The Regulation of Stress-Induced Proanthocyanidin Metabolism in Poplar

by

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B. Sc., University of Victoria, 2003

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Supervisory Committee

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Abstract

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Proanthocyanidins (PAs) are polymeric phenolic chemicals produced by many plant species that may contribute to protection of tissues against biotic and abiotic stress conditions. In poplar (*Populus* spp.) leaves, PA biosynthesis is rapidly activated by insect herbivore damage, indicating that PAs may be an inducible chemical defence. In this study, the expression of PA biosynthetic genes was monitored in poplar leaves exposed to several stress stimuli. The PA pathway was shown to be rapidly activated at the level of gene transcription by stresses such as elevated light, UV-B irradiation and infection of leaves by a biotrophic fungal parasite. A transcription factor gene of the R2R3 MYB type, *MYB134*, was found to be co-activated with PA biosynthetic genes under these stress conditions. When overexpressed in transgenic poplar, this gene induced a strong, specific activation of the PA pathway, indicating that it might function as a regulator of stress-induced PA metabolism in poplar. MYB134 was shown to bind to promoter fragments of PA biosynthetic genes which contained a conserved DNA *cis*-element found in the promoter regions of many other phenylpropanoid genes including putative MYB134-regulated genes. A global transcriptome analysis of leaves of the *MYB134*-overexpressing poplar plants confirmed that the PA pathway activation was complete and specific, and led to the identification of a number of putative novel PA biosynthetic and regulatory genes. These results indicate that stress-responsive PA and flavonoid metabolism in poplar may be regulated by a complex system involving both positive and

negative regulation. Preliminary results are also presented related to analysis of PA functions in transgenic plants, and the use of *MYB134* to engineer PA metabolism in plants other than poplar. This study provides insight into the regulatory mechanisms controlling stress-induced PA metabolism, and expands our understanding of roles that this biological response may play in poplar.

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List of Abbreviations

4CL	4-Coumarate CoA-Ligase
3GT	3-Glucosyl Transferase
ABC	ATP-Binding Cassette
ACC	Acetyl-CoA Carboxylase
ANR	Anthocyanidin Reductase
ANS	Anthocyanidin Synthase
AT	Acyltransferase
BAN	*BANYULS* (encoding ANR)
BHLH	Basic-Helix-Loop-Helix
C3'H	*p*-Coumaroyl-CoA 3'-Hydroxylase
C4H	Cinnamate 4-Hydroxylase
CaMV	Cauliflower Mosaic Virus
CCR	Cinnamoyl-CoA Reducatse
cDNA	Complementary DNA
CDS	Coding Sequence
CHS	Chalcone Synthase
CHI	Chalcone Isomerase
COMT	Caffeate-O-Methyltransferase
CTAB	Cetyltrimethylammonium Bromide
DFR	Dihydroflavonol Reductase
DMACA	Dimethylaminocinnamaldehyde
DPI	Days Post-Inoculation
DW	Dry Weight
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
F3H	Flavanone 3-Hydroxylase
F3'H	Flavonoid 3'-Hydroxylase
F3'5'H	Flavonoid 3' 5'-Hydroxylase
FG	Flavonol Glycoside
FLS	Flavonol Synthase
FNS	Flavone Synthase
FTC	Forest Tent Caterpillar
FW	Fresh Weight
GT	UDP-Glucose:Flavonoid Glucosyltransferase
GST	Glutathione S-Transferase
GUS	β-Glucuronidase

HCD	Hydroxycinnamic Acid Derivative
HL	High Light
HPLC	High Performance Liquid Chromatography
HPTII	Hygromycin Phosphotransferase II
IFR	Isoflavone Reducatse
IFS	Isoflavone Synthase
JA	Jasmonic Acid
LAR	Leucoanthocyanidin Reductase
LC-MS	Liquid Chromatography-Mass Spectrometry
LPI	Leaf Plastochron Index
MATE	Multi-Drug and Toxic Compound Extrusion
mDP	Mean Degree of Polymerization
MRP	Multidrug Resistance-Associated Protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOS	Nopaline Synthase
NPTII	Neomycin Phosphotransferase II
OMT	O-Methyl Transferase
PAR	Photosynthetically Active Radiation
PA	Proanthocyanidin
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PG	Phenolic Glycoside
PPO	Polyphenol Oxidase
ROS	Reactive Oxygen Species
SAM	S-Adenosyl Methionine
TDS	Tannin Deficient Seed
TT	Transparent Testa
UFGT	UDP-Glucose Flavonoid Glucosyltransferase
UV-B	Ultraviolet-B
$UV\text{-}B_{be}$	Biologically Effective Ultraviolet-B

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A forest tent caterpillar (FTC, *Malacosoma disstria*) on a trembling aspen (*Populus tremuloides*) leaf on the campus of the University of Victoria. Leaves of aspen and poplar produce a variety of physical, chemical, and biochemical defences to limit herbivore damage. Proanthocyanidins (PAs) are polyphenolic chemicals that are synthesized rapidly in trembling aspen leaves that have been damaged by FTC and other herbivores. The present study is aimed at understanding how this response is controlled at the level of gene expression.

Chapter 1: Introduction

1.1General introduction

Plants are faced with a multitude of herbivores, pathogens, competitors, and adverse conditions. The corresponding array of selective pressures has led to the evolution of numerous physiological and biochemical adaptations, including the production of many specialized chemicals or secondary metabolites that play crucial roles in plant life history strategies. Secondary metabolites serve many essential functions in plants, including protection against biotic and abiotic stresses, providing structural support, serving as signalling molecules, and mediating ecological interactions. The evolution of plant chemistry has been influenced by a complex group of selective forces, and in many cases, individual plant secondary metabolites may serve multiple adaptive functions.

Proanthocyanidins (PAs), also known as condensed tannins, are one group of secondary metabolites that are produced in a wide variety of vascular plants, including many important crops (Haslam, 1977; Chavan and Salunkhe, 1989; USDA, 2004). PAs are produced in many plant tissues, and are particularly abundant in the leaves and other tissues of many long-lived woody plants. In herbaceous plants, PAs are often produced in seeds but not vegetative tissues. PAs are one class of plant tannins, a heterogeneous group of phenolic compounds characterized by the ability to bind and precipitate protein. This property has long been exploited for the tanning of animal hides (Haslam, 1989). The two major classes of tannins are the hydrolysable tannins, including the gallotannins and ellagitannins, and the biochemically unrelated PAs.

Some plants allocate significant resources to PA production. PAs may protect vegetative tissues and seeds against biotic and abiotic stress (Rhoades and Cates, 1976; Dixon et al., 2005; Paolocci et al., 2007). As components of human and animal diets, PAs and other flavonoids are valued for their health-promoting properties. Epidemiological studies have linked the consumption of flavonoids and PAs to reduced risk of cancer and

heart disease (Cutler et al., 2008; Mursu et al., 2008). The health benefits of dietary flavonoids and PAs may be attributable in part to their strong antioxidant capacity and radical scavenging ability (Fuhrman and Aviram, 2001; Aron and Kennedy, 2008). In leguminous forage crops, PAs are an important agronomic trait; PAs present at moderate levels can bind to dietary protein and prevent pasture bloat in ruminants, a condition that can lead to major agricultural losses through death and reduced productivity of grazing livestock. PAs are thus important for plant, animal, and human health and understanding and manipulating their biosynthesis through plant genetic engineering or plant breeding may have wide ranging applications.

PA polymers are synthesized from flavonoid precursors. The flavonoid biosynthetic pathway has been well studied, although the final biosynthetic steps leading to PA polymer synthesis have not been elucidated. Regulation of flavonoid biosynthesis is also well studied and involves several interacting regulatory proteins that control the expression of flavonoid biosynthetic genes during development and in response to stress. Regulation of the branch of the flavonoid pathway leading to PAs has been investigated in relatively few species, but appears to involve the same classes of transcription factor proteins that regulate other flavonoid branch pathways. Genetic engineering of PA metabolism using regulatory factors that coordinately control multiple biosynthetic genes may be a useful strategy for producing enhanced, stress-tolerant plants and could also contribute to our understanding of PA biochemistry.

Populus (poplars, cottonwoods, and aspen, hereafter referred to collectively as poplar, unless otherwise specified) is a widely distributed tree genus of significant economic and ecological importance. Poplar, as the third plant genome to be sequenced, is also an important model organism in plant biology. Poplars produce PAs in leaves, roots, bark, and a variety of other tissues. Damage to leaves by insect feeding causes a rapid accumulation of PAs in some poplar species. This rapid PA accumulation is mediated by the transcriptional activation of genes encoding PA biosynthetic enzymes (Peters and Constabel, 2002; Tsai et al., 2006a). Up-regulation of PA biosynthesis following herbivore damage indicates that PAs may function as a chemical defense against herbivores in poplar. PAs have been hypothesized to protect plants by reducing the digestibility of plant tissues to herbivores (Swain, 1979; Feeny, 1969), although other

mechanisms through which PAs and other tannins can protect plants from herbivores may also be important (Appel, 1993; Steinly and Berenbaum, 1985; Barbehenn and Martin, 1994). Other functions for poplar PAs have also been proposed, including UV screening (Stevens and Lindroth, 2005), oxidative stress protection (Haikio et al., 2009), and defence against opportunistic pathogens (Stevens and Lindroth, 2005). Additionally, PAs in poplar leaves can alter the environment in which trees are growing by slowing the decomposition of dropped leaves, thereby affecting soil nutrient dynamics and influencing ecosystem processes and community structures (Schweitzer et al., 2004; Whitham et al., 2006). It is likely that PAs play multiple interrelated roles in poplar and other plants.

The present research examined stress-responsive PA metabolism in poplar at the molecular level. The expression of poplar PA biosynthetic genes in stress-treated leaves was profiled and correlated with the expression of putative regulatory genes of the *R2R3 MYB* class. Evidence is presented showing that the gene *MYB134* may be involved in the regulation of stress-induced PA metabolism. Identifying components of the PA regulatory system in poplar may permit genetic engineering of the pathway in poplar and other plants. This may be useful for identifying novel PA regulatory and biosynthetic genes and investigating the biological functions of PAs.

1.2 Overview of flavonoid and PA biosynthesis

1.2.1 The biosynthesis of flavonoids

Phenylpropanoids are a vast and diverse group of plant secondary metabolites that are involved in many important processes, including structural support, defence against pests and pathogens, protection from UV light and oxidative damage, pigmentation used in attracting pollinators and seed dispersers, regulation of seed germination, acclimation to abiotic stress conditions, intercellular signalling, mediating the establishment of symbiotic mycorrhizal fungal and rhizobial associations, and desiccation resistance (Forkmann, 1991; Harborne, 1994; Bohm, 1998; Dixon, 1999; Lewis and Davin, 1999; Winkel-Shirley, 2002; Gould, 2004; Antunes et al., 2006; Peer and Murphy, 2006).

Flavonoids are a subclass of phenylpropanoids that play many vital roles in plants. They are a diverse group of metabolites with over 6000 different compounds identified (Lepiniec et al., 2006).

The flavonoid biosynthetic pathway has been well characterized through biochemical and genetic analyses and flavonoid biosynthetic enzymes exhibit high conservation between plant species (Dixon and Pavia, 1995; Quattrocchio et al., 1998; Dong et al., 2001; Abrahams et al., 2002). The first enzymatic step of the phenylpropanoid biosynthetic pathway is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). PAL converts the amino acid phenylalanine and sometimes tyrosine (in monocots), produced through the shikimic-chorismic acid pathway, into cinnamic acid (Fig. 1-1). PAL requires no cofactor for activity. The reaction releases an ammonium ion which is recycled back into phenylalanine and tyrosine synthesis through glutamate (Weisshaar and Jenkins, 1998). The simple 9 carbon phenolic product, cinnamic acid, is the precursor for all phenylpropanoid molecules.

The core of the general phenylpropanoid pathway continues with an aromatic ring hydroxylation catalyzed by an oxygen requiring, NADPH-dependant cytochrome P450 enzyme, cinnamate-4-hydroxylase (C4H), to produce *p*-coumaric acid (Fig. 1-1). *p*-Coumaric acid constitutes the starting point for the synthesis of the coumarins and furanocoumarins, feeding deterrent and antimicrobial molecules that are in some cases

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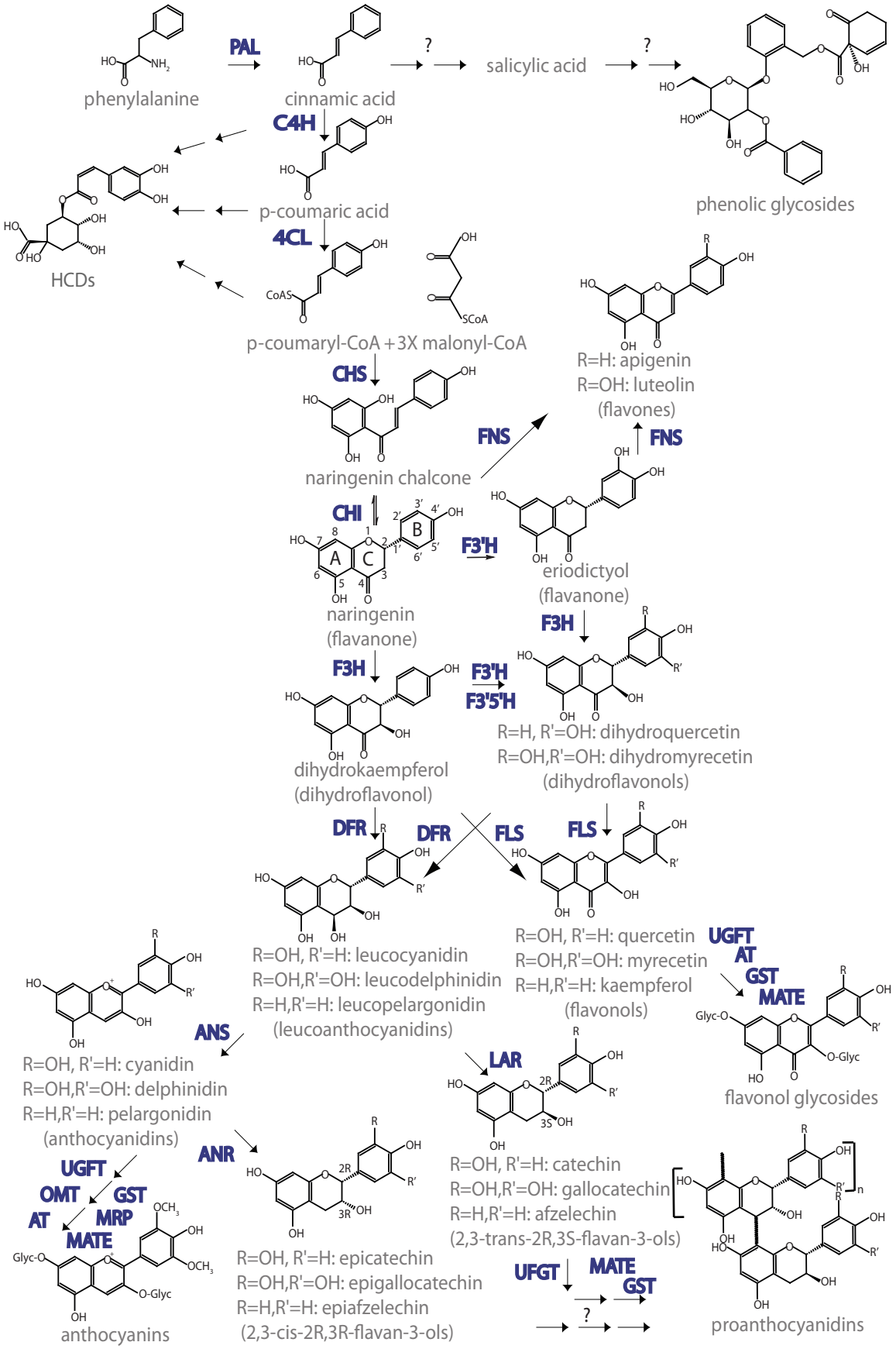
Figure 1-1. Biosynthetic pathway for the production of flavonoids, proanthocyanidins, and other classes of small phenolic compounds produced in poplar leaves (HCDs are represented by chlorogenic acid and phenolic glycosides by tremulacin). The flavanone naringenin illustrates the basic 15 carbon flavonoid structure with three rings (A, B, and C) showing the standard flavonoid numbering system including the C-2 and C-3 carbons at which PA starter units differ in stereochemistry. Enzymes are shown in blue. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; AT, acyltransferase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; MATE, multidrug and toxic compound extrusion transporter; MRP: multidrug resistance-associated protein transporter; UFGT, UDP-glucose:flavonoid glucosyltransferase; OMT, O-methyltransferase; GST, glutathione S-transferase.


phenylalanine
PAL
cinnamic acid
?
salicylic acid
?
phenolic glycosides
C4H
p-coumaric acid
4CL
HCDs
p-coumaryl-CoA +3X malonyl-CoA
CHS
naringenin chalcone
FNS
R=H: apigenin
R=OH: luteolin
(flavones)
FNS
CHI
F3'H
eriodictyol
(flavanone)
naringenin
(flavanone)
F3H
F3H
F3'H
F3'5'H
R=H, R'=OH: dihydroquercetin
R=OH,R'=OH: dihydromyrecetin
(dihydroflavonols)
dihydrokaempferol
(dihydroflavonol)
DFR
DFR
FLS
FLS
R=OH, R'=H: leucocyanidin
R=OH,R'=OH: leucodelphinidin
R=H,R'=H: leucopelargonidin
(leucoanthocyanidins)
R=OH, R'=H: quercetin
R=OH,R'=OH: myrecetin
R=H,R'=H: kaempferol
(flavonols)
UGFT
AT
GST
MATE
flavonol glycosides
ANS
R=OH, R'=H: cyanidin
R=OH,R'=OH: delphinidin
R=H,R'=H: pelargonidin
(anthocyanidins)
LAR
R=OH, R'=H: catechin
R=OH,R'=OH: gallocatechin
R=H,R'=H: afzelechin
(2,3-trans-2R,3S-flavan-3-ols)
UGFT
OMT
GST
AT
MRP
MATE
anthocyanins
ANR
R=OH, R'=H: epicatechin
R=OH,R'=OH: epigallocatechin
R=H,R'=H: epiafzelechin
(2,3-cis-2R,3R-flavan-3-ols)
UFGT
MATE
GST
?
proanthocyanidins

activated by UV radiation (Buchanan et al, 2000; Harborne, 1988). The last step in the general phenylpropanoid pathway leading to the production of precursors for flavonoid synthesis is a coenzyme-A (CoA) ligation catalyzed by the ATP-requiring enzyme 4-coumarate-CoA ligase (4CL), to produce *p*-coumaryl-CoA. 4CL enzymes can also activate of a variety of substituted cinnamic acids (e.g., caffeic acid (3,4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), 5-hydroxyferulic acid, and sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid)) (Hu et al., 1998; Harding et al., 2002). These hydroxycinnamoyl-CoA thiol esters may be used in the biosynthesis of lignans and monolignols through a series of hydroxylation, methylation, and dehydration reactions (Humphreys and Chapple, 2002).

The first committed step of the flavonoid pathway is catalyzed by chalcone synthase (CHS), a polyketide synthase which catalyzes the stepwise condensation of three molecules of malonyl-CoA, with one molecule of *p*-coumaryl-CoA to generate the 15 carbon flavonoid compound tetrahydroxychalcone or naringenin chalcone (Stafford, 1990; Winkel-Shirley, 2001). The malonyl-CoA is produced from acetyl-CoA by a cytosolic acetyl-CoA carboxylase (ACC) enzyme (Buchanan et al., 2000). Chalcone isomerase (CHI) then catalyzes a stereospecific ring closure isomerization generating 5,7,4'-trihydroxyflavanones (Fig. 1-1; Knogge et al., 1986; Jahne et al., 1993). The 15 carbon diphenylpropane flavonoid molecule is organized into three rings, an aromatic 'A' ring condensed to a heterocyclic 'C' ring, with an aromatic 'B' ring attached at position 2 of the C ring (Fig. 1-1). The positions of substituents are indicated by the numbering system shown in Fig. 1-1, with primed numbers used for the B ring.

From the flavanones, there are three major branches of flavonoid metabolism: isomerization to the isoflavonoids (in which the B ring is attached at position 3 instead of 2) catalyzed by the isoflavone synthases (IFS); introduction of a double bond between C-2 and C-3 catalyzed by flavone synthase (FNS) enzymes; or, a C-3 hydroxylation to produce the dihydroflavonols (Fig. 1-1; Stafford, 1990a). The C-3 hydroxylation is catalyzed by the Fe^{2+}- requiring, α-ketoglutarate-dependant diooxygenase, flavanone 3-β-hydroxylase (F3H) (Pelletier and Shirley, 1996; Koes et al., 2005). Hydroxylations of the B ring may be catalyzed by the NADPH-dependant cytochrome P450 monooxygenases flavanoid-3'-hydroxylase (F3'H) and flavanoid-3'5'-hydroxylase (F3'5'H) (Fig. 1-1;

Schoenbohm et al., 2000; Bogs et al., 2006). In petunia, a cytochrome b_5 protein, DIF-F, is required for F3'5'H activity (de Vetten et al., 1999) and a grapevine homologue of this protein (CytoB5) is co-expressed with *F3'H* and *F3'5'H* in a variety of grape tissues (Bogs et al., 2006), indicating that cytochrome b_5 proteins may be necessary for F3'H and F3'5'H function.

The dihydroflavonols, dihydrokaempferol, dihydroquercetin, and dihydromyricetin, depending on the B ring hydroxylation (Fig. 1-1), are substrates for the flavonol synthases (FLS), α-ketoglutarate-dependant diooxygenases that catalyze the formation of a C2-C3 double bond to yield the flavonols kaempferol, quercetin, and myricetin (Graham, 1998; Routaboul et al., 2006). These are usually glycosylated by UDP-glucose:flavonoid glucosyltransferases (UFGTs) to give the flavonol glycosides, which are stored in the acidic central vacuole of plant cells (Pelletier et al., 1997; Lukacin et al., 2001). The dihydroflavonols are also substrates for the NADPH-dependant reductase, dihydroflavonol reductase (DFR), which produces the leucoanthocyanidins (flavan-3,4-diols), such as leucocyanidin, leucodelphinidin, and leucopelargonidin (Fig. 1-1; Stafford, 1990a). DFR and other flavonoid reductases such as ANR (see Section 1.2.2) may exhibit substrate specificity, preferring dihydroflavonols with specific B ring hydroxylation patterns (Johnson et al., 1999, 2001; Xie et al., 2004).

Leucoanthocyanidins may be converted into the colored, achiral anthocyanidins, pelargonidin (4'-monohydroxylated B ring), cyanidin (3'4'-dihydroxylated B ring) and delphinidin (3',4',5'-trihybroxylated B ring) through the action of an α-ketoglutarate-dependant diooxygenase known as both leucoanthoyanidin diooxygenase (LDOX) and anthocyanidin synthase (ANS) (Fig. 1-1; Pelletier et al., 1997; Abrahams et al., 2003). Anthocyanidins may then be converted into the anthocyanins through the addition of sugar residues by UFGTs (e.g., 3- and 5-glycosyl and 5-rhamnosyltransferases) (Koes et al., 2005). Furthermore, the diversity of anthocyanins is increased through methylation by O-methyltransferases (OMT) and acylation (e.g., with acetyl, *p*-coumaroyl, and caffeoyl residues) of the glucose moiety by acyltransferases (AT) (Mol et al., 1998; Koes et al., 2005).

Anthocyanins may be transported into the central vacuole through several mechanisms (Grotewold and Davies, 2008). Accumulation of anthocyanins and other

flavonoids in the vacuole may be mediated by direct transport across the tonoplast (vacuolar membrane) or accumulation in ER-derived vesicles, which fuse into the central vacuole or sub-vacuolar compartments (Goodman et al., 2004; Poustka et al., 2007; Gomez et al., 2009). Genetic analyses have revealed that a number of Phi type glutathione-S-transferases (GSTs) (Edwards and Dixon, 2005) such as maize (*Zea mays*) BZ2 (Marrs et al., 1995), petunia AN9 (Alfenito et al., 1998), grapevine (*Vitis vinifera*) GST1 and GST4 (Conn et al., 2008), and carnation (*Dianthus caryophyllus*) FL3 (Larsen et al., 2003) are involved in vacuolar accumulation of anthocyanins. These may conjugate anthocyanins with glutathione to facilitate transport (Marrs, 1996) or, alternatively, function as flavonoid carrier proteins or 'ligandins' (Mueller et al., 2000). Mueller et al. (2000) were unable to detect a flavonoid-glutathione conjugating activity for petunia AN9 but found that AN9 could bind to flavonoids. The authors speculate that AN9 binds directly to anthocyanins and escorts them to vacuolar transporter proteins. GST-bound or glutathione-conjugated anthocyanins may be transported into the vacuole by members of the multidrug resistance-associated protein (MRP) subfamily of ATP-binding cassette (ABC) transporters, also termed GS-X pumps because of their high affinity for glutathionated compounds (Rea, 1999; Goodman et al., 2004; Klein et al., 2006). This is supported by the observation that a mutation in the maize MRP transporter gene, *MRP3*, results in a reduction and mislocalization of anthocyanin pigments. MRP3 is also co-regulated with other anthocyanin biosynthetic genes and localized to the tonoplast (Goodman et al., 2004).

A second mechanism for anthocyanin transport involves transporters of the multidrug/toxic compound extrusion (MATE) type. Overexpression of the tomato (*Lycopersicon esculentum*) anthocyanin regulatory gene *ANT1* (see Section 1.5) resulted in an up-regulation of anthocyanin pathway genes such as *CHS*, *CHI*, *DFR*, and *UFGTs*, together with the MATE transporter gene *MTP77*, which was hypothesized to be involved in anthocyanin transport into the vacuole (Mathews et al., 2003). Gomez et al. (2009) recently characterized two grapevine MATE transporters, anthoMATE1 and anthoMATE3, that function as tonoplast-localized H^+-dependent transporters of acylated anthocyanins. There are 58 MATE transporter genes in arabidopsis (*Arabidopsis thaliana*), and it is possible that they are involved in the transport and sequestration of a

number plant secondary metabolites (Omote et al., 2006), including other flavonoid metabolites (see below).

1.2.2 The biosynthesis of PAs

The late steps of the flavonoid pathway leading to PA biosynthesis have been poorly characterized until recently and many unresolved issues remain (reviewed by Marles et al., 2003; Xie and Dixon, 2005). PA polymers are composed of flavan-3-ol units that may have either 2,3-*cis* or 2,3-*trans* stereochemistry (Fig. 1-1). The configuration of C-2 is typically R, while that of C-3 may be R or S (e.g., 2R,3R-(-)-epicatechin and 2R,3S-(+)-catechin; Fig. 1-1). Feeding experiments with ^{14}C-labelled phenylalanine and cinnamic acid have shown that PA polymers are synthesized by sequential addition of "extender" units to the lower flavan-3-ol units (Jacques et al., 1977; Stafford et al., 1982). PA monomers may be referred to as procyanidins, prodelphinidins, and propelargonidins, corresponding to the B-ring hydroxylation of the anthocyanidin monomers released in catalyzed acid hydrolysis of PAs (Porter et al., 1986).

PA polymer length may be described as the mean degree of polymerization (mDP), which can range from dimers up to long polymers of dozens of extender units (Hernes and Hedges, 2004; Haslam, 1989; Stafford, 1990a). The proportion of 2,3-*cis*- and 2,3-*trans*-flavan-3-ol terminal and extender units, mDP, and degree of B-ring hydroxylation of PA polymers can exhibit considerable variation between species (Ayres et al., 1997; Hernes and Hedges, 2004). Subunits with 2,3-*cis* stereochemistry were found to be the most abundant PA extension unit in one study of PAs from 30 plant species (Foo and Porter, 1980). Proanthocyanidin polymers are primarily formed from $4\rightarrow6$ or $4\rightarrow8$ interflavan linkages (either α or β type) of the flavan-3-ol units (Fig. 1-1), but additional linkage types (e.g., ether interflavanyl C2-O-C7 and C2-O-C5 linkages) as well as branching have been documented in some species (Ferreira et al., 2005). Additionally, PAs may be derivatized by *O*-methylation, *O*-acylation, and *C*- and *O*-glycosylation. Esterification of the C-3 oxygen of the flavan-3-ol monomers with gallic

acid is common in some PA types (He et al., 2008). It is unknown how differences in mDP and linkage patterns in PAs among plants are determined.

An important model for genetic analyses of flavonoid and PA metabolism is the synthesis of PAs in the seed coat (testa) endothelial cells of arabidopsis. Arabidopsis seeds accumulate both PAs and flavonols (Lepiniec et al., 2006). PAs accumulate in seed coat endothelium, where they function to strengthen the seed coat and protect the endosperm and embryo. As endothelial cells die during seed desiccation, the normally colourless, vacuolar PAs are released and oxidized. These oxidized PAs cross-link polysaccharides and phenolics within the cell wall, giving the seed a brown color (Shirley et al., 1995; Pourcel et al., 2005; Kitamura et al., 2004). This brown seed coloration provides a useful means by which to visually identify mutants in PA biosynthesis, and numerous PA biosynthetic and regulatory genes (see Section 1.5) have been identified in arabidopsis through mutant screens for seeds lacking brown oxidized PAs. These include numerous *transparent testa* (*tt*) and *tannin-deficient seed* (*tds*) mutants (Shirley et al., 1995; Abrahams et al., 2002; Lepiniec et al., 2006).

The origin of the 2,3-*cis*-flavan-3-ols long remained a mystery, since all of the flavonoid metabolites that are precursors to PA synthesis exhibit a 2,3-*trans* stereochemistry (Fig. 1-1). Recently, the enzyme anthocyanidin reductase (ANR), which acts on the anthocyanidins produced by ANS to form the 2,3-*cis*-flavan-3-ols, was characterized in arabidopsis and the model legume *Medicago truncatula* (Xie et al., 2003, 2004). Analysis of an arabidopsis seed coat PA mutant led to the identification of the enzyme with anthocyanidin reductase activity. Mutations in the *BANYULS* (*BAN*) gene in arabidopsis cause accumulation of red anthocyanins rather than PAs in seed coat endothelia (Albert et al., 1997). This phenotype and the sequence similarity of the predicted BAN protein to DFR led to the hypothesis that *BAN* encodes an enzyme that reduces leucoanthocyanidins to 2,3-*trans*-flavan-3-ols (Devic et al., 1999). However, Xie et al. (2003) could not detect activity using leucoanthocyanidins as substrates for recombinant BAN. The authors attempted to functionally characterize the *BAN* genes by constitutive overexpression in transgenic tobacco. Flower petals of the transgenic tobacco lacked the characteristic pink coloration that normally results from anthocyanin accumulation. Staining of petals with the PA- and flavan-3-ol-specific stain

dimethylaminocinnamaldehyde (DMACA) (Feucht and Treutter, 1990) and hydrolysis of extracts in acidified butanol (Porter et al., 1986) revealed the presence of PAs, although the precise identity and localization of the reacting compounds was not determined. The authors proposed that the BAN enzyme produces 2,3-*cis*-flavan-3-ols from achiral anthocyanidin precursors, and reported that both recombinant BAN enzymes could convert cyanidin, pelargonidin, and delphinidin to epicatechin, epigallocatechin, and epiafzelechin, respectively. They subsequently showed that the enzymes exhibited different substrate preferences (Xie et al., 2004). In support of the placement of ANR downstream of ANS in the PA biosynthetic pathway (Fig. 1-1), it was also shown that the arabidopsis *tds4* mutant is deficient for a functional *ANS* gene (Abrahams et al., 2003). Subsequent to this analysis, *ANR* genes have been identified in a number of species, and ANR activity in plant extracts, expression of functional recombinant ANR enzyme, or ANR function in transgenic tobacco has been reported for several species (Punyasiri et al., 2004; Bogs et al., 2005; Pfeiffer et al., 2006; Gagne et al., 2009; Singh et al., 2009).

Formation of the 2,3-*trans*-flavan-3-ols (Fig. 1-1) is catalyzed by enzymes with leucoanthocyanidin reductase (LAR) activity (Tanner et al., 2003). The enzymatic formation of (+)-catechin and other 2,3-*trans*-flavan-3-ols from flavan-3,4-diol precursors through an NADPH-dependant reduction that removes the C-4 hydroxyl group was first demonstrated in extracts from Douglas-fir and other plants (Stafford and Lester, 1984; Kristiansen, 1986; Tanner and Kristiansen, 1993). The first *LAR* gene was recently cloned from the PA-rich legume silverleaf desmodium (*Desmodium uncinatum*). Tanner et al. (2003) purified LAR activity from leaves, obtained sequences of tryptic peptides from the purified protein by mass spectrometric analysis, amplified a partial sequence using degenerate primers, and cloned a putative *LAR* transcript sequence from a cDNA library. The *D. uncinatum* LAR protein is a member of the same protein superfamily as ANR and DFR but is more closely related to isoflavone reductases (IFR, NADPH:isoflavone oxidoreductase that reduce isoflavones to isoflavanones) and IFR-like proteins, such as chick pea (*Cicer arietinum*) IFR (Tiemann et al., 1991) and a loblolly pine (*Pinus taeda*) phenylcoumaran benzylic ether reductase (PCBER) (Gang et al., 1999; Tanner et al., 2003). Recombinant *D. uncinatum* LAR protein was shown to convert leucocyanidin, leucodelphinidin, and leucopelargonidin to catechin,

gallocatechin, and afzelechin respectively (Tanner et al., 2003; Fig. 1-1). *LAR* gene sequences have since been isolated from additional species, and functionally characterized in some cases (Bogs et al., 2005; Pfeiffer et al., 2006).

Formation of oligomers and polymers from flavan-3-ol initiation units remains poorly understood, and both enzymatic and non-enzymatic mechanisms have been proposed. *In vitro* synthesis of PAs using flavan-3,4-diols or their derivatives as electrophiles and flavan-3-ols as nucleophiles (Creasy and Swain, 1965; Stafford and Lester, 1982; Hemingway and Laks, 1985) has suggested possible mechanisms of PA precursor condensation (He et al., 2008). It has been hypothesized that extension units of 2R,3S stereochemistry could be derived from leucoanthocyanidins *via* C-4 carbocations or quinone methide intermidiates while 2R,3R extension units may arise from quinone methides derived from achiral anthocyanidins or from 2R,3R quinone methides produced from leucoanthocyanidins through a flav-3-en-3-ol intermediate (Jacques et al., 1977; Marles et al., 2003; Hemingway and Laks, 1985; Hemingway and Foo, 1983). So far, neither the enzymes responsible for the production of these intermediates nor the chemical intermediates themselves have been isolated in plants. It has also been hypothesized that 2R,3S and 2R,3R extension units are formed from flavan-3-ols, produced by ANR, LAR, or another flavonoid reductase, that are oxidized by a polyphenol oxidase (PPO)-like enzyme. PPOs are members of a class of copper-containing glycoprotein that catalyze the reduction of O_2 to H_2O with a concomitant oxidation of phenolic substrates. Flavan-3-ols may be oxidized to quinones that are converted to carbocations via coupled non-enzymatic oxidation (Xie and Dixon, 2005). Determining the chemical intermediates for PA extension units and the enzymes or mechanisms involved in polymerization, as well as their subcellular localization, remains an important unresolved issue in PA biochemistry.

The enzymes of flavonoid biosynthesis are cytosolic and thought to be organized into ER-associated “metabolons” or multi-enzyme complexes. Arabidopsis *CHS*, *CHI*, *F3H*, and *DFR* have been shown to be co-localized on the ER, and the complex is thought to be anchored by cytochrome P450 proteins such as F3’H and C4H (Winkel-Shirley, 1999; Saslowsky and Winkel-Shirley, 2001; Jorgensen et al., 2005). Studies using light and electron microscopy have shown that in some cases PAs are synthesized

in vesicles that bud off the ER and then fuse with the large central vacuole, where PAs accumulate (Bauer and Walkinshaw, 1974; Parham and Kaustinen, 1977; Rao, 1988; Stafford, 1990), although PA precursors may be also be transported directly into the vacuole from the cytoplasm (Debeaujon et al., 2001). The accumulation of PAs in cell walls has also been reported (e.g., Streit and Fengel, 1995).

A PA-deficient arabidopsis mutant, *tt12*, implicates the involvement of a MATE–type transporter in vacuolar PA polymer accumulation (Debeaujon et al., 2001; Marinova et al., 2007). The arabidopsis *TT12* gene encodes a tonoplast-localized MATE transporter. Microscopic examination of the endothelial cells of immature *tt12* seeds stained with vanillin, which reacts with flavan-3-ols and PA terminal units to form a red pigment, revealed an accumulation of lower levels of (-)-epicatechin or PAs that were localized to the cytoplasm rather than the vacuole (Debeaujon et al., 2001). Marinova et al. (2007) proposed that TT12 transports glycosylated (-)-epicatechin into the vacuole *in vivo*. However, although yeast vesicles expressing TT12 were able to transport the anthocyanin cyanidin-3-O-glucoside, no transport of glycosylated flavonols, glycosylated (+)-catechin, (+)-catechin aglycone, or a procyanidin dimer was detected (Marinova et al., 2007). TT12-mediated transport of cyanidin-3-O-glucoside was shown to be through an H^+-antiport mechanism. (+)-Catechin-3-O-glucoside was shown to inhibit cyanidin-3-O-glucoside transport by TT12 and taken together with the *tt12* mutant phenotype, a role in PA precursor transport was hypothesized (Marinova et al., 2007).

(-)-Epicatechin glycoside, the predicted TT12 substrate in arabidopsis, which lacks an *LAR* gene and produces only (-)-epicatechin-based PAs (Abrahams et al., 2002), has not been tested. Since the chemical identity of the PA polymer extension precursor remains to be determined, it is also possible that the *in vivo* substrate for TT12 transport is an unknown PA biosynthetic intermediate. Although TT12 was localized to the tonoplast, it is unknown whether TT12 functions to transport PA precursors directly into the vacuole or into the ER-derived pro-vacuoles that fuse with the central vacuole. *TT12*-like genes have been identified in several additional species, supporting a conserved role for these transporters in PA metabolism (Mathews et al., 2003; Chai et al., 2009), although functional characterization of these additional TT12-like proteins is lacking. Supporting the role of an H^+-flavan-3-ol-glycoside antiporter in PA precursor transport,

a mutation in the arabidopsis *AHA10* gene, encoding a H^+-pump that may be involved in establishing a proton gradient across the tonoplast, results in compromised seed coat PA accumulation (Baxter et al., 2005).

Glycosylation of (-)-epicatechin, proposed by Marinova et al. (2007) to be important for TT12-mediated transport, has recently been demonstrated by the product of a gene that is co-regulated with PA biosynthetic genes in *M. truncatula* (Section 1.6.3). Pang et al. (2008) identified a glycosyltransferase gene, *UGT72L1*, that is highly expressed in the PA-accumulating seed coat. Recombinant UGT72L1 was able to glycosylate (-)-epicatechin, and very weakly (+)-catechin and cyanidin (Pang et al., 2008).

Additional proteins involved in PA metabolism have been identified through analysis of arabidopsis *transparent testa* mutants. In the *tt10* mutant, seed coat browning is delayed, and the un-browned testa contain higher levels of soluble (-)-epicatechin, and procyanidin dimers, trimers, and tetramers (Pourcel et al., 2005). Pourcel et al (2005) hypothesized that the *TT10* locus encodes a PPO that catalyzes the enzymatic oxidation of PAs in seed coat endothelia, and that the delayed browning was the result of their eventual autoxidation by O_2. The authors determined that *TT10* encodes a putative laccase-type PPO that is expressed in developing seed coat endothelia and predicted to be secreted into the apoplast. In immature seeds in which *TT10* is strongly expressed but oxidative browning has not yet occurred, addition of (+)-catechin or (-)-epicatechin resulted in overnight browning in wild type and *tt4* (i.e., mutants lacking a functional *CHS*) but not *tt10* testa. LC-MS analysis of extracts from *tt4*, which produces no flavonoids, *tt10* and wild type seeds after incubation with (-)-epicatechin revealed that TT10 activity results in the accumulation of quinone methide (-)-epicatechin dimers and trimers with unusual and additional interflavan linkages compared to typical PAs. The authors hypothesize that the apoplastic TT10 laccase mediates oxidation of the (-)-epicatechin and PAs released during seed desiccation, although this was not experimentally investigated.

Kitamura et al. (2004) isolated a *transparent testa* mutant (*tt19*) that also failed to produce the anthocyanins that normally accumulate in the basal region of the arabidopsis stem. The *tt19* mutants exhibited light brown testa that eventually became as dark as wild

type during long term storage. Microscopic examination of seed coat endothelial cells stained with vanillin revealed that staining was restricted to small vacuole-like structures rather than the large central vacuoles characteristic of wild type cells. After maturation, wild type endothelia stained only weakly, while red staining was observed throughout *tt19* mutant endothelial cells. *TT19* was found to encode a Phi type GST sharing some sequence similarity with petunia AN9. *AN9* overexpression complemented the anthocyanin accumulation defect in *tt19* plants, but not the transparent testa phenotype. The authors hypothesized that TT19 functions in a similar fashion to AN9 in mediating vacuolar anthocyanin transport, but may function differently in PA metabolism. The vanillin staining showed that PA precursors accumulated in membrane bound compartments and that oxidation during seed desiccation was delayed, suggesting that PA precursors may be transported into membrane bound vesicles by TT12 while TT19 acts at the vesicle trafficking stage of PA production in arabidopsis seed coat endothelia.

An analysis of flavonoid transport in anther tapetum cells has also contributed to the understanding of TT12 and TT19 function (Hsieh and Huang, 2007). The tapetum is the innermost of four layers of cells enclosing the microspores which mature into pollen in anthers. These cells are characterized by storage organelles containing compounds such as flavonoids that are discharged after programmed cell death to coat mature pollen. Flavonoids are stored in unique ER-derived tapetal vesicles known as tapetosomes. Hsieh and Huang (2007) showed with staining and co-localization with the ER marker calreticulin that in *Brassica napus*, flavonoids (primarily quercetin and kaempferol glycosides) appeared to first accumulate in the ER network and then in spherical particles which were likely the tapetosomes. Interestingly, in arabidopsis *tt12* and *tt19* mutants, flavonoids did not accumulate in tapetosomes but instead accumulated in the cytosol at lower levels. Tapetosome formation itself was unaffected. This suggests that TT12 and TT19 are both necessary for the transport of flavonoid metabolites across the ER membrane into the lumen (Hsieh and Huang, 2007), and it is possible that they play a similar role in the transport of PA precursors to the central vacuole through ER-derived compartments in other cell types.

