenex, Torrance, CA, USA). The reaction products were eluted in 2 ml methanol:acetonitrile (50:50), and 100 μl was injected onto a reverse-phase Luna C18(2) column (250 x 60 mm, 5 μm) (Phenomenex) connected to a Beckman Coulter System Gold HPLC. The products were separated with a linear elution gradient from 90% solvent A (0.5% methanol in 0.01M phosphoric acid, v/v) to 95% solvent B (100% acetonitrile) over a 60 minute period with a flow rate of 1.5 ml min^{-1}. Reaction products were monitored at 370 nm (quercetin/other flavonoids) and 365 nm (kaempferol) on a System Gold 168 diode array detector. Products were quantified using integrated peak areas and pre-determined calibration curves for each of the substrates. For anthocyanidin reaction products, samples were centrifuged for 10 min at 13400g and the supernatant was filtered through a micro filter (Phenomenex). 100 µl of sample was injected onto the column and the products were separated with the same linear elution gradient. Anthocyanins were monitored at 520 nm.

Enzyme kinetics

To measure the reaction rates of the recombinant PtUGT1 enzyme for acceptor products, the concentration of quercetin and kaempferol was varied from 3 µM to 400

μM with 5 mM UDP-galactose as the donor substrate. For PtUGT2, 2,4,5-trichlorophenol (TCP) (3 μM – 10 mM) was used as the acceptor product, with UDP-glucose as the donor sugar. Assays were performed in microcentrifuge tubes as described above and analyzed using the Brooks kinetic package (Brooks, 1992) to determine values for Km and Vmax.

Phylogenetic analyses and molecular modelling

Poplar sequences were downloaded from the US Department of Energy (DOE) Joint Genome Institute (JGI) *Populus trichocarpa* genome version 1.1 (http://genome.jgi psf.org/Poptr1_1/Poptr1_1.home.html). Poplar UGT sequences were obtained by carrying out local BLAST searches using downloaded protein databases, using a Hidden Markov Model produced from 40 functionally characterized plant UGTs, using the REGEXP command available for MySQL with various combinations of the PSPG box motif found in all UGT sequences, and searching the JGI database with various glycosyltransferases terms as search items. All sequences obtained were manually examined, and aligned with ClustalW. Sequences were grouped into distinct groups by comparison with Arabidopsis groupings (Li et al., 2001).

Multiple alignment of flavonoid glycosyltransferase amino acid sequences was performed using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) from sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein/). Phylogenetic trees were generated using MEGA 4.0 (Tamura et al., 2007). Genetic distances were estimated using the pairwise distance amino acid substitution matrix. 1000 bootstrap replicates indicate the level of support for the data in each node.

For molecular modeling, PtUGT1 and PtUGT2 sequences were submitted to PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/; Kelley and Sternberg, 2009) for comparative modelling with solved UGT crystal structures. Pymol (http://www.pymol.org/) was used for further examination of the generated models.

3.3 Results

Identification of a UDP-galactose-flavonoid glycosyltransferase and a UDP-glucose-flavonoid glycosyltransferase from poplar

Two UGTs were previously identified as highly upregulated on microarray data of poplar tissue stressed by *M. medusae* (Miranda et al., 2007). Using the published *P. trichocarpa* genome, forward and reverse primers were designed to amplify both of these genes which we named PtUGT1 and PtUGT2. The PtUGT1 gene (1434 bp) encoded a predicted protein of 463 amino acids, with a calculated MW of 51.1 kDa, while the PtUGT2 gene (1353bp) encoded a predicted protein of 450 amino acids with a MW of 50.2 kDa. Sequence analysis and manual inspection of these UGT genes confirmed they contained the PSPG motif and thus belong to the family 1 glycosyltransferase group (Figure 3.1). Additional sequence analysis further grouped both genes within the UGT78 family of UGTs. Furthermore, phylogenic analysis placed the two sequences within group F of the UGT superfamily (Li et al., 2001; Bowles et al., 2005; Gachon et al., 2005).

To further help characterize the proteins, their amino acid sequences were aligned with other functionally characterized group F UGTs (Figure 3.1). There is a high level of sequence conservation near the C-terminal end of the proteins especially at the PSPG box. Additionally, several key amino acid residues essential for the activity of the

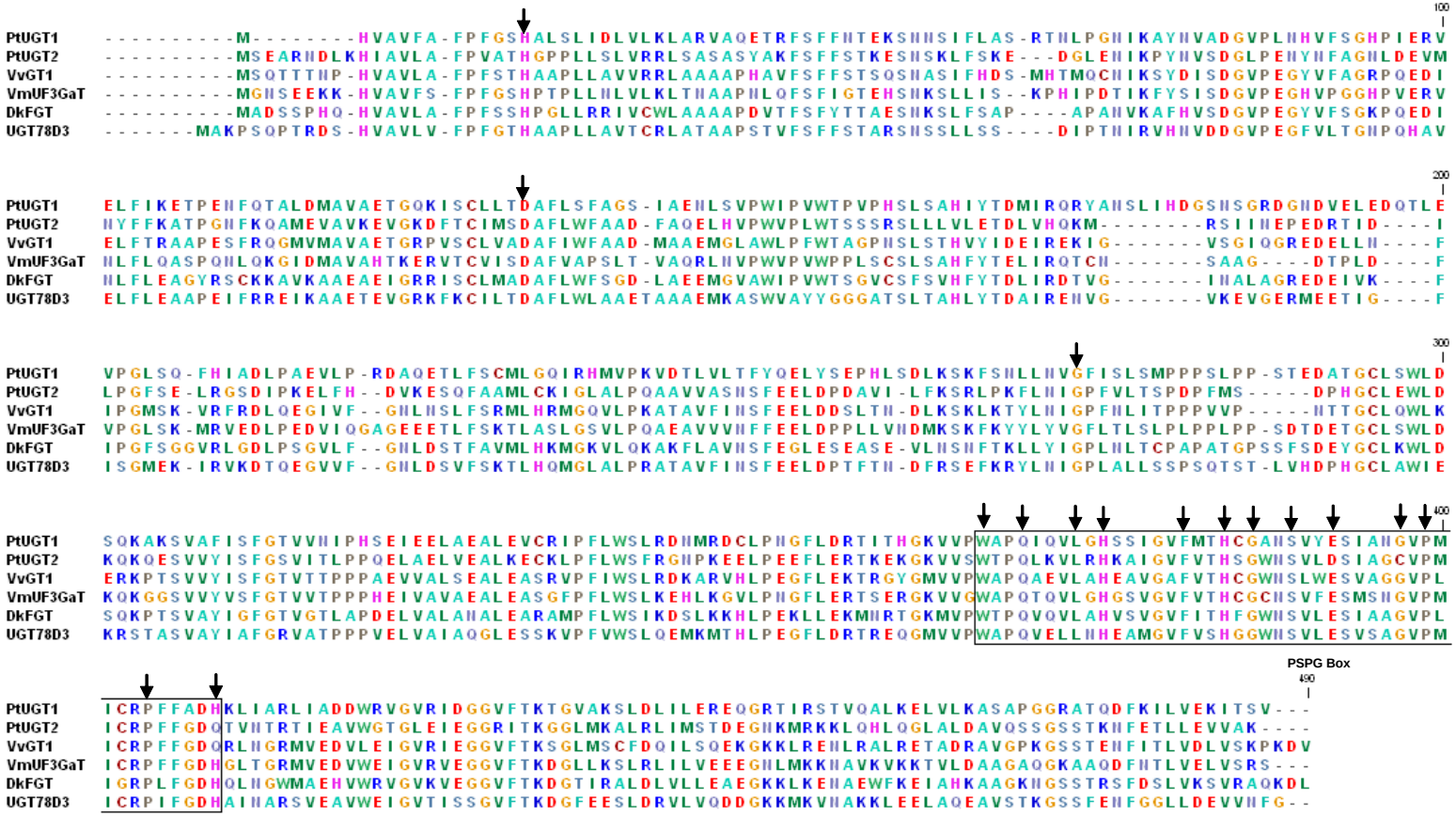


Figure 3.1: Amino acid sequence alignment of the two poplar UGT genes examined in this study with other previously characterized UGTs (belonging to group UGT78) whose catalytic activities have been experimentally proven.
The 44 amino acids boxed in black denote the consensus plant secondary product glycosyltransferase (PSPG) motif located near the C-terminus end, indicating the two poplar proteins are indeed UGTs. Arrows indicate key residues for donor and substrate binding. Dashes indicate gaps in the sequence for best alignment. Abbreviations of sequences: PtUGT1 and PtUGT2 from this study; VvGT1, UGT from *Vitis vinifera* (Ford et al, 1998); VmUF3GaT, UGT from *Vigna munga* (Mato et al, 1998); DkFGT, UGT from *Diospyros kaki* (Ikegami et al, 2009); UGT78D3, UGT from *Arabidopsis thaliana* (Jones et al, 2003).

protein and needed for proper donor and acceptor substrate binding to the active site are conserved (Wang, 2009). For example, the amino acid residues indicated with arrows in Figure 3.1 of the PSPG box have been found to interact directly with the UDP-sugar of the donor molecule (Offen et al., 2006; Osmani et al., 2008; Osmani et al., 2009). Furthermore, using point mutation to alter amino acid residues, Kubo et al. (2004) found that the last residue within the PSPG box determined sugar donor specificities of the UGT enzymes. In PtUGT1, the last residue in the PSPG box is a histidine and this amino acid residue is consistently found in UGTs that take UDP-galactose as the sugar donor. In PtUGT2, the last residue is a glutamine, and this amino acid predicts UDP-glucose to be the sugar donor.

In order to help obtain information on potential substrates of the new poplar UGTs, we constructed an unrooted phylogenetic tree of amino acid sequences that includes other biochemically characterized flavonoid-specific UGT enzymes (Figure 3.2). When placed within this phylogeny, the two poplar sequences group with UGTs that glycosylate the 3-hydroxyl group of the acceptor substrates, classifying both our poplar UGTs as 3-UGTs. Among the characterized UGTs, PtUGT1 displays greatest similarity (50%) with the UGT gene from *Vigna mungo* (VmUF3GaT) (Ishikura and Mato, 1993; Mato et al., 1998). By contrast, PtUGT2 was most similar to a UGT gene from grape (VvGT1) (Offen et al., 2006).

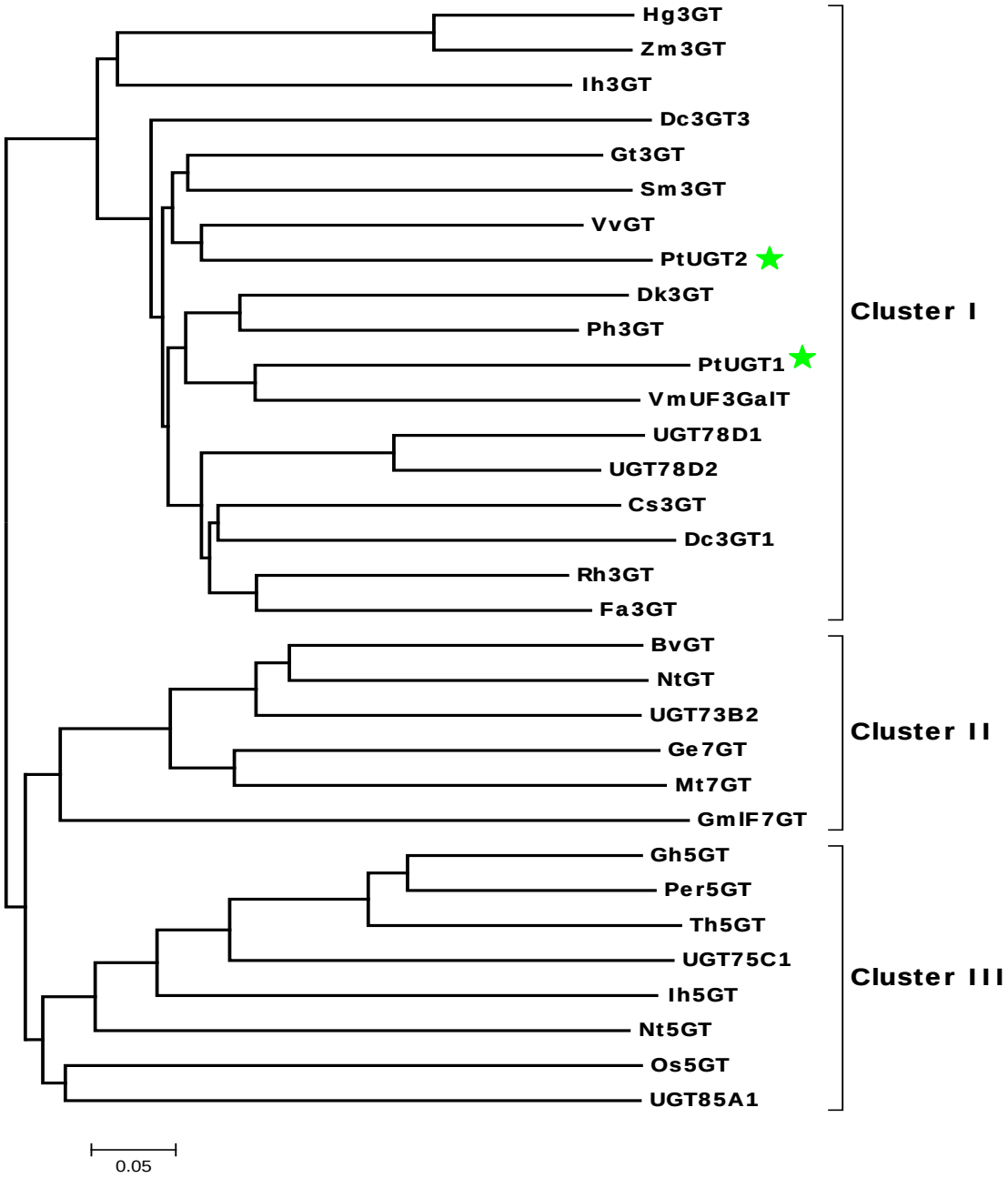


Figure 3.2: Phylogenetic relationship of poplar UGTs with various other plant UGTs.

Phylogeny of functionally characterized flavonoid glycosyltransferases and the poplar UGTs examined in this study. The enzymes are clustered according to the position at which they attach sugar residues to flavonoids: cluster I comprises UGTs that add sugars at the 3-O- positions; cluster II UGTs add sugars at the 7-O position; and cluster III UGTs add sugars at the 5- O position. Protein sequences were aligned with ClustalW and a Neighbour-joining tree was produced using MEGA software version 4.0 (Tamura et al., 2007). Abbreviations with GenBank accession numbers are on following page. Scale bar represents the number of amino acid differences. Stars indicate the PtUGT1 and PtUGT2 genes of interest.

Figure 3.2 Abbreviations:

BvGT - *Beta vulgaris* UDP-glucose:flavonoid-O-glucosyltransferase (AAS94329), Cs3GT - *Citrus sinensis* UDP-glucose-flavonoid-3-O-glucosyl transferase (AAS00612), Dc3GT1 - *Dianthus caryophyllus* UDP-glucose:flavonoid 3-O-glucosyltransferase (BAD52003), Dc3GT3 - *Dianthus caryophyllus* UDP-glucose:flavonol 3-O-glucosyltransferase (BAD52005), Dk3GT - *Diospyros kaki* flavonoid 3-O-galactosyltransferase (BAI40148), Fa3GT - *Fragaria x ananassa* Anthocyanidin 3-O-glucosyltransferase (Q66PF5), Ge7GT - *Glycyrrhiza echinata* isoflavonoid glucosyltransferase (BAC78438), Gh5GT - *Glandularia x hybrida* UDP-glucose:anthocyanin 5-O-glucosyltransferase (BAA36423), GmIF7GT - *Glycine max* UDP-glucose:isoflavone 7-O-glucosyltransferase (BAF64416), Gt3GT - *Gentiana triflora* UDP-glucose flavonoid 3-O-glucosyltransferase (Q96493), Hg3GT - *Hordeum vulgare* Anthocyanidin 3-O-glucosyltransferase (P14726), Ih3GT - *Iris x hollandica* Anthocyanidin 3-O-glucosyltransferase (BAD83701), Ih5GT - *Iris x hollandica* Anthocyanin 5-O-glucosyltransferase (BAD06874), Mt7GT - *Medicago truncatula* UDP-glucosyl transferase UGT73K1 (AAW56091), Nt5GT - *Nicotiana tabacum* UDP-glucose:salicylic acid glucosyltransferase (AAF61647), NtGT - *Nicotiana tabacum* phenylpropanoid:glucosyltransferase (AAB36652), Os7GT - *Oryza sativa* Japonica Group UDP-glycosyltransferase (BAD69345), Per5GT - *Perilla frutescens* var. *crispa* UDP-glucose:anthocyanin 5-O-glucosyltransferase (BAA36421), Ph3GT - *Petunia x hybrida* UDP-galactose:flavonol 3-O-galactosyltransferase (AAD55985), PtUGT1, PtUGT2, Rh3GT - *Rosa hybrid cultivar* UDP-glucose: flavonol 3-O-glucosyltransferase (BAE72453), Sm3GT - *Solanum melongena* (eggplant) UDP-glucose flavonoid 3-O-glucosyltransferase (Q43641), Th5GT - *Torenia hybrid* cultivar Anthocyanin 5-glucosyltransferase (BAC54093), UGT73B2 - *Arabidopsis thaliana* UDP-glucosyltransferase 73B2 (NP_567954), UGT75C1 - *Arabidopsis thaliana* Anthocyanin 5-O-glucosyltransferase (NP_193146), UGT78D1 - *Arabidopsis thaliana* Flavonol-3-O-glucoside L-rhamnosyltransferase (NP_564357), UGT78D2 - *Arabidopsis thaliana* Flavonoid 3-O-glucosyltransferase (NP_197207) UGT85A1 - *Arabidopsis thaliana* UDP-glycosyltransferase 85A1 (Q9SK82), VmUF3GaT - *Vigna mungo* Flavonoid 3-O-galactosyl transferase (BAA36972), VvGT - *Vitis vinifera* UDP glucose:flavonoid 3-o-glucosyltransferase (AAB81683), Zm3GT - *Zea mays* Anthocyanidin 3-O-glucosyltransferase (P16167).

Molecular cloning of poplar UGT genes

The full length cDNAs of PtUGT1 and PtUGT2 were cloned into a His-tag fusion expression vector and expressed in M15 *E. coli* cells. The major proportion of the protein was produced as insoluble inclusion bodies, but after optimization experiments, sufficient soluble protein could be obtained from the lysate for affinity purification using the His-tag. Purification of both recombinant UGT proteins on Ni-NTA resin resulted in a single band on a Coomassie gel (Figure 3.3a and b). The molecular weight of the expressed proteins was as expected (51 kDa for PtUGT1 and 50 kDa for PtUGT2). Western blotting was also performed using a His-tag antibody, and again, only one band of the expected size was seen for each poplar UGT (Figure 3.3c). This indicated that full length purified protein had been obtained, and was suitable for subsequent enzymatic assays.