1.3 Investigation of the PA pathway in poplar

1.3.1 Poplar as a model system for investigating defence phenylpropanoid metabolism

Poplars are dioecious, deciduous trees that have a wide distribution in the Northern Hemisphere (Bradshaw et al., 2000). These trees are important components of numerous ecosystems, ranging from subtropical to boreal forests. Poplars are members of the Salicaceae family (Class Magnoliopsida, Order Malpighiales), which also includes willows (*Salix* spp.). There are approximately 30 poplar species divided among six sections, which are *Abaso*, *Aigeiros* (which includes Eastern cottonwood, *P. deltoides*), *Leucoides*, *Populus* (which includes the European aspen, *P. tremula*, white poplar, *P. alba*, and the North American trembling aspen, *P. tremuloides*), *Tacamahaca* (which includes black cottonwood, *P. trichocarpa*), and *Turanga*. Interspecifc hybridization within sections, both naturally and for breeding or research purposes, is common (e.g., *P. tremula x alba* and *P. tremula x tremuloides*), and also occurs between sections in some cases (e.g., *P. trichocarpa x deltoides*) (Eckenwalder, 1985, 1996; Hamzeh and Dayanandan, 2004; Hamzeh et al., 2006).

Poplar has long been an important model species in tree biology (Bradshaw et al., 2000; Taylor, 2002; Cronk, 2005; Taylor, 2002; Yin et al., 2004; Tsai et al., 2006b; Jansson and Douglas, 2007). Recently, the genomic sequence of black cottonwood (clone "Nisqually 1") was published (Tuskan et al., 2006), enhancing the utility of poplar as a model system for molecular biology and genomics-based studies of trees (Sjodin et al., 2009). The poplar genome is of relatively modest size for a plant genome, roughly four times larger than arabidopsis, with ~450 Mb of sequence predicted to contain approximately 45,500 genes (Tuskan et al., 2006). The value of poplar as a model system is enhanced by genomics resources such as large expressed sequence tag (EST) collections (Schrader et al., 1998; Bhalerao et al., 2003; Kohler et al., 2003; Nanjo et al., 2004; Schrader et al., 2004) and microarray platforms such as the Treenomix 15,500 element cDNA microarray (Ralph et al., 2006), and the Umeå Plant Science Centre's

POP2 25,000 cDNA microarray (Sterky et al., 2004; Taylor et al., 2005). More recently, whole genome arrays such as the Affymetrix GeneChip® Poplar Genome Array have been developed. This array contains 61,251 probe sets representing more than 56,000 transcripts and predicted genes, produced using mRNA and EST sequences from 13 *Populus* species and hybrids as well as the predicted *P. trichocarpa* Nisqually 1 gene set (Affymetrix, 2005).

As a species that must cope with large numbers of pests and pathogens whose populations may go through hundreds or thousands of generations in the lifespan of a single individual or clone, poplar is an excellent model for studying plant defense and co-evolution between pests and pathogens and their hosts (Whitham et al., 1996). Numerous insect species have been found on the foliage of poplar species, and major outbreak defoliators such as the forest tent caterpillar (FTC, *Malacosoma disstria*) and the large aspen tortix (*Choristoneura conflictana*) can cause large scale damage to commercially valuable poplar species (Davidson and Prentice, 1968; USDA-Forest-Service, 1979; Lindroth and Hwang, 1996a). Poplars have evolved a variety of constitutive and inducible defence mechanisms, including defensive secondary metabolites (Section 1.3.2), biochemical defences (e.g., defensive proteins such as digestion inhibitors and enzymes), physical defences (e.g., defensive structures such as trichomes), and ecological defences (e.g., attraction of parasitoids) (reviewed in Constabel and Major, 2005; Philippe and Bohlmann, 2007).

Poplar species are an excellent system in which to study the regulation of phenylpropanoid biosynthesis and the involvement of transcription factors in mediating defensive flavonoid accumulation or other defence responses. Abundant publicly available mRNA transcript sequence data from a variety of stress-treated poplar tissues (e.g., Nanjo et al., 2004; Ralph et al., 2006) and the Nisqually 1 genome sequence can be utilized in the identification of regulatory genes, determination of biosynthetic gene family sizes, and analysis of promoter regions of stress-responsive genes. Furthermore, well established protocols for *in vitro* propagation and *Agrobacterium tumefaciens*-mediated transformation of poplar facilitate the study of gene function through constitutive expression or silencing of a gene of interest (Han et al., 2000; Busov et al., 2005).

1.3.2 PAs and other soluble phenolics in poplar

The phenolic metabolism of poplar is more complex than that of the primary model plant species, arabidopsis. The levels of PAs and other phenolic metabolites can vary considerably both between and within species (Hemming and Lindroth, 1995), a characteristic than can be exploited in analyses of phenylpropanoid regulation in poplar. Poplar leaves typically accumulate several classes of phenolic metabolites, including several phenolic glycosides (PGs), numerous hydroxycinnamic acid derivatives (HCDs) and their esters, and flavonoids such as flavonol glycosides, anthocyanins, and PAs (Bendz and Haglund, 1968; Pearl and Darling, 1968; Klimczak et al., 1972; Palo, 1984; Lindroth and Hwang, 1996a; Warren et al., 2002; Fig. 1-1). PGs and PAs are generally the most abundant soluble phenolics in poplar leaves (Lindroth and Hwang, 1996b).

Among the non-polymeric flavonoids, flavonol glycosides are particularly abundant in poplar leaves. For example, black cottonwood produces a number of quercetin and kaempferol glycosides in leaves (Warren et al., 2003), and aspen (*P. tremuloides* and *P. tremula x tremuloides* hybrids) leaves have been reported to contain quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside (Kinsley and Pearl, 1967), as well as myricetin and isorhamnetin (3'-methoxy-4'-hydroxy-flavonol) glycosides (Haikio et al., 2009). Under some conditions, poplars produce anthocyanins such as cyanidin-3-rhamnosylglucoside, cyanidin-3-glucoside and cyanidin-3-galactoside (Bendz and Haglund, 1968; Chang et al., 1989), particularly in juvenile leaves and senescing leaves in the fall (Keskitalo et al., 2005). Numerous flavonoid aglycones, including various chalcones, dihydrochalcones, flavanones, flavones, flavan-3-ols and dihydroflavonols have been documented in poplar bud exudates, which have been studied extensively due to the usefulness of their chemical composition for chemosystematics and chemotaxonomy (Greenaway et al., 1991a; Greenaway et al., 1992b; Valant-Vetschera, 2006; Tsai et al., 2006a and references therein). Members of these flavonoid classes also include biosynthetic precursors for PA production and are thus expected to be produced in cells synthesizing PAs, although they may not accumulate to detectable

levels. In leaves of one hybrid aspen (*P. tremula x tremuloides*) clone used in the present study, extensive HPLC and LC/MS analysis could detect no monomeric flavonoids other than flavonols (Susan Marles, Agriculture and Agri-Food Canada, unpublished data), although (+)-catechin was detected in a separate analysis of equivalent leaves from saplings of the same genotype (Riitta Julkunen-Tiitto, University of Joensuu, Finland, unpublished data) and in leaves of several other hybrid aspen clones (Haikio et al., 2009). Additional flavonoids, particularly flavones and flavone glycosides, have been identified in the leaves of some poplar species (Kinsley and Pearl, 1967; Pearl and Darling, 1971, Pearl and Darling, 1977; Greenaway et al., 1991b; Greenaway et al., 1992a).

Numerous quinate or shikimate esters of hydroxycinnamic acids and their derivatives (particularly coumaryl-, caffeoyl-, and feruloyl-quinate conjugates) accumulate in poplar leaves (Pearl and Darling, 1968; Pearl and Darling, 1971; Tsai et al., 2006a). For example, 14 different hydroxycinnamamoyl-quinate esters were detected in leaves of one *P. tremula x alba* hybrid clone (Tsai et al, 2006a). In most poplar species, chlorogenic acid (3-Ocaffeoyl-quinate, shown in Fig. 1-1) is the most abundant HCD in leaves. Consistent with the diversity of HCDs produced in poplar, the *hydroxycinnamoyl-CoA quinate/shikimate hydroxycinnamoyl transferase* (*HCT*) gene family is expanded in poplar in comparison to arabidopsis (Tsai et al, 2006a), which only produces hydroxycinnamoyl-quinate conjugates as substrates for C-3 hydroxylation in lignin biosynthesis (Hoffmann et al., 2003).

The PGs are a group of glycosylated phenolics that are based on the compound salicin (*o*-hydroxymethylphenyl,β-D-glucopyranoside). PGs are abundant in leaves of poplar and willow species; in trembling aspen, they can constitute more than 14% of leaf dry weight (DW). The salicin/PG biosynthetic pathway has not been characterized. Salicin is thought to be derived from salicylic acid produced from phenylpropanoid pathway-derived (i.e., cinnamate-derived *o*-coumaric acid or *o*-hydroxylated carboxybenzene (benzoic acid)) or shikimate pathway-derived (isochorismate) precursors (Zenk, 1967; Metraux, 2002; Tsai et al., 2006a). Common PGs in trembling aspen foliage include salicin, tremuloidin (salicin benzoylated at C-2 of the glucopyranose moiety), salicortin (salicin esterified with a 1-hydroxy-6-oxo-2-cyclohexene-1-carboxyloyl group *ortho* to the glycosylation on the benzene ring), and tremulacin (salicin that is both

benzoylated and esterified with the cyclohexanenone group; the structure of tremulacin is shown in Fig 1-1) (Clausen et al., 1989; Lindroth and Hwang, 1996a; Pearl and Darling, 1970; Thieme and Benecke, 1971). Numerous additional PGs have been identified in various poplar and willow species. PGs are potent anti-herbivore chemicals, and numerous studies finding significant negative correlations between foliar PG levels, or PGs in artificial diets, and herbivore feeding choice or performance have been reported (Reichardt et al., 1988; Reichardt et al., 1990; Lindroth and Bloomer, 1991; Arteel and Lindroth, 1992; Hemming and Lindroth, 1995; Hwang and Lindroth, 1997; Hwang and Lindroth 1998; Lindroth et al., 1997; McDonald et al., 1999; Osier et al., 2000a; Osier and Lindroth, 2006; Donaldson and Lindroth, 2007). Leaf PG concentrations can exhibit considerable within- and between-clone variation, affecting the suitability of poplar foliage for herbivores. One study reported a >10-fold between-clone variation for salicortin in leaves of 31 trembling aspen clones (Lindroth and Hwang, 1996b). While PGs have strong effects on some insect herbivore species, others possess mechanisms for detoxification or sequestration that make high PG foliage suitable for consumption (Hemming and Lindroth, 2000; Donaldson and Lindroth, 2004; Section 1.4.2).

Poplars produce PAs in many tissues, including leaves, stems, roots, and bark. In trembling aspen leaves, PAs have been shown to accumulate specifically in the palisade mesophyll cells on the adaxial (i.e., upper) side of the leaf as well as abaxial epidermal and spongy mesophyll cells (Kao et al., 2001; Tsai et al., 2006b). PAs accumulate at very high levels in leaves of some poplar species. For example, species such as trembling aspen and narrowleaf cottonwood (*P. angustifolia*) typically have leaf PA concentrations ranging from 10-20% DW or higher (Lindroth and Hwang, 1996b; Rehill et al., 2006). Levels of PAs also exhibit variability within species. Lindroth and Hwang (1996b) reported that PA concentrations in mature leaves of 31 wild trembling aspen clones varied 2.5-fold between clones. Significant genetic variability in leaf PA levels has been observed repeatedly for trembling aspen (Hwang and Lindroth, 1997; Osier and Lindroth, 2006; Madritch et al., 2009) and other poplar species (Bailey et al., 2006; Rehill et al., 2006; Harding et al., 2005). In a number of poplar species, leaf PA levels are higher in developmentally mature leaves compared to juvenile leaves, and can increase in mature

leaves over the course of each growing season (Osier et al., 2000b; Rehill et al., 2006; Lindroth et al., 2007).

Foliar PA concentrations in poplar are affected by environmental conditions. In addition to increases following herbivore defoliation (Section 1.4.3), leaf PA concentrations have been shown to respond to nutrient availability, light levels, CO_2 concentration, and O_3 exposure, although there is considerable variability in the level of response even within species (Kinney et al., 1997; Lindroth et al., 1997; Hemming and Lindroth, 1999; Lindroth et al., 2001; Osier and Lindroth, 2001; Holton et al., 2003; Harding et al., 2005; Liu et al., 2005; Haikio et al., 2009). Increased PA levels in leaves of trembling aspen grown in low nutrient soil or under elevated light have been reported in a number of studies (Hemming and Lindroth, 1999; Mansfield et al., 1999; Agrell et al., 2000; Osier and Lindroth, 2001; Osier and Lindroth, 2006). Similarly, growth under elevated CO_2 and O_3 levels have been found to cause increased leaf PA levels in trembling aspen in a number of studies (e.g., Kinney et al., 1997; Roth et al., 1998; Oksanen et al., 2001; Holton et al., 2003). Observations of increased PA production under elevated CO_2, reduced N availability, or elevated light support the hypothesis that phenolic metabolism responds to resource availability (Bryant et al., 1983; Bryant et al., 1985; Herms and Mattson, 1992; Section 1.4.3), but other effects may also be important.

PA chemical structure is likely an important determinant of biological activity, influencing chemical properties related to putative anti-herbivore functions (e.g., protein precipitation and oxidative activity) (Ayres et al., 1997; Barbehenn et al., 2006; Section 1.4.2) and nutrient cycling in soils (Kraus et al., 2003a; Maie et al., 2003; Nierop et al., 2006; see below). Several structural analyses of poplar PAs have been reported. These analyses indicate that different poplar species produce structurally distinct PAs. Ayres et al (1997) used ^{13}C-nuclear magnetic resonance (NMR) spectroscopy to study the PAs produced in leaves of trembling aspen and balsam poplar (*P. balsamifera*). Balsam poplar PAs were characterized by a composition of 61% procyanidin (*o*-dihydroxylated B-ring) monomers with 51% 2,3-*cis* stereochemistry, and an mDP of 5.5. Trembling aspen leaf PAs were composed of 43% procyanidin, with 64% 2,3-*cis* stereochemistry, and a mDP of 7. Hernes and Hedges (2004) reported the results of a survey of PA structure from numerous plant sources including black cottonwood leaves. Their study employed GC-

MS analysis of derivatized PA monomers after acid hydrolysis of plant extracts, a technique that does not allow discrimination between released PA units and free flavan-3-ols and flavan-3,4-diols if they are present. Black cottonwood leaf PAs were found to be composed of 77% procyanidin, primarily 2,3-*trans* initiator units but 65% 2,3-*cis* extension units, and an mDP of 5.32. Schweitzer et al. (2008) described preliminary results of PA analysis for several poplar species. In their analyses, trembling aspen PAs were found to be composed of 2,3-*cis* procyanidin/prodelphinidin and 2,3-*trans* prodelphinidin units, with polymer lengths ranging from 9-12 units. PAs from narrowleaf and Fremont cottonwood (*P. fremontii*) were composed of 2,3-*trans* procyanidin initiation units, but while Freemont cottonwood PAs have primarily 2,3-*cis* procyanidin extension units and lengths ranging from 1-6, narrowleaf cottonwood PAs were composed of 2,3-*cis* procyanidin and prodelphinidin extension units with polymer lengths ranging from 15-20. Schweitzer et al. (2008) suggested that environmental conditions resulting in altered PA levels in poplar leaves do not result in changes to polymer structure, but this requires experimental investigation.

Poplars are foundation species (i.e., dominant primary producers) in a number of ecosystems, and PAs in leaves, both on the plant and in the ground litter after they have been shed, can have important ecosystem effects (Bailey et al., 2005; Lindroth and Hwang, 1996a; LeRoy et al., 2006; Whitham et al., 2006). Considerable research has shown that PAs in shed leaves from poplar and other tree species can impact microbial communities and influence carbon and nitrogen cycling in soils (Bradley et al., 2000; Joanisse et al., 2007; Madritch et al., 2007; Madritch et al., 2009; Schweitzer et al., 2007; Schweitzer et al., 2008). PAs in forest litter can complex with proteins and other macromolecules, reducing rates of decomposition. Litter decomposition rates and annual rates of N mineralization and nitrification in soil have been shown to be significantly negatively correlated with poplar leaf PA concentrations (Schweitzer et al., 2004). These effects on microbial communities, litter decomposition, and nutrient cycling can affect the productivity of other producers in the ecosystem, consequently influencing ecosystem-scale processes. Traits that allow trees adapt to nutrient limited, high-PA soils, such as compensatory fine root production (Fischer et al., 2006) or specific mycorrhizal associations (Kraus et al., 2003b; Schweitzer et al., 2004), may confer a competitive

advantage to high PA-producing trees. The indirect effects of leaf PAs on soil properties and plant productivity may therefore be among the selective pressures influencing the evolution of PA accumulation patterns and PA structural characteristics in leaves of poplar and other species.

1.4 Stress-protective functions of flavonoids and PAs

1.4.1 Stress-induced flavonoid metabolism

The biological functions of PAs in plant tissues are not fully understood (Sections 1.4.2-1.4.4). PAs share many chemical properties with related flavonoid metabolites, including strong antioxidant capacity (Hagerman et al., 1998a; Bors and Michel, 1999) and absorption in the UV-B range (280-320nm) (Revilla et al., 1991). Additionally, like other flavonoids that typically accumulate in poplar leaves, such as flavonol glycosides and anthocyanins, PAs are sequestered in the large central vacuoles within plant cells. A consideration of the functions of related flavonoid products in plant stress responses may aid in understanding the roles of PAs in stress protection.

Numerous biological and environmental stress conditions induce the accumulation of phenylpropanoids and flavonoids in plants (Dixon and Paiva, 1995; Merzlyak and Chivkunova, 2000; Gopalakrishna et al., 2001; Stewart et al., 2001; Lillo et al., 2008). Many of the stresses that induce flavonoid accumulation in plants involve the production of increased levels of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) (Mittler, 2002). These highly reactive molecules can damage cellular components, including photosynthetic reaction centers, leading to photoinhibition (reduced photosynthetic capacity) (Powles, 1984; Trivedi et al., 1997). Plants have evolved a variety of systems to control ROS homeostasis. These include antioxidants such as ascorbate, glutathione, α-tocopherol, and carotenoids, and enzymes such as catalase, superoxide dismutase, thioredoxins, peroxiredoxins, and the enzymes of the ascorbate-

glutathione cycle (reviewed in Ajay et al., 2002; Gechev et al., 2005). It is becoming increasingly recognized that ROS are far more than just damaging byproducts of stress. ROS also play a fundamental role in stress signalling pathways mediating plant acclimation (Orozco-Cardenas et al., 2001; Foyer and Noctor, 2005; Gechev et al., 2005). Mechanisms that modulate ROS levels are therefore important components of plant stress-response signalling pathways (Vanderauwera et al., 2005). It has been hypothesized that some stress-induced flavonoids function to mitigate oxidative stress in cells through their light filtering and antioxidant capacities.

Anthocyanins are produced in leaves of many plants under a variety of stress conditions, including O_3 exposure, nutrient deficiency, drought, temperature extremes, salt stress, UV-B, and intense visible light (Harvaux and Kloppstech, 2001; Kimura et al., 2003; Diaz et al., 2006; Keutgen and Pawelzik, 2007; Roychoudhury et al., 2008; Hatier and Gould, 2009). Both nitrogen and phosphorous deficiency have been shown to induce anthocyanin accumulation (Ulrychova and Sosnova, 1970; Bongue-Bartelsman and Phillips, 1995; Scheible et al., 2004; Misson et al., 2005; Lea et al., 2007; Muller et al., 2007; Lillo et al., 2008). Under conditions of nutrient deficiency, energy collected by the light harvesting complex may be in excess of that which can be utilized by the photosynthetic machinery (Karpinski et al., 1999). Accumulation of anthocyanins under nutrient deficiency and other stresses may serve to control ROS production by screening excess light in a manner similar to the protective anthocyanin accumulation observed in senescing leaves in autumn (Hoch et al., 2000; Feild et al., 2001). It has also been hypothesized that anthocyanins may protect plant cells from oxidative stress by functioning as antioxidants (Lee and Gould, 2003; Philpott et al., 2004). This is supported by the observation that anthocyanins can accumulate in below ground tissues such as roots and tubers, where a light filtering role would not be relevant (Lewis et al., 1998; Philpott et al., 2009). The presence of anthocyanins has also been correlated with antioxidant activity in plant tissues (Gould et al., 2002; Gould et al., 2003; Neill and Gould, 2003; Peng et al., 2006). Like PAs, anthocyanins are valued as components of the diet because of the high antioxidant capacity characteristic of these molecules (Weisel et al., 2006; Elisia and Kitts, 2008; Spormann et al., 2008; Posmyk et al., 2009). Serving

multiple ecological and stress-protective roles, anthocyanins are an excellent example of multifunctional secondary metabolites (Gould, 2004).

UV-B radiation is well known as a stress that can induce the production of flavonoids in plant tissues. UV-B can damage DNA, proteins, and membranes both directly and through the production of ROS and free radicals (Surplus et al., 1998; Mackerness et al., 1999a; Mackerness et al., 1999b; Jordan, 2002). Many flavonoids, such as flavonols and PAs, absorb strongly in the UV-B range (Markham, 1982). Many plants accumulate UV-B absorbing flavonoids in epidermal tissues to prevent damaging UV-B radiation from reaching leaf interiors (Greenberg et al, 1997; Schmelzer et al., 1988; Hatier and Gould, 2009). Such a UV-B filtering role may have been the original function of flavonoids, in part facilitating the movement of plants to the terrestrial environment (Stafford, 1991; Rozema et al., 1997; Section 1.5.1). The flavonol glycosides (Fig. 1-1) are particularly important UV-B protective molecules (Ryan et al., 2002). Flavonols also accumulate in response to nutrient deprivation in arabidopsis (Stewart et al., 2001; Lea et al., 2007).

Flavonols and other flavonoids are essential for protecting many plants from the harmful effects of UV-B radiation, illustrated by the hypersensitivity to UV-B radiation exhibited by arabidopsis mutants lacking flavonoids (Li et al., 1993; Landry et al., 1995). In their study of tapetal cell flavonoid accumulation (Section 1.2.2), Hsieh and Huang (2007) found that pollen from *tt4, tt12*, and *tt19* plants that lacked pollen coat flavonoids germinated at substantially lower rates than wild type pollen after UV-B irradiation. Additionally, an arabidopsis mutant, *uvt1*, that exhibits increased *CHS* expression and accumulates elevated levels of UV-B absorbing compounds, including flavonoids, was found to be tolerant to UV-B irradiation levels that are lethal to the wild type (Bieza and Lois, 2001).

Like anthocyanins, flavonols may also protect plant tissues from UV-B and associated oxidative damage by functioning as antioxidants (Jordan, 2002). Ryan et al. (2002) found that in petunia, UV-B irradiation induced the biosynthesis of a higher proportion of the *o*-dihydroxylated flavonol quercetin than the monohydroxylated flavonol kaempferol, mediated by activation of the flavonoid B ring hydroxylase gene *F3'H* (Fig. 1-1). Similarly, increased quercetin over kaempferol under UV-B has also

been observed in *B. napus* (Olsson et al., 1998). Arabidopsis leaves normally accumulate kaempferol glycosides, but stresses such as low temperature and nutrient deprivation act synergistically to induce quercetin accumulation (Stewart et al., 2001; Lea et al., 2007; Lillo et al., 2008; Olsen et al., 2008). *o*-Dihydroxylated flavonoids are less effective at UV-B absorption but are more effective antioxidants than monohydroxylated flavonoids (Montesinos et al., 1995; Lavola et al., 1997), indicating that an increased quercetin to kaempferol ratio in UV-B exposed or stressed plants may reflect an antioxidant function for flavonols (Ryan et al, 2002). Like anthocyanins and PAs, flavonols are synthesized in the cytosol but accumulate primarily in the vacuole. In the cytosol, which has a neutral pH, flavonols would be far more effective antioxidants than under the acidic conditions of the vacuole (Ryan et al., 2002). Although a vacuolar location may not be ideal for an antioxidant function, some authors have argued that some reactive molecules, such as H_2O_2, which is an important second messenger in stress signalling with a half-life of 1 ms, would readily diffuse across the tonoplast where vacuolar flavonoids would be effective antioxidants (e.g., Hatier and Gould, 2009; Albert et al., 2009). Since flavonols, like anthocyanins and PAs, are synthesized in the cytosol, it is also possible that they serve an antioxidant function primarily in the cytosol, with flavonols being synthesized under elevated UV-B being enriched in the more effective dihydroxylated type, and a filtering function in the vacuole where they subsequently accumulate as glycosides (Ryan et al., 2002).

Stress-induced accumulation of phenylpropanoids in plant cells is typically mediated by transcriptional activation of genes encoding the corresponding biosynthetic enzymes (Hahlbrock and Scheel, 1989; Lozoya et al., 1991). Numerous examples of different stress conditions causing transcriptional activation of phenylpropanoid and flavonoid biosynthetic genes have been reported. For example, wounding and fungal infection of bean (*Phaseolus vulgaris*) hypocoytls has been shown to rapidly induce expression of *PAL* and *CHS* genes (Lawton and Lamb, 1987). Fungal elicitor treatment and exposure to UV-containing light induces *PAL* expression in parsley (*Petroselinum crispum*) cell culture (da Costa e Silva et al., 1993) and accumulation of UV-protective flavonoids in epidermal cells of parsley leaves following exposure of etiolated seedlings to UV-containing light was preceded by an accumulation of *CHS* transcripts (Schmelzer

et al., 1988). Up-regulation of *CHS* expression following wounding or application of the plant defence signal molecule methyl jasmonate (MeJa; see Section 1.5.7) has been demonstrated in a number of species (Schmid et al., 1990; Creelman et al., 1992; Tamari et al., 1995; Richard et al., 2000). In hybrid poplar (*P. maximowizii* x *trichocarpa*), O_3 and wounding were found to induce *PAL* expression, with greater induction in expression levels following both treatments in a more a O_3-tolerant genotype (Koch et al., 1998); *PAL* expression was also induced by wounding in trembling aspen bark (Thamarus and Furnier, 1998). Accumulation of flavonols under elevated UV-B is mediated by transcriptional activation of biosynthetic genes, including early general flavonoid pathway genes such as *CHS*, *CHI*, and *F3H* as well as the flavonol pathway-specific *FLS* (Ryan et al., 2002; Casati and Walbot, 2003; Owens et al., 2008). Additionally, a number of microarray analyses have shown that biosynthesis of anthocyanins in arabidopsis under nutrient deprivation or excess light is mediated by transcriptional activation of general flavonoid and anthocyanin biosynthetic genes (Scheible et al., 2004; Misson et al., 2005; Morcuende et al., 2007; Muller et al., 2007).

1.4.2 Stress-protective functions of PAs

As tannins, PAs are characterized by the ability to bind to and precipitate proteins (Stafford, 1990). This property has long been hypothesized to contribute to an anti-herbivore function of PAs (Feeny, 1968; Haslam, 1989). PAs can form strong molecular complexes with proteins and other macromolecules through hydrogen and covalent bonding (Bate-Smith and Swain, 1962; Chavan and Salunkhe, 1989). The ability of tannins to bind to and precipitate proteins is dependent upon polymer size and structure, pH, and protein structure (Hagerman et al., 1998b; Hagerman and Butler, 1981). High concentrations of compounds that reduce the nutritional value of plant tissue and inhibit digestion by insect larvae can potentially have a profound effect on their growth rate, limiting the damage that they do while feeding on a host plant over time (Rhoades and Cates, 1976). Such defences may also lengthen insect herbivore development times, rendering them more susceptible to predation and parasitism (Price et al., 1980). When

cells are destroyed by chewing herbivores, PAs are released from the vacuoles, freeing them to bind to plant proteins and herbivore digestive enzymes; they may also negatively impact herbivores by acting as feeding deterrents or toxins (Clausen et al., 1990).

The first research to establish a possible role for PAs in plant defence against insect herbivores was conducted by Feeny (1970), who observed that the number of lepidopteran pest species found on pedunculate oak (*Quercus robar*) leaves declines over the course of the season as leaf PA concentrations increase. This correlation between phenological changes in oak leaf chemistry and herbivore abundance led Feeny to propose that the seasonal build-up of PAs selected for spring-feeding herbivores. Feeny also showed that oak PAs could complex with proteins and inhibit digestion by the protease trypsin (Feeny, 1969) and that inclusion of PAs in an artificial diet fed to winter moth larvae (*Operophtera brumata*), an important oak pest in Europe, resulted in reduced larval growth rate (Feeny, 1968). These findings led Feeny (1970, 1976) and others (Rhoades and Cates, 1976) to hypothesize that PAs possess general anti-herbivore properties and function as a "broad spectrum" defence against insect herbivores by precipitating proteins and reducing the nutritional quality of leaves. However, this function has not always been supported experimentally, and the evidence for a role for PAs in insect herbivore defense is equivocal (Rossiter et al., 1988; Ayres et al., 1997; Forkner et al., 2004).

It has also been hypothesized that the anti-insect activities of some plant phenolics results from oxidative stress generated following enzyme-mediated oxidation or auto-oxidation of the phenolics in high pH insect guts rather than, or in addition to, protein precipitation (Felton et al., 1992; Appel, 1993; Bi and Felton, 1995; Hagerman et al., 2003). Oxidized phenolics can react with plant nutrients and insect proteins and can produce ROS that can damage herbivore tissues (Bi and Felton, 1995; Barbehenn et al., 2006, 2008). Plant oxidative enzymes such as PPO, which can oxidize *o*-diphenols to *o*-diquinones, may mediate the defensive functions of plant phenolics. Increased PPO activity and up-regulation of *PPO* genes following herbivore damage has been reported in a number of plants, including several tree species (Constabel, 1999; Haruta et al., 2001; Wang and Constabel, 2004; Ruuhola et al., 2008).

The composition of different phenolics in plant tissues may be very important in determining how specific metabolites function, and compounds such as PAs must be considered within the chemical context in which they occur (Moilanen and Juha-Pekka, 2008; Barbehenn et al., 2009). While several non-PA tannins such as the gallotannins and ellagitannins (Barbehenn et al., 2008; Moilanen and Juha-Pekka, 2008) exhibit high prooxidant activity in insect guts, PA levels have been found to be negatively correlated with oxidative activity (Barbehenn et al., 2006). In fact, Barbehenn et al. (2006) speculated that the antioxidant activity of PAs in insect guts could decrease the oxidative damage caused by co-ingested phenolics with high oxidative activity, such as hydrolysable tannins, and that this could explain reports of positive correlations between lepidopteran larval performance and PA levels. However, in an experiment in which sugar maple (*Acer saccharum*) leaves, which contain high levels of ellagitannins with high prooxidant activity, were coated with PAs and fed to gypsy moth (*Lymantria dispar*) caterpillars, no antioxidant effect in the midgut or improved larval performance was observed (Barbehenn et al., 2009). Similarly, addition of a mix of PAs from leaves of mountain birch (*Betula pubescens* ssp. *czerepanovii*) did not affect the growth retardant and feeding deterrent effects of the hydrolysable tannin vescalagin, when included in an artificial diet fed to two generalist herbivores (Roslin and Salminen, 2008). It should also be noted that in some trees, concentrations of PAs and levels of other phenolics such as hydrolysable tannins (Batesmith, 1977; Baldwin and Schultz, 1984; Baldwin et al., 1987) or PGs (Donaldson and Lindroth, 2007) have been found to be inversely related, which could explain positive correlations between PA levels and insect performance.

Correlations between tannin concentrations in plant tissues and herbivore performance or feeding preference support a role for PAs as anti-insect compounds, but the effects may be dependent on the particular species-species interaction, plant tissue, chemical context, and nutritional quality characteristics of plant tissue (Haukioja, 2003). Numerous studies suggest that PAs may be important for protection of plant tissues against herbivores in some contexts. For example, PA levels in groundnut (*Arachis hypogaea*) leaf bud petioles were found to be negatively correlated with groundnut aphid (*Aphis craccivoru*) fecundity (Grayer et al., 1992). In European beech (*Fagus sylvatica*) trees, PA levels in the inner bark were found to be negatively correlated with the degree

of infestation by the beech scale (*Cryptococcus fagisuga*) (Krabel and Petercord, 2000). High PA concentrations in mountain birch leaves were correlated with a small, marginally significant reduction in relative consumption rate and growth of the autumnal moth (*Epirrita autumnata*) larvae, but only in genotypes with low gallotannin concentrations (Kause et al., 1999). In another study of the same tree-insect interaction, numerous phenolic and nutritive characteristics were analyzed and only PA concentrations showed a weak but significant negative correlation with larval relative growth rate (Ossipov et al., 2001). Relative growth rate and pupal mass of autumnal moth were also negatively correlated with leaf PA concentrations in silver birch (*B. pendula*) grown under two fertilization levels (Mutikainen et al., 2000) and addition of purified PA to a diet of Alaska paper birch (*B. resinifer*) leaves resulted in significantly reduced survival and pupal mass of spear-marked black moth (*Rheumaptera hastata*) (Bryant et al., 1993). Following up on Feeny's observations of seasonal herbivore density and oak leaf PA levels, Forkner et al. (2004) analyzed seasonal insect herbivore abundance and species richness as well as foliar PA concentrations in both canopy and understory for two oak species (*Q. velutina* and *Q. alba*) in May, June, and August of one year. Out of thirty-eight insect species that were encountered at sufficient densities for analysis, two (*Acronicta increta* and *Attelabus* spp., both oak specialists) exhibited abundance levels that were negatively correlated with PA concentrations. Additionally, PA concentrations were negatively correlated with species richness for *Q. velutina* and *Q. alba*, but only in May and August respectively. It should be noted that hydrolysable tannins and other phenolics were not quantified in this study.

Although there are a number of examples of significant negative correlations between PA levels and herbivore feeding or performance, lack of discrimination between the effects of PAs and other tannins or phenolics has in some cases confused interpretation of the literature (Moilanen and Juha-Pekka, 2008). In some cases, reports of negative correlations between non-PA tannins or phenolics has been cited (e.g., by Mutikainen et al., 2000) as support for an insect-defence role for PAs in cases where PAs were in fact measured and found to have no significant effect (e.g., Rossiter et al., 1988; Sagers and Coley, 1995).

Although PAs appear to be important herbivore defence molecules in a context-dependant manner, it must be conceded that reduced larval performance on high PA diets does not constitute definitive evidence of an anti-herbivore function *in planta* or that herbivory was an important selective pressure affecting the evolution of PA accumulation patterns in plant tissues. Studies in which insect performance or feeding choice show no negative correlations with PA levels are also abundant in the literature. In a study in which purified mountain birch leaf PAs were included at a concentration of 15 mg g^{-1} DW in an artificial diet fed to two specialist (*Dichonia aprilina* and *Catocala sponsa*) and two generalist (*Acronicta psi* and *Amphipyra pyramidea*) species of moth larvae, no effect of PAs was detected (Roslin and Salminen, 2008). In a choice test in which weevils (*Phyllobius maculicornis*) were offered both relatively high PA silver birch foliage, resulting from growth under elevated CO_2, and leaves from untreated trees with lower PA levels, weevils showed a significant preference for the high PA tissue (Kuokkanen et al., 2003). Although small but significant increases in other phenolics make interpretation of this data difficult, the authors postulate that PAs may function as feeding stimulants in this context.

PAs exhibit considerable structural variability between different plants, and structural characteristics such as B-ring hydroxylation levels, types of linkage between monomers, branching patterns, and polymer length may be important for anti-herbivore activity (Clausen et al., 1990; Ayres et al., 1997). Ayres et al. (1997) analyzed PA structural diversity in 16 woody plants from six genera and tested whether structural difference effected anti-herbivore properties by monitoring growth and development of six insect species consuming control foliage or foliage coated with purified PAs (6% DW). PAs from the 16 plant species exhibited considerable structural difference, with procyanidin to prodelphinidin ratios ranging from 34:66 to 100:0, stereochemistry at C-3 relative to C-2 ranging from 100% *cis* to 70% *trans*, and mDP ranging from 4.7 to 15.6. Forty-five insect-PA combinations were tested in short term bioassays, and larval growth was negatively affected by PA addition in 35 cases, although only eight were significant. PAs from some species (e.g., *Salix alaxensis* and *Vaccinium vitis-ideae*) had no effect on growth rate for any insect species tested, while others (e.g., from *Betula resinifera* and *B. papifera*) were effective against more than one herbivore species; in general, increased B-

ring hydroxylation and molecular mass were correlated with greater anti-herbivore activity. In an accompanying control, the PG tremulacin was purified from trembling aspen leaves and applied to foliage fed to tiger swallowtail (*Papilio glaucus*) larvae at a concentration of 2% DW. Growth inhibition by this PG was greater than for any of the PA-insect combinations. The lack of a strong or significant anti-herbivore effect for PAs in the majority of combinations tested combined with the knowledge that PAs are produced by so many different plant species, at very high levels in some cases (e.g., poplars), led the authors to "doubt that selection imposed by folivorous insects can be the main explanation for the diversion of so much carbon, in so many species, away from plant primary metabolism" (Ayres et al., 1997).

The evidence for a role in herbivore-defence for PAs in poplar in particular is also mixed. As with other plants, the functions of poplar PAs may be dependent upon the tissue, context, and species-species interaction. Bryant et al. (1987) reported that large aspen tortrix larvae reared on artificial diets containing 0.5 % DW purified trembling aspen PA exhibited a small but significant reduction in weight at 5^{th} instar compared to controls. A study of cottonwood leaf beetle (*Chrysomela scripta*) performance on juvenile trembling aspen tissue from five clones with varying levels of PAs (3.8 - 6.2% DW) and PGs (15 - 22% DW) supports a role for PAs in defence against chrysomelid beetle species (Donaldson and Lindroth, 2004). Beetle performance was highly consistent between clones overall, but regression analysis revealed a significant (P = 0.011) effect of PA concentrations on larval growth rate. Cottonwood leaf beetle is a specialist herbivore adapted to high PG-containing poplar and willow species, and species with high PA levels in mature leaves may be unsuitable hosts for chrysomelid beetles (Gruppe et al., 1999). Poplar PAs may also affect mammalian herbivore feeding. Wild beavers showed a significant selection preference for branches with lower bark PA levels when offered a selection of branches from a 10-year old common garden of pure and hybrid cottonwoods (Bailey et al., 2004). Variation in PG levels had no effect on branch selection by beavers in this study.

A number of studies with lepidopteran larvae indicate that poplar leaf PAs are not important determinants of performance. Hwang and Lindroth (1997) assessed the performance of gypsy moth and forest tent caterpillar larvae feeding on 13 aspen clones

with variable PA and PG levels. Performance of both species was strongly dependent upon PG concentrations. Additional studies have confirmed that PGs are the most important variable for explaining difference in performance (Lindroth and Bloomer, 1991; Hwang and Lindroth, 1998) and feeding preference (Donaldson and Lindroth, 2007) of lepidopteran larvae. A number of studies in which foliar PA levels are altered due to elevated light levels, reduced nutrient availability, elevated CO_2 levels, and in response to defoliation have also failed to find a correlation between PA levels and insect performance (e.g., Lindroth et al., 1997; Hemming and Lindroth, 1999; Osier and Lindroth, 2001; Osier and Lindroth, 2004). However, there is evidence that higher PA levels may cause compensatory feeding by insects (Osier et al., 2000a; Osier and Lindroth, 2001), although the degree to which this is detrimental to the plant or the herbivore is difficult to determine.

1.4.3 Herbivore-induced PA metabolism in poplar and other trees

One line of indirect evidence supporting the hypothesis that PAs function to protect leaves of poplars and other trees from herbivory consists of observations of PA concentrations increasing in herbivore-damaged leaves or undamaged leaves on herbivore-defoliated trees. For example, defoliation of red oak (*Quercus rubra*) by gypsy moth results in increased leaf PA levels in protected leaves on defoliated trees (Schultz and Baldwin, 1982). Following a gypsy moth outbreak, damaged red oak and chestnut oak (*Q. prinus*) leaves had significanly higher PA levels than undamaged leaves, although this was mitigated by prior fertilization for chestnut oak (Hunter and Schultz, 1995). This study also reported that leaves on chestnut oak branches that had been enclosed in bags containing sufficient gypsy moth larvae to inflict leaf damage equivalent to 40% defoliation had significantly higher PAs after 3 weeks than control leaves on the same trees. Additionally, elevated foliar PA levels in years following defoliation has been documented. For example, defoliation of Alaska paper birch or mountain birch results in increased leaf PA levels the following year (Bryant et al., 1993; Kaitaniemi et al., 1998).

In general, changes in leaf phytochemical concentrations following herbivore damage may be classified as rapid induced resistance (RIR, i.e., accumulation of defensive chemicals within days of damage) or delayed induced resistance (DIR, i.e., elevated levels of phytochemicals the following growing season) (Haukioja et al., 1988; Bryant et al., 1993; Kaitaniemi et al., 1998; Stevens and Lindroth, 2005). Of course, using the term 'resistance' in this context may be misleading since such responses do not indicate that elevated metabolite levels are a direct response to herbivory or that they confer resistance (Haukioja, 1991). For PAs (and other phenolics), DIR and RIR are not necessarily correlated with increased resistance to herbivory or reduced herbivore performance (Osier and Lindroth, 2001). Other explanations for both DIR and RIR have been proposed, such as nutrient stress leading to altered carbon-nitrogen balance (Herms and Mattson, 1992; Bryant et al., 1993; Hunter and Schultz, 1995). The carbon-nutrient balance hypothesis predicts that under nutrient limited conditions, allocation to carbon-based defence metabolites will increase (see Section 1.3.2). Rather than representing an active defence response, the accumulation of carbon-based secondary metabolites such as PAs following defoliation may be a mechanism for storing surplus carbon under nutrient (primarily nitrogen) limiting conditions. Mitigation of both short-term and long-term herbivore-induced phenolic/PA accumulation in fertilized trees, where nitrogen limitation is ameliorated, is cited as support for this theory. In cases where defoliation leads to reduced performance of insects, it is unclear whether increases in carbon-based metabolites, decreased nutritive quality, or both, are responsible (Haukioja and Neuvonen, 1985).