Functional characterization of poplar UGT recombinant proteins

Substrate specificity

Recombinant PtUGT1 and PtUGT2 proteins were assayed for glycosyltransferase activity using sugar donors and acceptors as predicted by the phylogeny and primary sequence. Reaction products (glycosylated substrates) were monitored by HPLC, and appeared as new peaks in the reactions (Figure 3.4). UDP-glucose, UDP-galactose and UDP-glucoronic acid were tested as sugar donors, with twenty-six flavonoid and non-flavonoid substrates for both recombinant proteins (Table 3.1). Since the two UGT genes were upregulated under pathogen stress along with the induction of PAs, it was hypothesized that the flavan-3-ols, catechin and/or epicatechin, would be probable

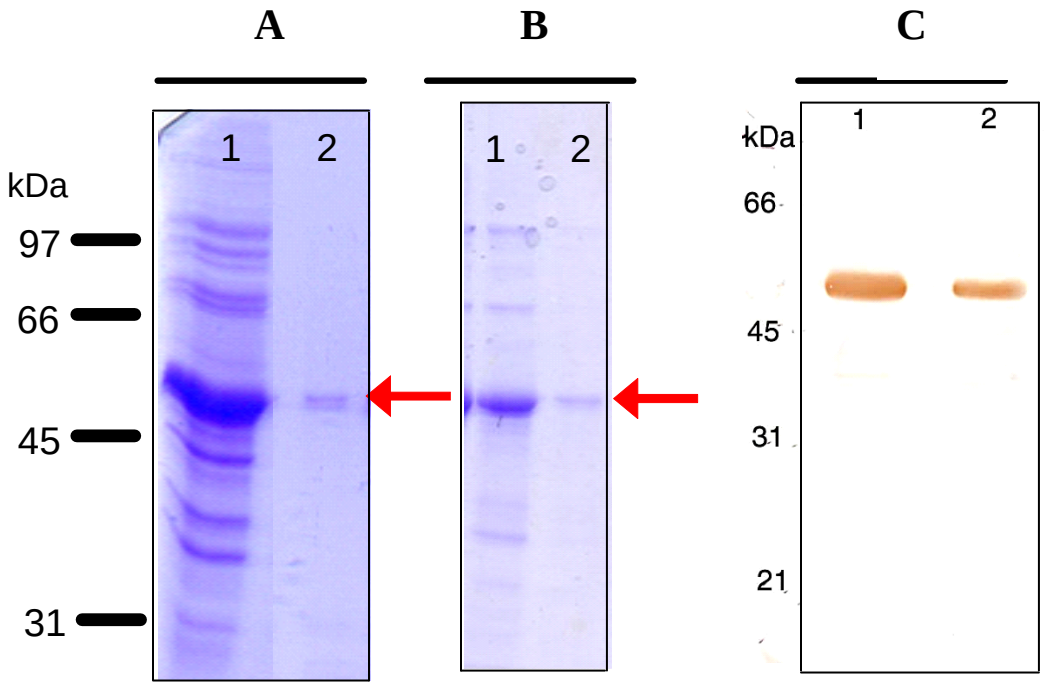


Figure 3.3: SDS-PAGE (A, B) and western blot (C) analysis of PtUGT proteins.
SDS-PAGE analysis of recombinant PtUGT1 protein (A) and PtUGT2 protein (B) to demonstrate purity. In both A and B lane 1 contains total induced protein in E. coli while lane 2 contains the PtUGT protein after purification on Ni-NTA resin that was subsequently used in all enzymatic assays. The red arrow indicated the UGT protein. Various protein Mr standards are shown (weights as marked). Western blot analysis (C) of PtUGT proteins were probed using an anti-HIS antibody. Lane 1 and 2 contain purified expressed recombinant PtUGT1 and PtUGT2 protein respectively.

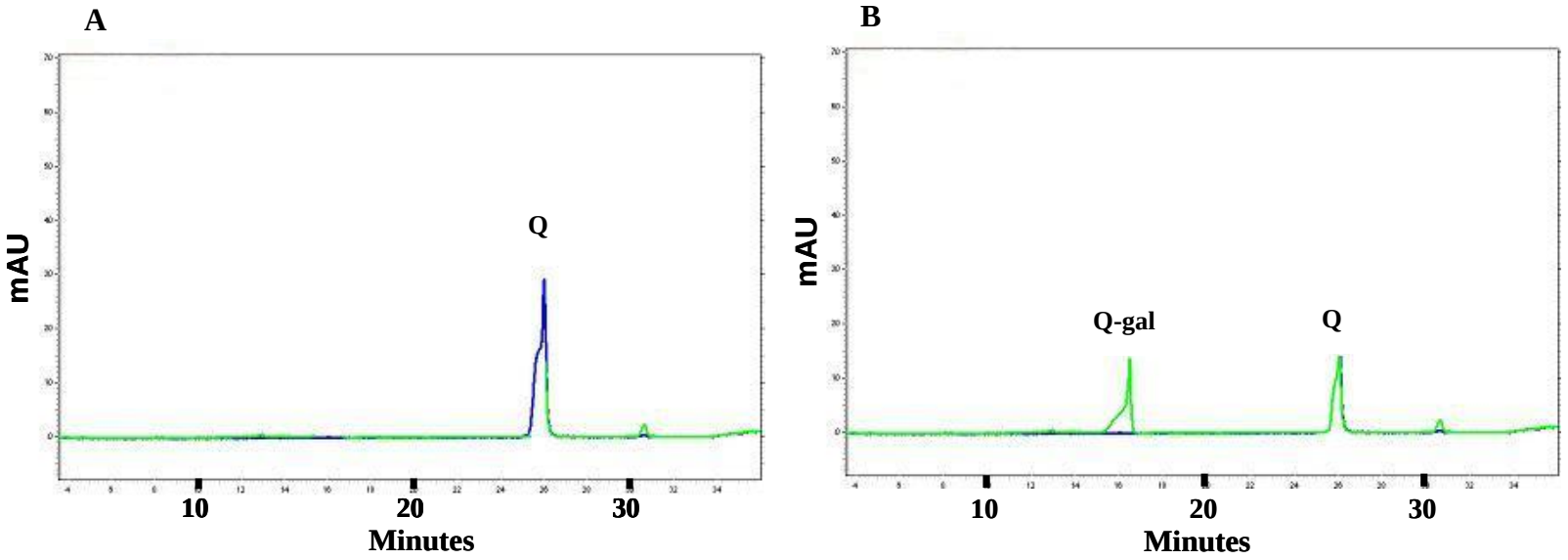


Figure 3.4: Representative HPLC elution profiles of the enzymatic assay catalyzed by recombinant UGT1 protein.
HPLC analysis of quercetin standard without the presence of recombinant UGT1 protein (The peak R_t= 26.1) (A). Reaction of UDP-galactose with quercetin in the presence of recombinant UGT1 protein (B). The new peak at R_t=16.3 is the quercetin galactoside formed by the enzyme. Q – quercetin, Q-gal – quercetin-galactoside.

Table 3.1: Phenolics and flavonoids assayed with PtUGT1 and PtUGT2 recombinant enzymes.
Check mark (✓) indicates activity occurred using that substrate, (X) indicates no activity.

Substrate tested	Activity with PtUGT1	Activity with PtUGT2
Anthocyanidins		
Cyanidin	✓	X
Malvidin chloride	X	X
Pelagonidin	X	X
Petunidin	X	X
Benzoate derivatives		
Benzoic acid	X	X
Dinitrosalicylic acid	X	X
Gallic acid	X	X
Protocatechuic acid	X	X
Salicylic acid	X	X
Salicin	X	X
Coumarins		
Esculetin	X	X
Flavonols		
Isorhamnetic	X	X
Kaempferol	✓	X
Myricetin	X	X
Morin	X	X
Quercetin	✓	X
Rutin	X	X
Flavanones		
Naringenin	X	X
Flavan-3-ols		
Catechin	X	X
Epicatechin	X	X
Hydroxycinnamic acids		
Caffeic acid	X	X
Chlorogenic acid	X	X
Ferulic acid	X	X
Sinapic acid	X	X
Other		
2,4,5 trichlorophenol (TCP)	X	✓
Tannic acid	X	X

substrates. However, only PtUGT1 had measurable activity with flavonoid substrates, and neither catechin nor epicatechin were substrates for the enzyme. PtUGT1 was only able to use the flavonols, quercetin and kaempferol, and the anthocyanidin cyanidin as substrates. Glycosylation of quercetin occurred at the 3 hydroxl position based on HPLC UV spectra analysis due to the hypsochromic shift of the UV max (Sun et al., 2007; Vogt et al., 1997; Kramer et al., 2003). The aglycone of quercetin displays a UV max at 372 nm, while glycosylated molecules can show UV shifts depending onto which residue the sugar is added. If glycosylation occurs at the 3 position, the UV max shifts to 354 nm. If the sugar is added at the 7 position, no hypsochromic shift is seen (UV max stays at 372 nm). If the 4' residue is glycosylated, a shift to 365 nm is seen. Since PtUGT1 displayed a hypsochromic shift of the UV max from 372 nm to 354 nm, it can be classified as a 3-UGT (see Appendix A). Furthermore, PtUGT1 activity was observed exclusively using UDP-galactose as the sugar donor, and neither UDP-glucose nor UDP-glucoronic acid was functional as a donor substrate. This result was consistent with the presence of histidine as the last residue of the PSPG box sequence, which predicts UDP-galactose as the sugar donor (Kubo et al., 2004).

In contrast to PtUGT1, PtUGT2 did not show activity with any of the flavonoid substrates tested. However, activity was detected with the xenobiotic TCP. UDP-glucose was the only sugar donor accepted by PtUGT2, as predicted by the presence of a glutamine residue in the last position of the PSPG box (Figure 3.1). Despite many attempts to measure enzyme activity for PtUGT2 using diverse flavonoids, no other substrates could be discovered.

Optimal pH conditions

Both enzymes displayed activity in a broad pH range, having 50% activity in buffers between pH 6.0 – 9.0. PtUGT1 activity drops quickly when the pH is shifted from its pH optimum (Figure 3.5). Maximal activity was found to occur at pH 7.8 for PtUGT1 and pH 7.9 for PtUGT2. It has been reported that when UGT enzyme assays were determined in a Tris buffered solution, the Tris inhibited the enzyme (Owens and McIntosh, 2009). However, no such reduction in activity was detected here.

Metabolite and ion effects on PtUGT1

A variety of metals were examined for their effects on the activity of PtUGT1 (Table 3.2). None of the tested compounds showed any clear activating effect on the enzyme. Cu and Co significantly reduced the relative activity of the enzyme, as has been previously seen in various other GTs (Vogt and Jones, 2000).

Kinetic analysis of the recombinant PtUGTs

The kinetic properties of PtUGT1 were determined using the flavonol acceptor substrates quercetin and kaempferol, and the donor sugar UDP-galactose. The properties for PtUGT2 were determined using TCP and UDP-glucose (Table 3.3). Michaelis-Menten kinetics were determined by varying the concentration of substrate under optimum assay conditions. The apparent Km and Vmax values were calculated from the Michaelis–Menten equation fitted to a non-linear least-squares regression kinetics program (Brooks, 1992). The PtUGT1 recombinant enzyme showed a relatively high affinity for both quercetin and kaempferol (Km = 27 uM and 24.5 uM respectively). For PtUGT2, the recombinant enzyme showed a low affinity for TCP (Km = 185 uM), which is similar to other UGTs having TCP glycosylating activity (Hall et al., 2011).

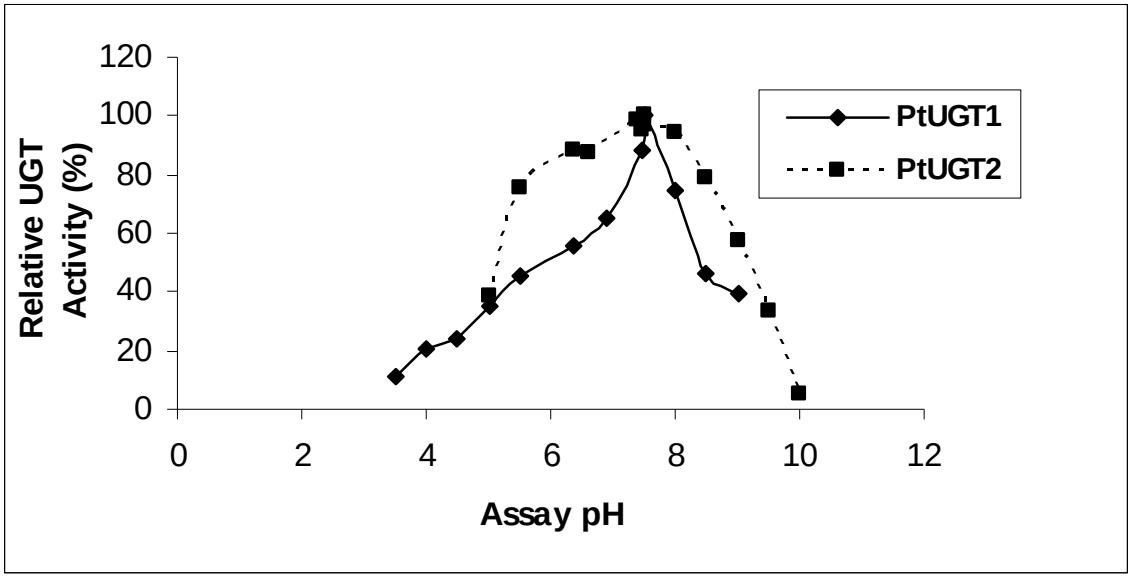


Figure 3.5: Purified recombinant PtUGT1 and PtUGT2 protein activity as a function of assay pH.
All values represent the means of n = 3 separate determinations and are reproducible to within ±10% of the mean value.

Table 3.2: Effect of various divalent metal cations on PtUGT1 activity.

Enzyme activity was assayed using UDP-galactose as the sugar donor and quercetin as the acceptor substrate. The enzyme activity is expressed relative to the control set at 100%. All values represent the means of n = 3 separate determinations.

Metal salt (5 mM)	Relative Activity (%)
KCl	90
LiCl	92
$MnCl_2$	71
$CaCl_2$	96
$MgCl_2$	88
NaCl	93
$CoCl_2$	1
$CuSO_4$	3

Table 3.3: Kinetic parameters of recombinant PtUGT1 and PtUGT2 proteins. The UDP-sugar donor was used at a concentration of 10 mM while the substrate concentration was varied. UDP-galactose was the donor for PtUGT1 and UDP-glucose for PtUGT2.

Substate	Km (uM)	Vmax (pKat/ug)
Quercetin	27	17.9
Kaempferol	24.5	26.4
TCP	185	115

Molecular modelling of poplar UGTs

The 3D crystal structure of five plant UGTs are publically available in the Protein Database (http://www.rcsb.org/pdb/home/home.do) (Osmani et al., 2009). When protein sequence alignment between sequences of interest and known models is high (above 40%), reliable predictions of the protein structure can be produced. The amino acid sequences of PtUGT1 and PtUGT2 are between 43% - 50% similar over the full length of the protein to both UGT78G1 and VvGT1, proteins whose structures have been determined. Therefore our poplar sequences were modelled using these structures with the online Phyre server. Figure 3.6 displays the ribbon diagram model of the structural alignment performed. The 3-D fold shows the conservation of the secondary structural elements between all three models. The models were further examined using Pymol software. Specifically, the size of the binding pocket was examined to potentially help elucidate some possible substrates, especially for PtUGT2. The substrate binding pocket of this enzyme was found to be slightly larger than the pocket seen in PtUGT1 (Figure 3.7). Since this may allow larger or modified flavonoids to act as substrates for PtUGT2, we hypothesized that isorhamnetin (3'-methyl-quercetin) might be a potential substrate. However, when this substrate was tested, again no glycosylation products could be detected (data not shown).

Examination of the large UGT family with in poplar

In order to place the two UGTs into a broader phylogenetic context, we used the publically available *P. trichocarpa* genome sequences to determine the total number of UGTs in poplar. Firstly, local BLAST searches of genome version 1.1 were performed

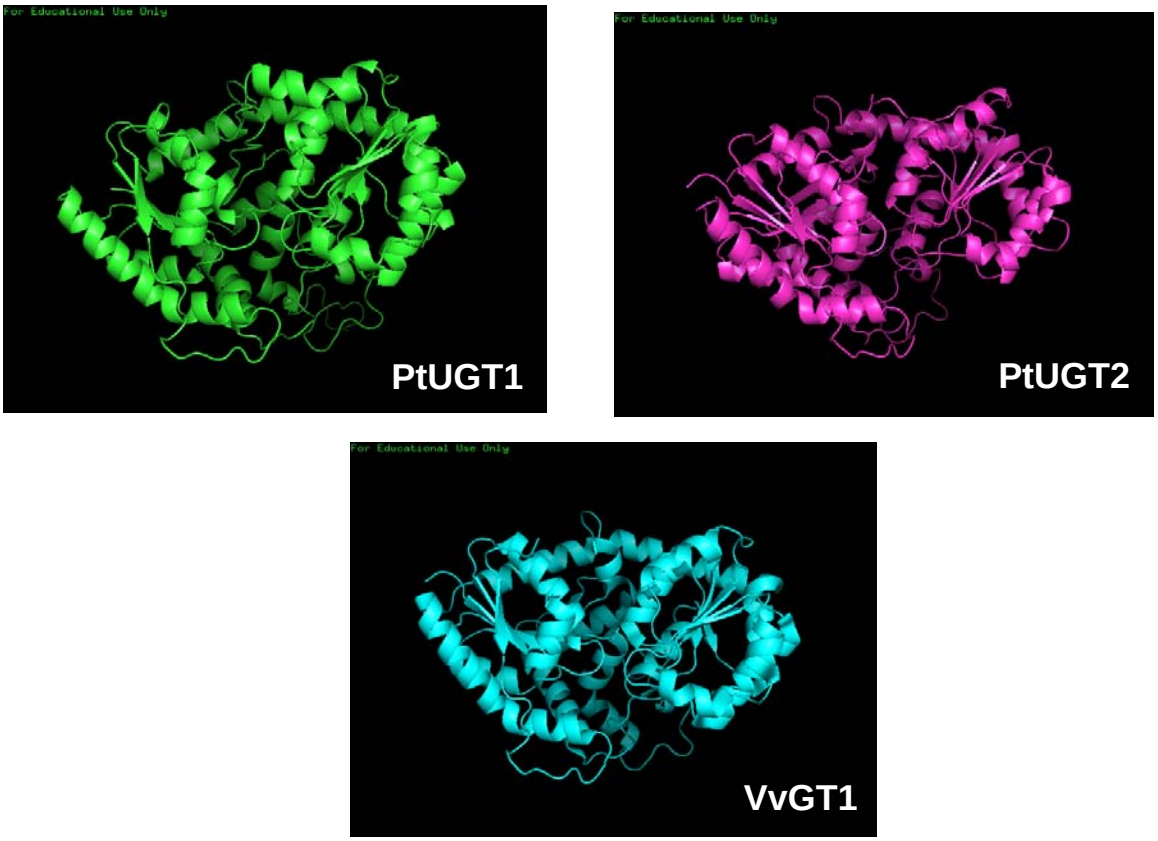


Figure 3.6: Ribbon diagram of modelled poplar UGTs structures after structural alignment with known UGTs using Phyre.
The crystal structures of the UGTs show the 3D folding elements of the secondary structure. α-helices are shown as arrows, β-strands are shown as ribbons, and connecting loops as string. VvGT1 is included as a reference model.

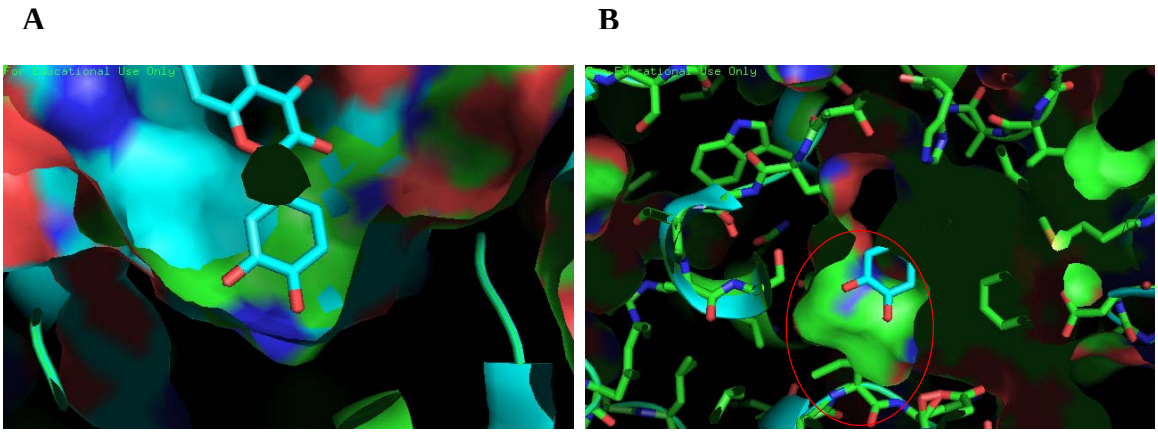


Figure 3.7: Molecular model of A) PtUGT1 and B) PtUGT2 showing the substrate binding pocket.
Using PyMol, the substrate binding pockets for PtUGT1 and PtUGT2 were modelled. The red circle on PtUGT2 emphasizes the larger binding pocket. Quercetin is depicted as the stick model in the binding pocket.

using the PtUGT1 sequence as an input sequence, and a total of 175 sequence hits were returned. To identify additional UGTs, a hidden Markov Model was produced using 40 functionally characterized UGTs as the input. The model is visualized in Figure 3.8. Using this model, of the 45555 poplar genes in the database, 172 sequences were identified that satisfied the E-value cutoff for significant hits. To identify additional UGTs in the poplar genome, the REGEXP tool was used. This algorithm is able to search for elements of the conserved PSPG box by searching the poplar genome for specific amino acids in specific orders. For example, the start of the PSPG box is a tryptophan (W) followed by two amino acids that vary, followed by a glutamine (Q). Using REGEXP, the motif WxxQ, where x signifies any amino acid, can be used to search the database. Using the PSPG box sequence and REGEXP, a variety of motifs were used to search the poplar genome (Table 3.4). The first four motifs used in the search begin at the start of the conserved PSPG-box of plant UGTs. Similar to BLAST searching and HMM modelling, 130-170 proteins were identified using these motifs. The last motif used corresponded to a region near the end of the PSPG box. Many more sequences were identified, yet not all were secondary product specific UGTs.

Overall, using all the above tools and manual verification, 171 unique UGT sequences were ultimately identified. These were named according to the system set out by the UGT Nomenclature Committee (Mackenzie et al., 1997) (see Appendix B). All identified poplar UGTs could be grouped into 14 phylogenetic clusters (A-N) based on highly supported boot strap values as defined by Bowles et al. (2005) (Figure 3.9). As stated previously, the two UGTs described in this study belong to the UGT78 family and group with phylogenetic cluster F. It had been expected that the two poplar UGTs would

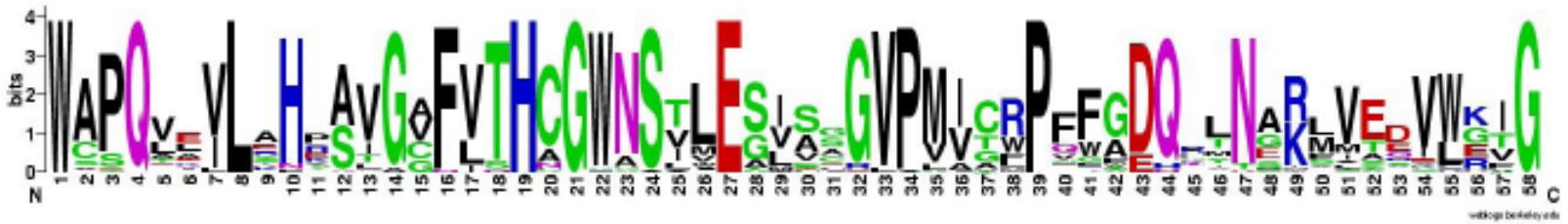


Figure 3.8: HMM model used to search the poplar genome for glycosyltransferases.
Forty previously characterized UGTs were used as models to produce the HMM model of a typical PSPG-box found in UDP-glycosyltransferases. Image was produced using the WebLogo tool found at http://weblogo.berkeley.edu/logo.cgi (Crooks et al, 2004).

Table 3.4: Sequence motif used to search the poplar genome using REGEXP.

The motifs used for searching the database are shown with '.' representing any amino acid.

Motif used for search	Number of sequence hits
'W..Q…L…….H'	170
'W..Q…L…….F..H'	150
'W..Q…L…….H.G…..E'	151
'W..Q…L…….F..H.G..S..E……..P….Q'	129
'G…..E……..P……G'	243

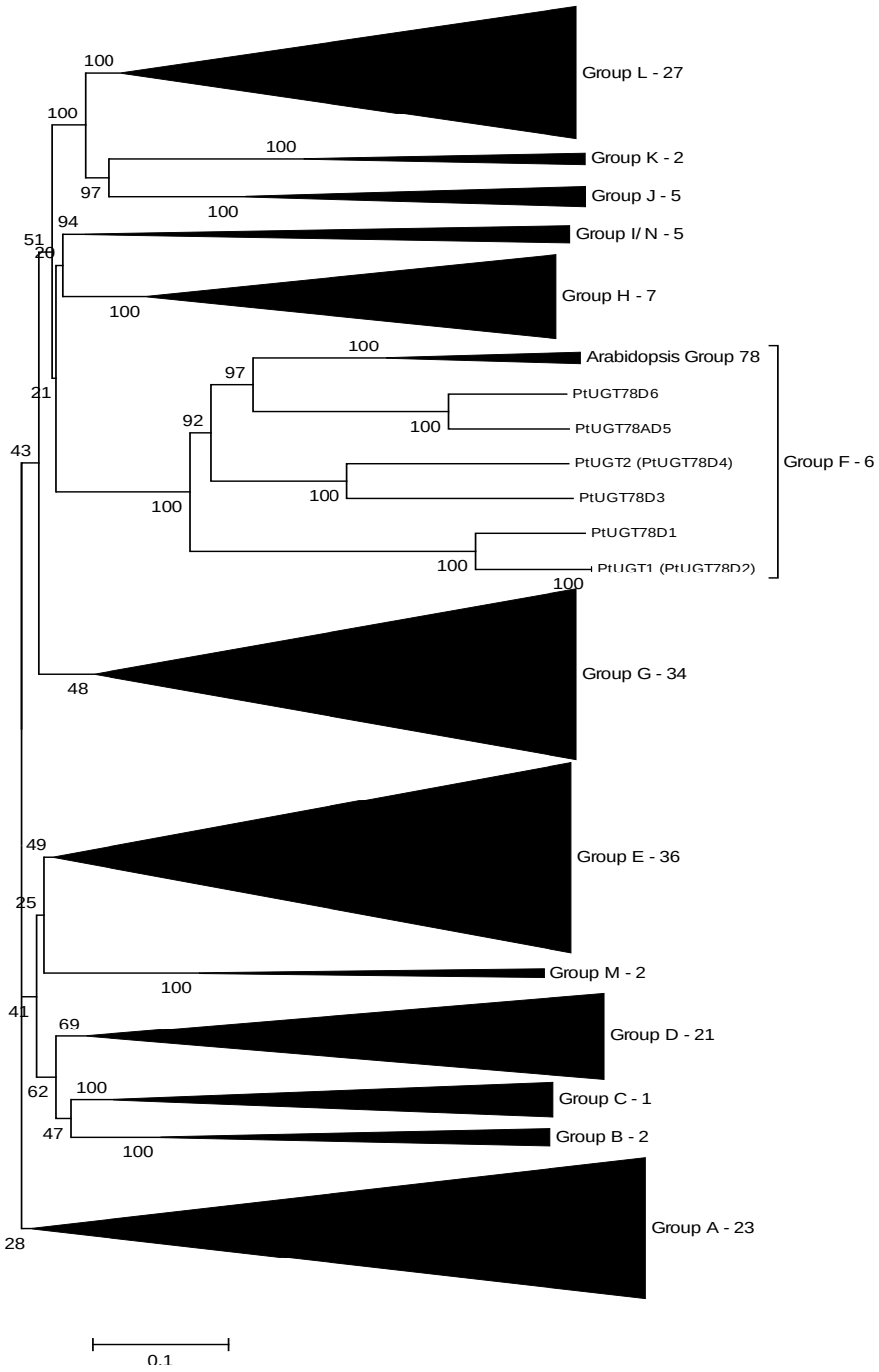


Figure 3.9: Phylogenetic analysis of UGT superfamily from Poplar.
The 171 sequences identified in our in silico analysis of the *P. trichocarpa* genome were used to construct an unrooted neighbour joining tree in MEGA. Boot strap values are indicated at the nodes. Sequences were grouped into phylogenetic clusters (A-N) as defined by Bowles et al. (2005) and total number of genes is indicated after the label. Group F is expanded to show the UGT78 family which contains the two genes examined in this study. Scale bar represents the number of amino acid differences.

group with other enzymes involved in the synthesis of PAs, such as the UGT from *Medicago truncatula* that glycosylates flavan-3-ols *in vitro* (UGT72L1; Pang et al., 2009). However, that enzyme falls into family UGT72 and further phylogenetic analysis placed it into group E, which is distinct from group F. Of the 171 unique UGT sequences in the poplar genome, five UGTs similar to UGT72L1 from Medicago and in the UGT72 family were discovered. These could be potential candidates for poplar UGTs that are directly involved in PA synthesis.

3.4 Discussion

The present work used molecular cloning and recombinant protein analysis to biochemically characterize two poplar UDP-glycosyltransferases. While they were originally hypothesized to be involved in the glycosylation of PA precursors based on co-expression with PA pathway genes, *in vitro* enzymatic analysis failed to find activity with flavan-3-ols. Rather our data point to a role for one of the UGTs in flavonol and anthocyanidin glycosylation. Furthermore, we searched the Populus genome to provide a more detailed analysis of all family 1 UGTs within poplar, and found that the two new UGT genes (PtUGT1 and PtUGT2) group with a clade of UGTs that only glycosylate at the 3-OH position.

Similarity of PtUGT1 and PtUGT2 with other flavonoid UGTs

Both poplar UGT proteins belong to glycosyltansferase family 1, as they possessed the conserved 44 amino acid PSPG motif at their C-terminal end. No signal sequences or targeting signals were identified on either of the poplar UGT sequences, suggesting they are found and function within the cytosol, which is similar to other plant

UGTs to date (Li et al., 2001). Within family 1, both poplar UGTs group into the UGT78 family. This family includes characterized UGTs from black soybean (UGT78K1; Kovinich et al., 2010), black bean (VmUF3GT; Mato et al., 1998), grape (VvGT1; Ford et al., 1998), persimmon fruit (DkFGT; Ikegami et al., 2009), petunia (F3GalT; Miller et al., 1999), and Arabidopsis (UGT78; Jones et al., 2003). All of these characterized enzymes have been found to be 3-UGTs like the poplar UGTs examined in this study. Furthermore, the recombinant UGTs from black soybean, grape and persimmon fruit are very similar in activity to PtUGT1 since they are all able to utilize flavonols and anthocyanidins as substrates. In contrast, the Arabidopsis and petunia UGTs are only able to glycosylate anthocyanidins. However, this grouping of PtUGT1 and PtUGT2 with the UGT78 family came as a surprise. We hypothesized that flavan-3-ols, which are precursors to the PAs, would be substrates for our UGTs based on previous co-expression and microarray data, and not the flavonols and/or anthocyanidins based on the phylogenetic grouping found. However, PtUGT1 did follow the phylogenetic predictions for substrate utilization as seen with the enzymatic assays, where as PtUGT2 followed neither our hypothesis nor the predictions.

Functional characterization of poplar UGTs

PtUGT1- a typical group F UGT with similar characteristics to others

Recombinant PtUGT1 was found to have catalytic activity towards both flavonols and anthocyanidins, with relatively high affinity for the flavonols quercetin and kaempferol, and no activity towards flavan-3-ols. The Km values of other biochemically

characterized UGTs using flavonoids as substrates have a wide range of values (from 0.9 – 400 uM) (Owens and McIntosh, 2009). The Km values of PtUGT1 for the two flavonols tested fall within the low end suggesting they are equally preferred as *in vitro* substrates and are likely to be the natural substrates for the enzyme *in vivo*. The pH optimum of 7.8 of PtUGT1 was similar to other group F UGTs and is well within the range of pH 7.0-8.0 seen in the majority of UGTs (Miller et al., 1999; Ogata et al., 2004; Owens and McIntosh, 2009). UDP-galactose was found to be the sugar donor and this corresponded with what was predicted through sequence analysis. Furthermore, PtUGT1 is phylogenetically most similar to a UGT from *Vigna mungo* (VmUF3GT) (Mato et al., 1998) and displays similar enzymatic activity to it; both are most active with kaempferol and quercetin and use UDP-galactose and not UDP-glucose as the sugar donor (Mato et al., 1998).

PtUGT1 is also similar to a characterized UGT from persimmon fruit. Ikegami et al (2009) used a suppression subtractive hybridization library on persimmon fruit tissue between a high PA cultivar and a low PA cultivar, and identified a flavonoid-UGT (DkFGT) in high PA containing fruit only. DkFGT belongs to the UGT78 family, similar to PtUGT1 from poplar. Enzymatic assays of the recombinant persimmon DkFGT showed similar substrate preference as seen with PtUGT1; it was able to use flavonols and anthocyanidins as substrates, and did not use the flavan-3-ols as was hypothesized. Furthermore, it used only UDP-galactose as the sugar donor, again similar to the poplar PtUGT1 enzyme (Ikegami et al., 2009). Thus, it seems that both poplar and persimmon UGTs are linked to the biosynthesis of PAs via co-expression, but that the *in vitro* activity does not reflect this.

PtUGT2 – an atypical group F UGT that does not show activity with predicted substrates

Recombinant PtUGT2, which is also found in group F UGTs, was only active against the xenobiotic TCP and not with any flavonoids tested. Being 50% similar to a grape UGT (VvGT1), it was hypothesized that similar substrates would potentially be used. VvGT1 is able to use a variety of sugar donors and flavonol substrates, none of which was seen with PtUGT2 (Offen et al., 2006). A similar finding was seen when a UGT (rVLOGT2) was cloned from Concord grape (*Vitis labrusca*). Using sequence analysis, rVLOGT2 was found to be highly similar to other anthocyanin 5-UGTs and flavonol 7-UGTs which are able to glycosylate anthocyanins and flavonols respectively. However, when the rVLOGT2 was tested, it was not able to add sugars to anthocyanins as expected, but was able to glycosylate flavonols and TCP, with TCP being the preferred substrate (Hall et al., 2011).

In order to find other potential substrates for PtUGT2, molecular models generated using Phyre and Pymol were examined. The substrate binding pocket was found to be larger in the PtUGT2 model when compared to PtUGT1 and it was speculated that a modified flavonoid on ring B could potentially be a substrate for the enzyme. The flavonol isorhamnetin contains a methyl group at position 3' of the B ring which would fit into the predicted pocket from the model. However, no activity was found with this substrate when it was used in the assay.

Since TCP was the only substrate found to be active with PtUGT2, it is hypothesized that it may have a possible role in xenobiotic detoxification. Detoxification of TCP by glycosylation with UGTs occurs in many plants. For example in Arabidopsis,

UGTs that glycosylate plant metabolites are also able to glycosylate xenobiotics with varying abilities (MeBner et al., 2003).

Our finding with PtUGT2 emphasizes the fact that using UGT amino acid similarity to determine potential functions of UGT proteins may not reveal correct substrates. Many similar UGTs have been found to have wide variety of differences in the molecules they can glycosylate. For example, in onion, two UGTs found in the same family (UGT73G1 and UGT73J1) have very different substrate specificity (Kramer et al., 2003). UGT73G1 was able to glycosylate various flavonoid substrates including flavones, flavonoid glucosides, flavanones, isoflavones and chalcones, while UGT73J1 only had activity with isoquercetrin (quercetin-3-glucoside) and genistein (an isoflavone). Hence, in order to truly determine enzyme activity, individual enzymes must be biochemically characterized.

Phylogenetic analysis of poplar UGTs identifies additional genes potentially involved in PA biosynthesis

Glycosyltransferases are found abundantly in plant species and genome analysis is a very useful tool in order to find UGTs. 171 unique UGT sequences were identified in poplar using a variety of bioinformatics tools available. This number corresponds well with what was expected based on similar values in other plant species (Li et al., 2001; Gachon et al., 2005; Kim et al., 2006a; Ko et al., 2006). Recently, a phylogeny reconstruction study was performed by Caputi et al. (2012) to help get an insight into the evolution of the UGT multigene family within plants. The authors performed BLASTP searches using the 44 amino acid PSPG motif, as well as HMMER-based sequence similarity searches to identify potential UGTs in all the genomes they tested. Using these

tools, Caputi et al. (2012) identified 178 poplar UGTs within version 2.0 of the poplar genome. This value is close to the 171 genes identified in poplar version 1.1 performed in this study. However, a previous review of plant family 1 glycosyltransferases (Yonekura-Sakakibara and Hanada, 2011) used Pfam searching of genomes, and identified 236 UGTs in version 2.0 of the poplar genome. This high estimate may be due to the inclusion of non-full length genes. For example, if a word search of "UDP + glycosyltransferase" is performed on the JGI version 1.1 database website, 256 sequences are returned, but many of the genes are not full length and/or would not encode functional proteins. It is suspected that the 236 genes identified by Yonekura-Sakakibara and Hanada may be due to the inclusion of some of these genes, or some newly annotated UGTs in version 2.0.