Both rapid and delayed herbivore- or damage-induced accumulation of PAs has been documented in poplar species, although the response is dependent on genotype and growth conditions. Trembling aspen is the most thoroughly investigated of the poplar species, although increased leaf PA levels following simulated herbivore damage of hybrid poplar and cottonwood has also been demonstrated (Arnold and Schultz, 2002; Tsai et al., 2006a). Roth et al. (1998) reported an accumulation of leaf PAs in trembling aspen caged in outdoor chambers with feeding FTC. Leaves on branch termini had sustained substantial damage. Compared to undamaged controls, leaf PAs in intact leaves from damaged trees were significantly higher after the first week of feeding and

continued to increase throughout the 4 week experiment to a maximum 2-fold increase over controls. However, the same group reported a second experiment in which artificial defoliation (50% leaf area removal) of second year trembling aspen from the same seed source growing in environmental control rooms had no significant effect on leaf PA levels six days after treatment (Lindroth and Kinney, 1998). The baseline PA levels were roughly 4 times higher in the first experiment, perhaps reflecting the different growing condition that may have influenced the responses. Osier and Lindroth (2001) also analyzed the effects of artificial defoliation on leaf chemistry for 4 different trembling aspen genotypes in their second growing season. Both severe (75%) and moderate (25%) defoliation induced significant increases in foliar PA levels in undamaged leaves of all four clones, but the response was ameliorated by prior fertilization. In another study with 12 genotypes of outdoor-growing, one year-old trembling aspen trees, a combination of FTC feeding and artificial wounding (75% leaf area removal over 1 week after FTC had been feeding for 10 days) resulted in a significant >2-fold increase in PAs in the damaged leaves, measure one week after defoliation (Stevens and Lindroth, 2005). Again, the induction was significantly lower for a treatment group that had been pre-fertilized. Delayed induced leaf PA level increases have also been documented for trembling aspen. One year after complete defoliation, Osier and Lindroth (2004) found that the same four aspen genotypes they had previously analyzed (2001) responded with increased foliar PA levels (mean ~20% increase compared to undefoliated controls), and that this increase was not ameliorated by fertilization.

Highlighting the importance of leaf ontogeny, source leaf-sink leaf connections, and systemic signalling for inducible PA metabolism in poplar species, Arnold and Schultz (2002) reported that immature sink leaves, but not mature source leaves, of hybrid poplar (*P. deltoides x nigra*) saplings responded to application of the plant stress hormone jasmonic acid (JA) (see Section 1.5.7) with a significant increase in PA levels. This was accompanied by ‘induced sink strength’, or enhanced phloem unloading, mediated by increased cell wall invertase activity. Increased carbon export from source leaves is likely also important (Babst et al. 2005). Carbon from connected source leaves is imported into induced sink leaves and converted to PAs, and the JA-induced PA accumulation does not occur if the sink tissue is removed (Arnold et al., 2004). Vascular

connections appear to be important for both assimilate movement for carbon redistribution and systemic defence signalling (Philippe and Bohlmann, 2007). Activation of putative systemic defenses in poplar in response to leaf wounding has been shown to be strongest in the sink leaf with a direct phloem connection to the wounded source leaf and to be dependent upon assimilate movement, indicating that a phloem mobile signal mediates systemic defence gene activation (Davis et al., 1991).

In conclusion, PA metabolism is activated by real or simulated herbivory in a number of poplar species, suggesting that increased PAs contribute to defence against attacking herbivores, but whether this up-regulation represents an active defence response is unclear. PAs are not “broad spectrum” anti-herbivore compounds, but do exhibit anti-herbivore activity in some contexts. Herbivory may have been a contributing selective agent leading to high-PA leaves and inducible PA metabolism in some poplar species and clones, but it is also possible that anti-herbivore activity may be only one property of a trait that has evolved to serve a variety of different functions in leaves of poplar and other plants.

1.4.4 Alternative functions for stress-induced PAs in poplar

The lack of a clear connection between PAs and herbivore deterrence has led to suggestions of alternative roles for PAs in leaves of poplar and other species. Many plant chemicals are multi-functional, and it is likely that PAs serve a variety of functions and that numerous selective pressures have influenced PA accumulation patterns in plant tissues. In addition to herbivore resistance, carbon storage, and impacts on con-specifics and other organisms through effects on soil nutrient cycling, leaf PAs have been proposed to confer resistance to a number of biotic and abiotic stress conditions. Stevens and Lindroth (2005) have suggested that the rapid up-regulation of PAs in poplar leaves following herbivore damage may contribute to the protection of damaged leaves from opportunistic pathogen invasion. This is supported by studies that have found negative correlations between plant tissue PAs and fungal pathogen infection (Iriti et al., 2005)

or fungal spore germination (Heil et al., 2002), indicating that protecting tissues against pathogen infection may be a relevant function for PAs in some contexts.

In addition to leaving damaged tissue susceptible to pathogen attack, defoliation by herbivores also allows more light penetration through the canopy to underlying leaves, and it has been suggested that herbivore-induced PA accumulation may contribute to the protection of leaves from UV-B (Stevens and Lindroth, 2005). Correlations between light levels and concentrations of PAs or other high molecular weight polyphenols have been observed in other plants (Mole et al., 1988), and PA concentrations in poplar leaves are strongly influenced by light levels (Hemming and Lindroth, 1999). Alonso-Amelot et al. (2004) reported that for two species of neotropical bracken fern (*Pteridium* spp.) growing on the Western Andes of Venezuela, PA concentrations increased with increasing altitude. They note that tropical mountains have the highest UV-B gradient with elevation in the world, and suggest that the significant correlation between PA concentrations and solar UV-B radiation indicates that PAs may play a UV-B filtering role in these species (Alonso-Amelot et al, 2004).

Related to this hypothesis, it is also possible that PAs may function as antioxidants *in vivo*. Like many other flavonoids, PAs exhibit strong antioxidant capacity *in vitro* (Hagerman et al., 1998a), and may contribute to stress resistance by scavenging oxygen radicals. PAs may also exert oxidative stress protective effects in some contexts through their ability to chelate metal ions (Yoneda and Nakatsubo, 1998; Soobrattee et al., 2005). Tea plants (*Camellia sinensis*) accumulate both flavan-3-ols, the precursors for PA biosynthesis, and polymeric PAs when exposed to water deficit conditions (Hernandez et al., 2006). Under these conditions, Hernandez et al. (2006) identified stable oxidation products of flavon-3-ols *in vivo*. Levels of both (-)-epicatechin quinone and (-)-epigallocatechin quinone sharply increased in tea leaves under water stress conditions, preceding an accumulation of PAs. The authors contend that this *in vivo* oxidation of PA precursors serves to protect plants from lipid peroxidation and other effects of oxidative stress (Hernandez et al., 2006). As has been suggested for flavonols, flavan-3-ols may have an antioxidant function in the cytosol where they are synthesized, while the metabolic end products accumulate in the vacuole where they may serve additional functions such as light filtering or defense against herbivores and pathogens.

The mechanism by which flavan-3-ol precursors are polymerized to form PAs is still unknown (Section 1.2.2). Hernandez et al. (2009) suggested that oxidation of flavan-3-ols may be an important antioxidant mechanism, and that radical scavenging may be in fact be an essential part of PA polymerization, with flavan-3-ol oxidation products being transported into the vacuole where they are polymerized to form PAs. In scavenging oxidizing radicals, flavan-3-ols and flavonols become aroxyl radicals (semiquinones) and then decay to quinones that may have pro-oxidant activities (Bors et al., 1990; Bors et al., 1994). However, polymerization of flavan-3-ol quinones through phenolic coupling reactions, with each monomer retaining the original number of reactive hydroxyl groups, results instead in enhanced antioxidant potential (Decolour et al., 1983; Quideau et al., 1995; Escribano-Bailon et al., 1996; Bors et al., 2001). A non-enzymatic oxidation mechanism would explain why no gene encoding an oxidative enzyme involved in PA polymerization has been identified despite extensive mutant screening using arabidopsis. An *in vivo* antioxidant function may also be related to up-regulation of the pathway following herbivore damage. Ralph et al. (2006) analyzed the transcriptional response of hybrid poplar (*P. trichocarpa x deltoides*) to FTC feeding using microarray analysis. FTC feeding caused an up-regulation of numerous oxidative stress response genes as well as a down-regulation of photosynthesis-related genes, indicating that increased oxidative stress may result from herbivore damage to poplar leaves.

O_3 exposure results in increased foliar PA concentrations in poplar (Holton et al., 2003; Agrell et al., 2005). O_3 can increase oxidative stress by degrading photosynthetic proteins and directly generating ROS (Pell et al., 1997; Brendley and Pell, 1998), and O_3 exposure can induce leaf senescence in poplar and other species (Pell et al., 1995; Pell et al., 1999). Haikio et al. (2007) identified hybrid aspen (*P. tremula x tremuloides*) clones that were both O_3-sensitive and O_3-tolerant, and reported that the O_3-tolerant group was characterized by higher levels of (+)-catechin, PAs, and a kaempferol glycoside derivative, while the O_3-sensitive clones had higher foliar PG concentrations (Haikio et al., 2009). When grown for two seasons under 1.5-fold greater-than-ambient O_3 levels, these hybrid aspen clones exhibited a general pattern of decreased PGs and increased PAs, (+)-catechin, and chlorogenic acid, leading the authors to suggest that PAs and (+)-catechin protect leaves under oxidative stress.

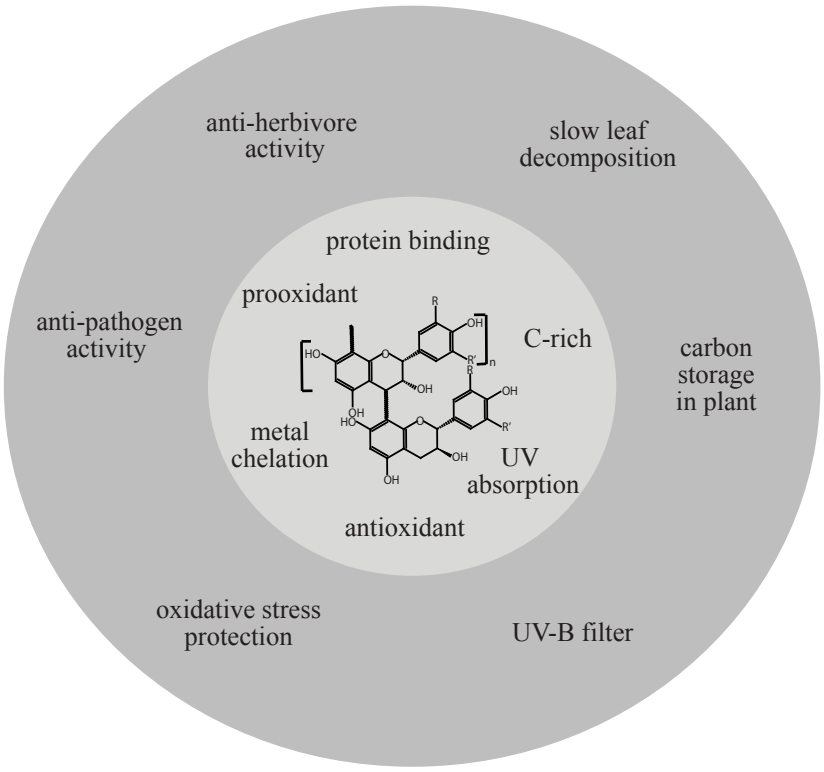


Figure 1-2. Chemical properties of PAs and possible functions in plants. The chemical properties of PAs (inner circle) may contribute to a variety of effects in tissues such as leaves (outer circle), which may contribute to plant fitness and be subject to natural selection, affecting the accumulation, distribution, and chemical structures of PAs in specific plants and tissues.

Several of the hypotheses to explain PA functions in plants stem from the chemical properties of PAs. For example, the ability of PAs to precipitate protein (i.e., the tannin properties) led to the hypothesis that they reduce the nutritional quality of plant tissues and confer protection against herbivores. PAs are carbon-rich compounds that bind to and precipitate protein, are redox active and have absorption spectra in the UV-B range. Figure 1-2 summarizes these chemical properties of PAs and how they might be related to biological activity in plant tissues that may in turn affect plant survival and fecundity and thus be subject to natural selection.

1.4.5 Transcriptional activation of PA biosynthetic genes in poplar

Elucidation of the molecular mechanisms mediating the up-regulation of PA metabolism under different stress conditions may contribute to the understanding of how PAs function in plants. Recent work has revealed that, like other flavonoid pathways, rapid increases in PA biosynthesis involve transcriptional control of genes encoding biosynthetic enzymes. The rapid accumulation of PAs following real or simulated herbivore damage in trembling aspen and other poplar species has been found to be mediated by the activation of phenylpropanoid and flavonoid pathway biosynthetic genes. Wound-induced accumulation of PAs in mature leaves of trembling aspen saplings was shown to be mediated by transcriptional activation of *PAL1*, *4CL2*, and *CHS1* and *DFR1* (Peters and Constabel, 2002). This up-regulation was found in both wounded leaves and upper unwounded leaves, implicating a phloem-mobile signal to activate PA metabolism systemically. *DFR1* was up-regulated following mechanical wounding of hybrid poplar (*P. trichocarpa x deltoides*) leaves as well, although the level of transcriptional activation was lower. It was fortuitous that the *PAL1* and *4CL2* genes were analyzed in this study, as a concurrently published study showed that while these gene family members are involved in PA metabolism, additional trembling aspen *PAL* and *4CL* genes are involved specifically in lignin metabolism and are not wound-responsive (Kao et al., 2002).

Kao et al. (2002) reported that in leaves of trembling aspen saplings, the expression of *PAL1* and *4CL2*, but not *PAL2* and *4CL1*, was up-regulated 24 hours after wounding with pliers, with a corresponding increase in leaf PA levels. Interestingly, the PAL1 and 4CL2 isoforms may be specifically involved in PA metabolism, both constitutive and inducible, while PAL2 and 4CL1 are specifically involved in the production of precursors for lignin biosynthesis. Constitutive expression of *PAL1* and *4CL2* was found to be strong in PA-producing tissues (e.g., emerging leaves), while *PAL2* and *4CL1* are more strongly expressed in tissues in which lignin metabolism is active (e.g., xylem). Additionally, expression of *PAL2* and *4CL1* overlaps that of a gene encoding the lignin biosynthetic enzyme, caffeate-O-methyltransferase (COMT). Hu et al. (1998) also found that *4CL1* is specifically expressed in lignifying xylem, while *4CL2* is expressed in leaf and stem epidermal layers. While both enzymes had relatively broad substrate specificities, recombinant 4CL1 but not 4CL2 could use 5-hydroxyferulic acid

as a substrate (a precursor for syringyl lignin synthesis) (Hu et al., 1998). Further supporting a specific role for 4CL2 in flavonoid metabolism, Harding et al (2002) showed in mixed substrate assays that competitive inhibition favored utilization of caffeic acid by recombinant 4CL1 and *p*-coumaric acid by 4CL2 (i.e., the presence of the flavonoid precursor *p*-coumaric acid inhibits the activation of other hydroxycinnamic acids by 4CL2).

Tsai et al. (2006a) reported an analysis of flavonoid and PA pathway gene expression in rapidly expanding sink leaves of hybrid cottonwood (*P. fremontii* × *angustifolia*), five days after mechanical wounding of source leaves (a treatment that induced a 2.5-fold increase in PA levels). The entire flavonoid pathway was up-regulated in these systemically wounded leaves, including PA-specific *ANR* and *LAR* genes. *PAL1*, *ANS2*, *F3H* and *CHI1* were among the most strongly up-regulated. Many of the flavonoid and PA biosynthetic enzymes in poplar are encoded by multi-gene families, compared to primarily single copy flavonoid genes in arabidopsis. The increased number of flavonoid gene copies could reflect the higher level of PA production in poplar compared to arabidopsis. In some cases, gene family members exhibited differential levels of activation. For example, *ANR1* was more strongly up-regulated than *ANR2*, *CHS1* and *CHS3* were more strongly induced than *CHS4*, and *LAR3* was more strongly activated than *LAR1* and *LAR2*. These differential levels of activation within multi-gene families in response to systemic wounding in hybrid cottonwood may indicate that gene family members play different roles in stress-responsive PA accumulation, perhaps mediated by different regulatory mechanisms.

Rapid transcriptional activation of the PA pathway following insect attack has been cited as evidence of an anti-herbivore function for PAs in poplar leaves (e.g., Peters and Constabel, 2002), but it is not known whether this response is specific to herbivore stress or part of a more general stress response. Stress-responsive regulation of the flavonoid pathway genes at the level of gene transcription is well established for flavonoid branch pathways such as anthocyanin and flavonol glycoside synthesis (Ryan et al., 2002; Winkel-Shirley, 2002; Kimura et al., 2003), and it appears that the PA pathway is similarly regulated under stresses such as insect damage and mechanical wounding of poplar leaves. The recent molecular analyses of PA pathway activation in

poplar following wounding and herbivory lay the foundations for investigations into the regulatory mechanisms controlling stress-induced activation of PA metabolism in poplar.

1.5 The regulation of flavonoid and PA metabolism

1.5.1 A conserved regulatory protein complex is involved in the regulation of anthocyanin and PA biosynthesis

The regulation of PA metabolism has been best characterized in the model plant arabidopsis, and recent analyses have revealed that the regulatory mechanisms involved are conserved in other plants. In arabidopsis, seed coat PA biosynthesis is controlled by the MYB transcription factor TT2, which functions within a transcription factor protein complex to initiate activation of target gene expression. TT2 functions together with a basic helix-loop-helix (BHLH) domain protein, TT8, and a WD-repeat protein, TTG1, to control the expression of late flavonoid and PA biosynthetic genes (Nesi et al., 2001; Baudry et al., 2004). PA metabolism is thus under combinatorial control (Wolberger, 1999) by multiple regulatory proteins that interact with each other and with conserved DNA *cis*-elements oriented in a specific manner within target gene promoters. MYB-BHLH-WDR complexes are involved in controlling anthocyanin metabolism in maize, arabidopsis and numerous other plants (Walker et al., 1999; Zhang et al., 2003), and the function of this protein complex may be conserved in higher plants (Ramsay and Glover, 2005).

MYB proteins comprise a very large family in plants, and the expansion of this family likely played an important role in the evolution and radiation of land plants. The evolution of land plants from marine ancestors required the development of a number of essential adaptations. Structural support, protection against intense light and UV-B radiation and associated oxidative stress, and control of dehydration were among the most important evolutionary developments required for movement into the terrestrial environment. Important traits like the biosynthesis and deposition of structural

phenylpropanoids such as lignin, accumulation of photo-protective pigments such as flavonoids in epidermal cells, and an epidermis that protects the plant from dehydration while permitting gas exchange and regulation of water loss as well as nutrient uptake and water acquisition from the soil, facilitated the colonization of land by plants. Duplication and neo-functionalization of regulatory genes likely played a key role in the evolution of these plant traits (Ramsay and Glover, 2005). Many of these adaptations later became diversified to serve new roles; for example, the evolution of phenolic chemicals to protect plants against herbivory. Transcription factors of the MYB class are one group of regulatory proteins whose expansion during plant evolution likely played a key role both in colonization of the land and in the subsequent radiation of higher plants (Rabinowicz et al., 1999).

MYB transcription factor proteins have been identified in a diverse group of eukaryotic organisms that includes plants, animals, fungi, and cellular slime molds (Martin, 1996; Dixon and Steele, 1999). Members of this protein family possess conserved amino-terminal MYB-domains, each approximately 50 to 52 amino acids in length. The MYB domain is a conserved DNA-binding domain that adopts a helix-helix-turn-helix conformation upon binding DNA in a sequence-specific manner (Oehler et al., 1990; Golay et al., 1994). This domain is repeated in tandem three times in animal MYB proteins (Fig. 1-3A), and this is thought to be the ancestral form (Perala, 1990). The MYB domain is characterized by conserved, regularly-spaced tryptophan residues that stabilize the helix-helix-turn-helix structure in a conformation similar to the DNA-binding domains of homeodomain proteins and the repressor protein of bacteriophage lambda (Ogata et al., 1994). The first MYB gene characterized was the oncogene v-Myb carried by the avian myeloblastosis virus, a truncated version of the vertebrate proto-oncogene c-Myb that arose through retroviral transduction (Oelgeschlager et al., 2001). The three repeated MYB-domains of the c-Myb protein are designated R1, R2, and R3 (Gonda, 1998). In c-Myb, the second and third α-helices of MYB repeats R2 and R3 are involved in binding DNA elements with the consensus sequence (C/T)AAC(G/T)G (Biedenkapp et al., 1988). C-Myb and related animal Myb proteins also contain a carboxy-terminal negative regulatory domain, and some members possess a central acidic transcriptional activation domain (Gonda et al., 1996; Lipsick et al., 2001; Fig. 1-3A).

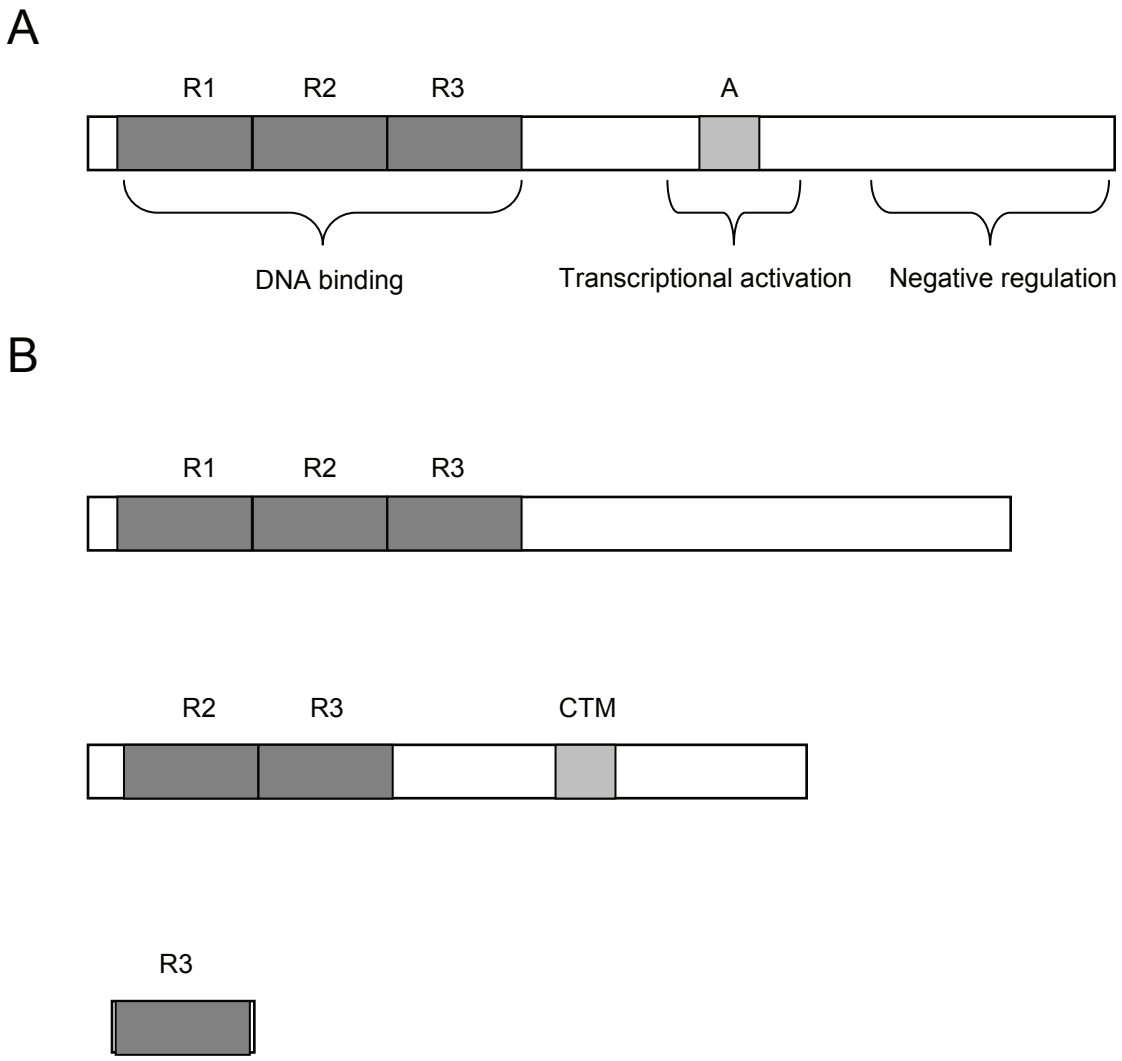


Figure 1-3. Schematic representations of different classes of MYB domain proteins. **A**. The vertebrate c-Myb with three MYB domains (R1, R2, and R3) , an acidic activation domain (A), and C-terminal negative regulatory region. **B**. Three classes of plant MYB proteins, the three repeat R1R2R3 MYB proteins, the two repeat R2R3 MYB proteins, and the single repeat R3 proteins. Outside the conserved DNA binding domains, plant R2R3 MYB protein sequences are highly variable, with the exception of short C-terminal motifs (CTM) that are shared between members of phylogenetically related MYB subgroups.

The MYB transcription factor family in animals is a small group of proteins, comprised of three members in vertebrates (Luscher and Eisenman, 1990; Ganter et al., 1999), whose normal functions include the regulation of cell proliferation and differentiation, primarily in hemopoietic tissues (Weston, 1990; Introna et al., 1994). In contrast, the plant *MYB* gene family has undergone a dramatic expansion and diversification of function (Martin and PazAres, 1997; Kranz et al., 1998; Dias et al., 2003). *MYB* genes are one of the largest groups of transcription factor genes in plants, with a large expansion of the family pre-dating the divergence of dicotyledonous and monocotyledonous plants. There are 126 members in arabidopsis (Stracke et al., 2001), 192 in poplar (Wilkins et al., 2009), and large families in rice (Jiang et al., 2004; Chen et al., 2006), maize (Rabinowicz et al., 1999), and grapevine (Matus et al., 2008).

Most plant MYB proteins contain two imperfect, tandem repeats of the MYB domain corresponding to the second and third repeats of the putative ancestral three-repeat Myb protein, and are therefore termed R2R3 MYB transcription factors (Stracke, 2001) (Fig. 1-3B). A relatively small number of one repeat (R3) and three repeat (R1R2R3) MYB proteins have also been characterized in plants (Kranz, 2000; Stracke, 2001; Carre, 2002; Fig. 1-3B). In R2R3 MYB proteins, amino acid sequences carboxy-terminal to the conserved MYB domains are highly variable, with the exception of short conserved motifs shared among small groups of proteins. Even MYB factors that are functionally equivalent when heterologously expressed in different species can have vastly divergent sequences outside of the DNA binding domain, although the conservation of short sequence motifs indicates that these motifs are likely important for function. R2R3 MYB transcription factors have been categorized into subgroups based on both MYB domain sequence and the short conserved C-terminal amino acid sequence motifs, initially using the arabidopsis MYB family and subsequently the more recently sequenced plant genomes such as rice and poplar (Kranz, 1998; Stracke, 2001; Jiang et al., 2004; Matus et al., 2008; Wilkins et al., 2009). Some of the conserved regions may function as transcriptional activation domains, analogous to the acidic domain of c-Myb, repression domains (Jin et al., 2000), or domains mediating interactions with other regulatory proteins (Jin et al., 1999).

Although many remain functionally uncharacterized, plant MYB transcription factors have been found to be involved in the regulation of a diverse group of primarily plant-specific developmental and physiological processes (Stracke, 2001). These include regulation of primary (Bender and Fink, 1998) and secondary metabolism (Borevitz et al., 2000), cellular differentiation (Noda, 1994) and determination of cell fate (Gonzalez et al., 2009), mediation of cellular responses to hormones (Gubler et al., 1999), regulation of circadian rhythms (Mizoguchi, 2002), and response to pathogen attack (Lee, 2001) and light stress (Feldbrugge, 1997). A number of the R2R3 MYB proteins whose functions have been characterized are involved in the regulation of phenylpropanoid metabolism (Mol, 1999) and epidermal cell fate (Ramsay and Glover, 2005). In arabidopsis, as will be described in detail below, different branches of flavonoid and phenylpropanoid metabolism, including anthocyanin, flavonol, PA, lignin, and sinapate ester biosynthesis, are regulated by different MYB proteins controlling the expression of distinct suites of target genes. In this system, MYBs function as both activators and repressors, with MYBs of the R2R3 class being important activators, while both R2R3 and R3 MYB proteins function as repressors.

The BHLH proteins with which MYB proteins such as arabidopsis TT2 interact are members of a class of transcription factor proteins characterized by an N-terminal basic α-helix and a C-terminal helix-loop-helix (HLH) domain, a signature that was first characterized in animal proteins such as Myc and MyoD (Murre et al., 1989). In canonical BHLH proteins, the 16 amino acid-long basic region binds DNA sequences with the consensus CANNTG (called the “E box”), while the helix-loop-helix region is in some cases involved in homo- and heterodimerization (Voronova and Baltimore, 1990; Murre et al., 1994). BHLH transcription factors often contain additional protein-protein interaction domains that are essential for their regulatory functions. The involvement of BHLH proteins with MYB proteins in the regulation of flavonoid metabolism was first elucidated in maize. The first MYB gene identified in plants, and also the first plant transcription factor characterized, was the maize *C1* gene (Cone et al., 1986; Paz-Ares et al., 1986). Early genetic screens showed that anthocyanin biosynthetic genes in maize were co-ordinately controlled by a small group of regulatory genes including C1 (Dooner and Kermicle, 1974; Dooner and Nelson, 1977; Dooner, 1983). The maize C1 protein

was later shown to activate anthocyanin production in the aleurone layer of the endosperm (Cone et al., 1993; Romero et al., 1998). Interaction with the BHLH transcription factor R1 was found to be necessary for this activation. Several R1 paralogues, including B1, Lc, and Sn function in a similar manner with anthocyanin regulatory MYBs in other maize tissues (Chandler et al., 1989; Ludwig et al., 1989; Tonelli et al., 1991; Goff et al., 1992; Consonni et al., 1993). A domain C-terminal to the BHLH domain that mediates dimerization was identified by Feller et al. (2006) and shown to be necessary for R1-dependent C1 function, possibly affecting the DNA-binding ability of C1. This dimerization domain is found in maize R1 and its paralogues as well as a number of homologous arabidopsis BHLH proteins including GL3, EGL3, and TT8. Recently, Hernandez et al. (2007) identified a protein that interacts with the HLH domain of R and likely contributes to the regulation of the maize *DFR* gene through modification of chromatin structure.

Goff et al. (1992) determined that a region of R1 located N-terminally to the BHLH domain mediates the interaction with a region within the MYB DNA-binding domain of C1. Grotewold et al. (2000) determined that the site of interaction in the MYB partner is within the second MYB domain repeat (R3) and that several amino acid residues are required to confer R-dependant activity. Unlike C1, the closely related maize MYB protein, P, which controls a different branch of flavonoid metabolism (see below), is not dependent on a BHLH partner to function. Substitution of six amino acids in P with the corresponding amino acids in C1 that mediate the interaction with R results in a mutated P protein (P*) that can interact with R, and activate C1 regulated genes in an R-dependent manner (Grotewold et al., 2000; Hernandez et al., 2004).

Building on the analysis of Grotewold et al. (2000), Zimmerman et al (2004) used the arabidopsis MYB family to identify the R3 sequence motif $[DE]Lx_2[RK]x_3Lx_6Lx_3R$ as being essential for the interaction of MYB proteins with R-like BHLH partners. Although the BHLH family is quite large in arabidopsis, with 162 members, only members of BHLH subgroup III (which includes the proteins TT8, GL3, and EGL3) are known to interact with TTG1 and MYB proteins involved in TTG1-dependant regulatory complexes (Bailey et al., 2003; Bernhardt et al., 2003; Heim et al., 2003; Zimmermann et al., 2004). After characterizing the conserved amino acid sequence signature required for

interaction with subgroup III BHLH proteins using analysis of known BHLH-interacting MYBs and single site deletion analyses, Zimmerman et al. (2004) identified the interaction motif in 14 arabidopsis R2R3 MYB proteins (PAP1, PAP2, MYB113, MYB114, MYB5, TT2, GL1, MYB23, WER, MYB82, MYB4, MYB3, MYB7, and MYB32) and six R3 MYB proteins (CPC, ETC1, ETC2, MYBL2, CPL3, and TRY). Interactions were tested for predicted BHLH-interacting proteins, and novel interactions were detected using yeast two hybrid assays. For example, the arabidopsis R2R3 repressor protein, MYB4, which regulates sinapate ester biosynthesis by functioning as a repressor of *C4H* expression (see below), was shown to interact with BHLH subgroup III members, including TT8.

In arabidopsis, the BHLH and WDR members of the MYB-BHLH-WDR regulatory complex are involved in controlling a number of different processes, while MYB proteins confer specificity to the complex. For example, TT8 and TTG1 interact with TT2 to control PA biosynthesis (Nesi et al., 201; Baudry et al. 2004) and the MYB protein PAP1 (or the paralogues PAP2, MYB113, and MYB114; Gonzalez et al., 2008) to control anthocyanin metabolism (Zhang et al., 2003; Baudry et al., 2006). Additionally, TTG1 and TT8 or the BHLH proteins GL3 or EGL3 (Zhang et al., 2003; Baudry et al., 2004) interact with the MYB proteins MYB4, GL1 (and MYB23), WER, and MYB5 to regulate sinapate ester biosynthesis, trichome initiation, root hair development, and seed coat epithelial cell development, respectively (Larkin et al., 1994; Lee and Schiefelbein, 1999; Wang and Chen, 2008; Li et al., 2009). A schematic model for TTG1 and TT8/GL3/EGL3-dependent regulatory pathways in arabidopsis involving R2R3 MYB proteins is shown in Fig. 1-4. MYB proteins are key determinants of target gene specificity for MYB-BHLH-WDR regulatory complexes, although there may be some level of functional redundancy with expression controlling *in planta* activity (Lee and Schiefelbein, 2001; Gonzalez et al., 2009).

The R2R3 MYBs involved in flavonoid regulation or characterized by the [DE]Lx_2[RK]x_3Lx_6Lx_3R motif (Fig. 1-5) belong to several classes or phylogenetic clades. Arabidopsis TT2 is a member of subgroups 5, N08, and C32, using the classification systems of Stracke et al (2001), Jiang et al. (2004), and Wilkins et al. (2009), respectively. It is also the only arabidopsis member of the TT2-Related Subclade defined

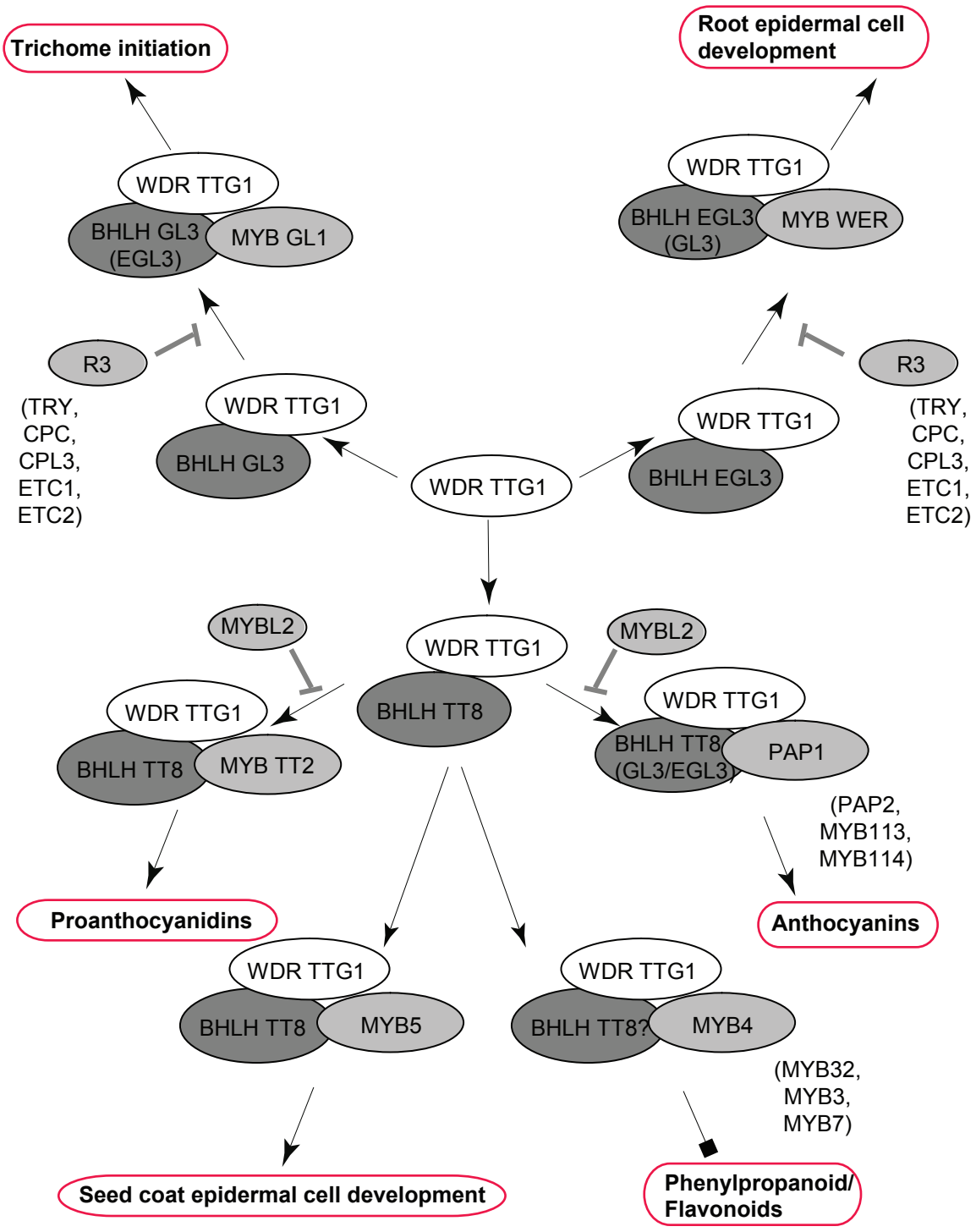


Figure 1-4. Schematic model of the TTG1 regulatory network in arabidopsis. As described in the text, MYB-BHLH-WDR complexes control flavonoid biosynthesis and epidermal cell development in arabidopsis. The WDR protein, TTG1, is involved with multiple BHLH proteins and MYB proteins to control these different processes. MYB proteins confer target gene specificity to the regulatory complexes. R3 MYBs (MYBL2, CPC, TRY, CPL3, ETC1, and ETC2) function as negative regulators, possibly by competing with R2R3 MYBs for BHLH interaction sites.

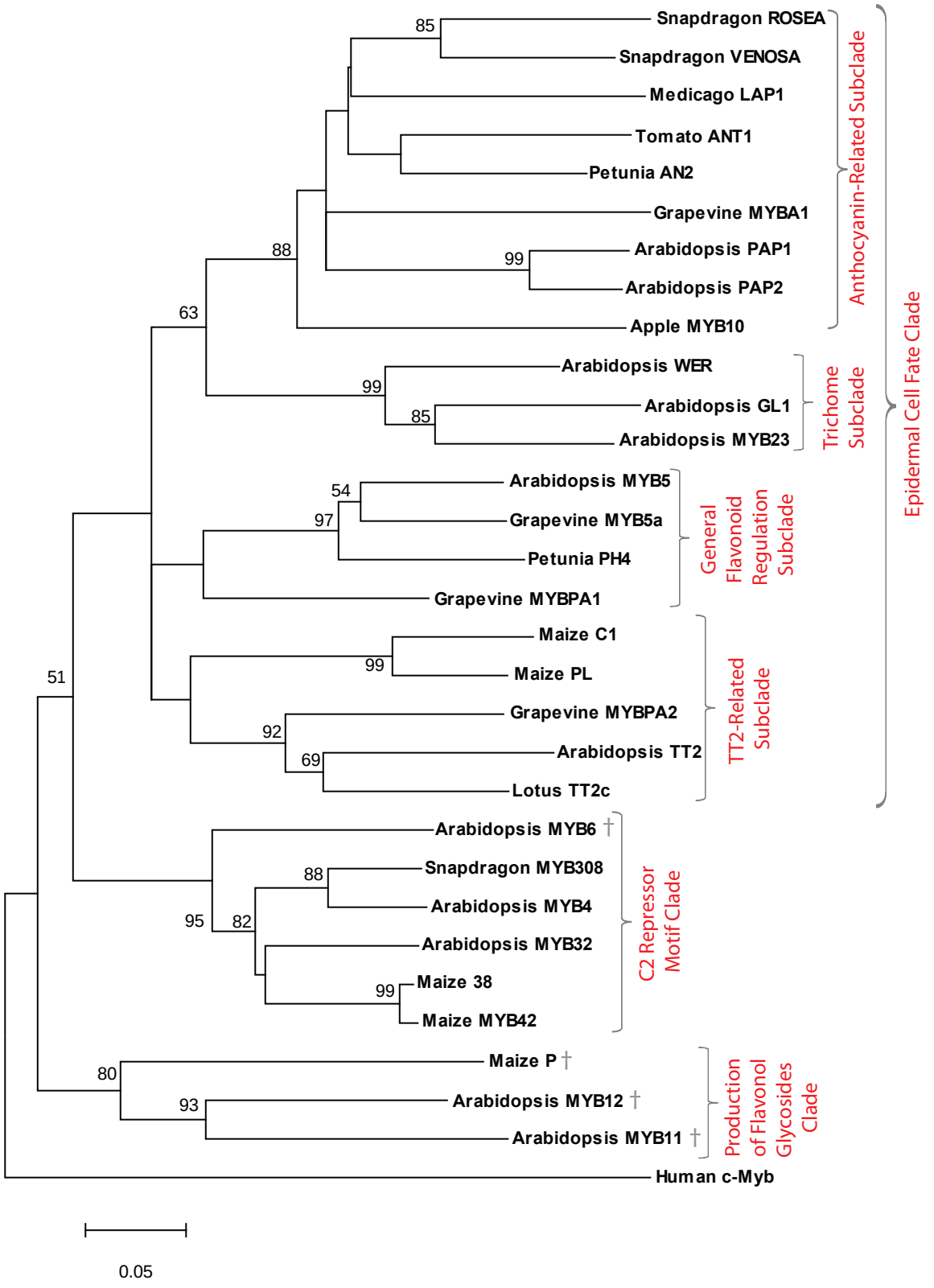
by Matus et al. (2008). Similarly, arabidopsis PAP1, together with PAP2, MYB113, and MYB114, is a member of Subgroup 6, N09, C27, and the Anthocyanin-Related Subclade. Arabidopsis MYB5 is not classified into a subgroup by Stracke et al. (2001), but is a member of subgroup N11 using the Jiang et al. (2004) system and C25 using the Wilkins et al. (2009) system, and a member of the General Flavonoid Pathway Regulation Subclade of Matus et al. (2008). Arabidopsis WER, MYB23, and GL1 are members of subgroup 15, G07, C45, and the Trichome Subclade. All of the above-listed proteins, together with MYB82 are classified into the Epidermal Cell Fate Clade by Matus et al. (2008). Arabidopsis MYB11, MYB12, and MYB111, which are not BHLH-interacting proteins, are members of subgroup 7, N13 and N14, C30 or the Production of Flavonol Glycosides Clade. Arabidopsis MYB4, together with MYB3, MYB7, and MYB32, is a member of subgroup 4 of Stracke et al. (2001) and C32 of Wilkins et al. (2009). Together with MYB6 and MYB8 (uncategorized by Stracke et al., (2001), and members of subgroup C2 of Wilkins et al. (2009)), which are not predicted to interact with BHLH partners, these MYBs constitute subgroup G05 (Jiang et al., 2004) or the C2 Repressor Motif Clade (Fig 1-5).

Additional BHLH-interacting members of these subgroups appeared to be involved in WDR-dependent regulatory complexes with activities that may not be present in arabidopsis. For example, the General Flavonoid Pathway Regulation Subclade member PH4 regulates vacuolar pH in petunia (Quattrocchio et al., 2006a), and grapevine MYB5a and MYB5b proteins function as general activators of multiple branches of flavonoid metabolism in developing grape (Deluc et al., 2006; Deluc et al., 2008; see Section 1.5.6).

R3 MYB proteins are also involved in the regulation of the processes controlled by MYB-BHLH-WDR complexes. R3 MYBs possessing the BHLH-interaction motif are thought to function as negative regulators by competing with R3R3 MYB proteins for interaction with BHLH partner proteins. The R3 MYB MYBL2 interacts with TT8 and functions as a negative regulator of anthocyanin and PA metabolism (Matsui et al., 2008; Dubos et al., 2008), while the functionally redundant R3 proteins TRY, CPC, CPL3, ETC1, and ETC2 function in an analogous manner in epidermal cell fate determination (Wada et al., 1997; Esch et al., 2004; Ryu et al., 2005; Tominaga et al., 2008) (Fig. 1-4).

WDR proteins, also known as WD40 proteins, are characterized by 40-43 amino acid regions repeated 4 to 16 times, each having an imperfectly conserved core region comprised of a glycine–histidine (GH) and a tryptophan–aspartate (WD) dipeptides separated by a partially conserved 26-28 amino acid region (Neer et al., 1994). The WD40 motif was first characterized in the G subunit of heterotrimeric G proteins, in which the WD40 repeats form a seven-bladed -propeller structure (Garcia Higuera et al., 1996; Smith et al., 1999). WDR proteins play a variety of regulatory roles, often facilitating protein-protein interactions (Neer et al., 1994; Ramsay and Glover, 2005). The first plant WDR protein characterized was the petunia AN11 protein (deVetten et al., 1997). *an11* mutant plants lack anthocyanin pigments, and homologous WDR proteins involved in the regulation of anthocyanin metabolism have been identified in several additional plants, including maize (PAC1; Selinger and Chandler, 1999; Carey et al., 2004), arabidopsis (TTG1; Walker et al., 1999), and *Perilla frutescens* (PFWD; Yamazaki et al., 2003). As described, the arabidopsis TTG1 protein mediates interactions between MYB and BHLH proteins involved in flavonoid metabolism as well as a number of developmental pathways, and homologous WDR proteins involved in cell fate determination (e.g., trichome initiation) have been identified in species such as cotton and Chinese cabbage (*Brassica rapa*) (Humphries et al., 2005; Zhang et al., 2009). However, it has been suggested that the MYB-BHLH-WDR regulatory complex evolved as a flavonoid regulatory complex, while involvement in cell fate determination was a later evolutionary development (Serna and Martin, 2006). A phylogenetically distinct group of R2R3 MYB proteins, including the snapdragon MIXTA protein, control a distinct epidermal cell development pathway that may pre-date the involvement of MYB-BHLH-

Figure 1-5. Phylogenetic analysis of R2R3 MYB transcription factors involved in flavonoid regulation of TTG1-dependent regulatory pathways. Neighbour-joining bootstrap tree constructed as described in Chapter 3 Materials and Methods (only bootstrap values higher than 50 are shown), using complete sequences of MYB proteins described in the text. Proteins that are not predicted to interact with BHLH partner are indicated by (†); all other proteins are predicted to be involved in WDR-dependant BHLH complexes. The clade and subclade designations of Matus et al. (2008) are shown. Human c-Myb included as an outgroup.


Snapdragon ROSEA
Snapdragon VENOSA
Medicago LAP1
Tomato ANT1
Petunia AN2
Grapevine MYBA1
Arabidopsis PAP1
Arabidopsis PAP2
Apple MYB10
Arabidopsis WER
Arabidopsis GL1
Arabidopsis MYB23
Arabidopsis MYB5
Grapevine MYB5a
Petunia PH4
Grapevine MYBPA1
Maize C1
Maize PL
Grapevine MYBPA2
Arabidopsis TT2
Lotus TT2c
Arabidopsis MYB6 †
Snapdragon MYB308
Arabidopsis MYB4
Arabidopsis MYB32
Maize 38
Maize MYB42
Maize P †
Arabidopsis MYB12 †
Arabidopsis MYB11 †
Human c-Myb
85
88
99
63
99
85
54
97
51
99
92
69
88
95
82
99
80
93
Anthocyanin-Related Subclade
Trichome Subclade
Epidermal Cell Fate Clade
General Flavonoid Regulation Subclade
TT2-Related Subclade
C2 Repressor Motif Clade
Production of Flavonol Glycosides Clade
0.05

WDR complexes in epidermal cell development in the rosid clade of dicotyledonous plants (Serna and Martin, 2006).

TTG1 functions in a transcriptional complex with the DNA-binding MYB and BHLH proteins. Baudry et al. (2004) showed using yeast two hybrid analysis that TT8, TT2 and TTG1 interact forming a ternary complex. In yeast three hybrid analyses, the addition of a third plasmid expressing TT2 or TT8 enhanced the stability of the TTG1-TT8 and TTG1-TT2 interaction, respectively, indicating that the three proteins form a complex *in vivo*. TTG1 does not appear to be necessary for DNA binding by TT2 and TT8, and may function by stabilizing the BHLH partner (Payne et al., 2000; Baudry et al., 2004). Supporting this function, overexpression of the maize BHLH gene R1 in *ttg1* arabidopsis mutants complements both the anthocyanin and trichome deficiencies (Lloyd et al., 2002).

Not all phenylpropanoid regulatory R2R3 MYB proteins are BHLH-WDR-dependent. For example, maize P and the arabidopsis MYB12, MYB11, and MYB111 proteins controlling flavonol metabolism (Stracke et al., 2007) as well as the arabidopsis MYB46, MYB58, and MYB63 proteins involved in the regulation of lignin biosynthesis (Zhong et al., 2007; Zhou et al., 2009) do not function within a MYB-BHLH-WDR complex. Fig. 1-6 presents a simplified model of the involvement of MYB proteins in the regulation of flavonoid and PA metabolism in arabidopsis. As will be described in the following sections, the involvement of MYB proteins in the regulation of flavonoid metabolism is highly conserved in plants, with proteins from distantly related plants often retaining functionality when heterologously expressed.

1.5.2 Regulation of the anthocyanin and flavonol pathways

Anthocyanin pigments serve essential roles in attracting pollinating and seed dispersing animals to flowers and to fruit (Harborne, 1993). Because of the high visibility of these compounds and of genetic lesions affecting their production, anthocyanins in flowers and other tissues are also effective at attracting plant geneticists. Beginning with the work of Gregor Mendel on pea (*Pisum sativum*) flower color (Brooker, 1999;

Fairbanks and Schaalje, 2007), anthocyanins have been a colourful model system for plant genetics as well as investigations of the biochemistry and regulation of plant secondary metabolism (Winkel-Shirley, 2001; Koes et al., 2005). Notably, an anthocyanin phenotype contributed to the discovery of RNA-mediated gene silencing in plants (Napoli et al., 1990; Jorgensen, 1995; Gonzalez, 2009). The anthocyanin biosynthetic pathway is one of the most well studied of plant secondary pathways and the MYB proteins involved in regulating anthocyanin biosynthesis are the most well characterized group of MYB proteins.

The maize *C1* locus controlling anthocyanin production in maize kernels was initially utilized by Barbara McClintock in her investigations that led to the discovery of transposable elements (McClintock, 1950; Cone et al., 1986; Buchanan et al., 2000; Chopra et al., 2006). C1 and paralogous MYB proteins such as PL, together with the BHLH protein R1 and its paralogues, regulate anthocyanin gene expression in a variety of maize tissues (Ludwig et al., 1989; Ludwig and Wessler, 1990; Cone et al., 1993b; Mol et al., 1996). C1 and paralogous MYB proteins are tissue-specific transcriptional activators of biosynthetic genes involved in anthocyanin production, including *CHS*, *F3H*, *DFR*, *ANS*, and *GST*, but not biosynthetic genes of the general phenylpropanoid pathway, such as *PAL* and *4CL* (Paz-Ares et al., 1986; Paz-Ares et al., 1987; Cone et al., 1993; Martin and Paz-Ares, 1997; Quattrocchio et al., 1998). The C-terminal region of C1 (i.e., not including the MYB domain) was shown to function as a transcriptional activation domain when fused to the GAL4 DNA-binding domain (Goff et al., 1991). The role of C1 in controlling flavonoid metabolism appears to pre-date the divergence of gymnosperms and angiosperm plants. A MYB protein from black spruce (*Picea mariana*) sharing high sequence similarity to maize C1 was shown to transactivate a maize anthocyanin biosynthetic gene promoter in an R1-dependent manner (Xue et al., 2003). The maize C1 protein is a member of the TT2-Related Subclade. Importantly, maize C1 was shown to strongly activate reporter gene expression from a 1496 bp arabidopsis *BAN* promoter fragment in arabidopsis protoplasts when transiently co-expressed with the maize *BHLH* gene *Sn*.

The closely related Anthocyanin-Related Subclade, which includes arabidopsis PAP1 and petunia AN2, is composed of numerous proteins that function as regulators of

anthocyanin biosynthesis in dicotyledonous plants (Table 1-1). Through a process of gene duplication and subfunctionalization, the Anthocyanin-Related Subclade genes may have evolved from TT2-Related Subclade-like ancestors, with TT2-Related Subclade genes evolving to become regulators of PA biosynthesis in dicotyledonous plants. Other evolutionary scenarios are also possible and characterization of additional C1-like proteins in other dicotyledonous plant species as well as additional monocotyledonous plants and gymnosperms should aid in clarifying the evolution of these regulatory systems. Because of the sequence similarity of C1 to PA regulators such as arabidopsis TT2, as well as the conservation of MYB protein function between species, it is very important to consider the functions of the well studied C1 gene when investigating the regulatory mechanisms controlling PA biosynthesis (Section 1.5.3).

In the regulation of flavonoid metabolism, the functions of MYB proteins are often conserved between even distantly related species. Expression of maize C1 together with a BHLH-type regulatory gene of the R1 family resulted in anthocyanin accumulation or flavonoid structural gene activation in arabidopsis, tobacco (Lloyd et al., 1992), and tomato (Bovy et al., 2002), as well as in petunia cell culture (Quattrocchio et al., 1993). Transformation of petunia with an R1 family member under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter resulted in anthocyanin accumulation, indicating that the mechanism of C1/PL interaction with R1-type BHLH regulators is conserved in petunia (Bradley et al., 1998). Anthocyanin-Related Subclade MYB proteins controlling anthocyanin metabolism have been described in numerous dicotyledonous plant species (Table 1-1). Petunia AN2 regulates a subset of flavonoid genes whose products act late in the anthocyanin biosynthesis pathway,

Table 1-1. R2R3 MYB transcription factors and known or putative target genes from selected plants. † The R2R3 MYB classification system of Matus et al (2008). ‡ Putative or proven target genes detected using methods such as expression profiling of mutants and overexpressors, as well as promoter binding and/or activation assays. Only flavonoid biosynthetic genes are included. Note that in many cases, direct activation is not proven. Italics indicates results of overexpression experiments in heterologous systems. *For a more extensive list and discussion of possible PAP1 target genes see Tohge et al. (2005) and Dare et al. (2008).

MYB	Species	Subgroup†	Target Genes ‡	References
Anthocyanin regulators				
C1	*Zea mays*	TT2-Related Subclade	CHS, F3H, DFR, *FLS*, ANS, UFGT, *RT*, GST, *ANR*	Cone et al., 1986; Paz-Ares et al., 1986; Paz-Ares et al.,1987 Quattrocchio et al., 1998; Baudry et al. 2004
MYBA	*Vitis* spp.	Anthocyanin-Related Subclade	PAL, CHS, CHI, F3H, ANS, UFGT, GST, OMT, MATE	Kobayashi et al., 2002; Cutanda-Perez et al., 2009
PAP1*	*Arabidopsis thaliana*	Anthocyanin-Related Subclade	PAL, 4CL, CHS, CHI, F3H, F3'H, DFR, ANS, UFGT, acyl-transferases, GST	Borevitz et al., 2000; Tohge et al., 2005; Comelli et al. 2008; Dare et al., 2008
ANT1	*Lycopersicon esculentum*	Anthocyanin-Related Subclade	CHS, CHI, DFR, UFGTs, MATE, GST	Mathews et al., 2003
MYB10	*Gerbera hybrida*	Anthocyanin-Related Subclade	PAL, C4H, CHS, F3H, F3'H, *DFR*, ANS, GST	Elomaa et al., 2003; Laitinen et al., 2008;
A	*Capsicum annuum*	Anthocyanin-Related Subclade	DFR, ANS	Borovsky et al., 2004
MYB3	*Gentiana triflora*	Anthocyanin-Related Subclade	F3'5'H, AT	Nakatsuka et al. 2008
Rosea1	*Antirrhinium* spp.	Anthocyanin-Related Subclade	F3H, F3`H, DFR, FLS, ANS, UFGT, MATE	Martin et al., 1991, Bartlett, 1989; Schwinn et al., 2006
Rosea2	*Antirrhinium* spp.	Anthocyanin-Related Subclade	CHI, F3`H	Schwinn et al., 2006
Venosa	*Antirrhinium* spp.	Anthocyanin-Related Subclade	CHI, F3H, F3`H, FLS, ANS, UFGT, MATE	Schwinn et al., 2006
MYB.Ph3	*Petunia*	Anthocyanin-Related Subclade	CHS	Solano et al., 1995
An2	*Petunia*	Anthocyanin-Related Subclade	CHS, DFR, RT, OMT	Quattrocchio et al., 1993
MYB1	*Ipomea nil*	Anthocyanin-Related Subclade	CHS, CHI, F3H, F3'H, DFR, ANS, UFGT, GST	Morita et al. 2006
MYB1	*Ipomoea batatas*	Anthocyanin-Related Subclade	CHS, CHI, F3H, DFR, ANS, UFGT	Mono et al. 2007
MYB10/MYB1	*Garcinia mangostana*	Anthocyanin-Related Subclade	DFR	Palapol et al. 2009
MYBA	*Malus x domestica*	Anthocyanin-Related Subclade	ANS	Ban et al. 2007
MYB10/MYB1	*Malus x domestica*	Anthocyanin-Related Subclade	*DFR, UFGT*	Epsley et al. 2007; Takos et al. 2007
LAP1	*Medicago truncatula*	Anthocyanin-Related Subclade	PAL, C4H, 4CL, CHS, CHI, F3'H, DFR, ANS, UFGT, AT, MATE	Peel et al. 2009
PA regulators				
TT2	*Arabidopsis thaliana*	TT2-Related Subclade	DFR, ANS, BAN, TT12, TT8, AHA10, *GT*	Nesi et al., 2001; Pang et al., 2009
MYBPA2	*Vitis vinifera*	TT2-Related Subclade	PAL, 4CL, CHS, F3H, F3'H, DFR, ANS, LAR, ANR, TT12	Terrier et al., 2009
TT2	*Lotus japonicus*	TT2-Related Subclade	ANR	Yoshida et al. 2008
MYBPA1	*Vitis vinifera*	General Flavonoid Regulation Subclade	PAL, 4CL, CHS, CHI, F3H, F3'H, F3' 5' H, ANS, LAR, ANR, TT12	Bogs et al., 2007; Terrier et al., 2009
Flavonol/Phlobaphene regulators				
P	*Zea*	Production of Flavonol Glycosides Clade	CHS, DFR, ANS	Grotewold et al., 1994
MYB12	*Arabidopsis*	Production of Flavonol Glycosides Clade	CHS, CHI, F3H, FLS	Mehrtens et al., 2005; Stracke et al., 2007
MYB11	*Arabidopsis*	Production of Flavonol Glycosides Clade	CHS, CHI, F3H, FLS	Stracke et al., 2007
MYB111	*Arabidopsis*	Production of Flavonol Glycosides Clade	CHS, CHI, F3H, FLS	Stracke et al., 2007
Flavonoid/Phenylpropanoid repressors				
MYB4	*Arabidopsis thaliana*	C2 Repressor Motif Clade	C4H, *4CL*, *CAD*, CHS	
MYB32	*Arabidopsis thaliana*	C2 Repressor Motif Clade	(DFR, ANS), COMT	Preston et al. 2004
MYB308	*Antirrhinum majus*	C2 Repressor Motif Clade	*C4H, 4CL, CAD, CHS*	Tamagnone et al. 1998
MYB330	*Antirrhinum majus*	C2 Repressor Motif Clade	*C4H, 4CL, CAD*	Tamagnone et al. 1998
38	*Zea mays*	C2 Repressor Motif Clade	DFR	Franken et al. 1994
MYB1	*Fragaria x ananasa*	C2 Repressor Motif Clade	*ANS*	Aharoni et al. 2001
Other flavonoid regulators				
VvMYB5a	*Vitus vinifera*	General Flavonoid Regulation Subclade	*PAL, C4H, 4CL, CHS*, CHI, *F3H*,F3'5'H, *DFR, LAR*, ANS, ANR	Deluc et al., 2006, 2008
VvMYB5b	*Vitus vinifera*	General Flavonoid Regulation Subclade	*CHS*, CHI, F3'5'H, LAR, ANS, LAR	Deluc et al., 2008

including those encoding DFR, 3GT, and GST (Quattrocchio et al., 1998), although *AN2* expression in maize can complement a mutation in *PL*, indicating that target gene specificity may be defined by different promoter structures in different species (Quattrocchio et al., 1993).

The arabidopsis *PAP1* gene, encoding a Anthocyanin-Related Subclade MYB protein homologous to AN2, was identified using an 'activation tagging' approach, in which plants were transformed with an *A. tumefaciens*-derived T-DNA (i.e., transfer DNA, the segment of DNA that is inserted into the host genome during *A. tumefaciens*-mediated transformation) carrying a CaMV 35S enhancer sequence at its right border (Borevitz et al., 2000). Mutants in which the *PAP1* locus was activated (*pap1-D* mutants) showed a large up-regulation of the phenylpropanoid pathway and anthocyanin accumulation in most tissues (Borevitz et al., 2000). In the *pap1-D* plants, expression of *PAL*, *CHS*, *DFR*, and a *GST* implicated in anthocyanidin transport was enhanced (Borevitz et al., 2000) (Table 1-1). Ectopic expression of *PAP1* in tobacco also resulted in anthocyanin accumulation (Zhou et al., 2008), illustrating the conserved function of this particular group of MYB genes between species.

In arabidopsis, increased *PAP1* expression has been shown to up-regulate the entire pathway leading to anthocyanin accumulation (Borevitz et al., 2000; Tohge et al., 2005a), although this regulation by PAP1 has not been proven to be direct and it is possible that other regulatory mechanisms are involved (Gonzalez et al., 2008). Promoter activation assays have shown that late anthocyanin biosynthetic genes, such as *DFR*, *ANS*, and *UFGTs*, are much more strongly activated than early flavonoid biosynthetic genes such as *CHS* and *F3H* (Dare et al., 2008). Additionally, arabidopsis *ttg1* and *BHLH* multiple mutants (i.e., *gl3*, *egl3* and *tt8*) show decreased expression of late but not early anthocyanin biosynthetic genes, as do RNAi knockdown lines in which expression of *PAP1* and the three paralogous arabidopsis Anthocyanin-Related Subclade *MYB* genes (*MYB113*, *MYB114* and *PAP2*) is reduced (Gonzalez et al., 2008). In many cases in other plants, BHLH- and WDR-dependant flavonoid regulatory MYB proteins appear to target the biosynthetic genes catalyzing later steps in the pathway (Table 1-1). Gonzalez et al. (2008) propose that late but not early flavonoid biosynthetic genes are direct targets of

WDR-dependent MYB transcriptional complexes. This may also be the case for WDR-dependent PA regulators (Section 1.5.6).

In maize, the MYB protein P activates the expression of a subset of flavonoid biosynthetic genes constituting a branch pathway leading to the production of phlobaphene pigments, a type of red colored 3-deoxy flavonoid produced through oxidation of flavan-4-ols that accumulate in maize kernel pericarp, silks and cob (Grotewold et al., 1991; Grotewold et al., 1994). Unlike C1 and PL, P does not interact with a BHLH-type protein (Grotewold et al., 1994). The P protein is a member of the Production of Flavonol Glycosides Clade, which also includes arabidopsis MYB12 (Hartmann et al., 2005; Mehrtens et al., 2005; Stracke et al., 2007; Matus et al., 2008). In transient transfection experiments, both arabidopsis MYB12 and maize P were shown to activate the arabidopsis *CHS*, *F3H*, and *FLS*, but not *F3'H* or *DFR* promoters (Mehrtens et al., 2005). In arabidopsis, MYB12 and the paralogues MYB11 and MYB111 control flavonol biosynthesis by targeting the early flavonoid biosynthetic genes together with *FLS* in different plant tissues (Stracke et al., 2007; Fig. 1-6).

Several studies have shown that the activation of flavonoid or anthocyanin biosynthesis by light exposure, UV-B, cold, dehydration, salt stress, or other stimuli is likely mediated by transcriptional activation of regulatory *MYB* genes, including both Anthocyanin-Related Subclade and TT2-Related Subclade regulators (Procissi et al., 1997; Piazza et al., 2002; Ithal and Reddy, 2004; Maeda et al., 2005; Takos et al., 2006; Ban et al., 2007; Cominelli et al., 2008). Chen et al. (2006) profiled the hormone- and stress-responsive expression of a large number of arabidopsis *R2R3 MYB* genes using reverse northern dot blot analysis. Members of the Anthocyanin-Related Subclade were induced in response to the plant stress signalling hormone JA (See Section 1.5.7), and *PAP1* was also induced by salt stress. Although the regulatory mechanisms controlling stress-responsive PA metabolism have not been elucidated, the observation that the coordinate up-regulation of biosynthetic genes involved in closely related branches of flavonoid metabolism involves MYB regulators that are also transcriptionally regulated suggests that co-expression analysis may be a useful approach for identifying MYB regulators of stress-induced PA metabolism.

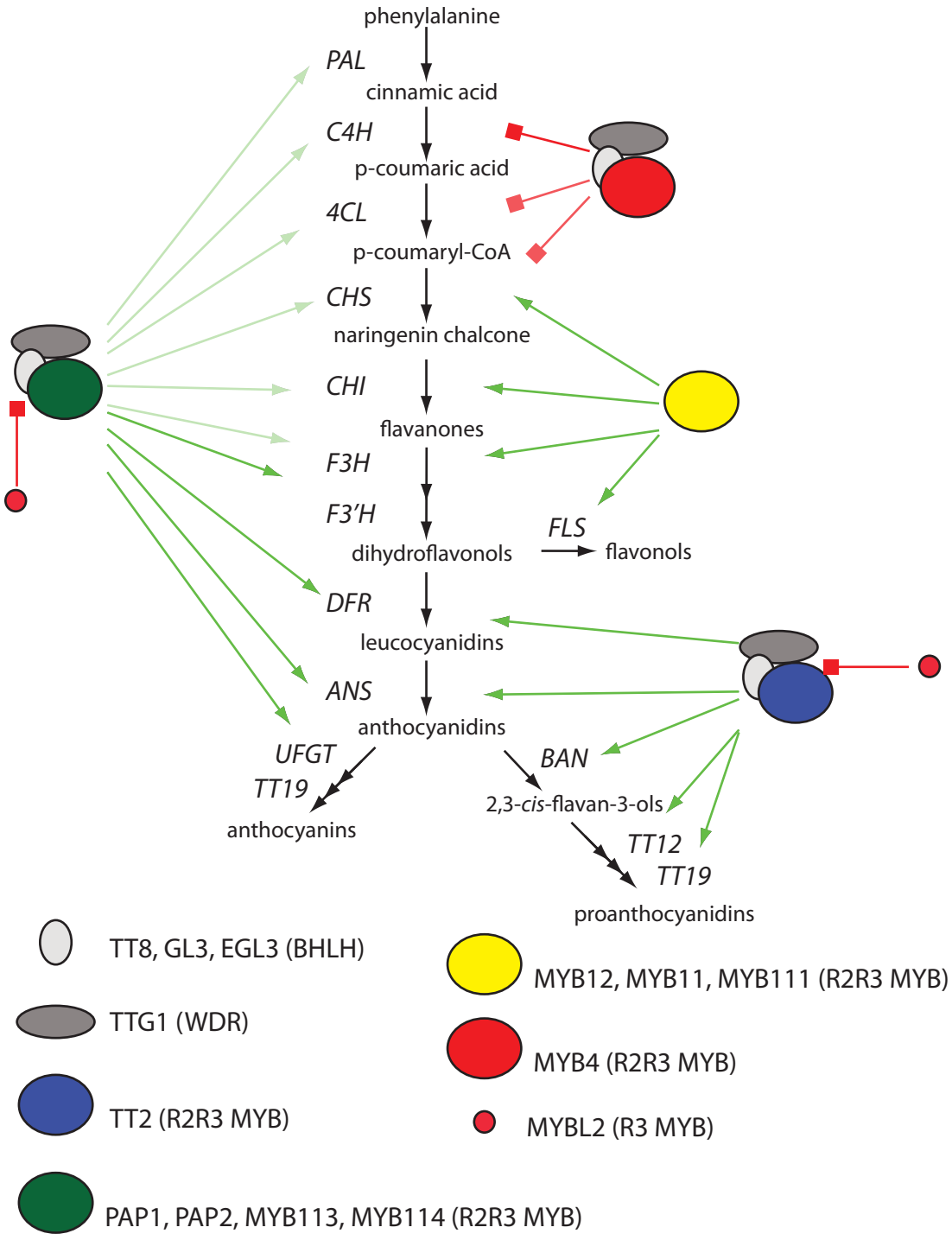


Figure 1-6. Schematic model of the regulation of flavonoid metabolism in arabidopsis by TTG1, BHLH proteins (TT8, GL3, EGL3), R2R3 MYB proteins PAP1 (and paralogues), TT2, MYB4, MYB12 (and paralogues), and an R3 MYB (MYBL2). Green arrows indicate gene activation functions, while red lines indicate negative regulatory functions. Lighter colored arrows indicate possible weaker activation, as described in the text.

1.5.3 Regulation of the PA pathway

Analysis of an arabidopsis seed coat PA mutant (Section 1.2.2) led to the identification of TT2, a MYB factor that controls seed PA biosynthesis. Arabidopsis *tt2* mutants show reduced or no seed coat expression of the late flavonoid and PA biosynthetic genes *DFR*, *ANS*, *ANR*, and *TT12*, but no loss of *CHS*, *CHI*, *F3H*, *F3'H*, or *FLS* expression (Nesi et al., 2001). Interestingly, arabidopsis TT2 appears to regulate the expression of *TT8* (Nesi et al., 2001; Tohge et al., 2005a; Baudry et al., 2006). PAP1 also appears to regulate *TT8* expression (Tohge et al., 2008), and ectopic expression of the petunia anthocyanin regulatory MYB gene *AN2* activates expression of the *TT8* homologue *AN1* (Spelt et al., 2000), indicating that regulation of BHLH cofactor expression by MYB factors may be conserved and that tissue specific expression patterns of anthocyanins and PAs may be controlled by MYB and WDR expression.

TT2-like R2R3 MYB factors appear to be involved in the regulation of PA metabolism in other plant species, as homologous genes have recently been described in *Brassica napus* (Wei et al., 2007), lotus (*Lotus japonicas*) (Yoshida et al., 2008) and grapevine (Terrier et al., 2009). Like arabidopsis TT2, the lotus TT2 and grapevine MYBPA2 are predicted to function in a MYB-BHLH-WDR complex. Lotus TT2 homologues are encoded by a family of several genes. All of the lotus TT2 proteins were shown to interact with arabidopsis TTG1 and TT8 in yeast two hybrid analyses and bombardment of arabidopsis leaf cells with constructs expressing the lotus TT2 genes as well as arabidopsis TTG1 and TT8 resulted in activation of a reporter gene fused to the arabidopsis *BAN* promoter (Yoshida et al., 2008). All of the functionally characterized TT2-like MYBs are characterized by a sequence motif with the consensus Vx_2IRTKA[IL]RC[SN], located C-terminal to the R2R3 MYB domain. The function of this motif is unknown.

Recently, another PA regulatory MYB gene, MYBPA1, belonging to a different MYB group was identified in grapevine (Bogs et al., 2007). Grapevine MYBPA1 is a member of the General Flavonoid Regulation Subclade, which includes arabidopsis MYB5, petunia PH4, and the grapevine MYB5a and MYB5b proteins (Section 1.5.2; Fig. 1-5). This MYB was isolated from an EST library made from developing grapes in which

PA biosynthesis is active. The MYBPA1 gene was shown to be co-expressed with the PA pathway genes, and MYBPA1 was shown in cotransfection assays to activate the arabidopsis *BAN* promoter and the grapevine *CHI*, *F3'5'H*, *LAR*, *ANS*, and *ANR*, but not anthocyanidin *UFGT*, promoters. *MYBPA1* expression in *tt2* arabidopsis complemented the mutation and also induced ectopic PA accumulation in seedlings. Interestingly, MYBPA1 is characterized by the [DE]Lx_2[RK]$x_3Lx_6Lx_3$R motif that mediates interaction with BHLH proteins but not the Vx_2IRTKA[IL]RC[SN] found in arabidopsis TT2 and related proteins (Bogs et al., 2007; Terrier et al., 2009). The characterization of this gene indicates that PA metabolism is controlled by at least two different types of R2R3 MYB proteins in grapevine and perhaps other species, although whether they function in the same cell types or under the same developmental or environmental conditions is not known.

In addition to the PA-specific MYB regulators of the TT2-Related Subclade and the General Flavonoid Regulation Subclade, the grapevine General Flavonoid Regulation Subclade members MYB5a and MYB5b appear to be general activators of flavonoid and PA metabolism. Overexpression of *MYB5a* in tobacco results in altered anthocyanin, flavonol, PA, and lignin levels (Deluc et al., 2006), and *MYB5b* overexpression likewise induces anthocyanin and PA accumulation in transgenic tobacco tissues (Deluc et al., 2008). *MYB5a*-overexpressing tobacco also exhibited indehiscent anthers, possibly caused by reduced anther endothecial cell lignification, caused by reduced expression of *caffeoyl coenzyme-A 3-O-methyltransferse* (*CCoAOMT*). Transient expression analysis showed that both proteins could activate expression from the arabidopsis *BAN* promoter and the grapevine *CHI*, *ANS*, *LAR*, and to a lesser extent *F3'5'H*, promoters (Deluc et al., 2008) (Table 1-1).

In addition to arabidopsis *TT2*, *TT8*, and *TTG1*, several other regulatory genes involved in controlling PA metabolism have been identified in arabidopsis using mutant screens. For example, the *TT1* gene encodes a novel zinc finger protein of the WIP family, although its role in PA regulation is yet to be determined (Sagasser et al., 2002). *TTG2* encodes a WRKY transcription factor that may represent a downstream target of MYB-BHLH-WDR complexes controlling PA biosynthesis as well as trichome and seed coat epithelial development (Johnson et al., 2002). The *TT16* locus encodes a MADS

domain protein that appears to play a role in seed coat endothelial cell development (Nesi et al., 2002).

1.5.4 Negative regulators of flavonoid metabolism

Several MYB proteins that function as negative regulators of phenylpropanoid and flavonoid metabolism have also been characterized. R2R3 MYB proteins of the C2 Repressor Motif Subclade, named for a conserved motif located carboxy-terminal to the DNA-binding MYB domain, are repressors rather than activators of phenylpropanoid and flavonoid biosynthetic genes. Maize MYB38 was found to completely inhibit C1-mediated activation of a reporter gene fused to the maize *DFR* promoter in co-transformation assays (Franken et al., 1994). Numerous additional C2 Repressor Motif Subclade R2R3 MYB proteins have since been shown to be involved in negative regulation of different branches of phenylpropanoid and flavonoid metabolism (Table 1-1). The snapdragon MYB308 and MYB330 proteins, when overexpressed in tobacco, down-regulated the general phenylpropanoid biosynthetic genes *C4H* and *4CL* as well as the gene encoding the lignin biosynthetic enzyme cinnamyl alcohol dehydrogenase (CAD) (Tamagnone et al., 1998). The maize C2 Repressor Motif Subclade MYB genes *MYB31* and *MYB42* also appear to function as repressors of lignin biosynthetic genes (Fornale et al., 2006; Sonbol et al., 2009).

Jin et al. (2000) used the sequence of snapdragon MYB308 to identify *MYB4* from among the arabidopsis R2R3 MYB genes, and then found it to function as a repressor of the phenylpropanoid biosynthetic gene *C4H*. When overexpressed in tobacco, arabidopsis MYB4 also repressed *C4H*, *4CL*, and *CAD* expression. Arabidopsis *myb4* mutants show a significant increase in foliar levels of the HCD sinapoyl malate, a major soluble phenolic compound in arabidopsis leaves. The authors showed that the increased sinapate ester accumulation conferred enhanced resistance to UV-B in *myb4* plants. Analysis of overexpressors and transfection assays revealed that MYB4 represses arabidopsis *C4H*, *4CL*, and *CHS* expression. The *MYB4* gene was shown to be down-regulated in response to UV-B exposure and mechanical wounding. Repression by

MYB4 may involve both binding site competition with activator MYBs (i.e., for both protein cofactors and DNA elements) as well as direct repression. A motif C-terminal to the MYB binding site with the consensus pdLNL$^{D}/_{E}$Lxi$^{G}/_{S}$ is conserved in C2 Repressor Motif Subclade MYBs, and has been shown by deletion and mutational analysis to be involved in repression by MYB4. Also, the C-terminal portion of MYB4 containing this motif confers transcriptional repression activity when fused to the GAL4 DNA-binding domain (Jin et al. 2000).

The arabidopsis C2 Repressor Motif Subclade family includes MYB3, MYB4, MYB6, MYB7, MYB8 and MYB32 (Kranz et al., 1998; Jin et al., 2000; Stracke et al. 2001). While all contain the predicted repressor motif, MYB3, MYB4, MYB7, and MYB32 are predicted to interact with BHLH partners, while MYB6 and MYB8 are not (Zimmermann et al., 2004). A *myb32* mutant was shown to be defective in pollen grain development and exhibited reduced expression of the lignin biosynthetic gene *caffeic acid o-methyltransferase* (*COMT*), but increased expression of *DFR* and *ANS*, in flowers (Preston et al., 2004). Interestingly, the *MYB32* promoter has also been shown to exhibit wound-induced expression in leaves of transgenic white clover (*Trifolium repens*) (Lin et al., 2003).

Given the size of the C2 Repressor Motif Subclade family in arabidopsis, it is possible that these putative repressor genes play uncharacterized roles in regulating other branches of phenylpropanoid metabolism including flavonoid production (see Section 1.6.3). The hormone- and stress-responsive expression profiling analysis of Chen et al. (2006) revealed that most members of the arabidopsis C2 Repressor Motif Clade were up-regulated by various plant hormones including jasmonic acid (See Section 1.5.7), the pathogen response-related signalling molecule salicylic acid (SA), as well as $CdCl_2$ and $NaCl_2$ stress treatments. Evidence that some C2 Repressor Motif Subclade members may function specifically as repressors of flavonoid biosynthetic genes comes from characterization of the strawberry (*Fragaria x ananasa*) *MYB1* gene. Overexpression of this C2 Repressor Motif Clade gene in tobacco resulted in a strong repression of *ANS* expression and reduced kaempferol and cyanidin glycosylation activity (Aharoni et al., 2001).

As mentioned above, R3 MYB proteins also function as repressors of MYB-BHLH-WDR complex-regulated target genes, possibly through competition with R2R3 MYBs for BHLH interaction sites. The TRY, CPC, CPL3, ETC1, and ETC2 R3 MYBs are involved regulating epidermal cell fate by affecting complexes involving the MYB proteins GL1, WER, and MYB23 (Wada et al., 1997; Lin and Schiefelbein, 2001; Wada et al., 2002; Bernhardt et al., 2003; Schiefelbein, 2003; Zhang et al., 2003; Ramsay and Glover, 2005; Ryu et al., 2005). Recently, the R3 MYB protein MYBL2 was shown function as a negative regulator of flavonoid biosynthesis (Dubos et al., 2008; Matsui et al., 2008). MYBL2 had been previously shown to interact with subgroup III BHLH proteins including TT8 (Zimmermann et al., 2004). Arabidopsis *mybl2* mutants exhibit elevated anthocyanin accumulation and elevated expression of *F3H*, *DFR*, *ANS*, *TT8*, and possibly *PAP1* (Dubos et al., 2008; Matsui et al., 2008). Expression of *MYBL2* under the *TT8* promoter resulted in a large decrease in seed coat PAs and a corresponding reduction in *CHS*, *F3H*, *F3'5'H*, *DFR*, *ANS*, *ANR*, and *TT8*, but not but not *TTG1* or *TT2*, expression (Dubos et al., 2008). In addition to being expressed in vegetative tissues, *MYBL2* is expressed in seed coat endothelial cells (Dubos et al., 2008), indicating that it plays a role in regulating both anthocyanin and PA metabolism in arabidopsis, through disrupting PAP1-BHLH-TTG1 and TT2-TT8-TTG1 complexes (Dubos et al., 2008; Matsui et al., 2008). Interestingly, MYBL2 expression is inhibited by light-stress, indicating this R3 protein may play a role in stress-induced flavonoid metabolism (Dubos et al., 2008).

In addition to the C2 Repressor Motif Subclade R2R3 MYB and single repeat MYB proteins, several additional negative regulatory mechanisms have been described in different plants species (reviewed by Quattrocchio et al., 2006b), including dominant negative mutants of R2R3 MYB and BHLH activators and an E3 ubiquitin ligase that may target GL3 (Downes et al., 2003; El Refy et al., 2004). This putative BHLH degradation-mediated negative regulatory mechanism is interesting in light of the proposed BHLH-stabilizing function of WDR proteins such as TTG1 (Payne et al., 2000; Baudry et al., 2004).

1.5.6 Phenylpropanoid and flavonoid gene promoters

Sequence-specific transcription factors function by binding to *cis*-acting regulatory elements and influencing gene expression by interacting with proteins such as the general transcription factor proteins involved in the initiation of transcription or proteins that alter chromatin structure. The DNA sequences bound by TT2 have not been elucidated, although the closely related maize C1 protein and numerous other MYB transcription factors have been shown to bind to A- and C-rich motifs known as AC elements that are present in the regulatory regions of numerous phenylpropanoid genes. AC element-like sequences, originally designated H-boxes with the core consensus CCTACC, were first identified in *PAL* and *CHS* genes and shown to be important for developmental and stress responsive expression (Lois et al., 1989; Loake et al., 1992; Yu et al., 1993). Canonical AC elements, AC-I (ACCTACC), AC-II (ACCAACC), AC-III (ACCTAAC), were defined in the minimal *PAL* promoter in bean, and shown to be necessary for vascular-specific expression (Hatton et al., 1995). Sablowski et al. (1994) identified a sequence designated Box P that was bound by a snapdragon MYB factor and characterized by an H-box core. Examination of a variety of plant phenylpropanoid and flavonoid promoter sequences that were available led to the identification of a P Box consensus, (A/C)ACC(T/A)A(A/C)C. Grotewold et al. (1994) showed that the maize P protein bound to a similar sequence, CC(T/A)ACC, within the maize *DFR* promoter. AC elements and related sequences have since been identified in the promoters of a wide variety of phenylpropanoid genes, including general phenylpropanoid genes (da Costa e Silva et al., 1993; Logemann et al., 1995; Bell-Lelong et al., 1997; Maeda et al., 2005; Haberer et al., 2006), genes involved in lignin metabolism (Patzlaff et al., 2003; Patzlaff et al., 2003; Gomez-Maldonado et al., 2004; Rogers and Campbell, 2004; Goicoechea et al., 2005; Zhou et al., 2009), and different branches of flavonoid metabolism, including general early (Solano et al., 1995; Faktor et al., 1997; Kobayashi et al., 1998; Koch et al., 2001) and late (Sainz et al., 1997; Lesnick and Chandler, 1998; Gollop et al., 2002; Shimada et al., 2007) flavonoid biosynthetic genes, as well as biosynthetic genes specific for flavonol biosynthesis (Hartmann et al., 1998; Hartmann et al., 2005). In addition to being involved in gene expression initiated by MYB transcriptional activators, AC

elements in phenylpropanoid gene promoters are also involved in repression of gene expression (Leyva et al., 1992; Hauffe et al., 1993; Hatton et al., 1995; Seguin et al., 1997).