The 171 sequences identified here were grouped and named according to the standard nomenclature as set out by UGT Nomenclature Committee. Using this phylogeny, PtUGT1 and PtUGT2 were compared with other UGTs in poplar. PtUGT1 and PtUGT2 are found within group F and this group contains four additional uncharacterized UGT genes in the poplar genome. Since PtUGT1 and PtUGT2 have very different substrate specificities, testing of these other four proteins may provide more clues as to the activities of the enzymes within this group. It is important to determine if they have overlapping activities or if each is specific in its substrate preference.

Group E, which contains UGTs similar to the flavan-3-ol UGT (UGT72L1) found in Medicago by Pang et al. (2008), was found to contain 36 unique UGTs within poplar. Of these, five were very similar to the Medicago UGT72L1 gene. It would be interesting to characterize these genes to determine if they indeed do glycosylate flavan-3-ols.

Connection of PtUGT1 and PA synthesis?

Based on co-expression analysis of fungal infected leaves and MYB overexpressing plants, it was hypothesized that the two poplar UGTs being examined were involved in the biosynthesis of PAs. However, *in vitro* functioning of the recombinant enzymes does not support this hypothesis. In fact, only one enzyme (PtUGT1) examined was actually found to be active against flavonoid substrates. PtUGT1 was shown to be active against flavonols and anthocyanidins, and not with the PA precursors. A similar finding using co-expression to identify PA related UGTs was seen in persimmon fruits as was mentioned previously (Ikegami et al., 2009; Agaki et al., 2009). It may be possible that these UGTs are not directly involved in the biosynthesis of PAs, but are involved in a feed-back regulation of the pathway. Studies have shown that high levels of flavonols could inhibit the activity of enzymes within the PA biosynthesis pathway (Xie et al., 2004). High levels of quercetin were shown to inhibit the activity of the key enzyme of PA biosynthesis, anthocyanidin reductase (ANR), in both Medicago and Arabidopsis. Thus, PtUGT1 and the persimmon UGT may be upregulated in high tannin plants to keep the levels of flavonols such as quercetin low within the cytosol in order to keep ANR active. In order to truly be able to figure out the function of PtUGT1 within poplar, the production of transgenic plants that alter the levels of the gene to test *in vivo* functioning may help shed more light on this issue.

Chapter 4: Downregulation of a flavonoid glycosyltransferase gene in transgenic poplar leads to decreases in the levels of proanthocyanidins

4.1 Introduction

Plants are able to synthesize a variety of secondary metabolites including alkaloids, isoprenes, glucosinolates, and phenolics (Croteau et al., 2000). One of the most intensely studied groups are the flavonoids; polyphenolic compounds found in all plant species. The core biosynthetic pathway of flavonoid synthesis is well understood, but those reactions which modify the basic structure and give rise to the tremendous diversity of flavonoids still need to be characterized. Over 9,000 different flavonoid compounds have been identified to date (Taylor and Grotewold, 2005). Although believed not to be required for normal plant growth, flavonoids have many functions that help with plant survival (Dixon and Pavia, 1995). Several functions of flavonoids are rooted in their ability to absorb light over a wide range of the light spectrum (Winkel-Shirley, 1996, Stafford, 1991). They have been implicated as 'sunscreens' for plants by absorbing UV-B light (Middleton and Teramura, 1993; Stapleton, 1992). Flavonoids also function as visual cues for animals. Anthocyanins, the most familiar group of flavonoids, absorb in the visible wavelength of light and are responsible for the bright colours seen in flower petals and fruits (Koes et al., 1994). Flavonoids have also been implicated in plant defense, including the isoflavonoids and the proanthocyanidins (PAs). PAs, also called condensed tannins, are toxic to many pathogenic fungi and can be both toxic and anti-nutritive to herbivores (Levin, 1976; Bauce et al., 2005; Barbehenn and Constabel, 2011).

PAs are high molecular weight polymers of flavan-3,4-diols and flavan-3-ols that are water soluble and have been found in many woody plant species (Barbehenn and Constabel, 2011). A unique characteristic of PAs is their ability to bind and form strong complexes with proteins and other macromolecules. As a defense against mammalian herbivores, PAs bind and inactivate the digestive enzymes, thus reducing the amount of available protein needed for growth. PAs appear to be directly toxic to insect herbivores through the production of high levels of reactive oxygen species, which can lead to lesions within the digestive tract (Barbehenn and Constabel, 2011). PAs are also known to have broad anti-microbial properties, which could be important in defense against pathogens, although to date this has little direct evidence.

Previous research has shown that wounding, insect attack, and light stress leads to the induction of tannin synthesis in poplar (Peters and Constabel, 2002; Miranda et al., 2007; Mellway and Constabel, 2009), as well as in some other tree species (Bryant et al., 1993; Lavola, 1998). More recently, when a MYB transcription factor believed to be the "master switch" for the PA pathway was overexpressed in transgenic poplar, the leaf tissue showed nearly a 40-fold increase in the levels of PAs (Mellway et al., 2009). Furthermore, northern analysis and microarray data of this tissue revealed that all other known PA synthesis genes (DFR, ANS, ANR, LAR) were also highly up-regulated. One gene, a poplar flavonoid glycosyltransferases (PtUGT1), was upregulated 45-fold in these plants; this co-regulation suggested a role for PtUGT1 in the PA pathway.

In the previous chapter, the cloning of the PtUGT1 gene was described and the *in vitro* analysis of recombinant PtUGT1 protein shown. Originally, it was hypothesized that the enzyme is directly involved in the production of PAs and glycosylates

monomeric PA precursor substrates (catechin and/or epicatechin), yet *in vitro* analysis did not corroborate this. PtUGT1 showed activity only with quercetin, kaempferol, and cyanidin, but not with catechin or epicatechin. However, we decided to further test the function of PtUGT1 *in planta* using transgenic RNA interference (RNAi) plants to downregulate gene expression and reduce levels of the UGT enzyme. Previous work in strawberry using RNAi to downregulate a UGT gene led to a significant reduction of the corresponding enzyme activity (Griesser et al., 2008; Schwab et al., 2011). The work in this chapter describes RNAi-mediated suppression of the PtUGT1 gene within poplar and its effects on flavonoid metabolites. Furthermore, expression analysis and phytochemical profiling of the PtUGT1 gene was performed in wild-type tissues to further help determine the function of this gene.

4.2 Materials and Methods

Plant material and chemicals

All chemicals used were obtained from Sigma (www.sigmaaldrich.com; Oakville, Canada) unless otherwise stated. Kanamycin, MS media, and phytoagar were obtained from Caisson Labs (www.caissonlabs.com; Utah, USA). Poplar clone *Populus tremula x alba* INRA 717-1-B4 was originally obtained from David Ellis (CellFor, Victoria, BC, Canada) and maintained in *in vitro* cultures in the Constabel lab. *P. trichocarpa* x *deltoides* clone H11-11 is maintained and propagated via greenwood stem cuttings.

Construction of the PtUGT1 suppression plasmid

An RNAi construct was generated to downregulate the expression of the PtUGT1 gene in poplar using the pKannibal vector (Helliwell and Waterhouse, 2003). For

construction of the plasmid, a 460 bp fragment from the 5' end of the PtUGT1 gene was amplified from an aspen cDNA library using two different sets of primers to facilitate cloning in the sense and antisense direction of the vector to form the characteristic hairpin loop. For cloning into the sense direction, the gene sequence was amplified using the primers 5'-GGGCTCGAGGAAAGATGGAGAGAGATTGAGTC-3' and 5'-GGGGGGTACCAGAGAAAACATGATTCAGCGGCAC-3' with XhoI and KpnI restriction sites (underlined) to introduce the sequence into the 5' end of the pyruvate orthophosphate dikinase (PDK) intron intermediate of the vector. For cloning into the antisense direction, the gene sequence was amplified using the primers 5'-GGGGGATCCGAAAGATGGAGAGAGATTGAGTC-3' and 5'-GGGAAGCTTAGAGAAAACATGATTCAGCGGCAC-3' with BamH1 and HindIII restriction sites (underlined) for introduction into the 3' end of the PDK region. Each fragment was cloned sequentially into the pKannibal vector. The complete vector containing both sense and anti-sense sequences was digested with NotI and cloned into a NotI digested pART27 binary vector yielding the PtUGT1-Kannibal-pART27 plasmid (Figure 4.1).

Transformation Protocol

Poplar genotype *Populus tremula* x *alba* clone INRA 717-1-B4 was transformed using *Agrobacterium tumefaciens* C58 (pMP90) cells, following a modified protocol from Ma et al. (2004). The binary plasmid (PtUGT1-Kannibal-pART27) was electroporated into pMP90 cells and the bacteria were incubated overnight in LB with rifampicin, gentamicin and spectinomycin. The next morning, cultures were spun down at 3500 rpm for 35 minutes and cell pellets were resuspended in induction media (MS

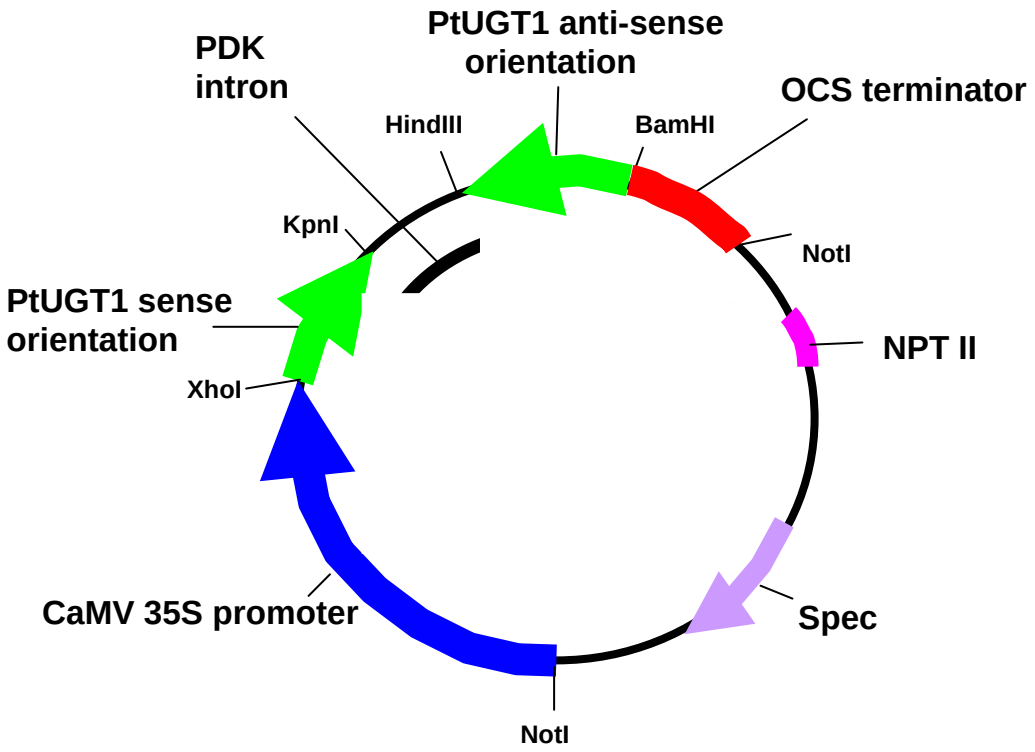


Figure 4.1: Vector map of the plant transformation vector PtUGT1-Kannibal-pART27 used to downregulate the PtUGT1 gene within poplar.
The RNAI pKannibal vector containing a sense and antisense 460bp gene fragment from PtUGT1 was ligated into pART27. The vector contains a Cauliflower Mosaic Virus promoter (CaMV 35S) and an octopine synthase (OCS) terminator. The neomycin phosphotransferase II (NPTII) gene and spectinomycin (Spec) allow for proper selection of transgenics.

with vitamins, 1.28 mM MES, 10 mM galactose and 50 μM acetosyringone) to an OD600 of approximately 0.5 AU. The bacterial cells were allowed to continue growing on a shaking incubator until the OD600 reached 0.6. Explant material (leaf pieces, internodal stem segments, and petioles) was excised from sterile tissue culture plantlets, wounded lightly with fine cuts with a scalpel, and co-cultured with the Agrobacterium suspension for 1 hour. Plant material was then blotted dry on sterile filter paper to remove excess bacteria and placed on solid MS media (5% (wt/vol) agar) supplemented with 5 μM N^6-(2-isopenteny1)adenine (2iP), and 10 μM α–naphthalene acetic acid (NAA) (MS+2iP+NAA). Explants were incubated in the dark for 2 days, after which they were transferred to new MS+2iP+NAA media containing cefotaxime sodium salt (250 mg/l), carbenicillin disodium salt (500 mg/l) and kanamycin (50 mg/l) to kill the agrobacterium, and incubated in the dark for 3 weeks. Emerging callus tissue was transferred to new media (MS media supplemented with 0.2 μM thiodiazurone (TDZ), 100 mg/l kanamycin, 250 mg/l cefotaxime and 500 mg/l carbenicillin) and placed in light to induce shoot formation. Once shoots began to appear, they were excised and placed on root inducing media (RIM: ½ MS media with 0.5 μM indole-3-butyric acid (IBA) and 25 mg/l kanamycin). Plants were continuously maintained on RIM. Multiple independently transformed lines were selected for based on their ability to grow on media containing kanamycin and were further confirmed via PCR analysis. Transgenic control plants were transformed with the empty pART27 binary plasmid.

RNA extraction, expression profiling and qPCR analysis

Total RNA was extracted from leaves using the CTAB method as described previously (Haruta et al., 2001). To remove genomic DNA, total RNA preparations (5

μg) were treated with amplification grade DNAse I (Invitrogen) according to the manufacturer's recommendations and precipitated with isopropanol. The RNA preparations were then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). For first strand cDNA synthesis, DNAse-treated RNA (1 μg), oligo dT_{20} primer and Superscript II reverse transcriptase (Invitrogen) was used according the manufacturer's recommendations. Absence of genomic DNA was confirmed by PCR analysis on the corresponding no reverse-transcriptase controls. Real-time quantitative PCR analysis was performed using the Mx3005P QPCR system (Stratagene) as described previously (Zifkin et al., 2011). Reactions were prepared using the QuantiTect SYBR Green PCR master mix (Qiagen) with gene-specific primers (667 nM) (Forward Primer: 5'-GCACAGTCCAAGCCCTTAAA-3' and Reverse Primer 5'-GTTACACCGATGTGATCTTTTCC-3') and cDNA template (10 ng) in a final reaction volume of 15 μl. Primers were designed using Primer3 software available online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky, 2000)). All reactions were run for an initial denaturation step at 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 30 s, 55^{o}C for 30 s and 72^{o}C for 30 s. Gene-specific amplification products were confirmed for each set of primers by a melt curve analysis (95°C for 1 min, 58-95°C for 30 s in one degree increments and 95°C for 30 s), which yielded a single peak at a temperature between 76-81°C. Gene-specific amplification products were also confirmed using agarose gel electrophoresis. For each primer set, multiple PCR products were separated on a 1.5% agarose gel, purified and two products were subjected to DNA sequencing. PCR primer efficiencies were calculated using the LinReg PCR program as described by Ruijter et al. (2009). Standard curves were

generated from the initial linear phase of each amplification plot. Each curve was manually inspected and outliers with an r^2 value of less than 0.980 were excluded from the efficiency calculation. All primer sets were determined to amplify with an efficiency of approximately 100%. Therefore, the relative transcript abundance was calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001) and normalized using both ubiquitin (AUBQ (forward primer 5'- TGTACTCTTTTGAAGTTGGTGT -3', reverse primer 5'- TCCAATGGAACGGCCATTAA -3'); Mohamed el al., 2010) and elongation factor (EPSF (forward primer 5'-GGCATTAAGTTTTGTCGGTCTGT-3', reverse primer 5'-GCGGTTCATCATTTCATCTGG-3'); Coleman et al., 2008) mRNA transcripts.

Plant growth conditions and stress treatments

For tissue survey profiling, hybrid poplar clone H11-11 was maintained under a 16h photoperiod at the University of Victoria Bev Glover Greenhouse, as described previously (Major and Constabel 2006). Two month old, greenhouse-grown trees were used for all experiments and leaves were designated according to the Larson and Isebrands (1971) leaf plastochron index (LPI) numbering system. The index leaf (LPI 0) was defined as the first developed leaf which had a lamina length > 20 mm. Subsequent leaves were numbered basipetally thereafter. Leaves collected for all experiments had their midveins removed and samples were frozen directly in liquid nitrogen and stored at -80^{o}C. For tissue and organ collection, apical and young leaves refer to the apical meristem and LPI 0 to 4. Mature leaves refer to LPI 9 to 14, and petioles were harvested from these leaves. Bark and wood were collected by separating the green tissues from the wood. Roots were collected from root tips. Dormant buds were collected from outdoor-

grown *P. trichocarpa* clone Nisqually-1 at the University of Victoria Centre for Forest Biology compound. Catkins and immature fruit were collected from wild *P. trichocarpa* located on campus at the University of Victoria.

Prior to phenotypic analysis, young *in vitro* grown plants were transferred to soil and allowed to acclimate to lower humidity in a mist chamber. After 3 weeks, young plants were potted in soil containing fertilizer and grown under a 16 hour photoperiod as described previously (Major and Constabel, 2006). For outdoor "light stress" experiments, potted 2 month-old greenhouse-grown transgenic and control trees were placed outdoors at the University of Victoria Centre for Forest Biology Compound and after 9 days mature leaves (LPI 9-14) were collected. Leaves collected for all experiments had their midveins removed, were frozen directly in liquid nitrogen, and stored at -80^{o}C.

HPLC analysis

For HPLC analysis, approximately 50 mg of frozen leaf tissue was ground in liquid nitrogen and extracted overnight in 10 mL of 80% methanol on an orbital shaker. The next morning, extracts were centrifuged at 4000g for 5 minutes to remove solid leaf tissue. The methanol extract was cleaned using a Strata-X 33 um solid-phase extraction column, according to the manufacturer's instructions (Phenomenex). The extracted compounds were eluted in 2 ml of methanol:acetonitrile (50:50, v/v) and 100 µl was injected onto a reverse-phase Luna C18(s) column (250 x 60 mm, 5 um) (Phenomenex) connected to a Beckman Coulter System Gold 126 Solvent Module with a System Gold 168 diode array detector. The compounds were separated with a linear elution gradient from 90% solvent A (0.5% methanol in 0.01 M phosphoric acid, v/v) to 95% solvent B (100% acetonitrile) over 60 minute period with a flow rate of 1.5 ml min^{-1}.

Proanthocyanidin assay

Proanthocyanidins were assayed using the acid-butanol assay, as described by Porter et al. (1986). Methonalic plant extracts (0.5 ml) were added to 2 ml of butanol-HCl reagent (95:5 v/v butanol: concentrated HCl). To this, 66.75 µl of iron reagent (2% w/v $NH_4Fe(SO_4)_2$ in 2N HCl) was added and the solution was vortexed. 1 ml of solution was removed prior to heating and used as the blank. The remaining solution was heated at 95^{o}C for 40 minutes. The solution was allowed to cool to room temperature for approximately 30 minutes before reading its absorbance at 550 nm. Total PAs were calculated by subtracting the unheated blank sample OD from the heated sample OD. Purified trembling aspen PAs were provided by Lynn Yip (University of Victoria, Constabel lab) and used as a standard.

Phenolic assay

Total phenolics were assayed using a modified Folin-Ciocalteau method, previously described by Singleton and Rossi (1965). 20 µl of the 80% methanol extracted supernatant from the PA assay was added to 100 µl of Folin-Ciocalteau reagent. The solution was mixed by gentle flicking. To this, 500 µl of 20% of Na_2CO_3 was added, followed by 380 µl of H_2O. The solution was vortexed and incubated at room temperature for 20 minutes, and the absorbance was read at 735 nm. Tannic acid and gallic acid were used as standards.

Anthocyanin assay

Approximately 50 mg of plant tissue was ground and anthocyanins were extracted in 1.5 ml of 0.1% HCl in methanol. The samples were left shaking overnight on a

rotationally shaker at room temperature. The next morning, samples were centrifuged at 13000 rpm for 10 minutes. The supernatants were recovered and anthocyanin concentration was determined by absorbance measurements at 530 and 657 nm using a spectrophotometer. The concentration of anthocyanins was calculated using the formula: Concentration (anthocyanins) = $Abs_{530} - 0.25\ Abs_{657}$; to compensate for chlorophyll absorption at 530 nm according to Mancinelli (1990).

4.3 Results

Expression analysis of PtUGT1 gene within poplar

Since little is known about the expression pattern of PtUGT1 in various tissues of poplar, we conducted *in silico* analysis using EST databases and online expression profiling programs. Using PopulusDB, an online searchable database for EST libraries, and using the poplar gene models for PtUGT1, only three ESTs were identified; one in each of bark, male catkins, and root tissues. Further online searching was performed using the Affymetrix gene chip-based PopGenIE cDNA eFP browser to view the PtUGT1 expression profile in *P. trichocarpa* (www.popgenie.org/tool/cdna-efp-browser). Expression of the PtUGT1 gene was again seen to be limited as it was only expressed in root meristem and in leaf tissue (leaves 8 and 10). Other online expression experiments using wood, senescence, and various other stress series (cold stress, drought, ozone) tissues showed no expression of PtUGT1. Manual searching in microarray datasets performed in the Campbell lab (University of Toronto) on various drought-stressed tissue also detected no expression of the PtUGT1 gene. Therefore, these abiotic stresses appeared not to stimulate expression of PtUGT1.

To obtain more detailed expression profiles of the PtUGT1 gene, a tissue survey in poplar was conducted using RT-PCR and quantitative RT-PCR. Total RNA was extracted from a series of organs and tissue samples harvested from greenhouse-grown hybrid poplar saplings and trees. Overall, expression of the PtUGT1 gene was seen in all tissues tested, with highest levels of transcript in young apical leaves, female catkins, buds and fruit tissue (Figure 4.2a,b). This data only partially correlated with the expression pattern found using online databases, but could be due to using different Populus species. Surprisingly, higher levels of gene expression was detected in the UVIC female catkin samples in the RT-PCR and qPCR data, unlike the online databases which showed expression in male but not female catkins.

Phenolic chemical profiles of poplar tissues

The same tissues from the gene expression survey were analysed for total phenolics, PAs, and anthocyanins using standard spectrophotometric assays. Total phenolic levels were high within all tissues tested, with highest levels found in the bud tissue and the lowest levels in the stem periderm and fruit (Figure 4.3a). Leaves and catkins also contained high levels of phenolics. By contrast, anthocyanins were found in a limited number of tissues (Figure 4.3b). High levels were found in the male catkins (likely due to their deep red colour), as well as in the bud tissue. Anthocyanins were also found in lower levels in the fruit, female catkins, root and apical leaf tissues. Similar to anthocyanins, PAs were also found in a limited number of tissues. By content, PAs were most abundant in female catkins, followed by male catkins. Root tissue was also found to contain moderate levels of PAs, as did bud and fruit tissue. Leaves, petioles and bark tissue showed low but detectable level of PAs (Figure 4.3c).

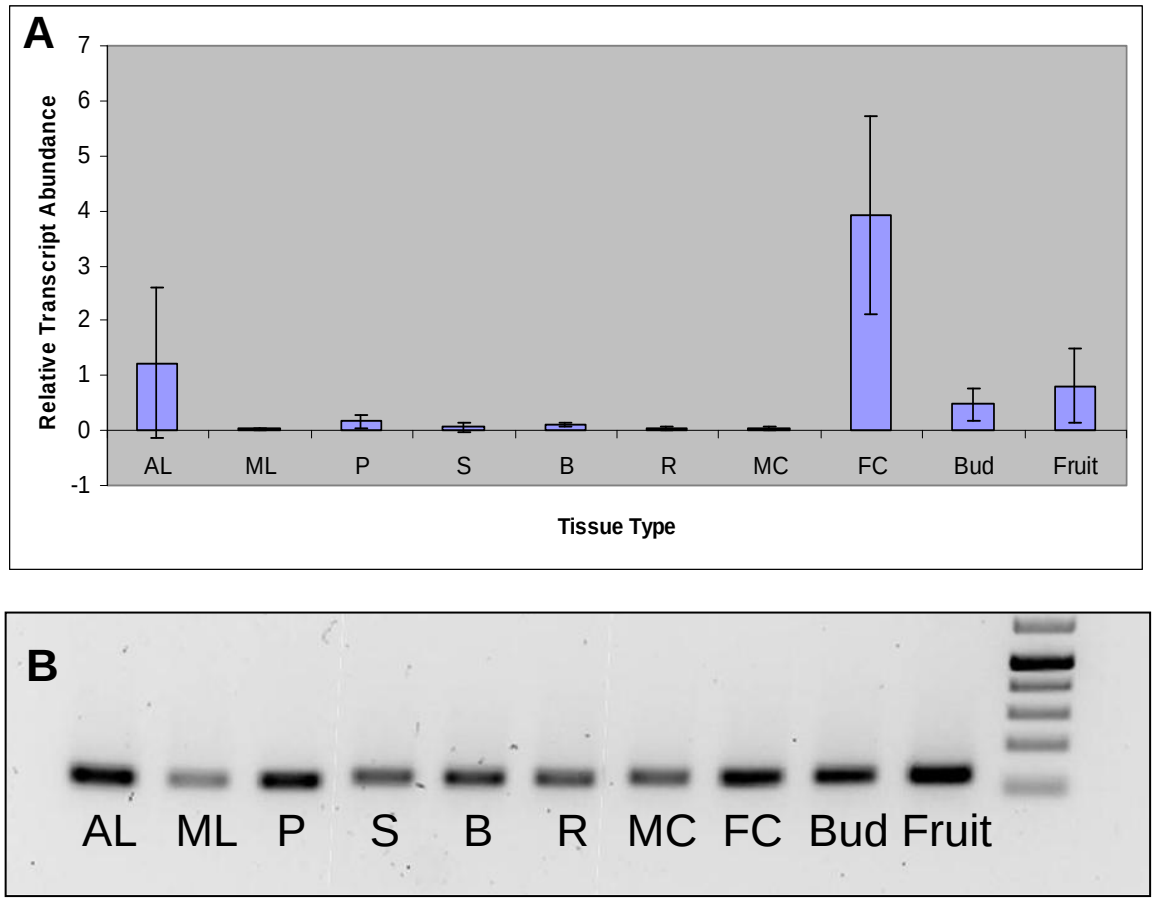


Figure 4.2: Expression profiling of PtUGT1 in various plant tissues.
A) Relative transcript abundance of *PtUGT1* measured using real-time quantitative PCR for various organs and tissues collected from wild *P. trichocarpa* (fruit, male and female catkins and outdoor buds) and greenhouse-grown *P. trichocarpa* x *P. deltoides* (clone H11-11). Expression levels relative to ubiquitin is shown. Data represents similar results from two biological replicates. All values represent the means of n = 3 separate samples. B) RT-PCR agarose gel showing expression of PtUGT1 in tissues and organs from poplar. Tissue was obtained from greenhouse grown hybrid poplar H11-11 (AL- apical leaves, ML- mature leaves, P- petiole, S- stem, B- bark, R- root) and from outdoor grown *P. trichocarpa* (MC – male catkin, FC – female catkin, B- dormant bud, Fruit – immature fruit).

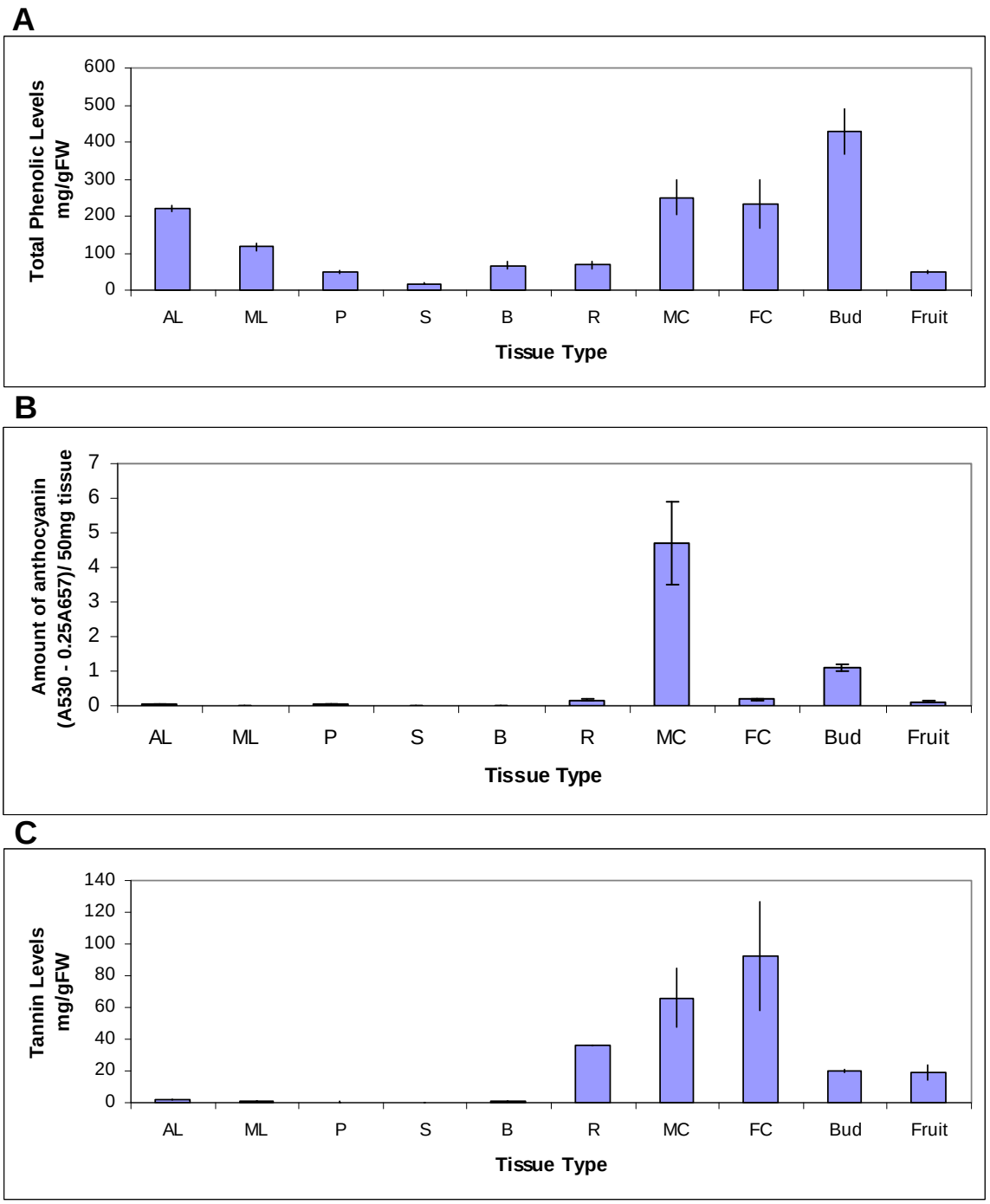


Figure 4.3: Phytochemical profile of various plant tissues from greenhouse grown and outdoor grown poplars.

A) Total phenolic levels determined using a modified Folin-Ciocalteau method. B) Total anthocyanins levels found in the tissues using a specrorphotometic assay. C) Proanthocyanidin levels as determined using the butanol-HCl method. Sample labels are AL- apical leaves, ML- mature leaves, P- petiole, S- stem, B- bark, R- root) and from outdoor grown P. trichocarpa (MC – male catkin, FC – female catkin, B- dormant bud, Fruit – immature fruit). All values represent the means of n = 3 separate samples.

Expressing the RNAi-PtUGT1 vector within poplar

To downregulate the PtUGT1 gene in poplar, an RNAi vector for *Agrobacterium* mediated plant transformations was constructed (Wesley et al., 2001; Wesley et al., 2003; Helliwell and Waterhouse, 2003). Multiple independently transformed lines of poplar (*P. tremula x alba* clone INRA 717-1-B4) were generated and moved into the greenhouse. To determine the expression level of the PtUGT1 gene within the knockdown poplars, real time quantitative PCR analysis was performed on mature leaf tissue RNA. Over forty independent transgenic lines were screened from which four lines (LKP 4, LKP 14, LKP 15, and LKP 17) with a pronounced reduction of the PtUGT1 transcript could be identified (Figure 4.4). Thus, the target of the RNAi construct was effectively downregulated in several of the transgenic lines. These four lines were multiplied and propagated for further analysis. Plants were grown and maintained under standard greenhouse conditions to identify any visible phenotypes, yet no physical phenotype was seen in any transgenics grown within the greenhouse (Figure 4.5a,b). The transgenic plants also had similar growth rates to that of non-transgenic control plants (no significant difference was found) (Figure 4.5c).

Outdoor light stress causes changes in the phytochemical composition of the knockdown plants

Levels of the PtUGT1 gene are normally found at low levels in greenhouse growing poplars. However, it was previously observed that moving plants out of the greenhouse into natural sunlight significantly increases the level of PtUGT1 within poplars (Mellway, 2009). Furthermore, high light intensity and/or UV light is also known to increase the levels of PAs in poplars. To confirm this, potted control poplar

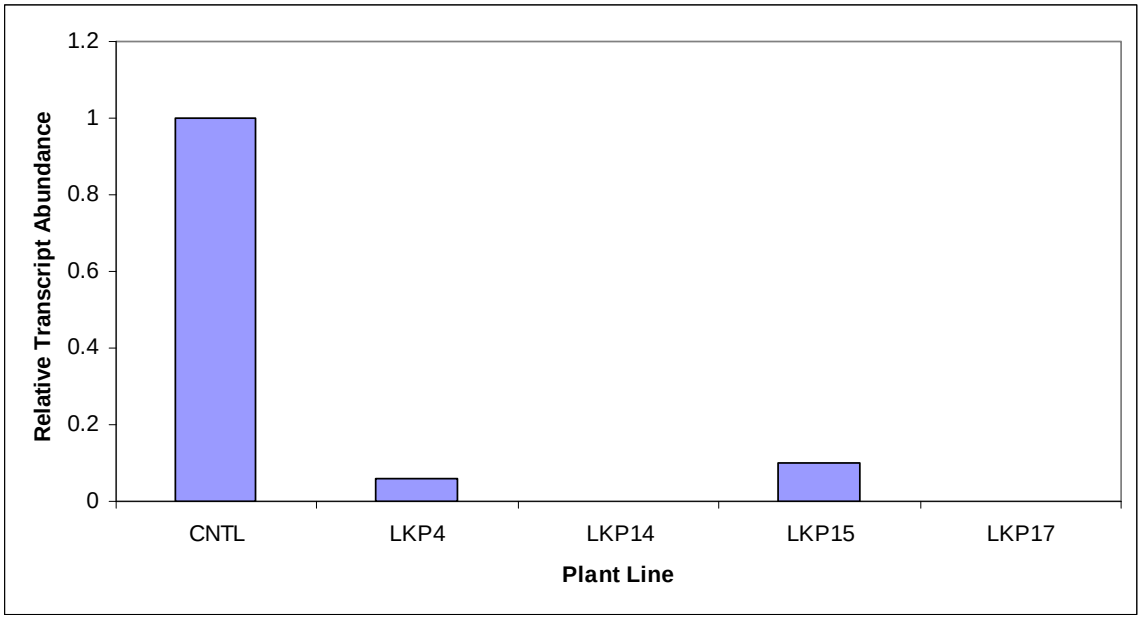
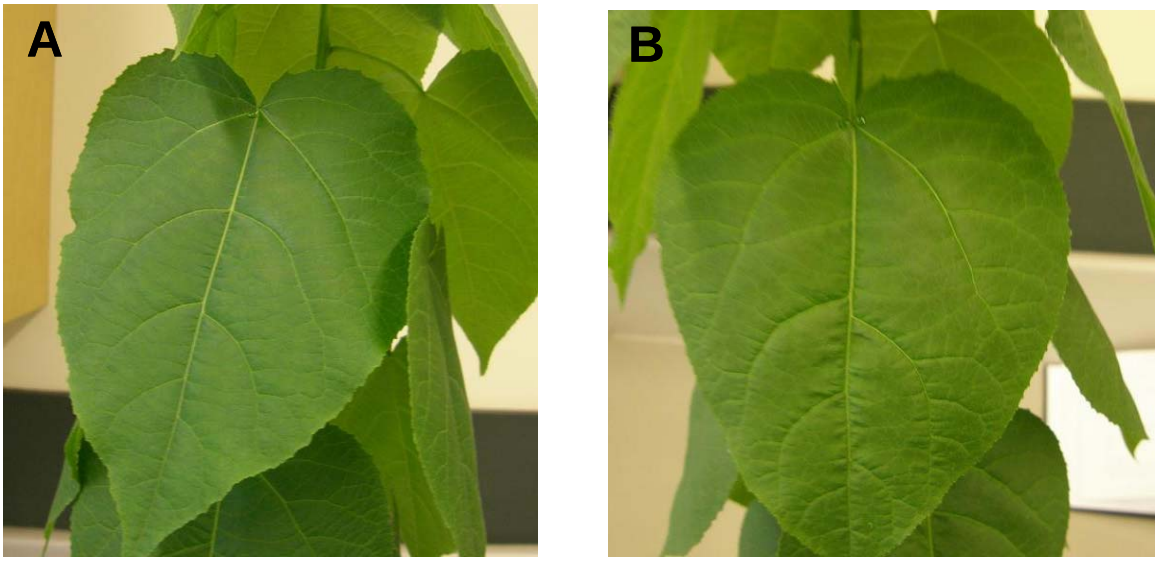


Figure 4.4: Real-time quantitative PCR analysis of *PtUGT1* in RNAi knockdown lines (LKP) following 9 days of UV stress.
Relative transcript abundance compared to ubiquitin is shown. The control value represents an average of four biological replicates (CNTL, relative expression set to 1). For the RNAi knockdown lines (LKP4, LKP14, LKP15, LKP17) at least three biological replicates were measured.