In maize, both P and C1 bind to two *cis*-elements within the *DFR* promoter, a proximal ACCTACCAACC sequence and a distal AACTACC sequence (Sainz et al., 1997). Either site is sufficient for activation of reporter gene expression by P or C1 together with R1 in transient transformation assays in maize cells. P binds to the proximal site with high affinity, while C1 binds both sites with relatively low affinity. Interestingly, unlike P, C1 was shown to have very broad DNA sequence binding specificity. Although C1 bound sequences with the consensus A(C/A)C(T/A)A(C/A)C with the highest affinity, recombinant C1 protein also recognized several sequences with two mismatches relative to the consensus (Sainz et al., 1997). It has also been proposed that sequences matching the c-Myb consensus are important for C1-mediated activation of the maize anthocyanidin *UFGT* and *GST* genes (Roth et al., 1991; Bodeau and Walbot, 1996).

In between the two C1/P binding sites in the maize *DFR* gene is a conserved element that is present in a number of maize anthocyanin biosynthetic gene promoters, called the anthocyanin response element (ARE) (Tuerck and Fromm, 1994; Lesnick and Chandler, 1998; Pooma et al., 2002; Hernandez et al., 2004). While both P and C1 can activate DFR expression, C1 is dependent on R1, while P is not. Hernandez et al. (2004) showed that the P* protein, which can activate *DFR* expression independently of R1, exhibited R1-enhanced transcriptional activity that was abolished when the ARE was mutated. In maize cells but not in yeast, R1 is essential for activation of a promoter containing only a GAL4-binding site by a C^{MYB}-$GAL4^{DBD}$-$C1^{C\text{-}term}$ chimeric protein. These finding led to the hypothesis that R1 has two functions: interaction of R1 with C1 relieves C1 from the effects of an unknown inhibitor protein that is present in plant cells but absent in yeast; and, R1, or unidentified R1-interacting protein(s), bind to the ARE and enhance the activity of C1 and P* (Hernandez et al., 2004). An ARE-like sequence has also been identified in the *DFR* promoter of the dicotyledonous plant, *Gerbera hybrid* (Elomaa et al., 2003). Interestingly, c-Myb and BHLH consensus sites are located within this sequence.

In arabidopsis, minimal *CHS*, *CHI*, *F3H*, and *FLS* promoters required for light-induced expression in arabidopsis protoplasts contain both AC elements and sites matching a consensus bound by BZIP class transcription factors (Hartmann et al., 2005). Mutation of either site reduces light-induced reporter gene expression, suggesting that MYB and BZIP proteins act synergistically to activate expression of these flavonol biosynthetic genes. Activation by the maize MYB P protein is dependent on the AC elements only. C1 and the R1 paralogue, Sn, can activate expression from the arabidopsis *CHS* minimal promoter, but this activation is strongly reduced when a site matching the consensus of a BHLH binding site, designated the R-response element (RRE), is mutated. The RRE is also present in the *CHI* and *F3H*, but not *FLS*, promoters, suggesting a mechanism by which different BHLH-dependent (e.g., maize C1) and BHLH-independent (e.g., maize P and arabidopsis MYB12) MYB factors that recognize identical *cis*-elements may control different branches of flavonoid metabolism. However, the mechanism by which the BHLH-dependent PAP1 and TT2 proteins regulate the two branches of late flavonoid metabolism leading to anthocyanin and PA metabolism has not been resolved.

Functional dissection of the arabidopsis *BAN* promoter by Debeaujon et al. (2003) revealed that the -148 to -62 region was sufficient for reporter gene expression in PA-accumulating endothelial cells. This domain was functional in both orientations, and was termed the PA enhancer. This enhancer domain contains motifs that match the c-Myb consensus (CNGTTR) and BHLH consensus, as well as two AC element-like motifs, one of which is similar to the high affinity P binding site of the maize *DFR* promoter. A similar motif was also found in the promoter of the arabidopsis *DFR* gene (Debeaujon et al., 2003). The actual site(s) of TT2 binding within the *BAN* PA enhancer was not determined. Recently, Dare et al. (2008) identified a *cis*-element with the consensus (C/T)CNCCAC(A/G)(A/T)(G/T) in the promoters of PAP1-regulated genes, including *DFR*, *ANS*, *UFGT*, and *MYB114*, although it was not determined whether PAP1 interacts directly with this motif.

In summary, AC elements bound by MYB transcription factors are components of a variety of phenylpropanoid gene promoters, including genes of flavonoid, general phenylpropanoid, and lignin metabolism. The target gene specificity of MYB proteins is

likely determined by both DNA sequence recognition specificity and interactions with other regulatory proteins that may also interact directly with DNA. AC elements and c-Myb consensus sites may also play a role in the regulation of other genes regulated by MYB-BHLH-WDR complexes: both classes of *cis*-element have been implicated in the regulation of *GL2*, a regulatory target of the GL1-GL3/EGL3-TTG1 complex involved in trichome initiation (Szymanski et al., 1998; Wang and Chen, 2008).

1.5.7 Regulation of the regulators and upstream signalling

The accumulation of flavonoids appears to be primarily determined by the expression of regulatory genes controlling suites or subgroups of biosynthetic genes (Quattrocchio et al., 2006b), but very little is known about the upstream signalling pathways and regulatory mechanisms involved in controlling the expression of these regulators in development or in response to different stresses (Endt et al., 2002; Gonzalez, 2009). Analyses of the maize *C1* promoter have reveled sequences that are involved in controlling *C1* expression (Scheffler et al., 1994; Kao et al., 1996). A class of proteins called B3-domain transcription factors may play a role in regulating *C1* and other flavonoid regulatory genes (Suzuki et al., 1997; Tsuchiya et al., 2004; Quattrocchio et al., 2006b). As mentioned above, a number of *MYB* (e.g., arabidopsis *MYB114*) and *BHLH* (e.g., arabidopsis *TT8*) genes are regulatory targets of flavonoid regulatory R2R3 MYB factors. Espley et al (2009) described a 23-bp tandem repeat sequence in the promoter of the apple *MYB10* gene that is present in red foliage varieties. This minisatellite-like structure is bound by MYB10, suggesting that ectopic anthocyanin accumulation is caused by autoregulation of the *MYB10* locus.

A number of hormones (e.g., ABA, ethylene, JA) as well as sugars have been implicated in the signalling systems controlling anthocyanin biosynthesis (Paek et al., 1997; Jiang et al., 2003; Sheoran et al., 2006; Hung et al., 2008; Loreti et al., 2008). Light induction of anthocyanin biosynthesis involves cryptochrome and phytochrome photoreceptors (Wade et al., 2001; Matsumoto et al., 2003; Giliberto et al., 2005). JA signalling appears to be involved in a number of stress-induced flavonoid accumulation

responses. JAs are oxylipin defense signalling molecules involved in local and systemic responses to herbivory, pathogen infection, ultraviolet radiation, O_3 and other stresses (Li et al., 2002; Farmer et al., 2003; Chico et al., 2008; Yoshida et al., 2009). JA-based responses (reviewed by Chico et al., 2008) involve the targeted ubiquitination and 26S-proteasome-mediated degradation of repressor proteins (called JAZ proteins), releasing transcription factors that control downstream responses (Chini et al., 2007; Thines et al., 2007; Shoji et al., 2008). One target of JAZ protein repression is the arabidopsis MYC2 protein, which acts as both a transcriptional activator and repressor in mediating JA-responsive gene expression (Boter et al., 2004; Lorenzo et al., 2004; Lorenzo and Solano, 2005). Among the MYC2-regulated genes in arabidopsis are oxidative stress resistance genes and herbivore-defence genes. When germinated in the presence of JA, *MYC2*-overexpressing arabidopsis seedlings exhibit a strong anthocyanin accumulation, while anthocyanin accumulation is abolished in *myc2* mutants (Dombrecht et al., 2007). In a genome-wide expression profiling analysis of MeJa-treated wild type and *myc2* arabidopsis, Dombrecht et al. (2007) found reduced MeJa-inducible expression of numerous transcription factor genes in *myc2* plants, including *PAP1* and *EGL3*, suggesting a direct mechanism for JA-mediated stress-induced activation of flavonoid metabolism.

JA-mediated regulation of flavonoid regulatory genes may be conserved in numerous plant species including poplar. Jasmonates appear to be important in defence signalling in poplar (Constabel et al., 2000; Haruta et al., 2001; Haruta et al., 2001; Arimura et al., 2004; Wang and Constabel, 2004; Lawrence et al., 2006) and MeJa treatment has been shown to induce flavonoid biosynthetic gene activation and PA accumulation in poplar (Arnold and Schultz, 2002; Peters and Constabel, 2002). JA biosynthetic genes have been shown to be up-regulated in poplar leaves following herbivory (Ralph et al. 2006). In arabidopsis, JA-induced expression of JAZ repressor genes appears to provide a negative feedback loop controlling the JA-response pathway, and identification of possible poplar JAZ homolgues as wound- and FTC regurgitant-induced (Major and Constabel, 2006) suggests that this mechanism may be conserved in poplar.

1.6 Metabolic engineering of flavonoid and PA metabolism

1.6.1 Metabolic engineering of plant secondary metabolism

The possibility of identifying transcription factors that control entire pathways is motivating many studies in plant biotechnology, since such regulators would be valuable for the metabolic engineering of plants for both plant and human health (Dixon, 2005; Sharma and Dixon, 2006; Yu and McGonigle, 2005). Metabolic engineering involves manipulating biochemical networks in transgenic plants in order to quantitatively or qualitatively modify the composition of specific metabolites. Expressing genes encoding biosynthetic enzymes, particularly those representing rate-limiting biosynthetic steps, at high levels in plant tissues is one approach that has been used to engineer plant secondary metabolite pathways (Dixon et al., 1998; Jung et al., 2000; Yu et al., 2000; Sreevidya et al., 2006). However, this approach suffers from a number of limitations. Biosynthetic pathways are often regulated by feedback mechanisms and homeostatic controls, and the effects on product accumulation of overexpressing a single enzyme are often limited (Martin, 1996). An extension of this strategy is to engineer multiple biosynthetic pathway genes into a plant (Liu et al., 2007). Similarly, chimeric fusion proteins catalyzing more than one biosynthetic reaction have been successfully used to engineer plant secondary metabolism (Tian and Dixon, 2006). Metabolic engineering strategies have also been designed to manipulate competition between branch pathways for common substrates. Overexpressing the key enzyme(s) for one branch pathway in mutant or antisense-suppressed plants lacking a branch pathway that would be competing for precursor molecules has result in significant gains in product accumulation (Liu et al., 2002; Yu et al., 2003). Combining biosynthetic gene overexpression with stress conditions that activate the corresponding pathway has also been a successful strategy for increasing metabolite accumulation in engineered plants (Yu et al., 2000; Deavours and Dixon, 2005).

Transcription factors that coordinately regulate multiple pathway genes are of interest for plant metabolic engineering applications. Secondary metabolism pathway

genes are often coordinately regulated, and identification of transcription factors that regulate genes encoding multiple biosynthetic steps could offer advantages over strategies targeting enzyme-encoding genes (Tian et al., 2008). One advantage of this strategy is that if master regulators of specific branches of metabolism can be identified, flux can be increased through a metabolic pathway even when all the required biosynthetic steps have not been identified (Martin, 1996; Dixon and Steele, 1999).

1.6.2 Applications of flavonoid pathway engineering

Products of the flavonoid pathway are of particular interest for metabolic engineering applications such as enhancement of plant stress resistance and increased nutritional or 'nutraceutical' value of food (Schijlen et al., 2004; Truetter, 2005). There is evidence from *in vitro* and animal model research as well as epidemiological studies indicating that dietary flavonoids might confer protection against a variety of diseases, including cancer and cardiovascular diseases (Renaud and de Lorgeril, 1992; Hou et al., 2004; Seeram et al., 2004; Aron and Kennedy, 2008; Mursu et al., 2008). Health benefits resulting from the high antioxidant activity, antibacterial and antiviral activities, antimutagenic and antitumoral properties, and antiinflammatory effects of flavonoid metabolites could be enhanced through metabolic engineering of food plants for elevated levels of health-promoting bioactive flavonoids (Dixon and Steele, 1999).

The PA pathway is of particular interest as a target for plant metabolic engineering. PAs are very potent antioxidants and have powerful free radical scavenging capacity (Bagchi et al., 1997, 2002; Khanna et al., 2002; Nandakumar et al., 2008). Numerous studies with tumor models have shown potential anti-cancer effects of PAs through such mechanisms as cell cycle arrest in tumor cells, increased apoptosis, reduced proliferation, and anti-angiogenesis, and numerous molecular targets have been identified (Agarwal et al., 2004; Ariga, 2004). The antioxidant and antiinflammatory properties of PAs are thought to contribute to the prevention of cardiovascular disease through such mechanisms as inhibiting the oxidation of low density lipoproteins and lowering plasma cholesterol (Aron and Kennedy, 2008). PA-rich foods such as blueberry, blackberry,

cranberry, black current, and tea are widely recognized as having possible health benefits, including prevention of a number of cancers and heart disease (Seeram et al., 2006; Neto, 2007). Other health benefits that have been associated with the consumption of PAs or flavan-3-ols include those resulting from their antimicrobial, antiviral, antiparasitic and neuroprotective properties (Ray et al., 2000; Bagchi et al., 2001; Aron and Kennedy, 2008). Metabolic engineering of the PA pathway could have numerous applications for the development of health-promoting foods.

In addition to the development of nutritionally enhanced foods, there is considerable interest in PA pathway engineering in forage plants for ruminant pasture. Pasture bloat is a condition in which excessive amounts of gas build up in the rumen of ruminant animals such as cattle and sheep. It is a major problem in agriculture, leading to yearly losses of millions of dollars in Canada through death and reduced productivity of livestock (McMahon et al., 2000; Popp et al., 2000). Leguminous forages such as alfalfa and clover (*Trifolium* spp.) have a high potential for increasing weight gain in comparison to traditional forages but are also a major cause of pasture bloat (Coulman et al., 2000; Waghorn and McNabb, 2003). These forages are highly digestible and relatively high in protein, which is degraded by rumen microorganisms causing the production of excessive gas and foam that becomes trapped in the rumen (Min et al., 2006). Microbial degradation of forage proteins reduces protein utilization, limiting potential economic gains from the use high quality leguminous forages, and also causes the build-up of ammonia, which must be converted to urea in the liver (McMahon et al., 2000). Leguminous forages such as birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) that contain moderate levels of PAs in foliage do not cause pasture bloat, since PAs bind to proteins and slow microbial degradation leading to improved protein utilization by the animal (Lees, 1992). Unfortunately, these relatively PA-rich leguminous pasture forages have a lower sustainability and productivity than alfalfa and clover; introducing or increasing PA production in forage plants is thus an important objective for PA pathway engineering (Sharma and Dixon, 2005; Xie et al., 2006).

Since the biological functions of PAs are thought to include protection against pests and pathogens, improving plant stress resistance is another area where metabolic

engineering of the PA pathway may be of value. Improving stress resistance through engineering of PA metabolism in poplar may be of considerable value as poplar becomes increasingly important as a source of cellulosic ethanol. The conversion of plant sugars to ethanol is set to play a major role in meeting the growing energy demands and reducing our reliance on fossil fuels (McCown et al., 1996; Sims et al., 2006). Finding new feedstocks for bioenergy is a major priority in the development of this industry (Lemus and Lal, 2005). Poplar is recognized as a valuable future energy crop for the production of ethanol from wood cellulose (Ragauskas et al., 2006). In fact, the development of poplar as a renewable energy source was a major motivation for selection of this organism for genome sequencing by the US Department of Energy (Tuskan et al., 2006). Plantations of poplar and other trees for biofuel production have a significant capacity for carbon sequestration and are considered to be potentially important in mitigating greenhouse gas emissions (Lemus and Lal, 2005). PAs are highly stable, carbon-rich compounds that slow decomposition in the soil, and in addition to increasing productivity through enhanced stress resistance, metabolic engineering of PA metabolism in poplar may be useful for increasing the carbon sequestration capacity of plantation forests. Plantations of engineered trees with enhanced growth and processing characteristics, as well as improved pest and pathogen resistance and increased sequestration of degradation-resistant carbon compounds in shed organs, may play an important role in meeting the goals of reducing fossil fuel consumption and net greenhouse gas emissions.

1.6.3 Metabolic engineering of flavonoid and PA metabolism

The functional conservation of the transcriptional regulators of flavonoid metabolism allows for the possibility of heterologous expression of regulators from model species in plants of agricultural importance. The anthocyanins are one important target of metabolic engineering that have been intensively investigated. Several groups have attempted to produce transgenic tomato fruit with high levels of anthocyanins using heterologous expression of conserved anthocyanin regulators. Overexpression of the maize anthocyanin regulators *C1* (MYB) and *Lc* (BHLH) in tomato resulted in

accumulation of the flavonol kaempferol and naringenin, but not anthocyanins (Bovy et al., 2002). Mathews et al. (2003) identified a tomato MYB gene *ANT1* encoding a homologue of the arabidopsis *PAP1* gene and successfully up-regulated the anthocyanin pathway in skin, but not flesh, of tomato fruit. Overexpression of snapdragon *MYB* and *BHLH* genes *Rosea1* and *Delila*, however, led to high levels of anthocyanin accumulation in both skin and flesh of tomato fruit, reaching levels (up to 2.83 ± 0.46 mg/g) comparable to high anthocyanin-containing berries such as blueberry and blackberry (Butelli et al., 2008). A cancer-susceptible p53-knockout mouse line fed a dietary supplement from these high anthocyanin-accumulating tomatoes exhibited a significantly increased lifespan in comparison to those fed a wild type tomato dietary supplement (Butelli et al., 2008).

Transcription factor genes have also been utilized to engineer PA metabolism. Constitutive expression of *TT2* in arabidopsis does not induce plant-wide PA accumulation, indicating that PA synthesis is controlled by the tissue- and cell type-specific expression patterns of additional regulatory genes (Nesi et al., 2001). When overexpressed together with the arabidopsis anthocyanin regulatory gene *PAP1* and the maize BHLH gene *Lc*, high levels of PAs were synthesized in vegetative tissues of arabidopsis, but seedlings died within two weeks of germination (Sharma and Dixon, 2005). The grapevine PA regulatory R2R3 MYB protein, MYBPA1, unlike arabidopsis TT2, was found to regulate both early and late flavonoid and PA biosynthetic genes (Bogs et al., 2007). Constitutive expression of MYBPA1 in the arabidopsis *tt2* mutant not only complemented the PA-deficient seed phenotype but also induced ectopic (but not plant-wide) PA production (Bogs et al., 2007). However, levels of MYBPA1 expression sufficient for high PA accumulation similarly resulted in seedling death. Overexpression of the grapevine General Flavonoid Regulation Subclade genes *MYB5a* and *MYB5b* in tobacco resulted in ectopic accumulation of anthocyanins and PAs primarily in flowers (Deluc et al., 2005; Deluc et al., 2008). These results indicate that model plants such as tobacco and arabidopsis may not be an optimal system for studying PA pathway engineering in vegetative tissues.

Since arabidopsis TT2 is an activator of late, but not early, PA biosynthetic genes, but overexpression of *PAP1* is sufficient to activate strong anthocyanin production, Xie et

al. (2006) attempted to engineer PA metabolism in tobacco by overexpressing *PAP1* and the gene encoding the PA branch point enzyme ANR from *Medicago*. Tobacco vegetative tissues accumulated high levels of both anthocyanins and PAs, suggesting that this strategy may be useful for engineering PA metabolism in other species. Overexpressing just the *ANR* gene in *Medicago* resulted in a threefold increase in PAs, but only within anthocyanin-accumulating leaf spots (Xie et al., 2006).

BHLH proteins have also been employed in attempts to engineer PA metabolism in plants. Ray et al. (2003) transformed alfalfa with the maize *BHLH* gene *Lc*. PAs accumulated in alfalfa leaves, but only under light stress conditions in which *LAR* was up-regulated. Similarly, overexpression of *Lc* in petunia results in enhanced anthocyanin accumulation, but only under high light (Albert et al., 2009). Li et al. (2007) overexpressed *Lc* in apple (*Malus x domestica*) and found significantly increased accumulation of leaf anthocyanins, flavan-3-ols, and PAs, mediated by activation of *CHS*, *F3H*, *DFR*, *LAR*, and *ANR*. The maize BHLH gene *Sn* was used to enhance anthocyanin and PA accumulation in the birdsfoot trefoil, a legume that produces PAs in leaves (Robbins et al., 2003; Paolocci et al., 2005; Paolocci et al., 2007). Similar to the findings of Ray et al. (2003), high light levels enhance PA accumulation in *Sn*-overexpressing lines. As described in Section 1.5.1, BHLH proteins tend to function in the regulation of multiple pathways, explaining why both anthocyanin and PA production are affected in these transgenic plants. MYB proteins have been found to confer target gene specificity to MYB-BHLH-WDR transcriptional complexes. For this reason, MYB regulators of the PA pathway may constitute more effective tools for metabolic engineering applications aimed at specifically altering PA composition. Analysis of MYB proteins involved in PA regulation in high PA-producing species such as poplar may lead to the identification of new genes for the genetic engineering of PA metabolism in species of agronomical importance.

1.6.4 Candidate gene identification using transcription factor overexpression

Many gaps still exist in our understanding of flavonoid biosynthesis and regulation. As model plant genomes have become available, it has become apparent that these genomes contain large numbers of functionally uncharacterized genes belonging to families involved in secondary metabolism (Saito et al., 2008). For example, the arabidopsis genome encodes 272 cytochrome P450 genes, 107 glycosyltransferase genes and 130 ABC protein genes, most of which have not been characterized (D'Auria and Gershenzon, 2005; Saito et al., 2008). Analysis of the poplar and arabidopsis phenylpropanoid biosynthetic gene families has revealed the presence of expanded families, often including members that are quite divergent from the characterized members (Hamberger et al., 2007). With the availability of an increasing number of microarray datasets for model plants such as arabidopsis, co-expression analyses, in which novel genes are identified as co-expressed with known biosynthetic pathway genes under various conditions, is one useful approach for the identification of candidate genes that may have novel functions in secondary metabolism pathways (Vanderauwera et al., 2005; Yonekura-Sakakibara et al., 2007; Ehlting et al., 2008; Saito et al., 2008; Yonekura-Sakakibara et al., 2008; Sauveplane et al., 2009).

A variation on this approach is based on the use of transcription factors to engineer secondary pathways in model plants followed by transcriptome profiling to identify putative novel target genes that are co-activated with the known biosynthetic genes. This approach has been successfully applied using overexpression of flavonoid regulatory genes, leading to the identification of a numerous candidate flavonoid biosynthetic and regulatory genes. For example, putative *PAP1*-regulated genes have been identified through expression profiling of *PAP1*-overexpressing arabidopsis plants using whole genome microarrays (Tohge et al., 2005a; Dare et al., 2008). In addition to known flavonoid biosynthetic genes involved in anthocyanin metabolism (Table 1-1), these studies also revealed that *PAP1*-overexpression up-regulates numerous putative novel anthocyanin regulatory and biosynthetic genes. For example, in addition to the activation of known anthocyanin regulators such as *TT8* and the Anthocyanin-Related Subclade gene *MYB114*, *PAP1*-overexpression resulted in increased expression of several additional classes of transcription factor genes (Dare et al., 2008). *PAP1*-overexpression also resulted in the activation of a number of genes that may have biosynthetic roles in

anthocyanin metabolism, including acyltransferases, *UFGT*s, a non-specific lipid transfer protein, a *DFR*-related gene, and an *IFR*-related gene (Dare et al., 2008).

Laitinen et al. (2008) activated the anthocyanin pathway in *Gerbera hybrid* by overexpressing the *G. hybrid* Anthocyanin-Related Subclade *MYB10* gene, and then profiled gene expression in multiple tissue types using a 9K *Gerbera* cDNA microarray. In addition to homologues of the known anthocyanin biosynthetic genes, *MYB10* overexpression activated expression of a gene with sequence similarity to the lignin biosynthetic gene *caffeoyl-CoA O-methyltransferase* (*COMT*) as well as an *R2R3 MYB* gene with homology to the C2 Repressor Motif Clade. Cutanda-Perez et al. (2009) used a similar approach with the grapevine Anthocyanin-Related Subclade *MYBA1* gene. Overexpression of *MYBA1* in grapevine hairy roots (using *Agrobacterium rhizogenes*, see Veena and Taylor, 2007) resulted in the accumulation of high levels of anthocyanins. Transcriptional profiling using a grapevine 14.5K probe microarray showed that in addition to homologues of known anthocyanin biosynthetic genes, *MYBA1* overexpression resulted in increased expression of numerous genes including several oxidoreductases, a gene related to *cinnamoyl-CoA reductase* (*CCR*), and an R2R3 MYB C2 Repressor Motif Clade gene.

This approach has also recently been applied to the PA pathway using PA-regulatory R2R3 MYB transcription factors. Terrier et al. (2009) overexpressed the grapevine PA regulatory MYB genes *MYBPA1* and *MYBPA2* in grapevine hairy roots, resulting in a 5-fold increase in PA levels. Microarray analysis using the grapevine 14.5K probe array revealed that 55 genes were up-regulated by both *MYB* genes. In addition to known PA pathway genes, both *MYBPA1* and *MYBPA2* overexpression resulted in increased expression of two *UFGT* genes, a glucose acyltransferase, two *CCR-related* genes, and a R2R3 MYB C2 Repressor Motif Clade gene.

Pang et al. (2008) overexpressed the arabidopsis *TT2* gene in *M. truncatula* hairy roots, resulting in PA, anthocyanin and flavonol accumulation. Affymetrix microarray analysis identified 422 *TT2*-activated probesets. Among the corresponding genes were *M. truncatula* homologues of PA pathway genes including *PAL, 4CL, CHS, CHI, F3H, F3'H, F3'5'H, DFR, ANR, LAR, AN*S, *TT12, TT19,* and *AHA10*, as well as a putative *FLS*, and homologues of arabidopsis *TT8* and *TTG1*. Genes with possible novel functions

in PA metabolism included two *UFGT*-like genes. In order to narrow in on candidates with possible novel functions in PA metabolism, the authors also profiled gene expression in the PA-rich *M. truncatula* seed coat and compared the results with available *M. truncatula* expression data for other organs (Benedito et al., 2008) to identify transcripts preferentially expressed in seed coats. This dataset was then compared with the *TT2*-activated gene set, leading to the identification of UGT72L1, which functions as a flavan-3-ol glycosyltransferase (Section 1.2.2)

In some cases, functional characterization of co-expressed candidate genes has led to the identification of new enzymes involved in anthocyanin and PA metabolism. For example, Tohge et al. (2005a) functionally characterized two *UFGT* genes that were up-regulated in PAP1-overexpressing arabidopsis through analysis of mutants, and Pang et al. (2008) showed recombinant UGT72L1 could glycosylate (-)-epicatechin, (-)-epigallocatechin, (+)-catechin and cyanidin. The use of microarray analysis for transcriptome profiling of whole plants, calluses, or hairy roots in which regulators of flavonoid biosynthetic pathways are ectopically expressed is clearly an effective strategy for identifying novel flavonoid biosynthetic and regulatory genes, but the approach does have some pitfalls. Pleiotropic effects resulting from transcription factor overexpression may lead to artifacts resulting from alterations to cell physiology, excessive metabolite accumulation or activation of indirect targets through the up-regulation of other transcription factors (Dare et al., 2008; Pang et al., 2008). Datasets with relatively small numbers of up-regulated genes and a high proportion of known pathway genes (e.g., Tohge et al., 2005a; Laitinen et al., 2008; Cutanda-Perez et al., 2009; Terrier et al., 2009) might be expected to contain a higher number of true regulatory targets than datasets with large numbers of de-regulated genes (e.g., Dare et al., 2008; Pang et al., 2008). Since MYB factors of both the TT2-Related and Anthocyanin-Related Subclades are involved in MYB-BHLH-WDR complexes, and both are known to activate expression of BHLH proteins that could function in complexes that regulate other processes such as epidermal cell development, it is highly likely that ectopic expression will result in off-target effects in whole tissue transcriptomes. These pitfalls may be addressed by such strategies as comparing de-regulated gene lists with datasets derived from high PA-producing tissues (e.g., Pang et al., 2008), analyzing target gene promoter structures for *cis*-elements

recognized by flavonoid regulatory MYB factors or directly testing gene activation using promoter activation assays (e.g., Dare et al., 2008).

1.7 Hypotheses, objectives and summary of key findings

It has been demonstrated that the insect damage-induced accumulation of PAs in poplar leaves follows the transcriptional activation of PA biosynthetic genes (Section 1.4.6). R2R3 MYB transcription factors are known to be important regulators of flavonoid metabolism in plants (Section 1.5.3). It was hypothesized that an R2R3 MYB regulator would play a role in regulating the wound-induced up-regulation of the PA biosynthetic pathway in poplar leaves. As a first step toward the characterization of the stress-responsiveness of the PA pathway at the molecular level, expression profiling was conducted under several stress conditions, both biotic and abiotic. The experiments reported in Chapter 2 were conducted with two overall objectives. The primary objective was to identify of a comprehensive set of PA marker genes and stress-responsive expression profiles that could be used to screen for potential regulators through co-expression analysis. This suite of genes would represent a set of putative targets of regulatory proteins that mediate stress-responsive PA pathway activation, and could therefore also be used to analyze the functions of candidate regulators. In order to accomplish this, the aim of the first part of the project was to identify and clone PA marker genes from poplar, and then study gene expression profiles under conditions that activate PA metabolism or other branches of flavonoid metabolism. A secondary objective of the analyses reported in Chapter 2 was to test the hypothesis that inducible PA metabolism is a specific response to wounding by studying how this suite of biosynthetic genes as well as leaf phenolic metabolite levels respond to stresses other than mechanical wounding, such as UV-B exposure and biotrophic fungus infection. Although the data reported are not intended to test hypotheses regarding PA functions, they do expand our knowledge of PA pathway responsiveness under stress conditions and point to future directions that such research might take.

In the second part of this study (Chapter 3), these PA marker genes and stress-responsive expression profiles were used to screen candidate regulatory genes for expression patterns that coincide with PA biosynthetic gene activation in leaves. Arabidopsis *TT2*, a developmental regulator of PA metabolism expressed in seed coat endothelia, was the only known PA regulatory *R2R3 MYB* gene when this study was initiated. Based on this, as well as the importance of transcriptional regulation in stress-induced flavonoid metabolism and reports of stress-responsive expression of putative flavonoid-regulatory *MYB* genes (Section 1.5.2), it was hypothesized that a stress-induced TT2-like R2R3 MYB protein regulates PA pathway activation in poplar. The first objective of the research reported in Chapter 3 was to identify *TT2*-like poplar genes using searches of poplar sequence databases. The second objective of this part of the study was to study stress responsive expression of the identified poplar *MYB* genes, followed by functional characterization of candidate genes.

In order to test the functions of the candidate regulators of stress-induced PA metabolism, constitutive overexpression in poplar using *A. tumefaciens*-mediated transformation was performed. This approach was chosen because in addition to contributing to functional characterization of the gene(s), it was hoped that overexpression of an activator of PA metabolism would cause alterations of the PA pathway gene expression in transgenic poplar. If such an effect was achieved, the corresponding regulatory gene would have a number of interesting applications. For example, it was hypothesized that a poplar *TT2* homologue could be used to identify novel poplar PA regulatory and biosynthetic genes (see Section 1.6.4). Additionally, the biological functions of PAs are still poorly understood (Section 1.4.4), and manipulation of the pathway in an important model species such as poplar would be a powerful experimental tool for evaluating proposed stress-protective functions.

Chapter 3 describes the identification of *MYB134* as a poplar *TT2*-like gene that is co-regulated with PA biosynthetic genes in stress-treated leaves. Constitutive expression of *MYB134* in transgenic poplar results in a strong, specific activation of PA pathway genes leading to a dramatic, plant-wide increase in PA concentrations. Analyses of *MYB134*-overexpressing poplar are presented together with additional experiments that indicate that the product of this gene may play a role in regulating the PA pathway. The

results of a leaf transcriptome profiling analysis following *MYB134* overexpression in poplar using the Affymetrix GeneChip® Poplar Genome Array are reported, including the identification of a number of genes that may represent novel regulatory or biosynthetic functions in PA metabolism. Additionally, preliminary results are reported to show that the use of transgenic trees with modified phenolic metabolism may be of value for research aimed at determining the biological functions of PAs in poplar.

Chapter 2: Molecular Analysis of Stress-Induced PA Metabolism in Poplar

Some of the work described in this chapter has been published in the following articles:

Mellway RD, Constabel CP (2009) Metabolic engineering and possible functions of proanthocyanidins in poplar. Plant Signaling & Behavior 4: 790-792

Mellway RD, Tran LT, Prouse M, Campbell MM, Constabel CP (2009) The wound-, pathogen-, and UV-B-responsive *MYB134* gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin metabolism in *Populus*. Plant Physiology 150: 924-941

Miranda M, Ralph SG, Mellway RD, White R, Heath MC, Bohlmann J, Constabel CP (2007) The transcriptional response of hybrid poplar (*Populus trichocarpa x P. deltoides*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Molecular Plant Microbe Interactions 20**:** 816-831

The data in Table 2-2 was contributed by Dr. Juha-Pekka Salminen (Department of Chemistry, University of Turku, Finland).

2.1 INTRODUCTION

PAs may be important plant defence molecules (Section 1.4), and poplars and other tree species commit significant resources to PA production in leaves (Section 1.3.2). In poplar, leaf PAs rapidly accumulate following wounding and herbivore damage, and recent analyses have shown that this is mediated by the activation of genes encoding biosynthetic enzymes such as PAL, CHS, and DFR (Section 1.4.6). The primary objective of the experiments reported in this chapter was to facilitate analyses leading to the identification of PA pathway regulators in poplar (Chapter 3). Since transcriptional regulators of stress-induced flavonoid metabolism acting directly upstream of biosynthetic gene activation are likely also transcriptionally activated following pathway-activating stimuli (Section 1.5.2), the expression profiles reported in this chapter form the basis for co-expression analyses aimed at identifying candidate poplar PA regulatory genes. These stress-induced PA biosynthetic genes are also intended to serve as markers to analyze the functions of putative PA regulators since they constitute a set of potential target genes, the expression of which could be analyzed following constitutive overexpression of putative PA regulatory factors (Chapter 3).

Poplar leaf PA levels are strongly affected by growth conditions such as O_3-exposure, elevated light, and nutrient limitation, indicating that the *in planta* functions of PAs may be broader than herbivore defence (Sections 1.3.2 and 1.4.4). It is unknown whether PA metabolism exhibits a rapid up-regulation upon initial exposure of poplar leaves to these stress stimuli. It has often been observed that exposure to one stress will enhance plant resistance to others, indicating that many stress-induced secondary metabolites are multifunctional or that they protect plants against a common component of multiple stress conditions (Ajay et al., 2002). Flavonoids closely related to PAs, such as anthocyanins and flavonols, have been found to accumulate under numerous stress conditions in plants (Section 1.4.1).

A secondary aim of the experiments reported in this chapter was to test the hypothesis that rapid up-regulation of PA pathway genes is a response that is specific to mechanical wounding and herbivory. The alternative, that multiple biotic and abiotic

stress conditions activate PA metabolism, would indicate that inducible PA metabolism in poplar has evolved to serve a different or broader function than defence against herbivory. However, no experiments were conducted to test the efficacy of poplar PAs as stress-protective molecules. A selection of marker gene transcripts for flavonoid and PA metabolism were cloned, and expression of these genes was profiled in response to wounding, elevated light, UV-B irradiation, and infection by a compatible parasitic fungal biotroph (*Melampsora medusae*, the causative agent of poplar leaf rust disease). Flavonoid and PA metabolite accumulation in stress-treated leaves was also profiled under these stress conditions.

Analyzing the molecular mechanisms mediating UV-B-inducible flavonoid accumulation in trees like poplar is important, given the increase in solar UV-B radiation reaching the Earth's surface due to the loss of stratospheric O_3 (Warren et al., 2002). Poplar species are major components of boreal and northern temperate biomes, where ground-level UV-B radiation is predicted to increase dramatically in the near future (Bjorn et al., 1998; Warren et al., 2003). Increased production of flavonoids such as flavonol glycosides and PAs may affect plant-herbivore interactions as well as decomposition of shed organs, and thus have ecosystem-scale consequences (Rousseaux et al., 1998; Veteli et al., 2002; Izaguirre et al., 2003; Veteli et al., 2003; Warren et al., 2003; Rousseaux et al., 2004; Julkunen-Tiitto et al., 2005). Although rapid up-regulation of the flavonol pathway following exposure to UV-B radiation has not been documented in poplar, it is a common plant response (Section 1.4.1). Furthermore, growth under prolonged conditions of elevated UV-B is known to result in increased accumulation of quercetin and kaempferol glycosides in poplar (Warren et al., 2003). Therefore, expression of a marker biosynthetic gene encoding a flavonol-specific enzyme FLS was also profiled in this study.

The basidiomycete, *M. medusae*, is an obligate biotrophic pathogen of poplar (e.g., *P. tremuloides*, *P. deltoides* and *P. trichocarpa x deltoides* hybrids) (Ziller, 1974; Karnosky et al., 2002; Newcombe et al., 1996). *M. medusae* is an important, wide-spread poplar pathogen that can cause significant yield losses in poplar plantations through premature leaf drop and loss of vigor (Walker and Hartigan, 1972; Widin and Schipper, 1981; Chen and Harrington, 2006). As a heteroecious (i.e., alternating between two

hosts), macrocyclic (i.e., producing five spore forms) rust, *M. medusae* has a complex life cycle involving a dikaryotic stage on a poplar host and a monokaryotic stage on a conifer host (Johnson and Kim, 2005; Bourassa et al., 2007). As a biotrophic parasite, *M. medusae* acquires nutrients from the living cells of susceptible hosts though specialized intercellular hyphal structures known as haustoria (Heath, 1997; Mendgen and Hahn, 2002; Glazebrook, 2005). A successful compatible infection by a urediniospore germling involves penetration of stomata by a structure called an appressorium, followed by the formation of a substomatal vesicle, an infection hypha, and the subsequent establishment of a haustorial mother cell from which the haustorium invades a host mesophyll cell (Heath, 1997; Mendgen and Hahn, 2002; Spanu, 2006; Rinaldi et al., 2007). Compatible biotrophic plant pathogens are able to successfully infect host tissues by escaping detection or suppressing host defenses (Schulze-Lefert and Panstruga, 2003). After penetration of the plant cell wall, the haustorium is thought to absorb plant nutrients through hexose and amino acid transporter proteins, facilitating continued hyphal growth (Schulze-Lefert and Panstruga, 2003; Both et al., 2005; Glazebrook, 2005). These hyphae then proliferate within the leaf parenchyma, producing more intercellular haustoria and ultimately the formation of uredinial pustules containing asexual urediospores on the abaxial leaf surface.

The experiments reported in this chapter establish a suite of marker genes and expression profiles for stress-induced PA pathway activation in poplar. It was determined that not only mechanical wounding of leaves, but light stress, UV-B irradiation, and *M. medusae* infection cause the activation of the PA pathway in poplar leaves. Poplar was also shown to respond to UV-B irradiation with a rapid activation of flavonol metabolism, with this response being much earlier than the UV-B-induced PA pathway up-regulation. The significance of these findings is discussed with reference to the possible *in planta* functions of stress-induced PA pathway activation in poplar.

2.2 MATERIALS AND METHODS

2.2.1 Plant growth conditions and stress treatments

The *P. tremuloides* clone A2 had been collected from the vicinity of Edmonton, Alberta, Canada (Haruta et al., 2001). *P. tremula x tremuloides* clone INRA 353-38 was provided by Richard Meilan (Purdue University, West Lafayette, IN), *P. tremula* x *alba* clone INRA 717-1-B4 by David Ellis (CellFor, Victoria, BC, Canada), and *P. trichocarpa* x *deltoides* clone H11-11 by Gary Radamaker (Washington State University, Pullman, WA). All clones were micropropagated *in vitro* on solid Murashige and Skoog medium (Sigma, St. Louis, MO), except clone H11-11, which was macropropagated from greenwood cuttings. Plants were maintained in the Bev Glover Greenhouse with fertilizer and light levels as described by Major and Constabel (2006). Twelve week-old plants were used for all stress experiments. All stress treatments were applied beginning at 10:00 am. Upon harvest of leaves, mid veins and necrotic tissue was removed and tissues were frozen in liquid nitrogen and stored at -80º C until analyzed. Leaves within the range of LPI 9-15 (leaf plastochron index, leaves number successively down from leaf 0, the youngest leaf with a lamina length of 2 cm; Larson and Isebrands, 1971) were used for all stress experiments.

For mechanical wounding experiments, leaf margins totaling approximately one fifth of the area of each leaf were crushed with pliers. For high light (HL)-exposure experiments, trees were moved from the greenhouse (mean maximum photosynthetically active radiation (PAR, 400-700 nm): 377 mol m^{-2} s^{-1}; biologically effective UV-B irradiance ($UV\text{-}B_{be}$): 0.26 kJ m^{-2} day^{-1}) into full natural sunlight during August in Victoria, BC (mean maximum PAR: 1655 mol m^{-2} s^{-1}; $UV\text{-}B_{be}$: 3.48 $kJ^{-1}m^{-2}$ day^{-1}). The wounding and light stress experiment shown was conducted in August 2005. For UV-B treatments, trees were acclimated for one week in a growth chamber (16/8 hr photoperiod, 19-25º C) equipped with F40T12 UV-B lamps (Phillips Lighting Company, Somerset, NJ) with pre-solarized cellulose acetate filters to block UV-C transmission. Plants were exposed to 0.21 kJ m^{-2} day^{-1} $UV\text{-}B_{be}$ before UV-B lamps were activated and 1.45 $kJ^{-1}m^{-2}$ day^{-1} $UV\text{-}B_{be}$ after activation. The UV-B experiment shown was conducted in October 2006. Measurements of $UV\text{-}B_{be}$ were made with an IL1700 radiometer equipped with an IL782A high gain photo-multiplier (International Light, Newburyport,

MA) using weighting factors from the Caldwell action spectrum normalized to 300 nm (Bjorn and Teramura, 1993; L'Hirondelle and Binder, 2002). Clone 353-38 or H11-11 were used for wounding, HL, and UV-B experiments as indicated. For *Melampsora medusae* infections, urediospores were collected from foliage and used to inoculate leaves of the susceptible clone H11-11 as described by Miranda et al. (2007). The experiment shown was conducted in October 2006 and was a replicate of an experiment conducted in March 2005.