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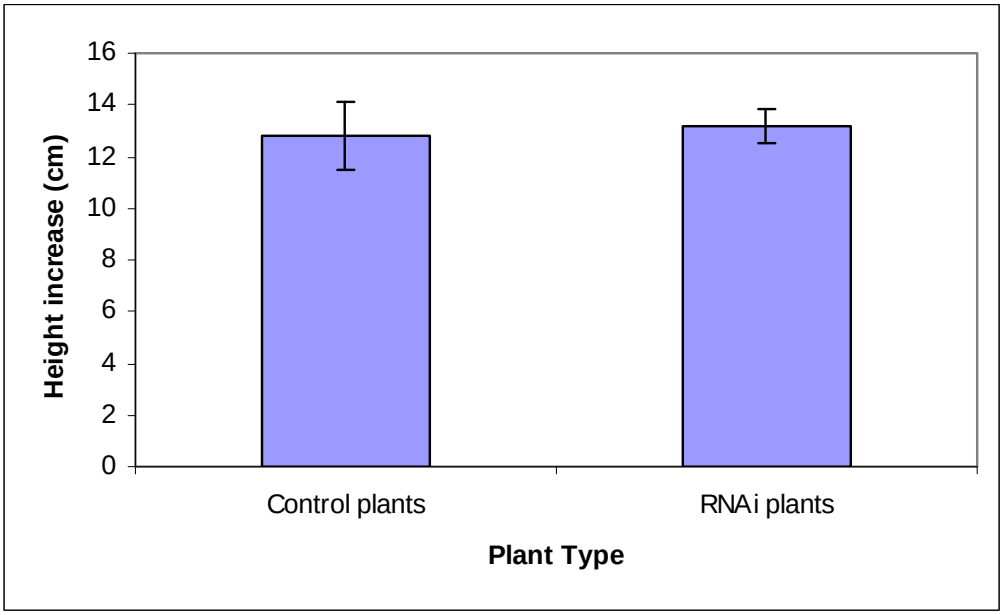

Figure 4.5: No visible phenotypic difference was seen in PtUGT1 knockdown poplars compared to control poplars.
A) Photograph of a typical control plant leaf. B) Photograph of a typical PtUGT1 suppressor plant leaf. Leaves were photographed on 2 month old trees that were grown in the greenhouse under normal conditions. C) No significant difference was seen in the height increase between control plants and knockdown plants grown in the greenhouse after a 10 day period. n=9.

plants were moved from greenhouse conditions to outdoor conditions in full sun at the University of Victoria Forest Biology Compound. After 9 days of high light induction, PA levels showed a 3 fold increase (Figure 4.6) and the levels of the PtUGT1 gene were also increased (data not shown). This indicated that the high outdoor light stress was activating the PA pathway as expected (Mellway et al., 2009). We hypothesized that a potential UGT-RNAi related phenotype would become more apparent under these conditions. Thus, PtUGT1-RNAi and empty vector transformed control plants were subjected to outdoor natural light for 9 days and the levels of phytochemicals (phenolics, anthocyanins and PAs) were examined (Figure 4.7).

Total phenolics in the RNAi lines were not significantly different from control plants; Student's t-test and Tukey-ANOVA test found no variation between the samples (Figure 4.7a). When levels of anthocyanins were examined however, RNAi lines LKP 4, LKP 14, and LKP 15 had statistically significantly lower levels of anthocyanins (Tukey-ANOVA test, $p<0.05$; Student's t test, $p<0.05$) compared to controls (Figure 4.7b). Visual inspection of LKP 17 plants showed an apparent lower level, but the high level of variation seen in the replicates led to a lack of significance.

PA levels in three of the four RNAi poplar lines contained significantly lower levels of PAs when compared to control plants (Figure 4.7c). Lines LKP 14, LKP 15 and LKP 17 contained approximately 50-60% lower levels of PAs (Tukey-ANOVA test: LKP 14-CNTL $p=0.03$, LKP 15-CNTL $p=0.03$, LKP 17-CNTL $p=0.01$; Student's t test: LKP 14 and LKP 15 $p<0.05$, LKP 17 $p<0.01$).

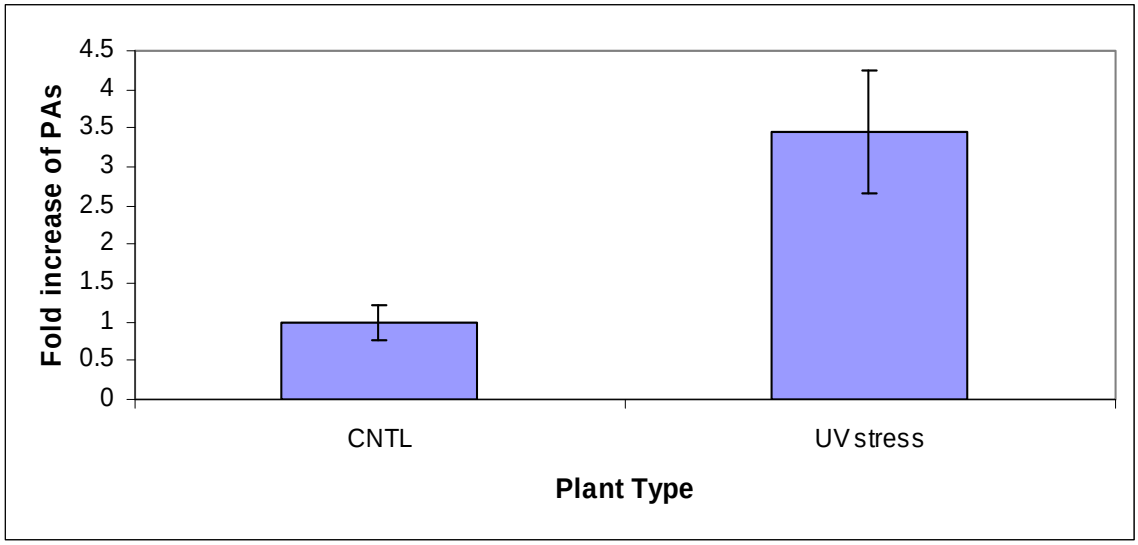

Figure 4.6: Outdoor light stress-induced accumulation of PAs in leaves of Populus. Wild type poplar plants were subjected to 9 days of outdoor light stress and levels of proanthocyanidins were analysed using the butanol-HCl test and compared to unstressed controls. Bars indicate the means of 5 trees per treatment with error bars indicating the standard error of means. Student's t test ($P<0.05$) shows significant difference.

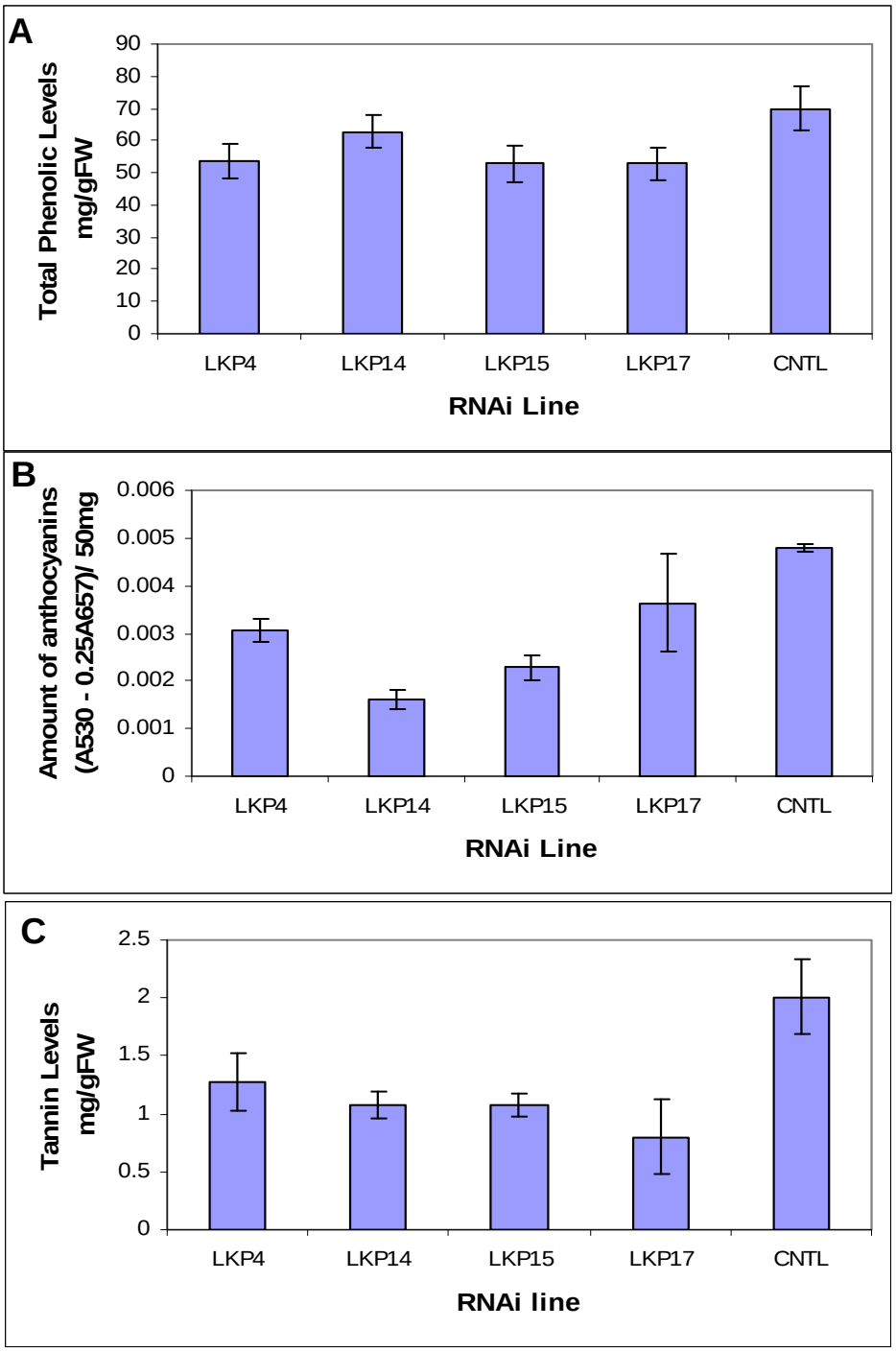

Figure 4.7: Phytochemical profiles of outdoor light stressed control and RNAi-PtUGT1 poplar plants.
Levels of phenolics (A), anthocyanins (B) and PAs (C) were determined using spectrophotometic assays (see Figure 4.3 and materials and method for details). All LKP lines values represent the means of n = 4 replicate samples, while control (CNTL) represent n=5.

Methanol extracts were also analysed on HPLC to determine whether downregulation of PtUGT1 expression led to a difference between separated phenolic compounds in RNAi lines and control plants (Figure 4.8). Integration of peak area for all flavonoid peaks was performed (see Appendix C). Peaks were defined and manually checked using absorption spectra to confirm they were flavonoid compounds. Surprisingly, no significant changes were found in the peaks of the various transgenic versus control plants analyzed.

4.4 Discussion

In order to better understand the function of PtUGT1, we investigated the expression of the poplar gene in wild type plant tissue and organs, as well as the effects of its downregulation using RNAi. Numerous transgenic poplar lines were produced using *Agrobacterium* mediated transformation, of which four lines showed substantially suppressed expression of PtUGT1. These lines were phytochemically characterized in more detail and shown to have significantly reduced PA levels.

Expression of PtUGT1in diverse poplar tissue in relation to PA and other phenolic levels

The PtUGT1 gene was found to be ubiquitously expressed at varying levels in all tissues tested by RT-PCR and qPCR, with highest expression occurring in the female catkin, leaf, bud and fruit tissues. Similarly broad expression profiles have been found for other flavonoid 3-UGTs that belong to the UGT78 family. Kovinich et al. (2010) found expression of UGT78K1 in black soybean to occur in all tissue (leaf, stem, root,

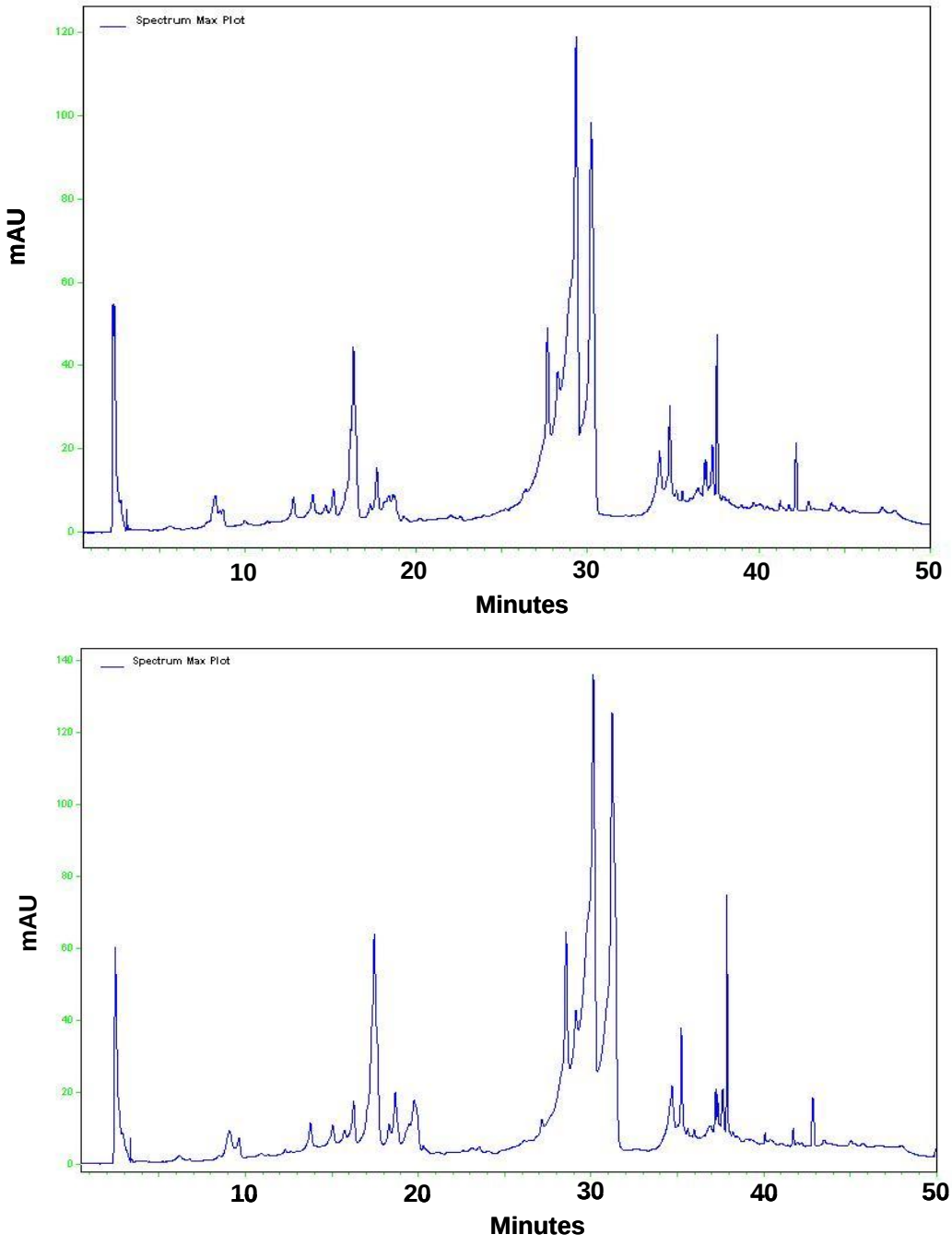

Figure 4.8: HPLC analysis of soluble phenolic metabolites in control (top) and PtUGT1 knockdown (bottom) leaf tissue extracts.
Representative maxplot chromatograms show each peak at its maximum absorption wavelength. Mature leaf tissue samples were obtained from 9 day outdoor light stressed control and transgenic plants.

flower bud pod, seed coat, and embryo) tested. Furthermore, a similar pattern was found in Arabidopsis for the UGT78D2 gene (expression in leaf, stem, buds, and roots was found) (Kubo et al., 2007). Thus a broad tissue expression profile of the PtUGT1 in poplar correlates with other plant species within the same UGT78 family.

Since previous data in the Constabel lab had suggested this gene may potentially be involved in the PA pathway, but phylogenetic analysis indicated that the gene may also be involved in flavonol and/or anthocyanin production (chapter 3), the various phenolic chemical classes in the different tissues were examined. If the PtUGT1 gene is involved in PA synthesis or anthocyanin synthesis, it would be expected that accumulation of the transcript should correlate with higher levels of PAs or anthocyanins. This is seen to some degree with PAs but not with anthocyanins. Therefore, the expression profiles are consistent with a potential role for PtUGT1 in the PA pathway (see below).

The levels of PAs are highest in female and male catkins, followed by roots, bud and fruit. The apical leaves also contain detectable levels of PAs. In quaking aspen (*P. tremuloides*), PAs have been found in the buds and catkins (Jakubas et al., 1989). PAs are also common in the fruit tissues of many trees including apple (Takos et al., 2006); peach (Senter and Callahan, 1990); and persimmons (Ikegami et al., 2009). PAs have also been detected in strawberry and avocado leaves (Stafford and Lester, 1980); willow tree leaves (Orians and Fritz, 1995); and in the roots of cypress and huckleberry plants (Kraus et al., 2004). However, it should be noted that while detectable, PAs were generally at a low concentration in the vegetative plant parts since these plants were grown in the greenhouse rather than outside.