2.2.2 RNA extraction and expression analysis

RNA for northern and PCR analyses was isolated from leaf tissue using the cetyltrimethylammonium bromide (CTAB) method as described by Haruta et al. (2001). Northern analysis was performed using ^{32}P-labeled DNA probes using standard protocols (Church and Gilbert, 1984; Sambrook and Russell, 2001). ^{32}P-labeled probes were synthesized with the Rediprime II labeling kit (Amersham, Sunnyvale, CA) using Qiaquick-purified (Qiagen, Mississauga, ON) DNA template fragments. For phenylpropanoid biosynthetic gene probes, cDNA sequence fragments were amplified from a cDNA preparation made from total leaf RNA from clone 353-38 using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Primers were designed using sequences available from the DOE Joint genome project database version 1.1 (http://genome.jgi-psf.org/Poptr1_1) or in GenBank (http://www.ncbi.nlm.nih.gov). For primer sequences, see Table A1-1. cDNAs were cloned into the vector pGEM-T Easy (Promega, Madison, WI). Sequencing to confirm insert sequences was performed by the Center for Biomedical Research Sequencing Facility (University of Victoria). The *DFR1* and *PAL2* probes were synthesized using the cloned fragments described in Peters and Constabel (2002).

2.2.3 Phytochemical assays and HPLC analysis

For HPLC analysis, 0.50g frozen leaf tissue was ground in liquid nitrogen and extracted for 4 hours in 10 mL 80% methanol. Extracts were centrifuged to remove solid debris and methanol was removed using a rotary evaporator, followed by clean-up with Strata-X 33 μm solid phase extraction columns, according to the manufacturer's instructions (Phenomenex, Torrence, CA). Compounds were eluted in 2 mL methanol:acetonitrile (1:1, v/v) and 30 μL was injected onto an HPLC system (Beckman Coulter System Gold 126 Solvent Module with a System Gold 168 diode array detector, Beckman Coulter, Inc. Fullerton, CA,) with a reverse phase Luna C18(2) column (250 x 60 mm, 5 μ; Phenomenex, Torrence, CA). Separation was performed with a linear elution gradient from 90% solvent A (0.5% methanol in 0.01 M phosphoric acid, v/v) to 40% solvent B (100% acetonitrile) over 30 min at a flow rate of 1.5 mL min^{-1}. The subclasses of phenolic compounds present in poplar leaves are well characterized and peaks can be assigned to different phenolic subclasses based on their distinctive UV/visible absorption spectra and quantified using representative standards (Markham, 1982; Maatta et al., 2001; Santos-Buelga et al., 2003; Mabry et al., 1970). All standards were from Sigma (St. Louis, MO) unless specified otherwise. Total HCDs were quantified as chlorogenic acid equivalents. Total flavonol glycosides were quantified as rutin (quercetin-3-rutinoside) equivalents. Phenolic glycosides were quantified using purified tremulacin, tremuloidin, and salicortin, provided by Thomas Clausen (University of Alaska). Proanthocyanidins were assayed using the acid-butanol assay, as described by Porter et al. (1986) and Peters and Constabel (2002). Purified trembling aspen proanthocyanidin was used as a standard, provided by Lynn Yip and Kevin Tam (University of Victoria). Relative levels of total soluble phenolics were determined using the Folin-Ciocalteau method, with gallic acid used as a standard (Singleton and Rossi, 1965).

For the phenolic analyses conducted by Dr. Juha-Pekka Salminen (Department of Chemistry, University of Turku, Finland), 20 mg leaf powder from each sample was extracted with 800 μl of acetone/water (7/3, v/v, containing 0.1% ascorbic acid) for 1 hour. The extraction was repeated a total of four times and the extracts were pooled. The acetone was removed with an Eppendorf concentrator 5301 (Eppendorf AG, Hamburg, Germany), and the remaining aqueous extract was freeze-dried. The freeze-dried sample was dissolved in 1400 μl of water, and filtered (0.45 μm). Using HPLC and a diode array

detector, salicylates were quantified as catechin equivalents (200 nm), flavonoids as quercetin equivalents (349 nm), chlorogenic acids as chlorogenic acid equivalents (315 nm), and coumaroylquinic acids as coumaric acid equivalents (315 nm) (Salminen et al. 1999). Total proanthocyanidins were estimated with the acid-butanol assay (Ossipova et al. 2001), using purified birch leaf proanthocyanidins as a standard.

2.3 RESULTS

2.3.1 Isolation of marker genes for PA biosynthesis and analysis of PA biosynthetic gene families in poplar

Although the only poplar flavonoid biosynthetic enzyme that has been functionally characterized is DFR1 (Peters and Constabel, 2002), the conservation of the pathway enzymes between species and the extensive genomics resources available for poplar make identification of the putative flavonoid and PA pathway genes feasible. Using consensus sequences from assemblies of publicly available EST sequences, a number of CDS sequences corresponding to PA pathway gene mRNA transcripts were PCR-amplified from *P. tremula x tremuloides* (clone 353-38) and cloned for use in expression analyses (Table A1-1). Sequences representing general phenylpropanoid metabolism (*4CL2*), early flavonoid metabolism (*CHS1*, *CHI1*, *F3H*), late flavonoid metabolism (*ANS1*), the PA branch of flavonoid biosynthesis (*ANR1*, *LAR1*, *LAR3*), and the flavonol branch of flavonoid metabolism (*FLS4*) were isolated. The poplar *ANR1* and *LAR1* sequences were confirmed to encode functional enzymes following recombinant expression in *E. coli*, catalyzing the formation of 2,3-*cis*-flavan-3-ols (i.e., (-)-epicatechin and (-)-epiafzelechin) from anthocyanidin precursors (i.e, cyanidin and pelargonidin) and a 2,3-*trans*-flavan-3-ol (i.e., (+)-afzelechin) from a leucoanthocyanidin (i.e., leucopelargonidin) precursor, respectively (Stefan Martens, Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Germany, unpublished data). Cloned fragments of poplar *PAL1* and *DFR1* that had been isolated in a previous study (Peters and Constabel, 2002) were also obtained.

Following the publication of the *P. trichocarpa* genome (Tuskan et al., 2006), the genome sequence was analyzed and the gene family sizes of phenylpropanoid and flavonoid genes involved in PA synthesis were determined (Table 2-1). The poplar genome has been found to contain more genes than arabidopsis, the primary plant model system, with gene families related to defense, disease resistance, cell wall biosynthesis, and metabolite transport expanded in poplar relative to arabidopsis (Tuskan et al., 2006). This is also the case for the PA pathway genes. While arabidopsis has single copies of *C4H*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, *ANS*, and *ANR* (Graham, 1998; Lepiniec et al., 2006), the poplar genome contains more than one copy of all of these except *CHI*, *F3H*, and *F3'H* (Table 2-1). Poplar contains two copies each of the late flavonoid pathway *DFR*, *ANS*, and *ANR*, and three copies of *LAR* (Table 2-1). Subsequent to the analysis shown here, Tsai et al. (2006a) published an analysis of the poplar phenylpropanoid gene families, including those involved in PA metabolism. Their analysis was consistent with the gene family sizes reported here and the gene naming system of Tsai et al. (2006a) is used throughout this report.

2.3.2 Wounding and intense light activate PA metabolism in poplar leaves

Different poplar species, as well as individual clones within species, are known to exhibit considerable phenotypic variation with respect to phenolic metabolism (Section 1.3.2). The accumulation of PAs following mechanical wounding or herbivore attack has been shown for a number of poplar species, and transcriptional activation of the *DFR1* gene was shown to precede PA accumulation in *P. tremuloides* clone A2 (Sections 1.4.3 and 1.4.6). However, this clone is not known to be amenable to *A. tumefaciens*-mediated transformation, an important technique for analyzing gene function and genetic engineering (Section 1.3.1). The primary model system employed in this study is the transformation-amenable hybrid aspen (*P. tremula x tremuloides*) clone INRA 353-38. A hybrid poplar genotype, the *P. trichocarpa x deltoides* clone H11-11, was also used in some analyses.

Table 2-1. Summary of phenylpropanoid and flavonoid gene families involved in PA biosynthesis in the *Populus trichocarpa* Nisqually 1 genome sequence, using the nolenclature of Tsai et al. (2006a).

Gene family	Gene[a]	Genome location[b]	Protein ID[b]
Phenylalanine ammonia-lyase			
	PAL1	scaffold 28	739479
	PAL2	chromosome 8	820249
	PAL3	chromosome 16	667815
	PAL4	chromosome 10	822571
	PAL5	chromosome 10	228016
Cinnamate 4-hydroxylase		chromosome 13	823837
	C4H1	chromosome 19	665925
	C4H2		
4-Coumarate-CoA ligase			
	4CL1	chromosome 1	639764
	4CL2	chromosome 19	665396
	4CL3	scaffold 121	827715
	4CL4	chromosome 18	262277
	4CL5	chromosome 3	758231
Chalcone synthase			
	CHS1	chromosome 14	572875
	CHS2	chromosome 1	814871
	CHS3	chromosome 1	814872
	CHS4	chromosome 3	554827
	CHS5	chromosome 3	554828
	CHS6	chromosome 3	554829
Chalcone isomerase			
	CHI1	chromosome 10	724846
Flavonoid 3'-hydroxylase			
	F3'H	chromosome 13	823742
Flavonoid 3',5'-hydroxylase			
	F3'5'H1	chromosome 9	557701
	F3'5'H2	chromosome 1	549386
Flavanone 3-hydroxylase			
	F3H	scaffold 57	424064

[a]annotations of Tsai et al. (2006a) ; [b]JGI *Populus trichocarpa* genome v1.1 (for genome locations, scaffold numbers are given where sequence is not yet assembled into chromosome linkage group).

Continued...

Continued from previous page.

Gene family	Gene[a]	Genome location[b]	Protein ID[b]
Flavonol synthase			
	FLS1	chromosome 19	665178
	FLS2	chromosome 2	551488
	FLS3	scaffold 135	582182
	FLS4	scaffold 135	828087
Dihydroflavonol reductase			
	DFR1	chromosome 2	710083
	DFR2	chromosome 5	206006
Anthocyanidin synthase			
	ANS1	chromosome 3	646527
	ANS2	chromosome 1	548547
Anthocyanidin reductase			
	ANR1	chromosome 4	831060
	ANR2	chromosome 11	834000
Leucoanthocyanidin reductase			
	LAR1	chromosome 8	656768
	LAR2	chromosome 5	566672
	LAR3	chromosome 15	835080

[a]annotations of Tsai et al. (2006a) ; [b]JGI *Populus trichocarpa* genome v1.1 (for genome locations, scaffold numbers are given where sequence is not yet assembled into chromosome linkage group).

As a first step toward characterizing stress-responsive flavonoid metabolism in these clones, and to determine if the isolated sequences would serve as useful markers of stress-responsive flavonoid pathway activation, expression of a subset of the flavonoid pathway genes was monitored in control and mechanically wounded leaves of clones 353-38 and H11-11 (Fig. 2-1[1]). In this experiment, RNA extracted from leaves of LPI 9-11 collected 24 hours after treatment was pooled from three separate trees per treatment. In addition to the mechanical wounding experiment, a set of H11-11 trees was moved from the greenhouse into full natural sunlight where they were exposed to elevated levels of both visible and UV-B radiation (together termed "high light", HL). Although rapid light-induced up-regulation of PA metabolism in poplar has not been reported, intense

[1] Note: the expression profiling of flavonoid and PA biosynthetic genes in systemically wounded rapidly expanding sink leaves of hybrid cottonwood (*P. fremontii* × *angustifolia*) reported by Tsai et al (2006a; Section 1.4.6) were published subsequent to the analyses reported in this chapter.

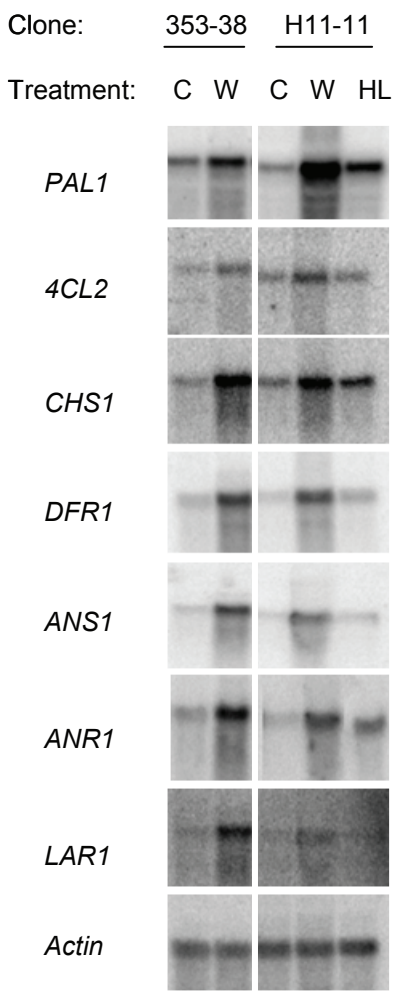


Figure 2-1. Northern analysis of PA pathway gene expression in response to wounding (W, 24 hr) and light stress (HL, 48 hr) in two hybrid poplar clones. 353-38: *P. tremula x tremuloides*; H11-11: *P. trichocarpa x deltoides*. For gene names, see Fig. 1-1. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 gene models listed in Table 2-1.

visible light and UV-B radiation are known to stimulate multiple branches of phenylpropanoid and flavonoid metabolism (Section 1.4.1). Expression of flavonoid pathway genes was monitored in leaves of the HL-exposed H11-11 trees (48 hours after initiation of the treatment). Mechanical wounding was found to activate expression of PA pathway genes in both the 353-38 and H11-11 clones (Fig. 2-1) for all genes tested (*PAL1*, *4CL2*, *CHS1*, *DFR1*, *ANS1*, *ANR1*, and *LAR1*). Some of the genes were also up-regulated in the H11-11 leaves exposed to elevated light, including the PA-specific gene *ANR1* (Fig. 2-1).

After establishing that the 353-38 clone responds to mechanical wounding with transcriptional activation of the PA biosynthetic pathway, gene activation following wounding was monitored over a short time series. Since the activation of PA pathway genes in leaves of clone H11-11 in response to elevated light represents a previously uncharacterized response, the 353-38 clone was also exposed to elevated light levels and PA pathway gene expression in leaves was monitored. For both treatments, gene expression was monitored at 1, 3, 6, 12, 24, and 48 hours after the beginning of the treatment, with leaves of LPI 9-11 from a separate tree harvested for each time point. Leaves from untreated control trees were also harvested at each time point. In this experiment, expression of a putative marker gene for flavonol metabolism, *FLS4*, was also monitored, since flavonols are known to be important plant sunscreens (Section 1.4.1).

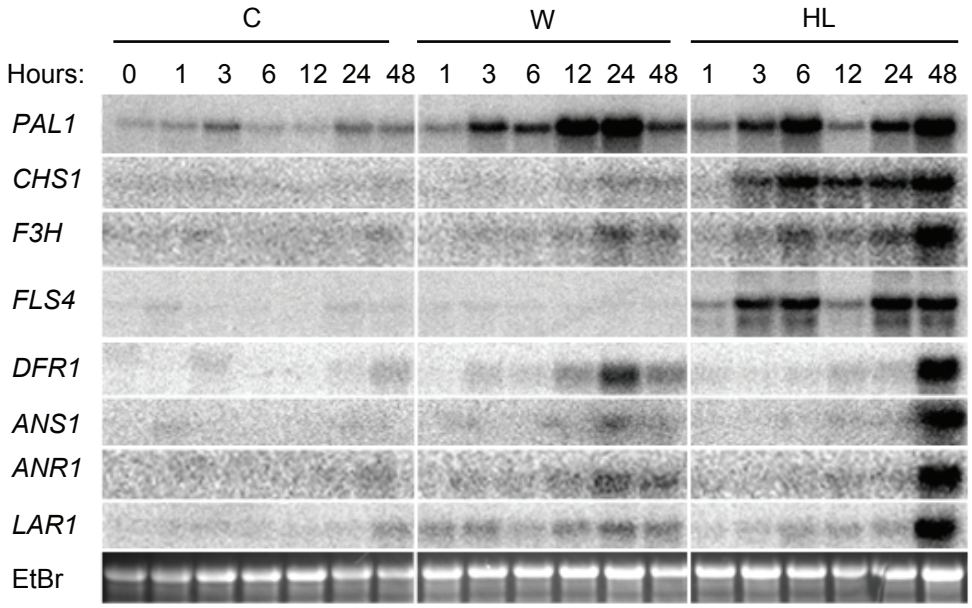


Figure 2-2. Phenylpropanoid and flavonoid gene expression in poplar leaves following wounding and exposure to high light. Northern analysis of transcript abundance over time in response to wounding (W) and exposure to elevated light (HL) in clone 353-38. For gene names, see Fig. 1-1 legend. EtBr, ethidium bromide-stained total RNA. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 gene models listed in Table 2-1.

Mechanical wounding of leaf margins resulted in the up-regulation of phenylpropanoid and flavonoid genes (Fig. 2-2). *PAL1*, the wound-inducible flavonoid-associated *PAL* gene (Section 1.4.5), was rapidly up-regulated (Fig. 2-2). General flavonoid biosynthetic genes, including *CHS*, *F3H*, *ANS1*, and the *DFR1* gene previously implicated in herbivore-induced PA accumulation (Section 1.4.5), were up-regulated within 24 hours of wounding, as were the PA-specific *ANR1* and *LAR1* genes. *FLS4* was not responsive to wounding (Fig. 2-2).

Analysis of gene expression in the HL-exposed plants revealed two distinct patterns of flavonoid structural gene activation (Fig. 2-2, right panel). A rapid activation of *PAL1*, *CHS*, *F3H*, and *FLS4* suggests that flavonol glycoside biosynthesis was up-regulated very early. The activation of flavonol biosynthetic genes exhibited a reduction at 12 hours, which may have been caused by the absence of the stimulus during the night or by circadian regulation. The early activation of flavonol biosynthetic genes was followed by a strong activation of PA biosynthetic genes by 48 hours, including the PA-specific genes *ANR1* and *LAR1* (Fig. 2-2). A corresponding increase in the expression levels of all of the early flavonoid pathway genes, with the exception of *FLS4*, was also observed at the 48 hour time-point, indicating a coordinated late activation of the full PA pathway (Fig. 2-2).

In order to better understand the response of 353-38 leaves to intense light, soluble phenylpropanoid metabolite compositions were quantified using HPLC (Fig. 2-3). A number flavonol glycosides were increased in HL-exposed poplar leaves (Fig. 2-3), and total flavonol glycoside concentrations were significantly increased in HL-exposed leaves (Fig. 2-3, 2-4A), corresponding to the strong, early activation of flavonol biosynthetic genes. Interestingly, two HCDs (chlorogenic acid or derivatives) were also present at higher concentrations in the HL-exposed leaves (Fig. 2-3, 2-4B). Intense light exposure did not have a significant effect on the concentration of phenolic glycosides (Fig. 2-4C). An accumulation of PAs following the HL-induced PA pathway activation was confirmed using acid-butanol PA assays (Fig. 2-4D). Although the analytical techniques employed in this study did not permit precise identification of individual flavonol glycosides, LC-MS analysis of equivalent leaves from this clone has shown that abundant flavonol glycosides include quercetin-3-O-glucoside, quercetin-diglycoside,

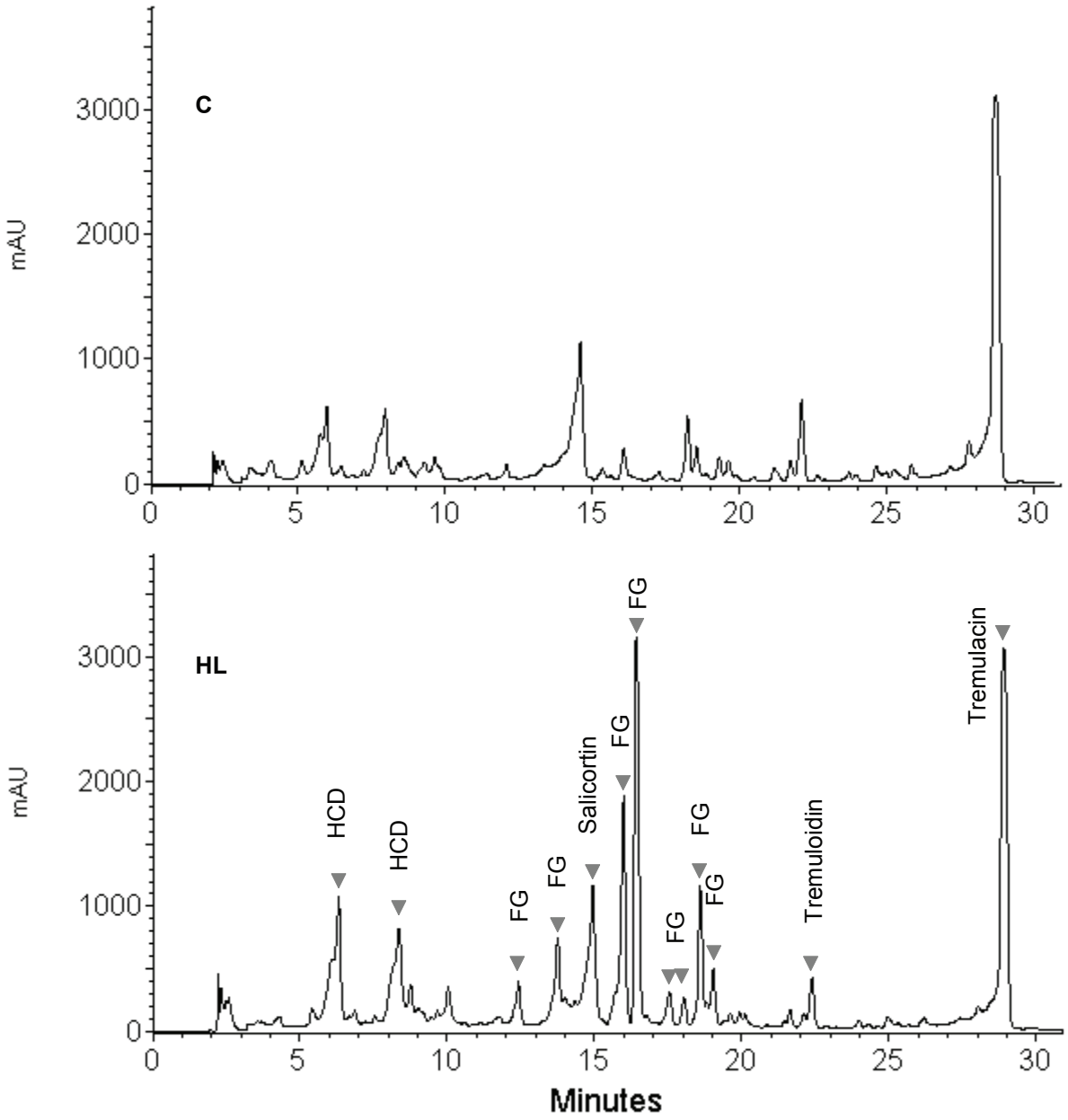


Figure 2-3. HPLC analysis of soluble phenolic metabolites in control and high light-exposed 353-38 leaf tissue. Representative maxplot chromatograms showing each peak at its λ_{max} for control (C) and high light-exposed (HL) leaf tissue (LPI 9-11). Differential peaks quantified and presented in Figure 2-4 are indicated with arrows. FG = flavonol glycoside, HCD: hydroxycinnamic acid derivative.

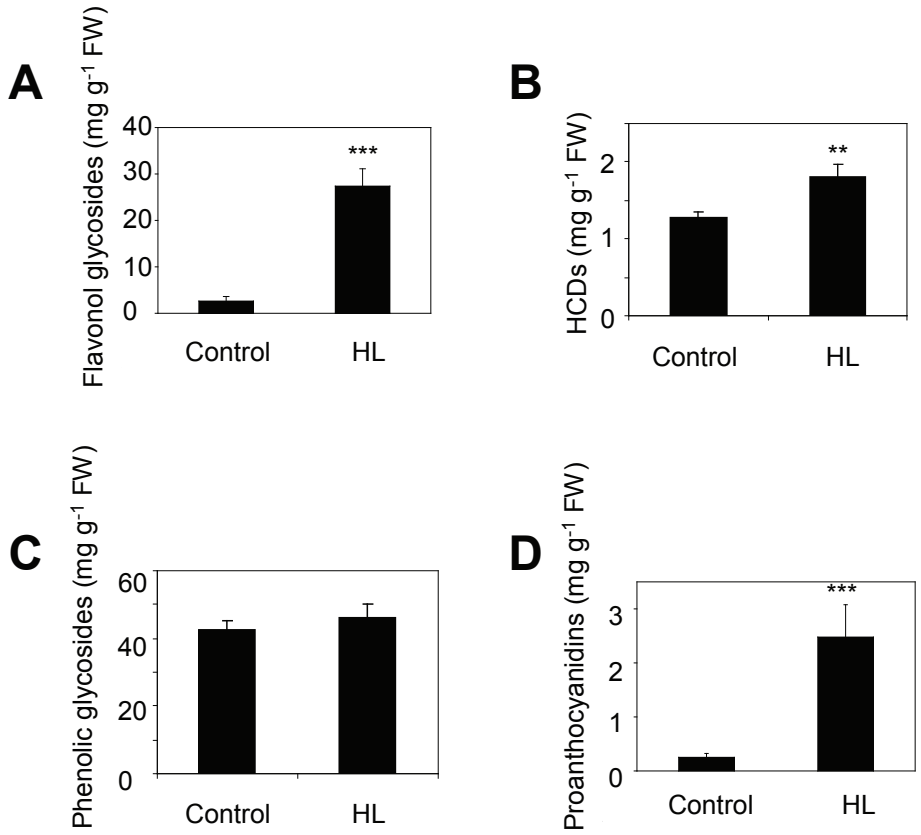


Figure 2-4. Analysis of PAs and other phenolics in poplar (clone 353-38) leaves after 7 days of HL treatment. Total flavonol glycosides (**A**), HCDs (**B**), and phenolic glycosides (**C**) quantified using HPLC (peaks quantified are annotated on the representative chromatograms in Fig. 2-3). PAs (**D**) quantified using the acid-butanol method. Bars indicate means of 4 (HPLC analysis) or 7 (PA analysis) trees per treatment, with error bars indicating the SE of mean. Asterisks indicate significant differences using Student's t test (** $P < 0.01$, *** $P < 0.001$).

quercetin-3-*O*-arabinoglucuronide, and kaempferol-3-O-glucuronide (Riitta Julkunen-Tiitto, University of Joensuu, Finland, unpublished data). Additionally, it has been determined that 353-38 produces compounds with absorption spectra matching acylated flavonol glycosides in leaves (R. Mellway and C. P. Constabel, unpublished data), and low levels of myricetin glycoside (Susan Marles, Agriculture and Agri-Food Canada, unpublished data).

An independent analysis of the phenolic composition of control and HL-exposed 353-38 leaves from the same experiment confirmed the large increase in flavonol glycoside concentrations as well as the smaller increase in HCDs (chlorogenic acid and coumaroylquinic acid and derivatives) (Table 2-2). In this analysis, nine different flavonol glycosides were identified in the 353-38 leaf tissue. All nine were increased in HL-exposed leaves, with three exhibiting very large increases (Table 2-2).

2.3.3 UV-B irradiation rapidly activates flavonol glycoside biosynthesis followed by a later activation of PA metabolism

To investigate the distinct HL-induced patterns of early and late flavonoid structural gene expression in more detail, flavonoid gene expression in poplar leaves exposed to increased UV-B irradiance in a growth chamber was examined. Northern analysis of gene expression revealed an activation of flavonol biosynthetic genes, including early flavonoid pathway genes and the flavonol marker gene *FLS4*, by 3 hours, followed by a later up-regulation of PA-specific biosynthetic genes by 48 hours (Fig. 2-5, center panel). As with the HL-exposed leaves, the PA specific genes *ANR1* and *LAR1* were strongly up-regulated at the 48 hour time-point together with the early pathway genes such as *PAL1* and *CHS1* (Fig. 2-5), indicating that the PA pathway exhibits a co-coordinate but late response to UV-B exposure.

HPLC analysis revealed a significant increase in flavonol glycoside concentrations (Fig. 2-6, 2-7A), corresponding to the rapid activation of *FLS4* and the early pathway genes. Unlike the HL treatment, in which leaves were exposed to elevated UV-B as well as intense visible light, UV-B exposure alone resulted in a small but

Table 2-2. HPLC analysis of phenolic compounds in leaves of control and high light (HL)-exposed poplar (clone 353-38) leaves. Data contributed by Juha-Pekka Salminen (Department of Chemistry, University of Turku, Finland).

Compound	Treatment	
	Control (mg g^{-1} DW)	HL (mg g^{-1} DW)
Salicylate 1	3.39	0.16
Salicylate 2	0.47	0.48
Salicylate 3	5.28	1.04
Salicylate 4 (salicortin)	17.19	29.33
Salicylate 5	4.66	0.81
Salicylate 6	0.45	0.65
Salicylate 7 (tremulacin)	12.06	13.73
Salicylate 8	0.08	0.04
Salicylate 9	0.28	0.25
Sum of salicylates	43.9	46.48
Chlorogenic acid or derivative 1	3.64	6.03
Chlorogenic acid or derivative 2	0.00	0.00
Chlorogenic acid or derivative 3	0.27	0.17
Sum of chlorogenic acid or derivatives	3.92	6.20
Coumaroylquinic acid or derivative 1	0.23	0.30
Coumaroylquinic acid or derivative 2	0.36	0.62
Coumaroylquinic acid or derivative 3	0.52	1.22
Sum of coumaroylquinic acid or derivatives	1.11	2.14
Flavonoid glycoside 1	0.27	0.79
Flavonoid glycoside 2	0.03	0.29
Flavonoid glycoside 3	0.17	2.38
Flavonoid glycoside 4	0.04	0.06
Flavonoid glycoside 5	0.48	0.84
Flavonoid glycoside 6	0.44	10.12
Flavonoid glycoside 7	0.01	0.24
Flavonoid glycoside 8	0.24	4.40
Flavonoid glycoside 9	0.06	0.15
Sum of flavonoid glycosides	1.73	19.28
Total phenolics	50.6	74.1

significant reduction in total phenolic glycoside concentrations (Fig. 2-7C). A significant increase in PA concentrations was also detected, corresponding to the late activation of the PA biosynthetic pathway (Fig. 2-7D).

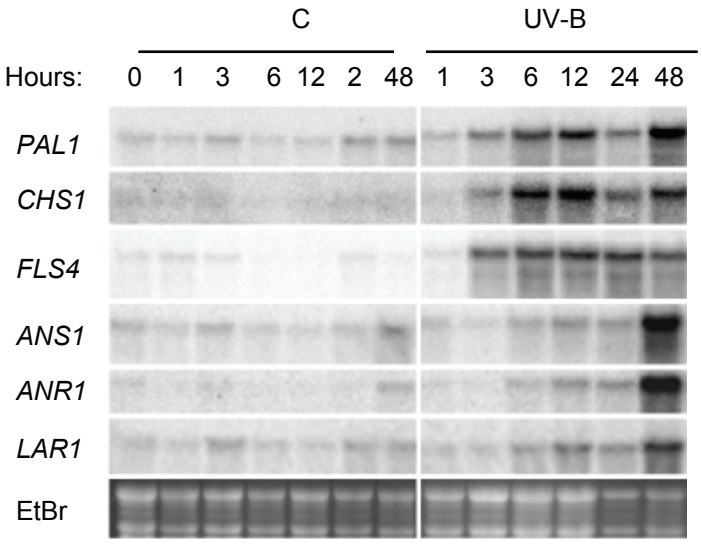


Figure 2-5. UV-B activation of the flavonoid biosynthetic pathway. Northern analysis of phenylpropanoid and flavonoid structural genes in control (C) and UV-B-treated (UV-B) poplar leaves (clone 353-38). For gene names, see Fig. 1-1. EtBr, ethidium bromide-stained total RNA. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 gene models listed in Table 2-1.

Since the objective of this research was to identify and analyze potential regulators of the stress-induced PA pathway, the stress-responsiveness of the PA pathway was also analyzed in the *P. trichocarpa* Nisqually 1 clone, for which a complete genome sequence has recently been published (Tuskan et al., 2006). Both wounding and UV-B exposure were found to induce a significant increase in leaf PA concentrations (Fig. 2-8A and B). Expression of the PA-specific marker gene *LAR3* was up-regulated in the mechanically wounded Nisqually 1 leaves (Fig. 2-9), indicating that this increase in PA concentrations is mediated by transcriptional activation of the PA pathway.

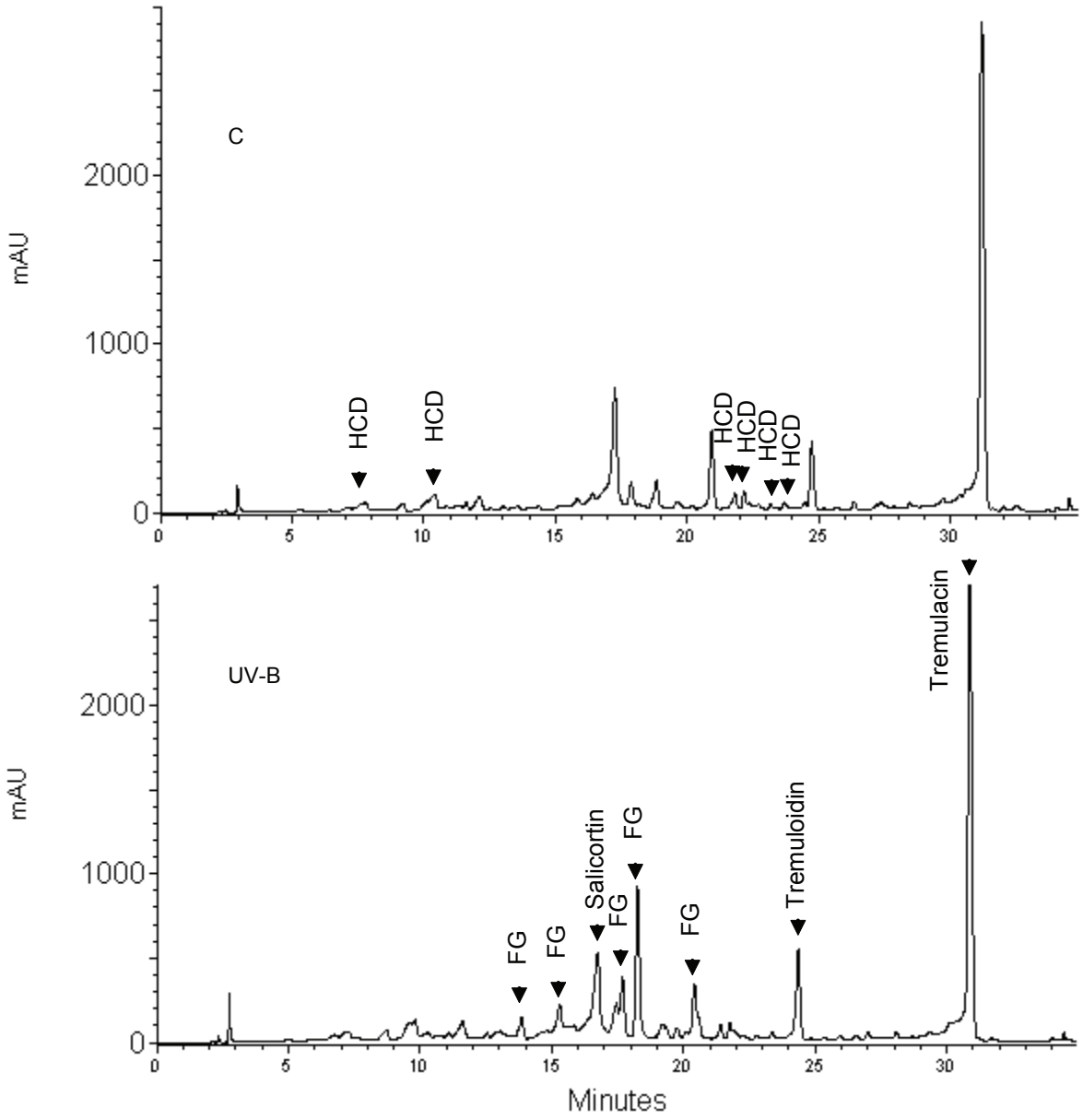


Figure 2-6. HPLC analysis of soluble phenolic metabolites in control and UV-B-exposed 353-38 leaf tissue. Representative maxplot chromatograms showing each peak at its λ_{max} for control (C, 7 days) and UV-B-exposed (UV-B, 7 days) leaf tissue (LPI 9-11). Differential peaks quantified and presented in Fig. 2-7 are indicated with arrows. FG = flavonol glycoside, HCD: hydroxycinnamic acid derivative. Note that peaks present on both chromatograms are only labeled on one or the other.

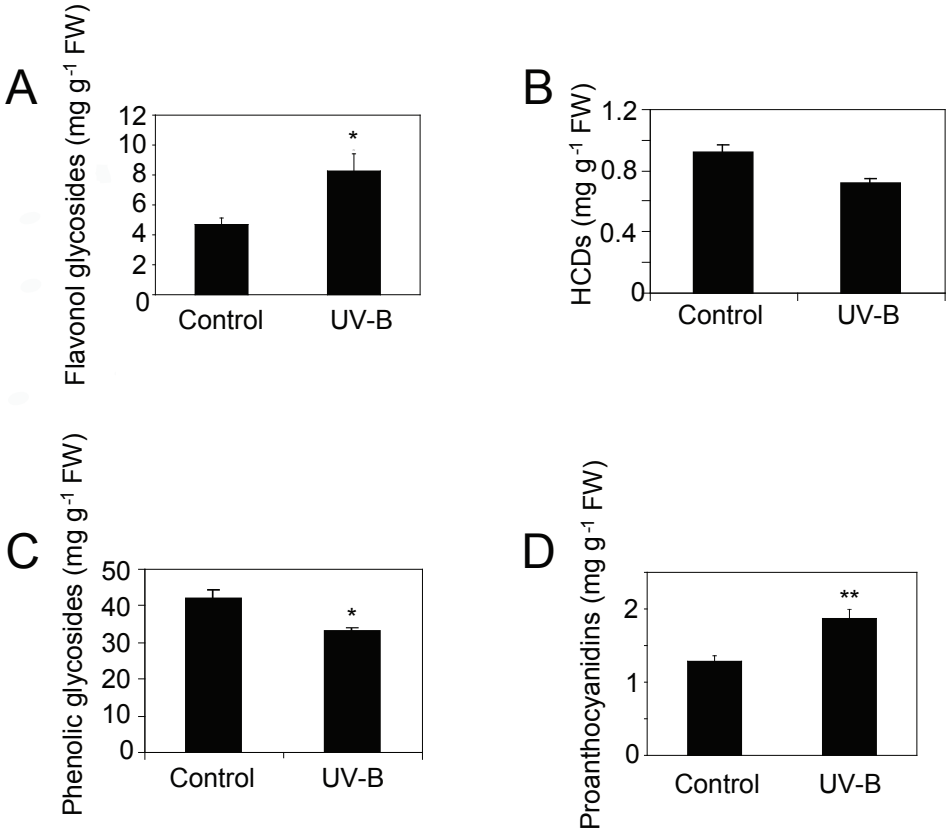


Figure 2-7. Phytochemical changes in control (C) and UV-B-treated (UV-B) poplar leaves (clone 353-38) after 7 days. Flavonol glycosides (**A**), HCDs (**B**), and phenolic glycosides (**C**) quantified using HPLC, and PAs (**D**) quantified using the acid-butanol method. Bars indicate means of 4 trees per treatment, with error bars indicating the SE of mean. Asterisks indicate significant differences using Student's t test (* $P < 0.05$, ** $P < 0.01$).

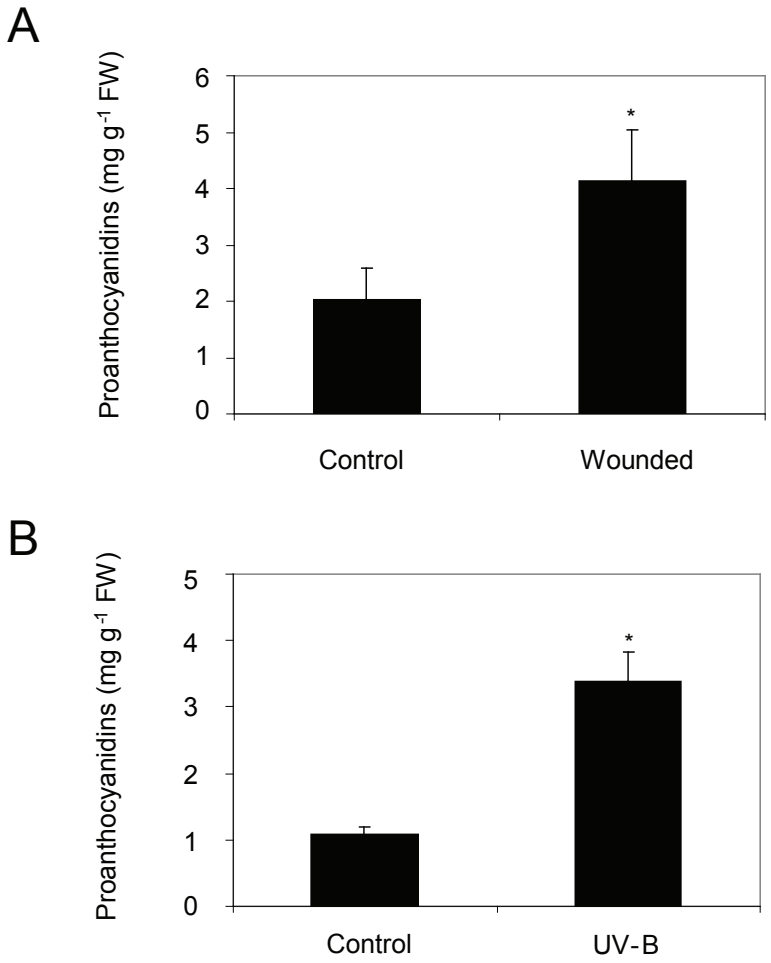


Figure 2-8. Stress-induced accumulation of PAs in leaves of *Populus trichocarpa* clone Nisqually 1. **A**. PA levels in control and wounded poplar leaves after 7 days. **B**. PA levels in control and UV-B-exposed (UV-B) poplar leaves after 7 days. Bars indicate means of 4 trees per treatment, with error bars indicating the SE of mean. Asterisks indicate significant differences using Student's t test (* $P<0.05$)

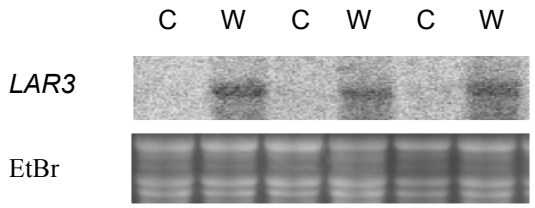


Figure 2-9. Stress-induced PA pathway in the *P. trichocarpa* Nisqually 1 clone monitored with expression of a PA-specific marker gene. Northern analysis of *leucoanthocyanidin reductase 3* (*LAR3*) expression in control (C) and wounded (W, 24 hours) leaves. EtBr, ethidium bromide-stained total RNA. *LAR3* CDS sequence used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 genome protein ID 835080.