When levels of PA accumulation were compared to the gene expression of PtUGT1 within the poplar tissues, only a partial similarity was found. The PtUGT1 gene was highly expressed in the female catkin, apical leaves, fruit and the bud tissue, where high levels of PAs were measured. However, expression of the gene is very low in the roots, yet they contain a significant amount of PAs. The reason for this discrepancy is not clear but could be related to differences in timing and developmental zones of the tissue sampled. Roots actively grow from their tip and can be divided into three anatomically distinct zones (Taylor and Peterson, 1999). The youngest area where the root is growing is called the white zone. This is followed by the tannin zone (also called the condensed tannin zone), and lastly the cork zone (Enstone et al., 2001). The root apical meristem is found in the white zone and this is where the root is actively growing. Approximately 20 mm from the tip of the root, the condensed tannin zone begins and here PAs start accumulating (Peterson et al., 1999). Within this zone, the cells of the cortex are mostly dead (Taylor and Peterson, 1999). In the tissue survey conducted here, the root samples collected consisted of mainly mature-aged roots, with few young actively growing root tips. It is likely that the root tips are more active in tannins synthesis, but that the older sections continue to contain high levels of PAs. This may account for the apparently low levels of gene expression seen in the root sample. If more fine young roots (<2 mm in diameter) had been collected, a different expression pattern may have been found. Another possible reason for the discrepancy between expression pattern and metabolite level for PAs in the roots may be flavonoid transport. Within Arabidopsis, flavonoids are able to be transported through out the plant, thus there is a

possibility that PAs may be synthesized elsewhere in the plant and transported to the roots for storage or possibly for defense (Buer et al., 2007).

Male catkins show the highest levels of anthocyanins, consistent with their dark red colour (Wilkins et al., 2009). Yet the PtUGT1 gene is poorly expressed in this tissue. Anthocyanin levels in the bud were the second highest in all tissue tested, followed by low, but detectable levels in the fruit, roots, and female catkins. All other tissues had very low levels of anthocyanins. Buds and fruits are generally all brightly coloured, and this is due to the accumulation of anthocyanins (Steyn, 2009). Also, anthocyanins have been found in roots of Myrtle plants (Solangaarachchi and Gould, 2001). When the anthocyanin levels in each tissue is compared to the gene expression of PtUGT1, as mentioned earlier, high expression occurs in the female catkins, apical leaves and fruit, but this does not coincide with the levels of anthocyanins. Hence, it seems that the PtUGT1 gene may not be primarily involved in anthocyanin accumulation. Overall, expression and phytochemical analysis of the poplar tissue survey suggests that PtUGT1 is indeed somehow involved in PA production within poplar plants. This conclusion was further strengthened by the RNAi experiments.

Downregulation of the PtUGT1 gene in poplar leads to reduced PA level

The RNAi vector was effective in reducing PtUGT1 expression in transgenic poplar although only for a subset of lines. Four transgenics with a significantly decreased level of the PtUGT1 gene were analysed in detail for physical and chemical phenotypes. These plants did not display any visible phenotype; plant growth and leaf appearance were indistinguishable from non-transgenic and empty vector control poplars. This lack of visible phenotype is consistent with previous studies which altered the expression of

genes involved in the flavonoid pathway. In MYB134 overexpressing poplar plants with high levels of PAs, no visible or growth phenotype was observed (Mellway et al., 2009). In strawberry, when CHS gene expression was decreased using RNAi, no phenotype was seen in the growth and leaf appearance of the transgenic plants compared to controls (Hoffman et al., 2006). Similarly in tomato with reduced CHS gene expression, the transgenic vegetative tissue showed a similar phenotype to non-transgenics (Schijlen et al., 2007). In all cases, despite the lack of visible phenotypes, phytochemical levels were impacted.

To investigate potential changes in leaf phenolics in the PtUGT1-RNAi poplars, the plants were first exposed to natural (outdoor) sunlight light. This treatment is known to enhance the flavonoid levels (Koes et al., 1994; Stapleton, 1992). After 9 days, the transgenics were analyzed for a chemical phenotype. Using the general Folin-Ciocalteau assay to determine levels of total phenolics, no significant difference was seen in the transgenics versus the control plants. By contrast, a spectrophotometric test for anthocyanins detected significantly lower levels of these compounds in three of the four PtUGT1 knockdown lines. Analysis of PA levels also showed significantly decreased levels in three knockdown lines, and this was originally hypothesized from previous co-expression data but not consistent with the biochemical data (chapter 3). The lower levels of anthocyanins observed in RNAi knockdown poplars is consistent with the phylogenetic analysis in chapter 3 which grouped PtUGT1 with others UGTs potentially involved in the pathways leading to flavonols and anthocyanins. Recombinant PtUGT1 protein displayed *in vitro* glycosylation activity with both those class of substrates (chapter 3). Originally we had suspected monomers of PA precursors (flavan-3-ols) to

be substrates of PtUGT1, but *in vitro* assays did not confirm this. Quercetin, kaempferol and cyanidin were found to be substrates of PtUGT1, with the sugar residues added at the 3-O position of the molecules (chapter 3). This specificity of PtUGT1 is consistent with other characterized enzymes that are found in the same UGT phylogeny group (group F). The closest homologue to PtUGT1 is the UGT gene from *Vigna mungo* (VmUF3GaT) (Ishikura and Mato, 1993; Mato et al., 1998) which shows *in vitro* activity with the flavonols quercetin and kaempferol glycosylating at the 3-O position. Furthermore, the closest homologue in Arabidopsis (UGT78D2, At5g17050) is also a 3-O glycosyltransferase that has been shown to be involved in anthocyanin production (Kubo et al., 2007). Additionally, other similar 3-UGTs in petunia and grape also are involved in anthocyanin production (Miller et al., 1999; Hall et al., 2011). Thus phylogenetically, as well as enzymatically, PtUGT1 is implicated to be involved in the flavonol and anthocyanin pathways.

However, since tissue expression analysis indicated that the PtUGT1 gene was more likely to be involved in PA rather than anthocyanin biosynthesis, and since RNAi plants also had reduced PA levels, how is a flavonol/anthocyanidin specific UGT able to influence PA levels *in planta*? It is hypothesized that the changes in the levels of PAs and anthocyanins within knockdown PtUGT1 plants may be due to the changes in the levels of early metabolites within the flavonoid pathway. For example, if the activity of PtUGT1 is suppressed, this could cause the *in vivo* flavonoid substrates it acts on to accumulate within the plant cell. Since the phenylpropanoid and flavonoid biosynthesis pathways are subjected to multiple levels of control, if these substrates build up, they can potentially act as feedback regulators to inhibit earlier enzymes within the pathway. For

instance, high levels of flavonols have been shown to inhibit the activity of enzymes within the flavonoid pathways. Specifically, the flavonol quercetin and flavanone naringenin are known to inhibit the activity of PAL, the first enzyme which leads to the synthesis of phenylpropanoids and flavonoids (Sarma and Sharma, 1999). If PAL has decreased activity, this would reduce the synthesis of downstream products including phenolics, flavonoids and anthocyanins. Thus, the decreased expression of PtUGT1 may indirectly be decreasing the activity of PAL or other phenylpropanoid/flavonoid enzymes and this may be one of the reasons that both anthocyanins and PAs were found to be decreased in the PtUGT1 RNAi plants.

In conclusion, this work is the first report we believe which shows that altering UGT expression can alter levels of PAs in transgenic plants. Previous studies involving UGTs utilizing RNAi technology have focused on genes involved in lignin biosynthesis to help in the production of plants for biofuels (Lee et al., 2009; Li et al., 2011), or on genes involved in anthocyanin synthesis leading to altered flower/fruit colouring (Griesser et al., 2008; Chen et al., 2011). In this work, RNAi was used to successfully decrease levels of PAs in poplar using a UGT of interest. Our work with poplars with a decreased expression of the PtUGT1 gene supports the hypothesis that this gene is somehow involved in the flavonoid pathway leading to the production PAs. Tissue survey analysis supports the idea of the gene being involved in PA biosynthesis since gene expression and the tissues in which PAs accumulate correlate with each other. Furthermore, a decrease in the expression of the gene using RNAi transgenics leads to a decrease in the PA levels in the plants. Future work that will help shed further information on the involvement of the PtUGT1 gene within the flavonoid pathway

includes the production of poplar transgenics with the PtUGT1 gene overexpressed. Such plants have already been produced (4 lines of overexpressing poplar (*P. tremula x tremuloides*)), but need to be further examined. Preliminary experiments on these plants have not revealed significant changes in flavonol, anthocyanin or PA levels compared to controls, yet more in depth examination must be performed.

Chapter 5: Overall conclusions and future directions

Being sessile organisms, plants are unable to relocate to a new environment if problems arise where they are rooted. They must evolve adaptations to cope with unavoidable stresses that may be imposed upon them. Poplars are an excellent model to study these adaptations since they are long lived plant species that will be subjected to many stresses over their lifespan. Poplars are predicted to have evolved a variety of defences to help combat these stresses, both biochemical defences and defensive secondary metabolites.

A wound induced acid phosphatase in poplar

One mechanism in plant defense is the induction of acid phosphatases (APs). APs are classically known to be induced under phosphate stress, but an AP was found to be induced following wounding and pest stress in the leaves of poplar. As presented in chapter 2, an AP was identified in leaves as being highly induced during the poplar herbivore defense response. The data reported is the first to show an increase in AP activity due to wounding plant tissue. This gene was molecularly characterized and time course analysis from wounding treatment showed rapid induction of the PtdAP1 gene systemically throughout the plant, which is typical for the induced herbivore response in poplar. Using western blotting and AP activity assays I was able to correlate the increase in gene expression to an actual increase in the levels of the AP protein within the wounded leaves. There was an approximate 2-3 fold increase in the amount of protein in wounded tissue, and this coincided with an increase in AP activity seen (also 2-3 fold increase). Our finding of an increased activity of AP in wounded poplar tissue implies a

role for the AP protein in plant defense. Yet, in order to fully understand the importance and function of this AP within poplar defense, pure protein would be helpful for further experiments. This could be accomplished by purifying the AP from wounded poplar leaf tissue. Since the PtdAP1 gene is highly expressed in wounded tissue, the risk of purifying other APs would be low. Another option is to produce recombinant protein using the PtdAP1 gene. This was attempted using various cloning and expression vectors in E. coli, but thus far it was not possible to get active recombinant protein (data not shown). APs are known to be glycosylated and expression within E. coli most likely does not allow for sugars to be added, which could impact proper folding. Using a different eukaryotic expression host may help. Functional recombinant soybean AP has been produced using the methyltrophic yeast *Pichia pastoris* (Penheiter et al., 1998). Insect cell lines have also been successfully in expressing APs (Vogel et al., 2002). The production of recombinant PtdAP1 protein would allow for more indepth biochemical analysis to be performed helping to elucidate its function. Also, the recombinant poplar AP protein could be used in insect feeding assays to assess whether it affects insect development and survival.

Two poplar UDP-glycosyltransferases- do they function in PA biosynthesis?

The main objective of chapters 3 and 4 of this thesis was to examine two UGT genes that were first identified from previous microarray data. The two genes followed similar induction patterns to other genes involved in the production of PAs and thus we hypothesized they were involved in PA biosynthesis and sought to test this hypothesis.

In 2008 while this work was in progress, Pang et al. identified a UGT from *Medicago truncatula* that was active specifically with flavan-3-ols, the precursors in the

production of PAs. Since it was suspected our poplar UGT genes were also involved in PA biosynthesis, we believed that our genes would be similar to this gene. Yet phylogenic analysis of the two poplar UGT genes did not group them with the Medicago UGT (UGT72 family). They were found to cluster with enzymes that are able to glycosylate flavonols and anthocyanidins (UGT78 family). Thus using this information, we tested a wide variety of substrates, including flavonols, anthocyanidins and flavan-3-ols. When active soluble recombinant protein was used in enzymatic assays, PtUGT1 was able to glycosylate quercetin and kaempferol (flavonols), and cyanidin (anthocyanidin), but not flavan-3-ols as we suspected. This substrate specificity correlated with the enzymes in the UGT78 family. By contrast, even though the other poplar gene PtUGT2 grouped phylogenetically with the UGT78 enzymes, it did not show any activity with those substrates. PtUGT2 was only able to glycoslate the xenobiotic TCP, which was used as an artificial test substrate. Thus, sequence analysis of UGTs cannot always be used to accurately predict substrate specificities of the enzymes. PtUGT1's *in vitro* substrates were correctly predicted, but PtUGT2 were not. This emphasizes that the relationship between sequence identity and substrate specificity is complex in UGTs and recombinant protein must be produced and tested to determine substrates.

Since PtUGT1 was most highly upregulated in both previous microarrays preformed in our lab, we produced transgenics for functional analysis. To do this, the pKannibal suppression vector was used and chapter 4 describes the findings from these plants. Levels of the PtUGT1 gene were very low in four lines produced (qPCR detected very low, if any, levels of the transcript after 40 cycles). These plants were outdoor light

stressed and the levels of the phytochemicals were examined. PA levels were decreased up to 50% in some of these plants and it seems that *in vivo*, the PtUGT1 gene influences the production of PAs within poplar though the mechanism is not clear.

Our finding of a UGT78 enzyme that is implicated in PA regulation via transgenics yet had unexpected *in vitro* activity is similar to studies done by Ikegami et al. (2009) and Akagi et al. (2009) on persimmon fruit. Persimmon fruits that accumulate large amounts of proanthocyanidins (normal non-PCNA (pollination-constant and non-astringent)) were compared to fruit that lose their ability produce PAs (PCNA type mutants). This was achieved using a suppression subtractive hybridisation analysis to identify differential expression of genes thought to be involved in PA biosynthesis. Agaki et al. (2009) identified many shikimate and flavonoid biosynthetic genes that were downregulated in the PCNA persimmon compared to the non-PCNA type. This co-expression analysis also identified a group UGT78 flavonoid 3-O-galactosyltransferase (DkFGT) which was clearly downregulated in the low tannin PCNA fruit. It was cloned, expressed in *E. coli* and the recombinant protein was shown to be able to glycosylated both flavonoids and anthocyanidins. Their finding mirrors what was seen with our PtUGT1 gene (chapter 3). Both the poplar and the persimmon genes had elevated expression in high PA producing plants, and both were found to utilize unexpected substrates not directly involved in PA biosynthesis. However, it seems that our PtUGT1 gene, and perhaps the DkFGT gene, are indeed somehow involved in PA biosynthesis *in vivo*. The use of recombinant proteins and *in vitro* experimentation although an important step may not provide a true sense of their *in planta* functions.

The results presented in chapter 3 and 4 of this thesis suggest that PtUGT1 is involved in the production of PAs. To further investigate how the PtUGT1 gene is involved in PA biosynthesis, the PtUGT1-RNAi poplars produced could be used for global transcriptome, proteomic and/or metabolomic profiling. Observed changes in genes, proteins, and/or metabolites between control and RNAi plants would provide valuable information on the role of PtUGT1 in this pathway. In chapter 3, the *in vitro* data suggest a role for PtUGT1 in flavonol glycosylation. As mentioned, flavonols are known to affect the activity of earlier enzymes within the flavonoid pathways, especially PAL (Sarma and Sharma, 1999). If PtUGT1 is indeed involved in flavonol glycosylation, its decreased expression would cause flavonoid aglycones to accumulate. Thus, analysis of transgenic plants using microarrays and proteomics could determine if changes in the gene/protein expression of flavonoid biosynthesis enzymes, such as PAL, occurs. If for example, PAL is indeed decreased in expression, then the *in vitro* data may indeed show correct substrates for PtUGT1 and may help to determine the true involvement of it within PA biosynthesis.

Since the levels of PAs were decreased in PtUGT1 knockdown plants, it would be valuable to overexpress this gene and see whether an increase in PA levels would occur. PAs are thought to have many health benefits for humans and the ability to increase the levels within various plants and even crop species would be beneficial for all. Also, increases in the levels of PAs within the feedstocks of cattle would help decrease bloat since PAs can bind to soluble proteins thus inhibiting their availability for microbes within the cow's rumen. Thus altering levels of PAs within plants can have very beneficial effects.

The original finding that the PtUGT1 and the PtUGT2 genes were upregulated under pathogen stress along with other PA biosynthetic genes suggests they are involved in the plant defense response. The production of PtUGT1-RNAi knockdown plants indicated that the gene is somehow involved in the PA biosynthetic pathway. However, when recombinant protein to PtUGT1 was made, substrates not directly involved in the PA pathway were found to be used. These knockdown plants will provide a great resource for examining this contradiction as well as for directly testing the role of the PtUGT1 gene and PAs in plant defense. For example, using these knockdown plants with lower PA levels, they can be infected with *Melampsora medusae* to determine if they become more or less suspectible to infection. Furthermore, feeding bioassays with insect herbivores using these plants will determine if the gene is also involved in defense against herbivory. These studies will help to further our knowledge about the involvement of PtUGT1 in plant defense.

Overall, the research within this thesis helps to provide more information in poplar defense against both abiotic and biotic stresses. Furthermore, the work described indicates that our knowledge of the biosynthetic pathway leading to PAs is not yet complete, and the UGTs described provide an opportunity for discovering unknown steps in the pathway.