2.3.4 Infection of leaves by the fungus *Melampsora medusae* activates the PA pathway in poplar

During the course of these analyses, a microarray dataset became available that profiled the response of the H11-11 clone to infection of leaves by a compatible strain of the poplar leaf rust (*M. medusae*) (Miranda et al., 2007). This study utilized the Treenomix 15.5K Poplar cDNA microarray to profile transcriptional changes at various time points after inoculation of leaves with fungal spores (Ralph et al., 2006). Among the deregulated genes, a number of flavonoid pathway genes were found to be up-regulated at the 6 day time point (Miranda et al., 2007). Examination of the corresponding sequences revealed that they included the PA specific genes *LAR* and *ANR*, and that the entire PA pathway was up-regulated with the exception of *DFR*. This indicated that *M. medusae* infection results in activation of the PA biosynthetic pathway in H11-11. This response constitutes an additional system with which to identify putative PA regulators through co-expression analyses, and the response was therefore investigated further. Northern analyses of gene expression confirmed activation of the PA biosynthetic pathway for all marker genes tested in an independent infection experiment (Fig. 2-10A and B). The PA-specific *PAL1* gene and the early flavonoid biosynthetic genes *CHS1* and *F3H* were up-regulated. The wound- and HL-induced *DFR1* gene, not present on the Treenomix 15.5K cDNA microarray, was also up-regulated, confirming that the entire

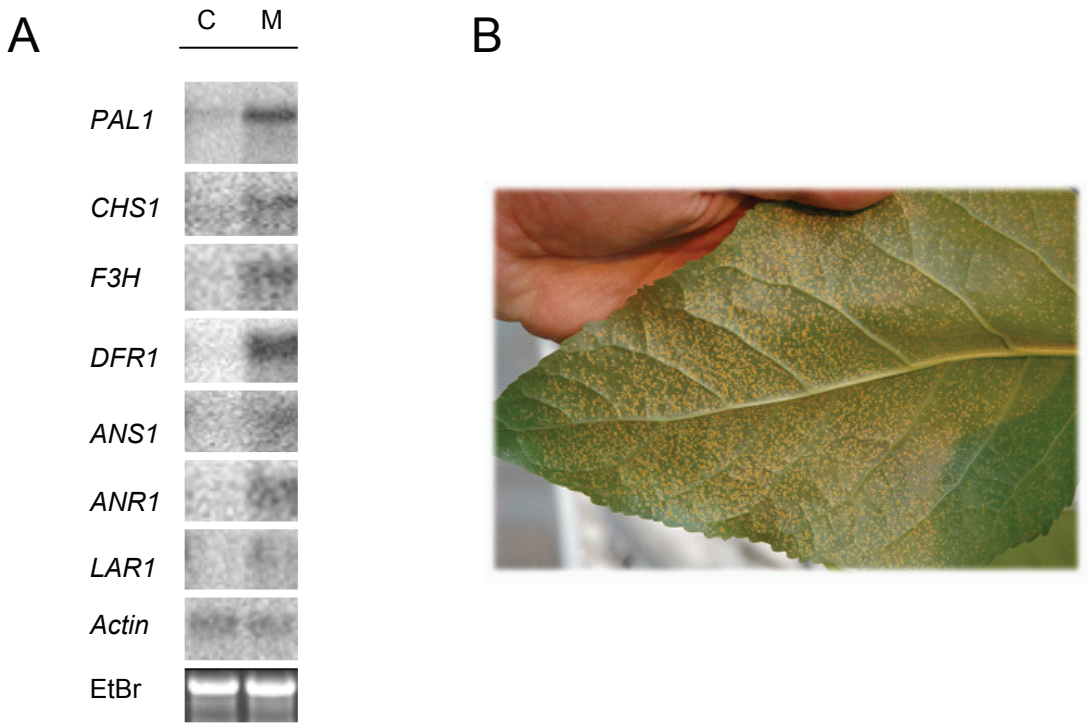


Figure 2-10. *M. medusae*-infection up-regulates PA metabolism in poplar. **A**. Northern analysis demonstrating up-regulation of flavonoid and PA pathway genes by *M. medusae* infection (6 dpi) of poplar (H11-11) leaves. RNA pooled from three control (C) and three infected (M) leaves. For gene names, see Fig. 1-1. EtBr, ethidium bromide stained total RNA. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 gene models listed in Table 2-1. **B**. Uredinial pustules containing asexual urediospores visible on the underside of a *M. medusae*-infected H11-11 leaf (9 dpi).

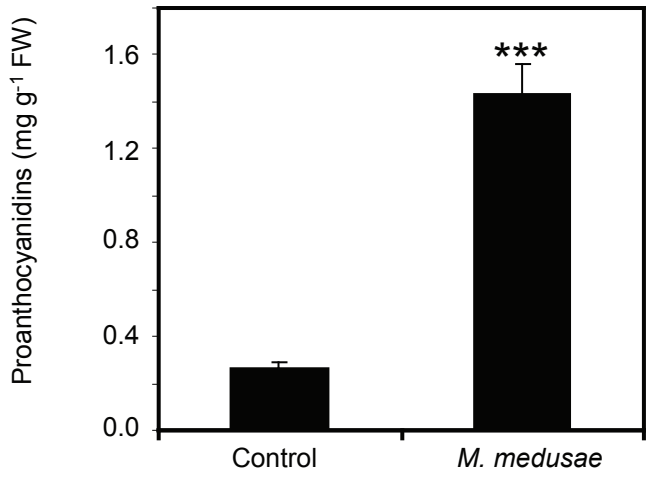


Figure 2-11. *M. medusae* infection leads to significant increase in foliar PA levels. PA levels in control and *M. medusae*-infected poplar leaves at 20 dpi. Bars show + SE of the mean of three control and three infected leaves. This represents a 5.3-fold increase (Student's t test, ***P < 0.001) and is representative of multiple independent replicates of the experiment.

PA pathway is activated by *M. meduase* infection (Fig. 2-10A). Likewise, the PA-specific *LAR1* and *ANR1* genes were activated (Fig. 2-10A). Corresponding to this strong activation of the entire PA biosynthetic pathway, a significant increase in PA concentrations in *M. meduase*-infected H11-11 leaves was detected (Fig. 2-11).

2.4 DISCUSSION

2.4.1 Marker genes for stress-responsive up-regulation of PA metabolism

The analyses described in this chapter establish a set of marker genes for stress-induced PA metabolism that can be used in expression analyses to identify genes that are co-activated with the PA pathway under different stress conditions. The observed metabolite profiles are consistent with the gene activation patterns observed, with significant increases in PA levels following HL-exposure, UV-B-exposure, and *M. medusae* infection, and significant increase in flavonol glycoside levels occurring only in HL- and UV-B-exposed leaves. The decrease in PG concentrations in UV-B exposed leaves may be indicative of a competition for substrates between the PG pathway and the up-regulated PA pathway, in agreement with reports of inverse relationship between PG and PA levels in poplar leaves (Section 1.4.2).

While transcriptional activation of flavonoid pathway genes such as *DFR1* has been associated with wound- and herbivore-induced PA metabolism (Section 1.4.6), the identification of *ANR1*, *LAR1*, and *LAR3* as stress-inducible flavonoid biosynthetic genes is important, as the products of these genes mediate entry of metabolites into the PA branch of flavonoid metabolism (Fig. 1-1). The different PA-activating stresses result in distinct activation kinetics for PA pathway genes. These different activation profiles under wounding, high light- and UV-B-exposure, and fungal biotroph infection constitute a useful system for the identification of genes that are co-regulated with the known PA pathway genes under stress.

It is apparent that in wounded leaves, the entire biosynthetic pathway leading to PA accumulation is up-regulated within one day of wounding (Figs. 2-1, 2-2). The one exception among the profiled genes is the flavonol synthase (*FLS4*), which shows no induction. Consistent with this, no accumulation of flavonols is observed in wounded tissue. In HL-and UV-B-exposed poplar leaves, there is a large difference in timing of activation of the early and late biosynthetic genes (Figs. 2-2, 2-5). Following initiation of the stimulus, there is a rapid and strong induction of the pathway up to and including *FLS4*. There is an induction of the PA pathway in these tissues, but transcripts are not strongly detected until after the first 24 hours (Figs. 2-2, 2-5). This strong, rapid up-regulation of flavonol metabolism may be useful for screening for regulators that are specific to PA metabolism and not involved in controlling other branches of flavonoid metabolism under stress conditions (Chapter 3). The two distinct expression patterns indicate that different regulatory mechanisms are likely functioning in controlling the coordinate light-induced activation of flavonol biosynthetic genes, and the later proanthocyanidin gene activation. Different regulatory mechanism controlling flavonol and PA metabolism in poplar is consistent with the different regulatory proteins controlling flavonol and late flavonoid metabolism in arabidopsis (Section 1.5.2, Fig. 1-6). It is conceivable that the UV-B or intense visible light itself is the signal that is perceived and initiates signalling events leading to activation of the flavonol biosynthetic genes, while the PA pathway activation occurs in response to secondary effects of prolonged exposure such as damage to cellular components (see below).

The activation of the PA pathway under elevated UV-B irradiation and following mechanical wounding in the *P. trichocarpa* clone Nisqually 1 is important because a complete reference genome is available for this clone (Tuskan et al., 2006). Since poplar can exhibit considerable variation in phenolic metabolism and defense responses between species and even between individual clones of the same species (Section 1.3.2), it is advantageous that these responses occur in Nisqually 1. Examination of the promoter structures of flavonoid biosynthetic genes and analysis of regulatory gene families using the poplar genome would be less relevant if Nisqually 1 did not exhibit stress-induced PA metabolism (Chapter 3). Another important outcome of this work was that the hybrid aspen clone 353-38 exhibits PA pathway activation following mechanical wounding and

other stresses. Unlike some aspen clones, this clone can be stably transformed using *A. tumefaciens* (Augustin et al., 2004), an important method that can be used to study gene function (Chapter 3).

The light exposure and fungal infection experiments reported here provide useful systems with which to analyze the expression of novel genes implicated in PA metabolism, such as putative regulators or genes predicted to serve novel roles in PA metabolism (Chapter 3). However, the data presented here must be interpreted with caution. Successful biotrophic fungal infection of leaf tissue results in significant metabolic restructuring and loss of nutrients to invading hyphae, and PA activation may be a secondary response (see below). Movement of plants outdoors into full sunlight is also complicated by the fact that other stresses may have been important in PA pathway activation in this experiment, such as increased wind exposure. The UV-B experiments were more controlled, but, again, prolonged exposure to this stress may have resulted is secondary effects that contributed to PA pathway activation. Because of these confounding variables, these experiments should not be interpreted as evidence of PA functions, but taken together they hint that the roles that PAs play in poplar may be broad.

2.4.2 New light on the stress-protective roles of PAs in poplar leaves

2.4.2.1 Activation of foliar PA metabolism in response to biotrophic rust fungus infection

Activation of the PA pathway genes following *M. medusae* infection occurs much later than the wound- or UV-B-induced responses, with significantly increased transcript accumulation not occurring until between 3 and 6 days post-inoculation (dpi) (Miranda et al., 2007). Inoculation of leaves with *M. medusae* spores results in the production of uredinial pustules containing asexual urediospores on the abaxial sides of leaves by 8 dpi (Miranda et al., 2007) (Fig. 2-10B). No macroscopic symptoms are visible before 4 dpi, but intercellular fungal hyphae and haustoria at infection sites are visible with

microscopic examination of leaves at 1 dpi (Miranda et al., 2007). By 3 dpi, extensive intercellular hyphae are visible, and by 6 dpi, hyphal masses are beginning to form under the abaxial epidermis at each infection site (Miranda et al., 2007). Gene expression profiling of leaves during this infection process using the Treenomix 15.5K cDNA microarray revealed several relevant gene expression patterns (Miranda et al., 2007). A number of pathogen response-related genes, including PR genes, β-1,3-glucanases, and chitinases, are up-regulated by 1 dpi and remain strongly up-regulated at 3, 6, and 9 dpi (Miranda et al., 2007). Genes associated with herbivore defence, such as those encoding Kunitz trypsin inhibitors and PPO (Constabel et al., 2000; Haruta et al., 2001) showed an early up-regulation (1 dpi) followed by strong down-regulation at later time points. Photosynthesis-related genes such as chlorophyll a/b binding proteins and photosynthetic reaction centre proteins clustered with genes that are significantly down-regulated after 3 dpi and are among the most strongly repressed genes at 9 dpi (Miranda et al. ,2007).

Activation of the PA pathway genes, including the PA-specific *ANR1* and *LAR1*, in *M. medusae* infected leaves does not occur until sometime after 3 dpi (Miranda et al., 2007). The activation of pathogen response-related genes by 1 dpi suggests that the fungus is rapidly perceived by the plant and that much later activation of the PA pathway may be a secondary response to the effects of infection. Networks of intercellular fungal hyphae and intracellular haustoria are quite extensive by 3 dpi, and it is possible that activation of the flavonoid pathway is a response to loss of nutrients and metabolic reprogramming of plant cells. Nutrient deprivation can lead to oxidative stress (Section 1.4.1), and nutrient limited growth conditions are known to cause increased PA accumulation in poplar (Section 1.3.2). Down-regulation of photosynthetic genes during *M. medusae* infection may result in reduced photosynthetic capacity and a consequent increase in ROS. Thus, up-regulation of PAs in *M. medusae*-infected leaves may not reflect a role in pathogen defense, but does suggest that the roles of PAs in poplar are broader than insect defense.

2.4.2.1 Activation of foliar PA metabolism in response high light and UV-B

The strong up-regulation of PA biosynthetic genes and accumulation of PAs following light stress and UV-B exposure was a novel result of these experiments. Rapid induction of PA biosynthesis following UV-B-stress has not been previously reported, although Lavola et al. (1998) reported that in birch (*Betula pendula*) saplings grown in growth chambers, elevated UV-B resulted in increased foliar PA levels. Based on the strong activation of the PA pathway following exposure of poplar leaves to intense light and UV-B, it could be speculated that, like flavonols and anthocyanins, PAs may function as protectants through UV-B filtering, as antioxidants, or both (Section 1.4.4).

In addition to UV-B and intense visible light, oxidative stress is also associated with pathogen attack and herbivory and is thus a common theme in the stresses that induce PA synthesis in poplar (Sections 1.4.1 and 1.44.4). Reactive oxygen species are known to be important signalling molecules in plants, and may be involved in cellular signalling under a wide variety of environmental stress conditions (Foyer and Noctor, 2005). Molecules which can affect the oxidative status of plants cells may play a role in signal modulation rather than simply stress resistance. Although an antioxidant function must be reconciled with the vacuolar location of PAs *in planta* (Edreva, 2005), a protective role against oxygen radicals could explain why this pathway is up-regulated under a variety of stress conditions.

Close and McArthur (2002) have proposed the oxidative pressure hypothesis to account for variation in levels of plant phenolics. These authors argue that protection from photodamage through antioxidant capacity is the primary function of many, if not most, plant phenolics. They contend that hypotheses stating that phenolics evolved primarily as defences against herbivores or as storage compounds for excess carbon are less consistent with observed ecological patterns than the hypothesis that they function to protect plants from photodamage. PA accumulation patterns in poplar leaves appear to be consistent with this theory, as nutrient and light conditions strongly affect PA concentrations, but varying PA concentrations often have limited or no effect on insect herbivore feeding preference or performance (Sections 1.3.2 and 1.4.2). The oxidative pressure hypothesis does not rule out secondary functions for phenolics in herbivore defense; in some cases, defence against herbivores or pathogens may be the primary function of particular classes of phenolics. In poplar, levels of phenolic glycosides exhibit

a strong negative correlation with lepidopteran larval performance (Section 1.3.2), indicating that an anti-herbivore role may be the primary function of these metabolites. In support of this, phenolic glycoside levels did not significantly increase under elevated UV-B or intense light. In fact, reduced phenolic glycoside levels were detected following UV-B exposure (Fig. 2-7C), indicating that under these conditions phenylpropanoid precursors might be shuttled into pathways for the production of phenolics that function in oxidative stress resistance rather than herbivore defence.

2.4.3 Conclusions and future directions

The analyses reported in this chapter establish that mechanical wounding of poplar leaves induces a coordinated transcriptional activation of the PA biosynthetic pathway, including PA-specific genes such as *LAR* and *ANR*. The PA pathway is activated under additional stress conditions, including UV-B irradiation and biotrophic fungal parasite infection, and biosynthetic genes show distinct activation kinetics under these stresses. The primary purpose of these experiments was to identify a set of marker genes and stress conditions which could be used both in the identification of putative PA regulators through co-expression analyses and in the functional analysis of such regulators using transgenic plants (Chapter 3). While the stress-responsive transcript accumulation profiles identified in these analyses constitute a useful system for such a strategy, examination of the poplar genome revealed that many PA pathway genes are represented by multiple copies; it will be interesting to determine the extent to which different flavonoid gene family members contribute to PA biosynthesis in different tissues and under different stress conditions.

These analyses showed that PA metabolism in poplar is activated by multiple stress conditions in addition to mechanical wounding. The experiments described here were not aimed at testing hypotheses related to the biological functions of PAs in poplar, and only suggest directions that future research might take. Do oxidative stresses condition such as O_3 exposure, drought, and toxic metals such as Zn, Cu, and Cd also rapidly activate PA metabolism in poplar? Treatment of poplar leaves with the herbicide

paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), which causes photooxidative stress in plants by producing superoxide radicals at photosystem I sites through reduction of O_2 (Jin-Hong and Choon-Hwan, 2003; Fedtke and Duke, 2004), would be a useful strategy to determine if PAs respond directly to oxidative stress. Research aimed at evaluating proposed stress-protective or other roles of PAs would benefit from transgenic plants with altered PA or phenolic levels. The ultimate goal of this research is to identify transcription factors involved in the regulation of stress-inducible PA metabolism, which might then be used to engineer the PA pathway in poplar and other plants. The molecular analyses of stress-induced PA metabolism described in this chapter constitute an essential first step toward this goal.

Chapter 3: An R2R3 MYB transcription factor, MYB134, is involved in the regulation of stress-inducible PA metabolism in poplar

Some of the work described in this chapter has been published in the following articles:

Mellway RD, Constabel CP (2009) Metabolic engineering and possible functions of proanthocyanidins in poplar. Plant Signaling & Behavior 4: 790-792

Mellway RD, Tran LT, Prouse MB, Campbell MM, Constabel CP (2009) The wound-, pathogen-, and UV-B-responsive *MYB134* gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin metabolism in *Populus*. Plant Physiology 150: 924-941

The data in Table 3-3 was contributed by Dr. Juha-Pekka Salminen (Department of Chemistry, University of Turku, Finland).

The data in Fig. 3-24 was contributed by Michael B. Prouse and Malcolm M. Campbell (Department of Cell and Systems Biologyand Centre for the Analysis of Genome Evolution and Function, University of Toronto, Canada).

The data in Fig. 3-36 was contributed by Megan M. Towns (University of Victoria).

Affymetrix GeneChip® Poplar Genome Array Microarray hybridizations were performed by the laboratory of Dr. Armand Séguin (Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre).

3.1 INTRODUCTION

PAs are thought to contribute to plant health through the protection of tissues against biotic and abiotic stress (Chapter 1). While some of the *in planta* functions of PAs are still a matter of debate, dietary PAs, like other flavonoids, are valued for their health-promoting properties. PAs are potent antioxidants and this attribute may contribute to the beneficial effects that have been attributed to the consumption of PA-rich foods (Ariga, 2004; Seeram et al., 2006). PAs in the diet have been linked to reduced risk of cardiovascular disease and certain cancers, and there is considerable interest in improving human health through increasing the consumption of PA-rich foods. While foods such as blueberries, blackberries, tea, red wine, chocolate and apples are excellent sources of dietary PAs (Seeram et al., 2006; Nandakumar et al., 2008), enriching the PA content in these foods or creating new "functional foods" or "nutraceuticals" with enhanced health promoting and disease preventative properties (DeFelice, 1992; Dillard and German, 2000) by up-regulating or introducing PA production through breeding or genetic engineering could have a positive impact on human health (Tohge et al., 2005b). Research aimed at engineering PA metabolism in plants has also been motivated by the goal of improving leguminous forage crops for ruminant consumption (Section 1.6.2).

There have been several attempts to up-regulate the PA pathway through genetic engineering using both model plant systems and plants of agricultural or agronomic importance (Section 1.6). Use of transcription factor genes to activate the PA biosynthetic pathway in whole or part has been the most successful strategy to date (Tian et al., 2008), but large, specific increases in PA synthesis in plant tissues have been difficult to achieve (Section 1.6.3). In part, this lack of success may be attributable to the use of model systems such as arabidopsis and tobacco, which do not accumulate appreciable levels of PAs in vegetative tissues. The most successful attempts to engineer PA metabolism in arabidopsis have resulted in seedling lethal phenotypes (Xie et al., 2006; Bogs et al., 2007). While arabidopsis has undoubtedly been an important model system for elucidating PA biosynthesis and regulation, attempts to metabolically engineer PAs in plants would benefit from a model system in which PAs are produced at high levels in a wider range of tissues.

Regulators of the flavonoid pathway are highly conserved in plants, and transcription factors often retain some function even between phylogenetically distant species (Section 1.5). Identifying PA regulators in a species like poplar, where the PA biosynthetic pathway is highly active in many tissues, may provide novel regulatory genes that could be used to engineer the PA pathway in poplar and other plants. The late steps in PA biosynthesis remain to be elucidated (Section 1.2.2) and transgenic plants in which the PA pathway is specifically activated by the overexpression of PA-regulatory transcription factors genes may be an effective strategy for the identification of novel PA biosynthetic and regulatory genes (Section 1.6.3).

Poplar trees commit significant resources to the production of PAs, although the functions of these metabolites in poplar tissues remain poorly understood (Section 1.4). Poplars are foundation species in a number of temperate ecosystems, and PAs produced by poplar can impact other organisms and influence associated populations and communities (Section 1.3.2). Therefore, engineering the PA pathway in poplar would have the added benefit of producing PA-modified poplar trees that may be useful for studying the *in planta* functions of these ecologically important compounds.

As described in Section 1.5, flavonoid biosynthetic genes such as those involved in PA and anthocyanin synthesis are regulated by MYB-BHLH-WDR transcription factor complexes. Activation of specific subsets of flavonoid pathway genes leading to PA or anthocyanin production is thought to be mediated by the specificity of the MYB partner within this complex (Section 1.5.2 and 1.5.3). The arabidopsis MYB factor TT2 was the first PA pathway regulatory MYB factor identified (Nesi et al., 2001), and the only known PA-specific regulator until recently (Section 1.5.3).

In order to better characterize stress-responsive PA metabolism in poplar, sequences representing *TT2*-like MYB genes were sought in poplar sequence databases. Expression of candidate MYB genes revealed that several *TT2*-like poplar genes exhibited increased expression under PA pathway-activating stresses. One of these genes, *MYB134* is predicted to encode a protein that shares high sequence similarity to arabidopsis TT2 within the MYB DNA binding domain as well as within a short sequence motif located carboxy-terminal to the MYB region. Evidence is presented to show that *MYB134* plays a role in regulating the stress-induced PA metabolism, including

co-expression of this gene with the PA pathway under multiple stress conditions and specific activation of PA biosynthetic genes in transgenic poplar in which *MYB134* is constitutively expressed. A global analysis of gene expression in leaves of *MYB134*-overexpressing poplar is presented, and candidate novel PA biosynthetic and regulatory genes are discussed. Additionally, preliminary studies with *MYB134*-overexpressing transgenic plants indicate that manipulation of the pathway using regulators such as MYB134 may be useful for analyzing the biological functions of PAs in poplar.

3.2 MATERIALS AND METHODS

3.2.1 Phylogenetic analysis, cloning of putative poplar PA-regulatory R2R3 MYB genes and plant transformation

Sequences analyses and primer design were performed using Vector NTI Advance, version 9.0 (Invitrogen, Carlsbad, CA). For multiple sequence alignment and phylogenetic analysis, sequences were aligned using ClustalW (Chenna et al., 2003). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with the minimum evolution test and p-distance model with 1000 boostrap replicates using the Molecular Evolutionary Genetics Analysis (MEGA) package version 3.1 (Kumar et al., 2004).

The coding sequences of *MYB134*, *MYB183*, and *MYB097* were PCR-amplified from a *P. tremuloides* (clone A2) cDNA library with primers (Table A1-2) containing restriction linker sites for subcloning into the vector pBI-524 between the double CaMV 35S promoter with alpha mosaic virus RNA4 transcriptional enhancer sequence and the *nopaline synthase* (*NOS*) terminator sequence (Datla et al., 1993; Wang and Constabel, 2004). Sequences were first cloned into pGEM-T Easy (Promega, Madison, WI) and sequencing was performed by the Centre for Biomedical Research Sequencing Facility (University of Victoria, Data A1-1). After subcloning into pBI-524, the overexpression cassette was then subcloned into the pRD400 binary plasmid carrying the *neomycin*

phosphotransferase II (*nptII*) gene for kanamycin resistance (Datla et al., 1992). The binary vector pRD400-MYB134 was transferred to the *A. tumefaciens* strain C58 (pMP90) (Koncz and Schell, 1986; Hellens et al., 2000). The pRD410 plasmid containing the *β-glucuronidase* (*GUS*) gene was used as a control construct (Datla et al., 1992). The coding sequences of *MYB183* and *MYB097* were PCR-amplified from a *P. tremuloides* (clone A2) cDNA library with primers (Table A1-1) containing restriction linker sites for subcloning into the vector pMJM (obtained from Armand Séguin, Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre), a modified pRT106 plant expression vector (Topfer et al., 1993) containing the maize ubiquitin promoter (Christensen et al., 1992) and a 35S terminator. This overexpression cassette was then subcloned into the pCAMBIA1305.1 binary vector (GenBank accession number AF354046; CAMBIA, Black Mountain, ACT, Australia) carrying the *hygromycin phosphotransferase II* (*hptII*) gene conferring hygromycin resistance. The binary vectors pCAMBIA1305.1-MYB097 and pCAMBIA1305.1-MYB183 were transferred to the *A. tumefaciens* strain C58 (pMP90). The 353-38 and 717-1-B4 clones were transformed using the method of Leplé et al. (1992) (pRD400-MYB134 and pRD410) and Han et al. (2000) (pCAMBIA1305.1-MYB097 and pCAMBIA1305.1-MYB183). Tobacco (*Nicotiana tabaccum*) clone ϕω, obtained from Dr. Santosh Misra (University of Victoria), was transformed using the method of Han et al. (2000). Positive independently transformed lines were identified by selection of shoots from separate explants on kanamycin- or hygromycin-containing rooting medium, and confirmed by PCR analysis.

3.2.2 Plant growth conditions and stress treatments

Plant growth conditions and stress treatments are described in Section 2.2.1.

3.2.3 RNA and DNA extraction and expression and Southern analysis

RNA extraction and northern analyses were performed as described in Section 2.2.2. For real-time PCR analysis, 25 ng of total RNA was treated with deoxyribonuclease I (DNAse I, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Five ng DNAse I-treated RNA was then was used for reverse transcription with SuperScript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Following validation experiments (as described by Qiagen, 2003), real-time PCR analysis was performed using a Stratagene Mx4000 (Stratagene, La Jolla, CA). Triplicate reactions were run on triplicate independent experiments. Reactions (15 μL) consisted of the QuantiTect™ SYBR® Green mix (Qiagen, Missisauga, ON) with 0.67 μM gene-specific primers and 6.25 ng of cDNA template per reaction. Primer sequences are given in Table A1-3. The amplification protocol was 95º C for 15 min, followed by 40 cycles of 94º C for 30 s, 55º C for 40 s, and 72º C for 60 s. Dissociation curves were obtained and reaction products visualized using agarose gel electrophoresis. Cycle threshold (Ct) values were determined by Mx4000 software and relative transcript abundances ($2^{-\Delta Ct}$) were determined after normalization to a constitutively expressed *ACTIN* gene.

Genomic DNA was extracted using the CTAB method, as described by Wang and Constabel (2004). Total DNA was digested with EcoRI, XbaI, and HindIII according to the manufacturer's instructions (New England Biolabs, Boston, MA). Southern blot analyses were conducted as described by Sambrook and Russell (200l) using ^{32}P-labeled DNA probes synthesized with the Rediprime II labeling kit (Amersham, Sunnyvale, CA) and Qiaquick-purified (Qiagen, Mississauga, ON) *nptII* DNA template fragments.

3.2.4 Phytochemical assays and HPLC analysis

HPLC and phytochemical analyses were performed as described in section 2.2.3.

3.2.5 Histochemical staining

Fresh plant material was placed into Tissue-Tek O.C.T. Compound Embedding Medium (Sakura Finetek, Inc., Torrence, CA) and left overnight in a -20º C freezer before slicing 20 μm (leaf) or 40 μm (petiole and stem) thick sections using a Microm HM 500 cryomicrotome (Francheville, France). PAs and flavan-3-ols were detected by staining sections for 5 min with DMACA (1% w/v in ethanol:6 N HCl, 1:1, v/v). Images were recorded using a Spot RT KE digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on a Zeiss Universal compound microscope (Thornwood, NY) for leaf blade sections or a Wild M420 macroscope (Heerbrug, Switzerland) for stem and petiole sections.

3.2.6 Promoter sequence analysis and electrophoretic mobility shift assays

Promoter sequences from the Nisqually 1 genome sequence were analyzed using the Plant *Cis*-Element Database (PLACE, http:// www.dna.affrc.go.jp/ PLACE/ signalscan.html) using the SIGNAL SCAN program web interface (Prestridge, 1991; Higo et al., 1998). Manual alignments were carried out using the Vector NTI Advance software package (Invitrogen, Carlsbad, CA). For identifying common motifs in promoter fragments bound by recombinant MYB134 (see below), the Bioconductor R package COSMO (Constrained Search for Motifs in DNA Sequences) was accessed at http://cosmoweb.berkeley.edu/intro.html (Bembom et al., 2009). 180 bp promoter fragments of poplar *PAL1*, *DFR1*, and *ANR2* were cloned into pGEM-T Easy (Promega, Madison, WI) and sequences confirmed by sequencing at the Centre for Biomedical Research Sequencing Facility (University of Victoria). Electrophoretic mobility shift assays were performed by Michael B. Prouse in the laboratory of Malcolm M. Campbell (University of Toronto). Recombinant MYB134 protein was produced in *E. coli* using the coding sequence cloned in frame into the NdeI and BamHI sites of the pET15b vector (Novagen, Madison, WI). Recombinant MYB134 protein was produced, extracted and affinity purified as described by Patzlaff et al. (2003). EMSA conditions were exactly as

described previously in Patzlaff et al. (2003) and Gomez-Maldonado et al. (2004) using recombinant MYB134 protein.

3.2.7 Forest tent caterpillar (FTC) bioassays

FTC bioassay experiments were conducted by Megan M. Towns (University of Victoria). FTC (*Malacosoma disstria*) egg bands were obtained from Barry J. Cooke (Canadian Forest Service, Edmonton, AB) and stored at 2º C until used. Egg masses were scraped to remove spumaline, sterilized with 1% commercial bleach for 3 min, and rinsed several times with ddH_2O prior to hatching. Larvae were reared on artificial diet prior to use. For choice experiments, three high PA 353-38 *MYB134*-overexpressor (lines 3-5) and three *GUS* control (lines 1-3) lines were compared. Leaves of LPI 10-20 where matched for age and size from paired plants. After rinsing in distilled water, 1.5 cm diameter leaf disks were cut using a cork borer. Five leaf disks, each from one transgenic line and one control line, were placed in alternate order along the periphery of a 9 cm Petri dish lined with moistened filter paper. To begin the experiment, larvae were chosen at random and placed into the centre of the dish. Four repetitions of each pairing were performed, for a total of twelve dishes per experiment. All choice experiments ran for two days. Larvae were hatched between six and fifteen days prior to the start of the experiments, and each dish contained between four and eight first instar larvae, depending on the experiment. Leaf disk area was measured using an LI-3100 Area Meter (Li-Cor, Inc., Lincoln, NB).

3.2.8 Affymetrix GeneChip® Poplar Genome Array analysis of *MYB134*-overexpressing poplar

Affymetrix GeneChip® Poplar Genome Array microarray hybridizations were conducted by the laboratory of Armand Séguin (Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre). For analysis of wild type 717-1-B4 and

MYB134-overexpressor (line 1) leaf tissue, labeled cRNA samples were hybridized to GeneChip arrays. Assessment of RNA quality, cRNA synthesis, labeling, hybridization, and data acquisition of Affymetrix poplar GeneChips were performed by the Functional Genomics Platform at the Genome Quebec Innovation Centre at McGill University (Montreal, QC, Canada). Data and statistical analyses for identification of differentially regulated genes were performed using FlexArray, a BioConductor R software package developed by M. Blazejczyk and associates (Genome Quebec, Montreal) (Gentleman et al. 2004). The raw data were adjusted for background signal and normalized using the Robust Multi-array Average (RMA) method (Irizarry et al. 2003). To identify differentially expressed genes, a Significance Analysis of Microarrays (SAM) was performed using the "siggenes" package of Bioconductor through FlexArray (Tusher et al. 2001). Only genes with a P value ≤ 0.01 and a fold change >2 or <0.5 were considered to be up- or down-regulated. The Affymetrix NetAffix analysis center, the poplar genome database, and BLAST searches were used to annotate deregulated genes. Gene annotations were also checked with the PopARRAY database (http://popgenome.ag.utk.edu/mdb/index.php). Annotations were done using the Arabidopsis Information Resource (TAIR) genome annotation, version 8 (http://www.arabidopsis.org/).

3.3 RESULTS

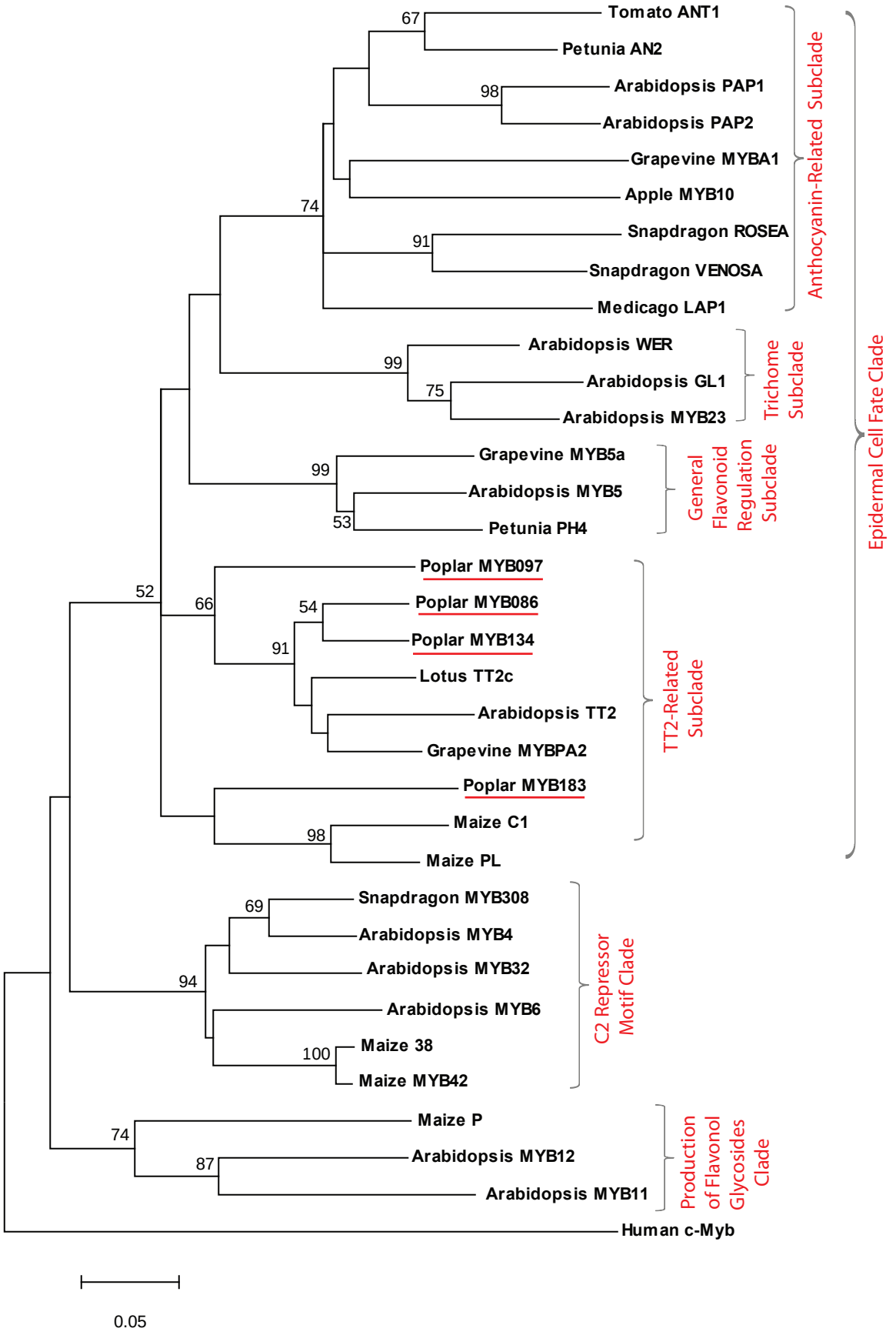
3.3.1 Identification of putative poplar PA regulators

Based on the knowledge that PA synthesis is activated by biotic and abiotic stresses (Chapter 2) and the central role of R2R3 MYB factors in regulating flavonoid metabolism (Chapter 1), a search for a transcriptional regulator of stress-responsive PA metabolism was initiated, focusing on poplar MYB genes with sequence similarity to the arabidopsis *TT2* gene. An EST sequence predicted to encode an R2R3 MYB domain transcription factor was identified in an EST library made from systemically wounded

poplar leaves (GenBank accession number CN192773; Christopher et al., 2004). The predicted protein encoded by this transcript exhibits high sequence similarity to arabidopsis TT2 as well as maize C1, both of which belong to the TT2-Related Subclade using the nomenclature of Matus et al. (2008) (Section 1.5). In order to identify additional candidate regulators of stress-induced PA metabolism in poplar, public poplar EST databases were searched for additional homologues of TT2. This search resulted in the identification of four genes predicted to encode R2R3 MYB transcription factors of the TT2-Related Subclade. cDNAs corresponding to these four candidate PA regulatory MYB genes were cloned from *P. tremuloides* clone A2*,* a genotype with particularly strong herbivore-induced PA accumulation (Peters and Constabel, 2002). With the completion of the *P. trichocarpa* genome sequence (Tuskan et al., 2006), the corresponding genomic sequences were identified and found to correspond to *P. trichocarpa* gene models *MYB097*, *MYB086*, *MYB134*, and *MYB183* (Wilkins et al., 2009). The genome contains an additional copy of *MYB086* (designated *MYB087*) with greater than 98% nucleotide identity within the coding sequences.

A phylogenetic tree was constructed using the predicted amino acid sequences of the R2R3 MYB domains of these and several additional putative flavonoid regulatory MYBs from poplar as well as a number of R2R3 MYB proteins from other species (Fig. 3-1). The four candidate MYBs (*MYB097*, *MYB086*, *MYB134*, and *MYB183*) are most closely related to members of the TT2-Related Subclade group rather than to members of

Figure 3-1. Phylogenetic analysis of putative flavonoid regulatory poplar R2R3 MYB domain proteins with selected R2R3 MYB domain proteins from other species. R2R3 MYB protein sequences were aligned using ClustalW and phylogenetic tree constructed using Mega 3.1 (Kumar et al., 2004) using minimum evolution test and p-distance model with 1000 boostrap replicates. Bootstrap values higher than 50% are shown. *Homo sapiens* c-Myb included as an outgroup. The Clade and Subclade designations of Matus et al. (2008) are indicated in red. The poplar MYB genes analyzed in this study are underlined in red. GenBank accession numbers are given in brackets: tomato (*Lycopersicon esculentum*) ANT1 (AAQ55181); petunia (*Petunia x hybrida*) AN2 (AAF66727); arabidopsis PAP1 (MYB75, AAG42001); arabidopsis PAP2 (Q9ZTC3); grapevine (*Vitis vinifera*) MYBA1 (BAD18977); apple (*Malus x domestica*) MYB10 (ACQ45201); snapdragon ROSEA1 (ABB83826); snapdragon (*Antirrhinum majus*) VENOSA (ABB83828); Medicago (*Medicago truncatula*) LAP1 (ACN79541); arabidopsis WER (WEREWOLF 1, NP_196979); arabidopsis GL1 (GLABROUS 1, P27900); arabidopsis MYB23 (NP_198849); grapevine MYB5a (AAS68190); arabidopsis MYB5 (Q38850); petunia Ph4 (AAY51377); lotus (*Lotus japonicus*) TT2c (BAG12895.1); arabidopsis TT2 (MYB123, Q9FJA2); grapevine MYBPA2 (ACK56131.1); maize (*Zea mays*) C1 (AAK09327); maize PL (AAB67721); snapdragon MYB308 (P81393); arabidopsis MYB4 (NP_850879); arabidopsis MYB32 (O49608); arabidopsis MYB6 (Q38851); maize 38 (P20025); maize MYB42 (CAJ42204); maize P (P27898); arabidopsis MYB12 (ABB03913); arabidopsis MYB11(CAB83111); human c-Myb (AAB49039).