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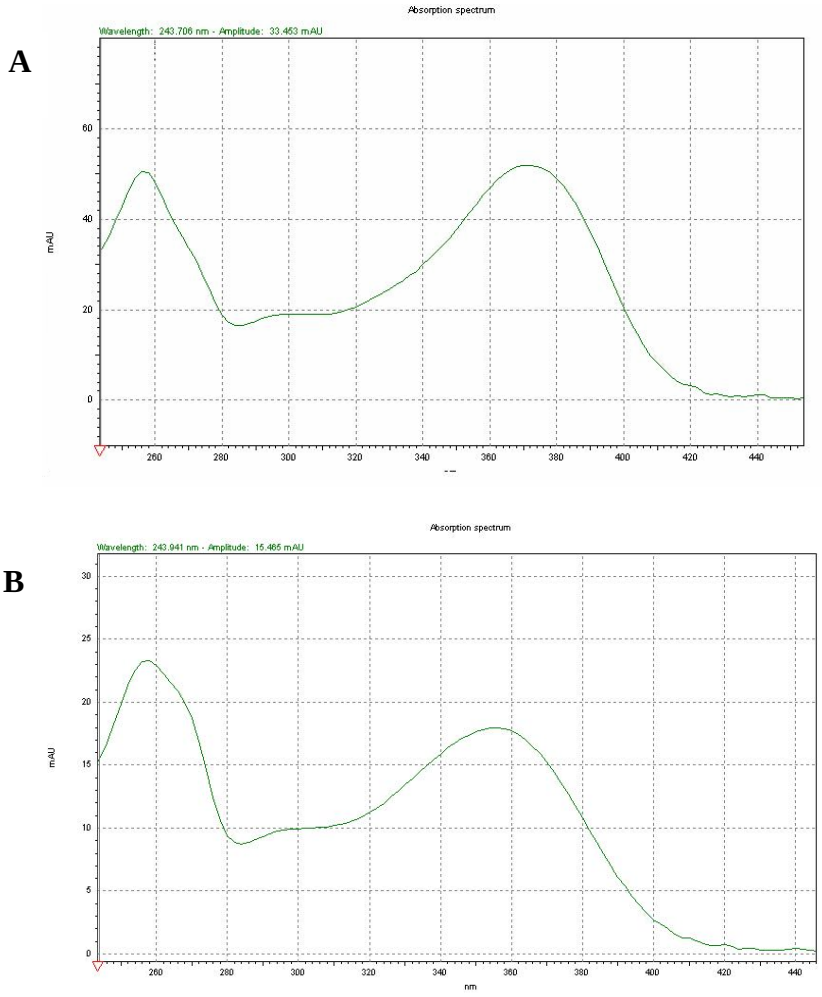
Appendix A: Hypsochromic shift showing 3-O glycosylation



Supplementary Figure A1: (A) UV spectra of pure quercetin with UV max seen at approximately 370 nm. (B) UV spectra of glycosylated quercetin with UV max seen at approximately 354 nm displaying UV shift.

Appendix B: List of UGTs identified from *Populus trichocarpa* genome version 1.1

Genes identified have been named according to the standards set out by MacKenzie et al., 1997. Poplar gene models follow in parentheses.

UGT71 family

PtUGT71B1 (grail3.1094000101 [Poptr1_1:670180])
PtUGT71B2 (gw1.1772.3.1 [Poptr1_1:275789])
PtUGT71B3 (eugene3.00060083 [Poptr1_1:560124])
PtUGT71B4 (eugene3.00160104 [Poptr1_1:575969])
PtUGT71B5 (eugene3.00060085 [Poptr1_1:560126])
PtUGT71B6 (eugene3.00160106 [Poptr1_1:575971])
PtUGT71B7 (eugene3.00060086 [Poptr1_1:560127])
PtUGT71B8 (eugene3.00160107 [Poptr1_1:575972])
PtUGT71B9 (eugene3.00060107 [Poptr1_1:560148])
PtUGT71B10 (eugene3.00160109 [Poptr1_1:575974])
PtUGT71B11 (eugene3.00160114 [Poptr1_1:575979])
PtUGT71B12 (eugene3.00160115 [Poptr1_1:575980])
PtUGT71B13 (eugene3.00060078 [Poptr1_1:560119])
PtUGT71B14 (eugene3.00160087 [Poptr1_1:575952])

PtUGT71C1 (eugene3.00091201 [Poptr1_1:557941])
PtUGT71C2 (eugene3.00160092 [Poptr1_1:575957])

UGT72 family

PtUGT72B1 (fgenesh4_pg.C_LG_XIV000386 [Poptr1_1:774476])
PtUGT72B2 (fgenesh4_pg.C_scaffold_40000171 [Poptr1_1:781421])
PtUGT72B3 (fgenesh4_pg.C_scaffold_40000170 [Poptr1_1:781420])
PtUGT72B4 (estExt_fgenesh4_pg.C_LG_II1536 [Poptr1_1:816627])
PtUGT72B5 (estExt_Genewise1_v1.C_LG_XIV1614 [Poptr1_1:731283])
PtUGT72B6 (estExt_Genewise1_v1.C_LG_XIV1619 [Poptr1_1:731288])
PtUGT72B7 (eugene3.00031105 [Poptr1_1:554472])

PtUGT72D1 (gw1.VII.3359.1 [Poptr1_1:219054])

PtUGT72E1 (gw1.VII.3365.1 [Poptr1_1:219060])
PtUGT72E2 (eugene3.00071041 [Poptr1_1:563103])

UGT73 family

PtUGT73B1 (eugene3.12010001 [Poptr1_1:580281])
PtUGT73B2 (fgenesh4_pg.C_LG_I002588 [Poptr1_1:752985])
PtUGT73B3 (fgenesh4_pg.C_LG_XVIII000577 [Poptr1_1:779209])
PtUGT73B4 (fgenesh4_pg.C_scaffold_147000009 [Poptr1_1:785422])

PtUGT73B5 (fgenesh4_pg.C_scaffold_147000010 [Poptr1_1:785423])
PtUGT73B6 (fgenesh4_pg.C_scaffold_70000221 [Poptr1_1:782873])
PtUGT73B7 (fgenesh4_pm.C_LG_I001042 [Poptr1_1:798039])
PtUGT73B8 (fgenesh4_pm.C_LG_IX000327 [Poptr1_1:804280])
PtUGT73B9 (fgenesh4_pm.C_scaffold_147000002 [Poptr1_1:811430])
PtUGT73B10 (grail3.0017021801 [Poptr1_1:642013])
PtUGT73B11 (gw1.1198.3.1 [Poptr1_1:264969])
PtUGT73B12 (gw1.316.1.1 [Poptr1_1:284426])
PtUGT73B13 (gw1.XIV.1213.1 [Poptr1_1:244470])
PtUGT73B14 (eugene3.00290281 [Poptr1_1:590098])
PtUGT73B15 (eugene3.00012366 [Poptr1_1:549925])
PtUGT73B16 (eugene3.00060425 [Poptr1_1:560466])
PtUGT73B17 (eugene3.00090672 [Poptr1_1:557412])

PtUGT73C1 (fgenesh4_pm.C_LG_XII000158 [Poptr1_1:806338])
PtUGT73C2 (grail3.0018007101 [Poptr1_1:646335])

PtUGT73D1 (eugene3.00280250 [Poptr1_1:589514])
PtUGT73D2 (gw1.XVI.3101.1 [Poptr1_1:257162])

UGT74 family

PtUGT74B1 (grail3.0005006601 [Poptr1_1:666418])

PtUGT74E1 (eugene3.01820023 [Poptr1_1:585760])
PtUGT74E2 (fgenesh4_pg.C_scaffold_1119000002 [Poptr1_1:790329])
PtUGT74E3 (fgenesh4_pm.C_LG_XIV000523 [Poptr1_1:807713])
PtUGT74E4 (fgenesh4_pm.C_scaffold_64000028 [Poptr1_1:810275])
PtUGT74E5 (gw1.3559.2.1 [Poptr1_1:285380])
PtUGT74E6 (gw1.696.6.1 [Poptr1_1:294507])
PtUGT74E7 (gw1.VII.465.1 [Poptr1_1:216160])
PtUGT74E8 (gw1.VII.484.1 [Poptr1_1:216179])
PtUGT74E9 (eugene3.00041395 [Poptr1_1:556682])
PtUGT74E10 (eugene3.00070072 [Poptr1_1:562134])

PtUGT74F1 (eugene3.13510003 [Poptr1_1:582116])
PtUGT74F2 (gw1.VII.1165.1 [Poptr1_1:216860])
PtUGT74F3 (estExt_Genewise1_v1.C_LG_VII0493 [Poptr1_1:718172])
PtUGT74F4 (eugene3.00070068 [Poptr1_1:562130])

UGT75 family

PtUGT75C1 (fgenesh4_pg.C_scaffold_155000048 [Poptr1_1:785827])
PtUGT75C2 (gw1.IV.3716.1 [Poptr1_1:198627])

PtUGT75D1 (fgenesh4_pg.C_LG_VI000465 [Poptr1_1:762155])
PtUGT75D2 (grail3.0021016401 [Poptr1_1:645347])
PtUGT75D3 (grail3.0021016501 [Poptr1_1:645348])
PtUGT75D4 (eugene3.00140929 [Poptr1_1:572884])
PtUGT75D5 (eugene3.00160459 [Poptr1_1:576324])

UGT76 family

PtUGT76B1 (fgenesh4_pg.C_LG_VI000312 [Poptr1_1:762002])

PtUGT76C1 (gw1.1107.6.1 [Poptr1_1:263864])
PtUGT76C2 (gw1.X.1927.1 [Poptr1_1:227230])
PtUGT76C3 (gw1.X.1932.1 [Poptr1_1:227235])

PtUGT76E1 (fgenesh4_pg.C_LG_IX001190 [Poptr1_1:768139])
PtUGT76E2 (eugene3.00011560 [Poptr1_1:549119])

PtUGT76F1 (eugene3.11070001 [Poptr1_1:579636])

UGT78 family

PtUGT78D1 (eugene3.00410022 [Poptr1_1:592179])
PtUGT78D2 (eugene3.16750001 [Poptr1_1:584786])
PtUGT78D3 (fgenesh4_pm.C_LG_IX000407 [Poptr1_1:804360])
PtUGT78D4 (gw1.951.3.1 [Poptr1_1:298709])
PtUGT78D5 (gw1.XVIII.2887.1 [Poptr1_1:262346])
PtUGT78D6 (eugene3.00090352 [Poptr1_1:557092])

UGT79 family

PtUGT79B1 (fgenesh4_pg.C_LG_VI001274 [Poptr1_1:762964])
PtUGT79B2 (fgenesh4_pm.C_scaffold_187000008 [Poptr1_1:811849])
PtUGT79B3 (gw1.64.3.1 [Poptr1_1:292351])
PtUGT79B4 (gw1.XIX.2318.1 [Poptr1_1:249918])
PtUGT79B5 (eugene3.00110645 [Poptr1_1:568631])
PtUGT79B6 (eugene3.00770001 [Poptr1_1:596027])
PtUGT79B7 (eugene3.01320001 [Poptr1_1:581747])

UGT80 family

PtUGT80A1 (gw1.II.379.1 [Poptr1_1:409044])
PtUGT80A2 (gw1.XIV.4120.1 [Poptr1_1:247377])

PtUGT80B1 (gw1.V.341.1 [Poptr1_1:204940])
PtUGT80B2 (estExt_fgenesh4_pm.C_LG_II0308 [Poptr1_1:830147])

UGT82 family

PtUGT82A1 (estExt_Genewise1_v1.C_LG_X5789 [Poptr1_1:726527])

UGT83 family

PtUGT83A1 (fgenesh4_pm.C_LG_IV000394 [Poptr1_1:800707])
PtUGT83A2 (estExt_Genewise1_v1.C_LG_X5789 [Poptr1_1:726527])
PtUGT83A3 (eugene3.00170469 [Poptr1_1:577727])
PtUGT83A4 (eugene3.00041122 [Poptr1_1:556409])

UGT84 family

PtUGT84A1 (gw1.IX.2384.1 [Poptr1_1:201919])
PtUGT84A2 (gw1.IX.2395.1 [Poptr1_1:201930])

PtUGT84B1 (eugene3.02050001 [Poptr1_1:587034])
PtUGT84B2 (gw1.IX.2389.1 [Poptr1_1:201924])
PtUGT84B3 (fgenesh4_pg.C_LG_IX000679 [Poptr1_1:767628])

UGT85 family

PtUGT85A1 (eugene3.04240002 [Poptr1_1:592440])
PtUGT85A2 (gw1.1589.3.1 [Poptr1_1:273663])
PtUGT85A3 (gw1.I.3549.1 [Poptr1_1:174949])
PtUGT85A4 (gw1.I.3572.1 [Poptr1_1:174972])
PtUGT85A5 (gw1.II.1756.1 [Poptr1_1:410421])
PtUGT85A6 (gw1.II.3843.1 [Poptr1_1:412508])
PtUGT85A7 (gw1.IV.1075.1 [Poptr1_1:195986])
PtUGT85A8 (gw1.VI.1161.1 [Poptr1_1:416788])
PtUGT85A9 (gw1.VI.1162.1 [Poptr1_1:416789])
PtUGT85A10 (gw1.VI.1163.1 [Poptr1_1:416790])
PtUGT85A11 (gw1.VI.1525.1 [Poptr1_1:417152])
PtUGT85A12 (gw1.VI.2360.1 [Poptr1_1:417987])
PtUGT85A13 (gw1.VII.1753.1 [Poptr1_1:217448])
PtUGT85A14 (gw1.VII.705.1 [Poptr1_1:216400])
PtUGT85A15 (gw1.XVI.867.1 [Poptr1_1:254928])
PtUGT85A16 (gw1.XVI.869.1 [Poptr1_1:254930])

PtUGT85A17 (gw1.XVI.905.1 [Poptr1_1:254966])
PtUGT85A18 (gw1.XVI.910.1 [Poptr1_1:254971])
PtUGT85A19 (gw1.XVI.921.1 [Poptr1_1:254982])
PtUGT85A20 (gw1.XVII.369.1 [Poptr1_1:258378])
PtUGT85A21 (gw1.XVII.379.1 [Poptr1_1:258388])
PtUGT85A22 (gw1.XVII.385.1 [Poptr1_1:258394])
PtUGT85A23 (gw1.XVII.389.1 [Poptr1_1:258398])
PtUGT85A24 (gw1.XVII.395.1 [Poptr1_1:258404])
PtUGT85A25 (gw1.XVII.400.1 [Poptr1_1:258409])
PtUGT85A26 (gw1.XVII.405.1 [Poptr1_1:258414])
PtUGT85A27 (fgenesh4_pg.C_LG_XVI000107 [Poptr1_1:776865])
PtUGT85A28 (fgenesh4_pg.C_LG_XVI000131 [Poptr1_1:776889])
PtUGT85A29 (fgenesh4_pg.C_LG_XVI000973 [Poptr1_1:777731])
PtUGT85A30 (eugene3.00160169 [Poptr1_1:576034])
PtUGT85A31 (estExt_Genewise1_v1.C_LG_V2740 [Poptr1_1:715868])
PtUGT85A32 (eugene3.00160153 [Poptr1_1:576018])
PtUGT85A33 (eugene3.00060224 [Poptr1_1:560265])
PtUGT85A34 (eugene3.00160160 [Poptr1_1:576025])

UGT86 family

PtUGT86A1 (gw1.163.8.1 [Poptr1_1:273974])
PtUGT86A2 (fgenesh4_pg.C_LG_IX001471 [Poptr1_1:768420])

UGT87 family

PtUGT87A1 (gw1.I.2249.1 [Poptr1_1:173649])
PtUGT87A2 (fgenesh4_pg.C_LG_IX000843 [Poptr1_1:767792])
PtUGT87A3 (grail3.0053022001 [Poptr1_1:641532])
PtUGT87A4 (eugene3.00090876 [Poptr1_1:557616])
PtUGT87A5 (fgenesh4_pg.C_LG_I002071 [Poptr1_1:752468])

UGT88 family

PtUGT88A1 (eugene3.00440275 [Poptr1_1:592940])
PtUGT88A2 (eugene3.01220019 [Poptr1_1:580586])
PtUGT88A3 (fgenesh4_pg.C_LG_IV001159 [Poptr1_1:759531])
PtUGT88A4 (fgenesh4_pm.C_LG_XVII000147 [Poptr1_1:808954])
PtUGT88A5 (grail3.0031007501 [Poptr1_1:661415])
PtUGT88A6 (grail3.0038022401 [Poptr1_1:648281])
PtUGT88A7 (grail3.0044019301 [Poptr1_1:678615])
PtUGT88A8 (gw1.494.4.1 [Poptr1_1:290393])
PtUGT88A9 (eugene3.00040272 [Poptr1_1:555559])
PtUGT88A10 (eugene3.00040275 [Poptr1_1:555562])

UGT89 family

PtUGT89A1 (eugene3.00101302 [Poptr1_1:566744])

PtUGT89B1 (eugene3.00120577 [Poptr1_1:569924])

UGT90 family

PtUGT90A1 (eugene3.00180593 [Poptr1_1:578369])

UGT91 family

PtUGT91A1 (eugene3.00181234 [Poptr1_1:579010])
PtUGT91A2 (eugene3.09560001 [Poptr1_1:597503])
PtUGT91A3 (fgenesh4_pg.C_LG_I000238 [Poptr1_1:750635])
PtUGT91A4 (fgenesh4_pg.C_LG_XIV000324 [Poptr1_1:774414])
PtUGT91A5 (estExt_fgenesh4_pm.C_LG_II0734 [Poptr1_1:830383])
PtUGT91A6 (eugene3.00120106 [Poptr1_1:569453])
PtUGT91A7 (eugene3.00170040 [Poptr1_1:577298])
PtUGT91A8 (estExt_fgenesh4_pm.C_LG_X0707 [Poptr1_1:833746])

PtUGT91C1 (fgenesh4_pm.C_LG_III000645 [Poptr1_1:800180])

UGT92 family

PtUGT92A1 (fgenesh4_pm.C_LG_I000127 [Poptr1_1:797124])
PtUGT92A2 (gw1.531.8.1 [Poptr1_1:290747])

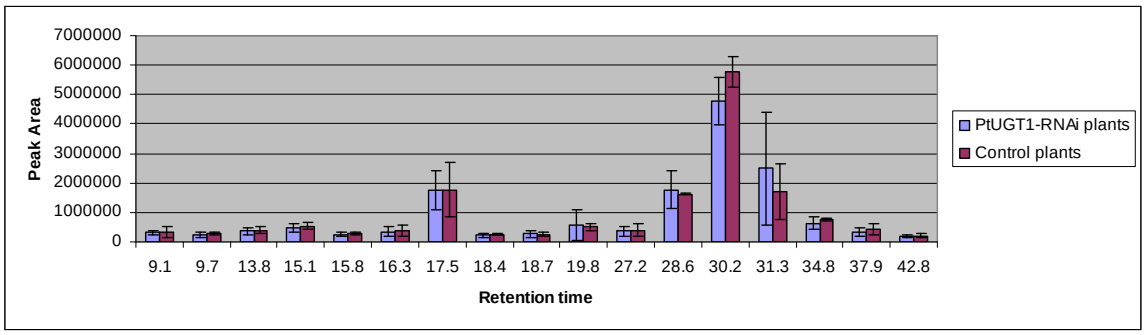
UGT93 family

PtUGT93B1 (fgenesh4_pm.C_LG_XVI000181 [Poptr1_1:808453])
PtUGT93B2 (eugene3.00060436 [Poptr1_1:560477])

UGT94 family

PtUGT94F1 (fgenesh4_pg.C_LG_VIII000208 [Poptr1_1:765194])

Appendix C: Peak area integration of soluble phenolic metabolites in control and RNAi poplar plants



Supplementary Figure C1: HPLC chromatograms (see Figure 4.8 for example of elution profile) of control and RNAi-PtUGT1 leaf tissue that have been integrated and peak area determined. Defined peaks were manually checked and confirmed using absorption spectra. No peaks were found to be significantly different between control and knockdown plants. Error bars represent standard error.