Tomato ANT1
Petunia AN2
Arabidopsis PAP1
Arabidopsis PAP2
Grapevine MYBA1
Apple MYB10
Snapdragon ROSEA
Snapdragon VENOSA
Medicago LAP1
Arabidopsis WER
Arabidopsis GL1
Arabidopsis MYB23
Grapevine MYB5a
Arabidopsis MYB5
Petunia PH4
Poplar MYB097
Poplar MYB086
Poplar MYB134
Lotus TT2c
Arabidopsis TT2
Grapevine MYBPA2
Poplar MYB183
Maize C1
Maize PL
Snapdragon MYB308
Arabidopsis MYB4
Arabidopsis MYB32
Arabidopsis MYB6
Maize 38
Maize MYB42
Maize P
Arabidopsis MYB12
Arabidopsis MYB11
Human c-Myb
67
98
74
91
99
75
99
53
52
66
54
91
94
69
100
74
87
Anthocyanin-Related Subclade
Trichome Subclade
General Flavonoid Regulation Subclade
TT2-Related Subclade
C2 Repressor Motif Clade
Production of Flavonol Glycosides Clade
Epidermal Cell Fate Clade
0.05

the other flavonoid regulatory MYBs within the Epidermal Cell Fate Clade (Fig. 3-1). These findings are congruent with a recently published phylogenetic analysis of the entire *P. trichocarpa* Nisqually 1 R2R3 MYB family (Wilkins et al., 2009). The Anthocyanin-Related Subclade is composed of MYB proteins that function as anthocyanin biosynthetic gene activators (Section 1.5.2, Table 1-1). Members of the C2 Repressor Motif Clade include negative regulators of phenylpropanoid and flavonoid metabolism (Section 1.5.4), while the General Flavonoid Regulation Subclade includes proteins involved in controlling vacuolar pH and flavonoid biosynthetic gene activation (Sections 1.5.2 and 1.5.3).

MYB134 and *MYB086* encode proteins containing a sequence motif similar to the Vx_2IRTKA[IL]RC[SN] motif located C-terminal to the R2R3 MYB domain in arabidopsis TT2 (Nesi et al., 2001) (Fig. 3-2). A similar conserved sequence motif is found in other TT2-Related Subclade proteins, including the recently identified lotus TT2 proteins (Yoshida et al., 2008), the grapevine MYBPA2 (Terrier et al., 2009), and several uncharacterized predicted MYB proteins including cotton (*Gossypium hirsutum*) MYB36 (Fig. 3-2), Japanese beech (*Fagus crenata*) MYB251 (GenBank accession number BAG75107) and apple MYB11 (Fig. 3-2) and MYB9 (GenBank accession number ABB84757). The [DE]Lx_2[RK]$x_3Lx_6Lx_3$R motif involved in the interaction of MYB proteins with BHLH partners (Section 1.5.1) is present in all four putative poplar PA regulatory MYBs, which indicates that like TT2 these MYBs likely require the presence of BHLH cofactors to function (Fig. 3-2).

3.3.2 Stress-responsive expression of poplar MYB genes

Sequence similarity and phylogenetic analysis indicate that poplar *MYB134* or *MYB086* are the most similar to arabidopsis TT2 and therefore likely to function in PA regulation. These genes shared highest sequence similarity with TT2 within the MYB domain and also shared a conserved sequence motif C-terminal to the MYB domain (Fig. 3-2). To identify putative regulators of stress-induced PA metabolism from among the *MYB* genes isolated, gene expression was monitored in leaves following exposure to PA-

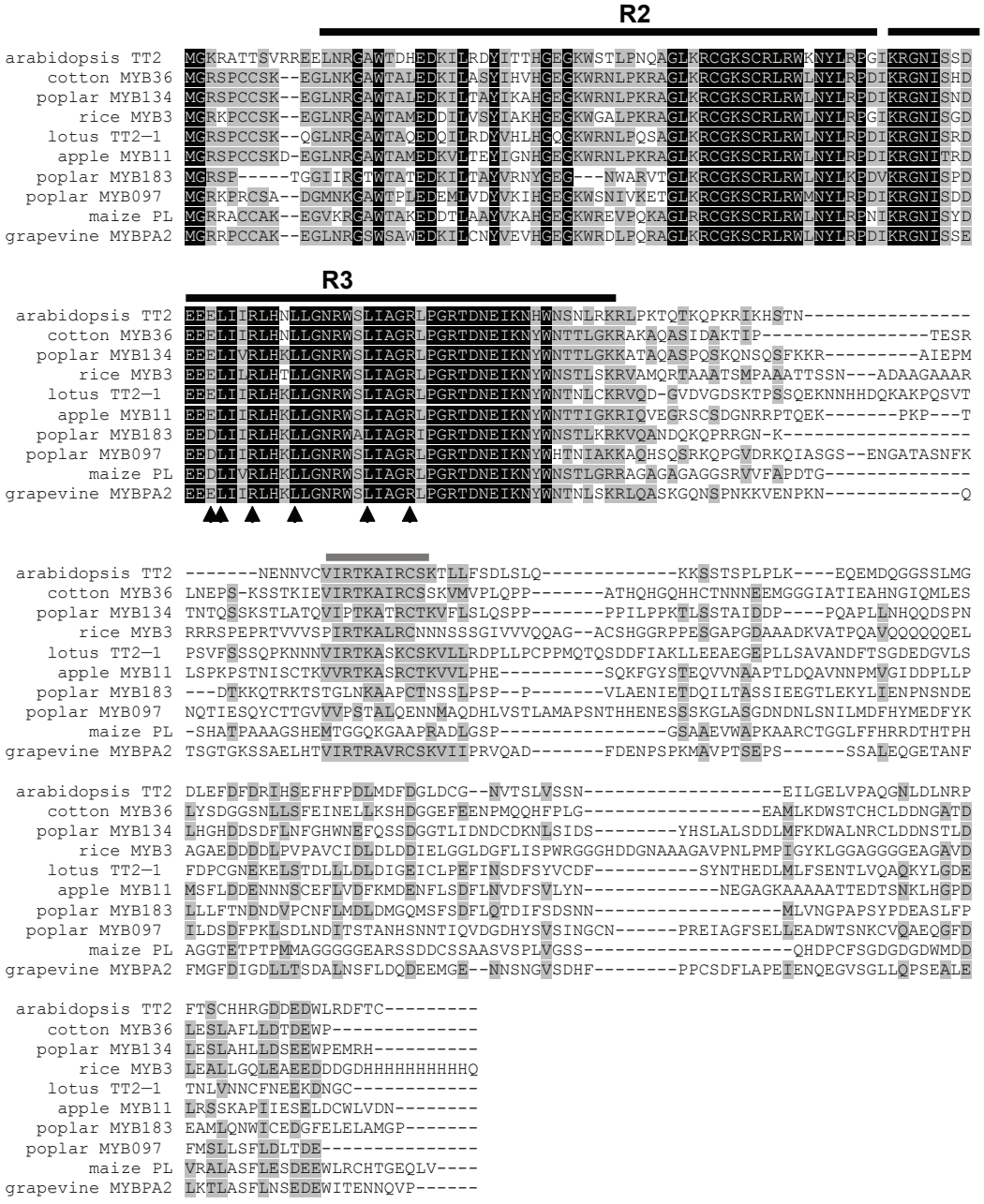


Figure 3-2. Multiple sequence alignment of poplar MYB protein sequences with the PA pathway regulators arabidopsis TT2 (GenBank accession numbers not given here are given in the Fig. 3-1 legend), grapevine MYBPA2 , lotus MYBTT2-1 (GenBank accession number BAG12893), as well as maize PL, and uncharacterized MYB proteins from rice (*Oryza sativa* MYB3, GenBank accession number BAA23339), apple (MYB11, GenBank accession number AAZ20431), and cotton (*Gossypium hirsutum* MYB36, GenBank accession number AAK19617) containing conserved motif(s) C-terminal to the MYB DNA-binding domain. Black-boxed residues are identical, while grey are similar amino acids. The residues involved in the interaction with bHLH cofactors are indicated by arrows. Grey bar indicates the Vx2IRTKA[IL]RC[SN] motif found in arabidopsis TT2 and rice MYB03, and the black bars indicate the R2 and R3 repeats of the MYB domain.

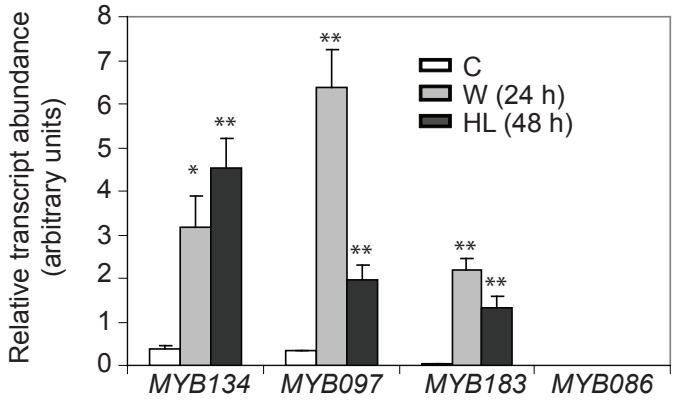


Figure 3-3. Real-time PCR analysis of MYB gene expression in control (C), mechanically wounded (W, 24 hour), and high light-exposed (HL, 48 hour) 353-38 poplar leaves. Relative transcript abundance (arbitrary units) calculated with three technical replicates on triplicate biological replicates of each sample. Asterisks indicate significant differences using Student's t test (* P<0.05, ** P< 0.01).

activating stress conditions. Real-time PCR was used to analyze transcript levels in wounded and HL-exposed 353-38 leaves for *MYB134*, *MYB183*, *MYB097*, and *MYB086*. Gene-specific primers were designed to amplify a fragment of each gene within the highly divergent 3' end of the sequence rather than the conserved 5' end, which encodes the MYB DNA binding domain (Appendix A, Table A1-3). *MYB134*, *MYB097*, and *MYB183* all exhibited significant wound- and HL-induced up-regulation, while *MYB086* exhibited a very low, constitutive level of expression (Fig. 3-3). *MYB086/87* expression was too low relative to the other genes to be visible with the scale of this graph, but was visible with gel electrophoresis (Fig. 3-4). In contrast to *MYB134*, which exhibits increased expression by 6 hours, *MYB086* is not responsive to wounding (Fig. 3-4).

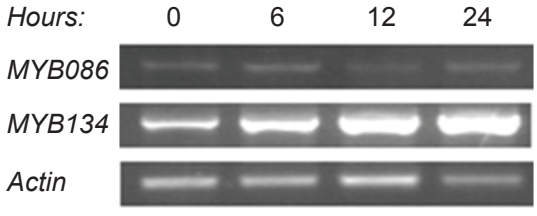


Figure 3-4. RT-PCR analysis of *MYB087*, *MYB134*, and *actin* transcript levels in control (0 hour) and mechanically wounded (after 6, 12, and 24 hours) 383-38 leaf tissue. DNAse-treated RNA extracted from leaves 9-11 and pooled from three separate trees used for cDNA synthesis for each reaction. PCR reactions with *MYB087*, *MYB134*, and *actin* gene-specific primers as described for real-time PCR analysis.

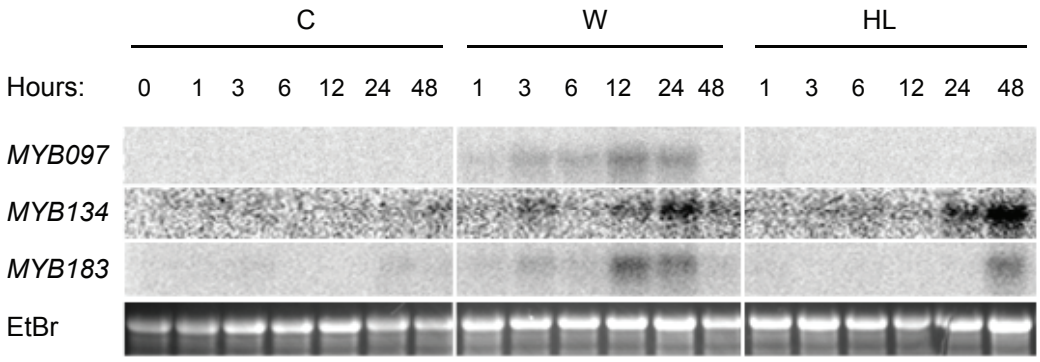


Figure 3-5. Time course of transcript levels of putative flavonoid regulatory MYB genes *MYB134*, *MYB097*, and *MYB183* in poplar leaves after wounding and exposure to elevated light. Northern analysis of putative PA regulatory *MYB* genes in control (C), mechanically wounded (W), and high light-exposed (HL) leaves at various time points after (beginning of) treatment. EtBr, ethidium bromide-stained total RNA.

To gain further insight into the stress-responsive expression of the poplar TT2-Related Subclade *MYB* genes, transcript abundance of the three stress-responsive genes was monitored at various time points following wounding and HL-exposure (Fig. 3-5). To facilitate direct comparison with PA pathway activation, *MYB* gene expression was analyzed in the same tissues and at the same time points as for the stress-responsive PA pathway gene analysis (Chapter 2, Fig. 2-2). All three genes were up-regulated by 3 hours after mechanical wounding, and also showed some up-regulation at 12 and 24 hours after wounding (Fig. 3-5). None of the *MYB* genes were co-activated with the rapidly induced *FLS4* gene in the HL-exposed leaves (Figs. 2-2 and 3-5). The *MYB134* was also up-regulated by 24 hours after the initiation of HL exposure, while *MYB183* exhibited weak up-regulation by 48 hours. The activation of *MYB134* at the 24 hour time point, which is well after the early flavonol pathway up-regulation but before the strong HL-induced PA pathway up-regulation, supports a role for this gene as a transcriptional regulator of PA pathway genes. Transcriptional activation of PA regulatory genes might be expected to precede up-regulation of the target genes.

In order to confirm this up-regulation of *MYB134* following wounding and HL-exposure, the wounding and HL experiment was replicated and expression of *MYB134* as well as a subset of PA marker genes (*PAL1*, *CHS1*, *ANS1*, and *ANR1*) was monitored. Expression of the *FLS4* gene was also analyzed. In this experiment, the level of up-

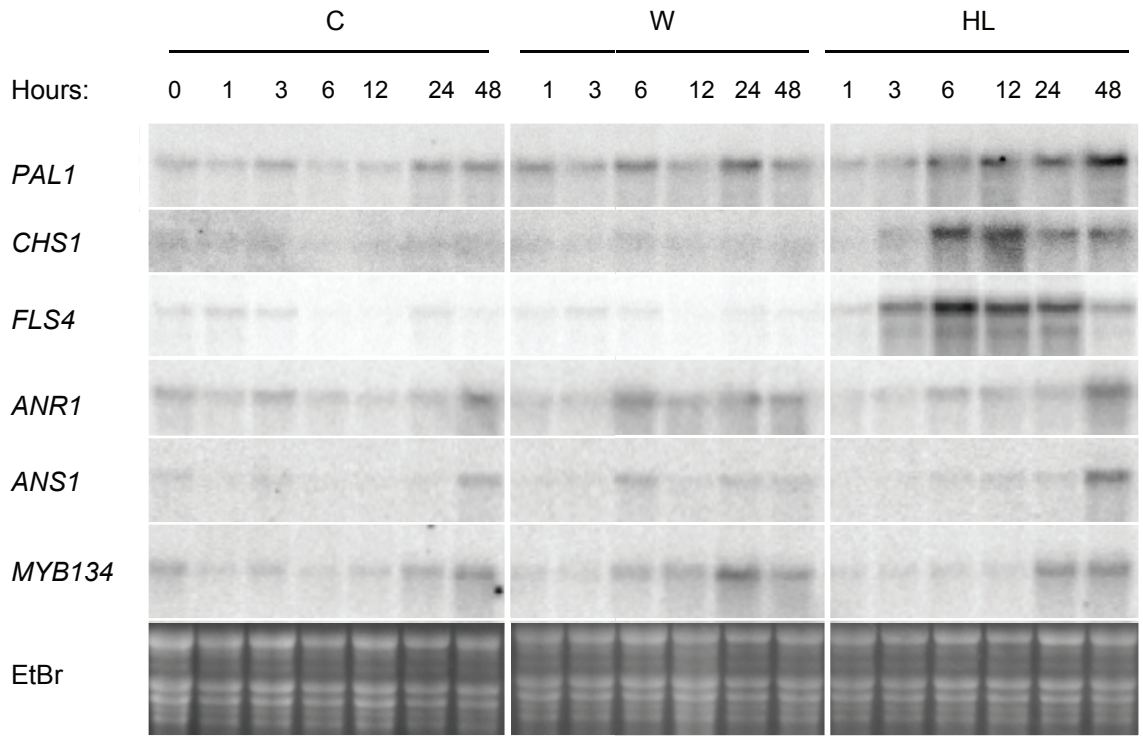


Figure 3-6. Northern analysis of PA pathway gene expression in response to wounding (W) and light stress (HL) in clone 353-38. Gene names given in Fig. 1-1 legend. EtBr, ethidium bromide-stained total RNA. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually 1 gene models listed in Table 2-1.

regulation of the PA pathway following wounding was very low (Fig. 3-6). However, *MYB134* was co-activated with the PA pathway genes following wounding, with a similarly low level of activation (Fig. 3-6). Once again, the *FLS4* gene was not wound-responsive (Fig. 3-6). Exposure of leaves to elevated light resulted in a rapid activation of *PAL1*, *CHS1*, and *FLS4* by 3 hours, but not *MYB134* or later flavonoid pathway genes (Fig. 3-6). Similar to the previous experiment, *MYB134* was weakly up-regulated by 24 hours followed by a weak activation of *ANS1* and *ANR1* by the 48 hour time point (Fig. 3-6).

Since UV-B exposure and infection of leaves by the fungal biotroph *M. medusae* were also found to induce PA pathway gene activation (Sections 2.3.3 and 2.3.4), expression of the three poplar TT2-Related Subclade genes was monitored in UV-B-exposed and *M. medusae*-infected leaves (Fig. 3-7). Of the wound- and HL-induced *MYB* genes, only *MYB134* was tightly co-induced with the late flavonoid biosynthetic genes

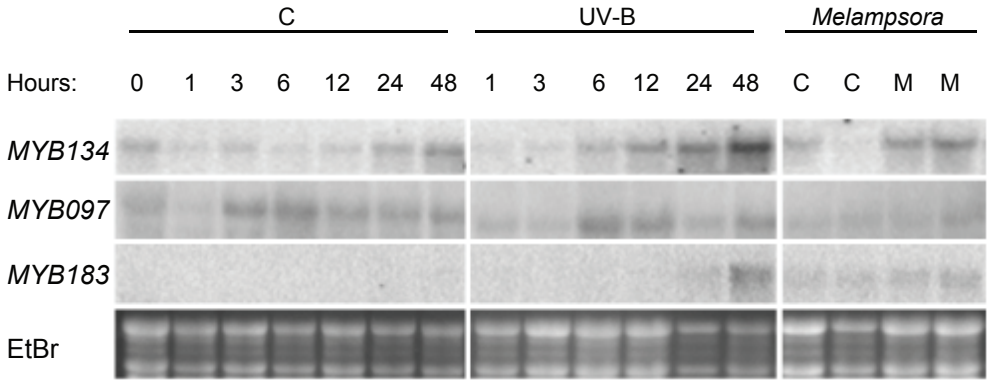


Figure 3-7. The effects of UV-B exposure and *M. medusae* infection on the expression of putative PA regulatory MYB genes *MYB134*, *MYB097*, and *MYB183*. Northern analysis of putative PA regulatory MYB genes in control (C) and UV-B-treated (UV-B) poplar leaves, as well as control and *M. medusae*-infected (M) leaves at 6 dpi. EtBr, ethidium bromide-stained total RNA.

under elevated UV-B, although *MYB183* exhibited a late up-regulation (Fig. 3-7). Similarly, only *MYB134* exhibited a clear up-regulation in *M. medusae*-infected leaves at 6 dpi (Fig. 3-7). Overall, these expression profiling data show that several members of the poplar TT2-Related Subclade exhibit some stress-induced co-activation with PA biosynthetic genes. However, *MYB134* expression was the most strongly correlated with PA activation under all conditions analyzed. Of the inducible MYB genes, the predicted protein encoded by *MYB134* also exhibits the highest homology to TT2 within the R2R3 MYB domain and shares a carboxy-terminal sequence motif. Based on these observations, it was hypothesized that MYB134 plays a role in regulating stress-induced PA metabolism in poplar leaves. Since PA biosynthesis occurs in numerous poplar vegetative tissues, expression of the *MYB134* gene was surveyed in a variety of tissues using RT-PCR. *MYB134* transcripts were detected in roots, bark, leaf petioles, and apical leaves (Fig. 3-8).

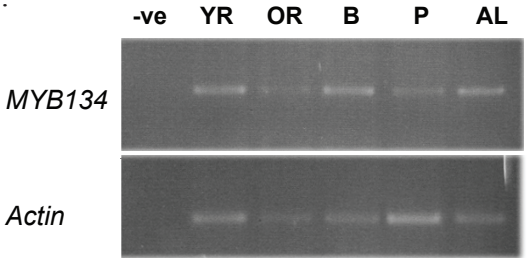


Figure 3-8. PCR analysis of *MYB134* expression in selected poplar tissues. cDNA synthesized from DNAse-treated RNA and reaction conditions and gene-specific primers as for real time PCR analysis, except that reactions were run for 28 cycles. YR: young root; OR: old root; B: bark; P: petiole; AL: apical leaves and shoot apex.

3.3.3 Effects of MYB134 overexpression in poplar on phenolic metabolism

In order to investigate the role of *MYB134* in the regulation of stress-induced PA metabolism, this gene was overexpressed in poplar under the control of a double cauliflower mosaic virus (CaMV) 35S promoter, a strong, constitutive promoter commonly used to express transgenes at high levels in plants (Gruber and Crosby, 1993). *β-glucuronidase* (*GUS*)-overexpressing lines were also produced as controls (Datla et al., 1992). Two genotypes, *P. tremula x tremuloides* clone INRA 353-38 and *P. tremula x alba* clone INRA 717-1-B4, were chosen for *A. tumefaciens*-mediated transformation. Multiple independently transformed lines were generated for both genotypes, and confirmed transgenics were moved to a greenhouse for analysis. For plants of both genotypes, grown and maintained under standard greenhouse conditions for up to six months, *MYB134* overexpression did not lead to any discernable gross phenotypic abnormalities (Fig. 3-9). However, analysis of PA levels in leaves revealed that *MYB134* overexpression resulted in a large increase in leaf PA concentrations (Fig. 3-10). One of the 353-38 *MYB134*-overexpressor lines exhibited a much lower but still significant (P = 0.003) increase in PA levels (353-38 *MYB134* line 1, Fig. 3-10). Concentrations of total soluble phenolics were also found to have strongly increased in leaves of all *MYB134*-overexpressing lines of both genotypes, with the exception of 353-38 *MYB134* line 1 (Fig. 3-11). Southern blot analysis using a probe complementary to the *nptII* gene present on the T-DNA revealed that this line contained five or six T-DNA insertions, compared to three or fewer copies for the other lines (Fig. 3-12). This indicates that the lower increase in PA levels in this line may be the result of silencing of the transgene due to the high number of T-DNA insertions (Tang et al., 2007).

To determine if tissues other than mature leaves also respond to *MYB134* overexpression with activation of the PA biosynthetic pathway, a tissue survey of PA levels in four high *MYB134* transgene-expressing lines was conducted (353-38 *MYB134*-overexpressor lines 2-5). A significant increase in PA concentrations was found in all tissues analyzed ($P < 0.01$), with the exception of young roots ($p = 0.055$) (Fig. 3-13). All

A

B

Figure 3-9. *MYB134* overexpression does not result in any discernable macroscopic phenotypic difference compared to control plants. **A**. Photograph of typical 353-38 *MYB134*-overexpressor leaf. **B**. Photograph of typical wild-type 353-38 leaf. Leaves photographed on 6 week old trees grown under standard greenhouse conditions.

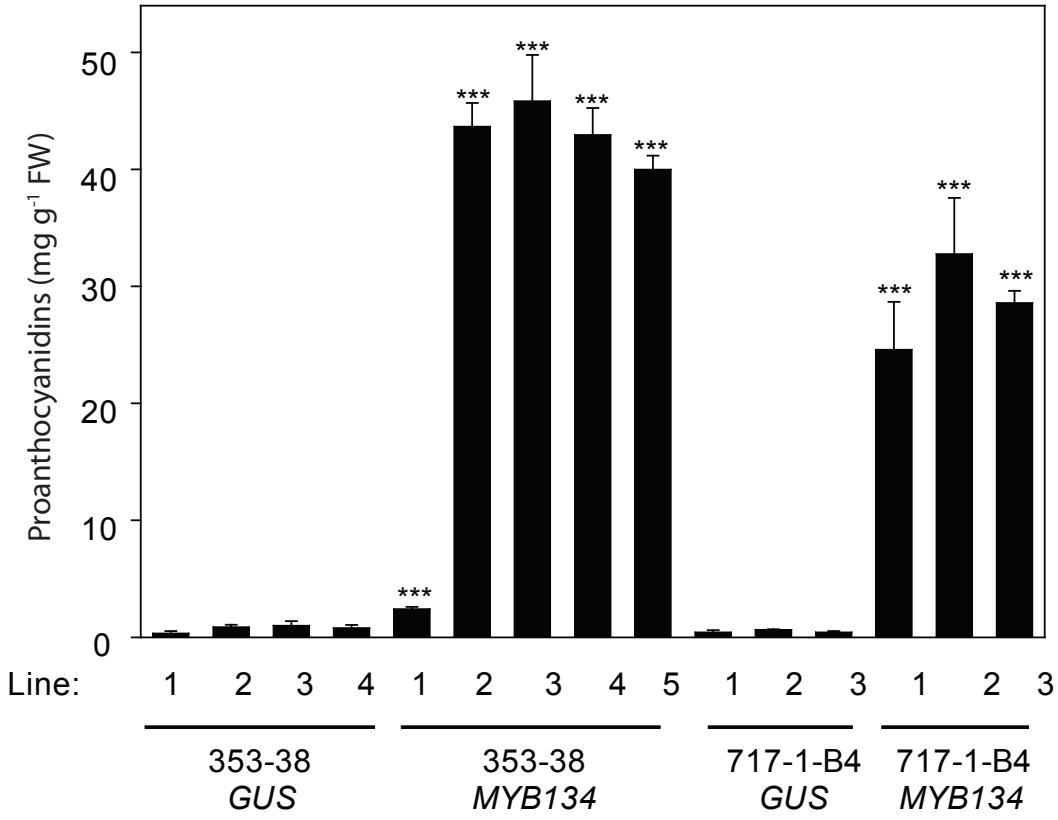


Figure 3-10. Effects of *MYB134* overexpression on PA concentration in poplar leaves. Graph shows PA concentrations in leaves of independently transformed *GUS*- and *MYB134*-overexpressor 353-38 and 717-1-B4 hybrid clones. Bars indicate means of at least three trees per line, with error bars showing ± SE. Asterisks indicate significance between the sum of control lines and the indicated *MYB134*-overexpressor line (Student's t test, *** P<0.001).

tissues analyzed exhibited a significant increase in concentrations of total soluble phenolics, measured using the Folin-Ciocalteau method (Table 3-1).

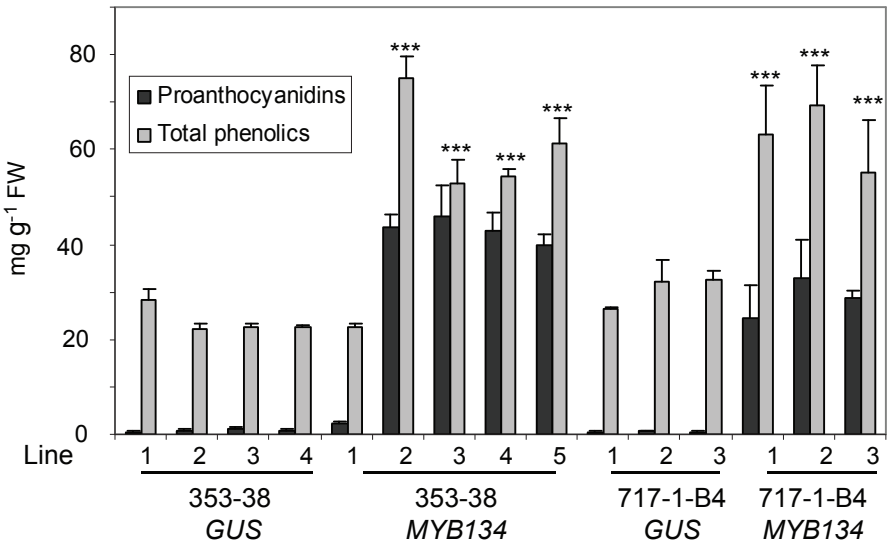


Figure 3-11. Effects of *MYB134* overexpression on total soluble phenolics in transgenic poplar leaves (with PA concentrations shown for comparison). Bars indicate means of at least three trees per line, with error bars showing ± SE. Asterisks indicate significance between all control lines and *MYB134*-overexpressor line (Student's t test, *** P<0.001).

In addition to the large increase in PA concentrations, HPLC analysis revealed that *MYB134* overexpression caused significant alterations to general phenolic metabolite profiles in 353-38 leaves (Fig. 3-14). The most pronounced effect was a significant reduction in PG concentrations. Total PG levels were reduced from a mean of 46.7 ± 12.2 mg g^{-1} fresh weight to 17.0 ± 1.9 mg g^{-1} fresh weight, and levels of all individual PGs (salicortin, tremuloidin, and tremulacin) reflect this change (Fig. 3-15A and B). *MYB134* overexpression resulted in a small but significant increase in concentrations of total flavonol glycosides (Fig. 3-16A). To determine whether *MYB134*-overexpression affected flavonoid B ring hydroxylation, flavonol aglycones were quantified using HPLC analysis of extracts after treatment with heat and acid to remove sugars from flavonols (Fig. 3-17). Interestingly, levels of the dihydroxylated flavonol quercetin were

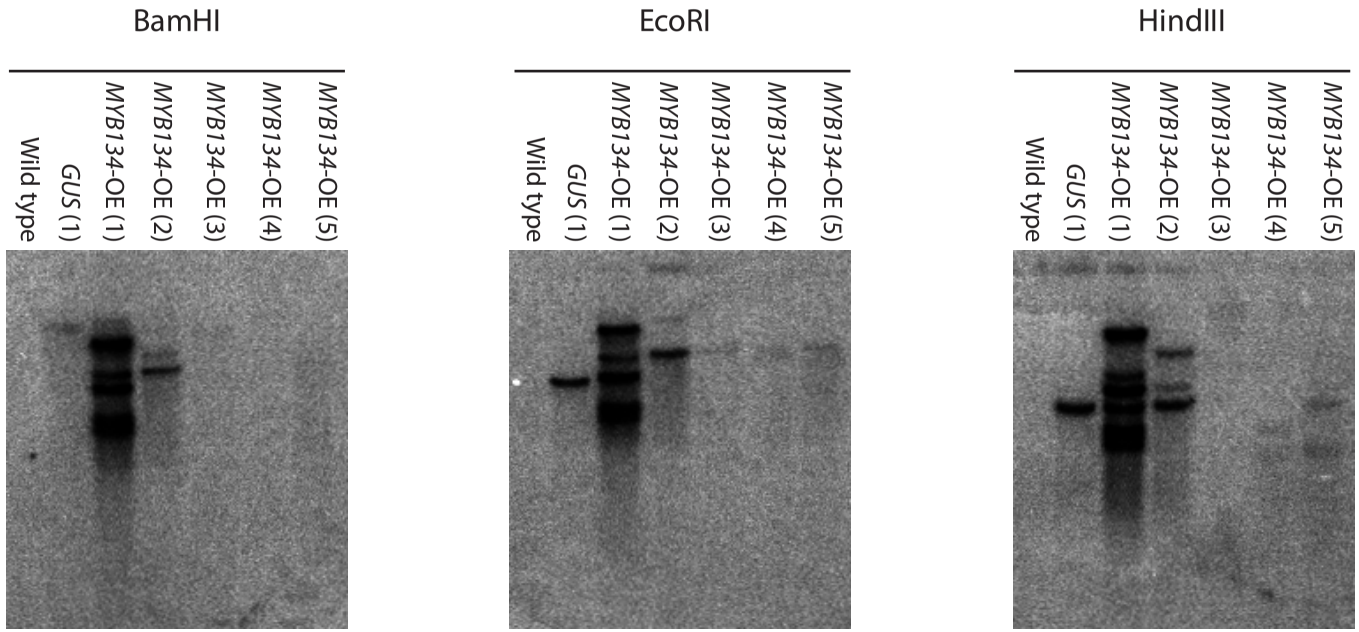


Figure 3-12. Southern blot analysis of transgene copy number in 353-38 plants transformed using pRD410 (70S::GUS) or pRDMYB134 (70S::MYB134) vectors. Untransformed wild type also included. Line shown in brackets. Total DNA digested with BamHI, EcoRI, or HindIII and hybridized with a ^{32}Plabeled probe for the *nptII* gene.

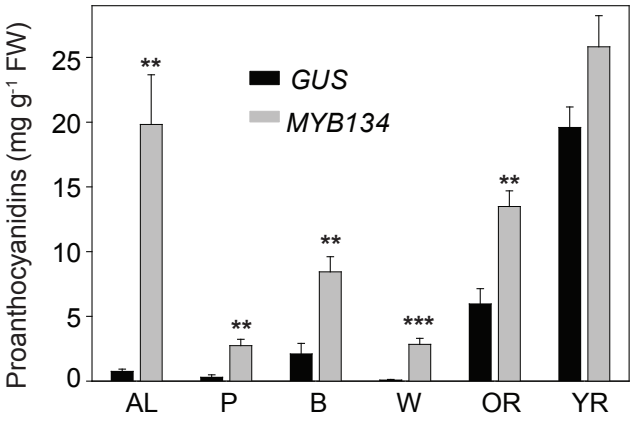


Figure 3-13. Effects of *MYB134* overexpression on PA concentration in transgenic poplar tissues. AL: apical leaves, P: petioles of leaves (LPI 9-11), B: bark, W: woody stem with bark removed, OR: old root (within 4 cm of shoot base), YR: young root (within 4 cm of tip). Bars indicate means of four independently transformed lines (353-38 GUS lines 1-4 and *MYB134*-overexpressor lines 2-5), with error bars indicating + SE of mean. Asterisks indicate significant differences using Student's t test (* P<0.05, ** P< 0.01, *** P<0.001).

Table 3-1. Percent increase in total soluble phenolics in tissues of high PA-accumulating 353-38 *MYB134*-overexpressors relative to *GUS* control plants. Error bars are SE of mean, n=6. Asterisks indicate significant difference between *MYB134*-overexpressors and controls using Student's t test (*P<0.05, **P<0.01, ***P<0.001).

Tissue	% increase ± SE
leaves (LPI9-11)***	99.4±10.2
apical leaves**	47.0±8.6
bark*	71.7±14.7
wood*	30.7±4.9
petioles***	54.9±3.6
young root*	39.1±12.2
old root*	68.2±20.9

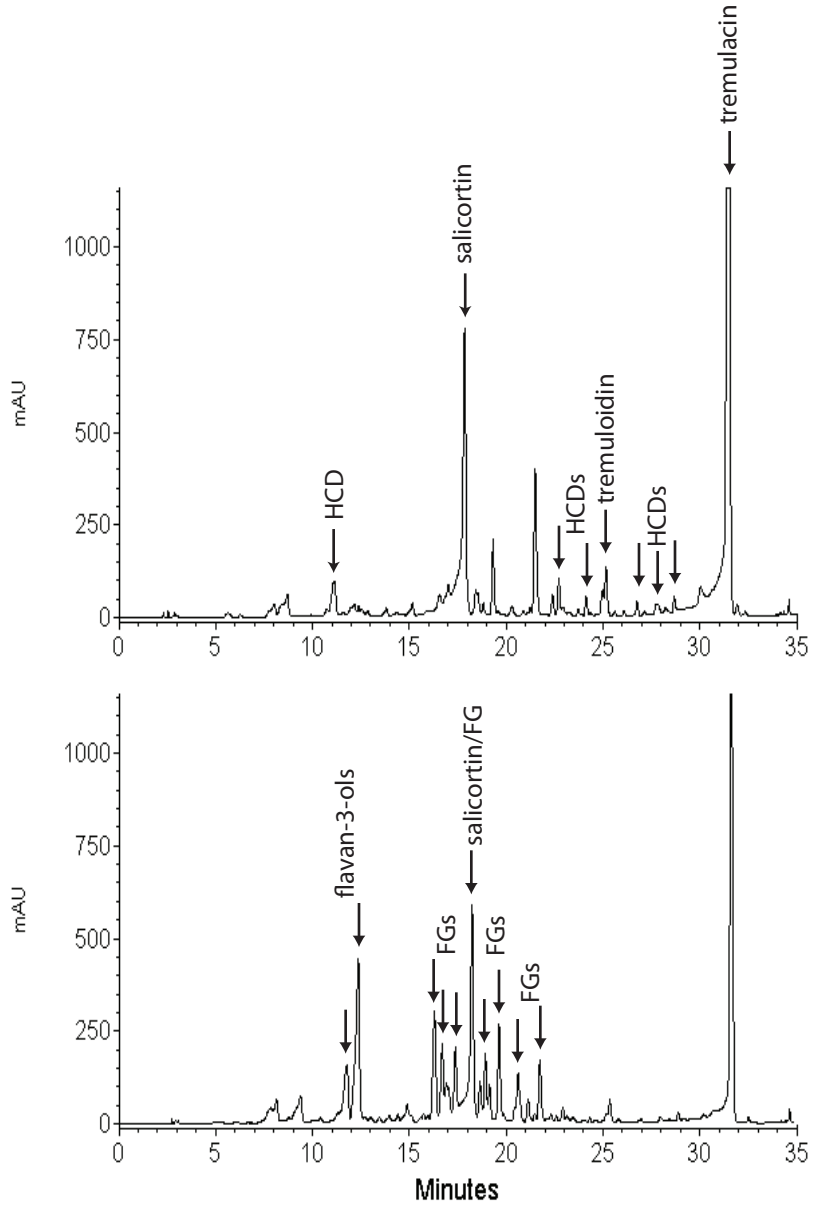


Figure 3-14. HPLC analysis of soluble phenolics in control 353-38 GUS control (top) and *MYB134*-overexpressor (bottom) leaf extracts. Representative maxplot chromatograms show each peak at its wavelength of maximum absorption (note that some peaks present in both chromatograms are labelled in one or the other and the tremulacin peak in the top chromatogram is truncated). FG: flavonol glycosides. Major phenolic glycosides (salicortin, tremuloidin, and tremulacin) are individually labeled.

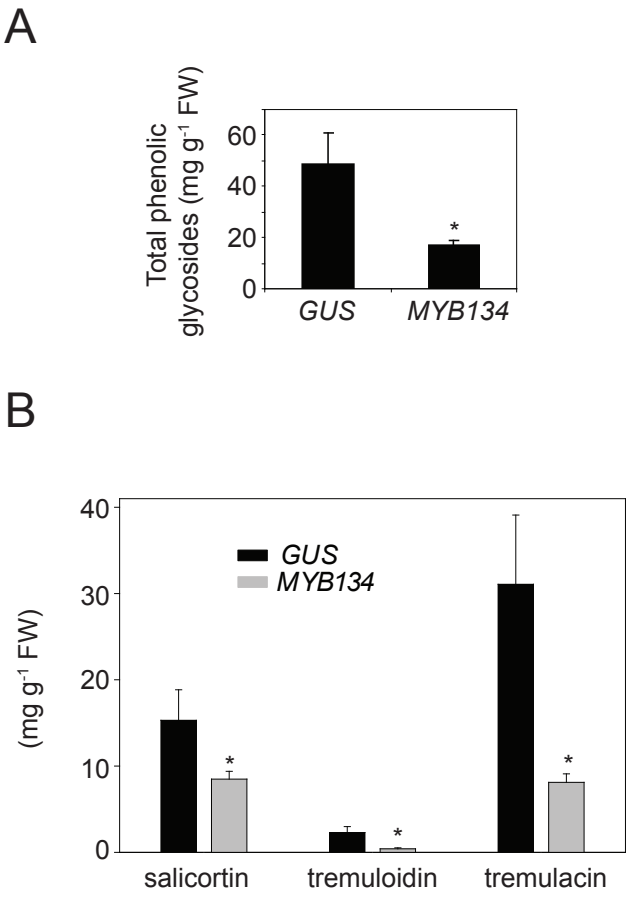


Figure 3-15. Analysis of phenolic glycoside (salicortin, tremuloidin, and tremulacin) concentrations in control 353-38 *GUS*- and *MYB134*-overexpressor leaf extracts. **A**. Total phenolic glycoside concentrations in leaves of 353-38 control (*GUS*) and *MYB134*-overexpressor (*MYB134*). **B**. Levels of three individual phenolic glycosides in 353-38 control (*GUS*) and *MYB134*-overexpressor (*MYB134*) leaves. For both **A** and **B**, bars indicate means of four independently transformed lines (*GUS* lines 1-4 and *MYB134*-overexpressor lines 2-5), with error bars indicating + SE. Asterisks indicate significant differences using Student's t test (* $P<0.05$, ** $P< 0.01$, *** $P<0.001$).

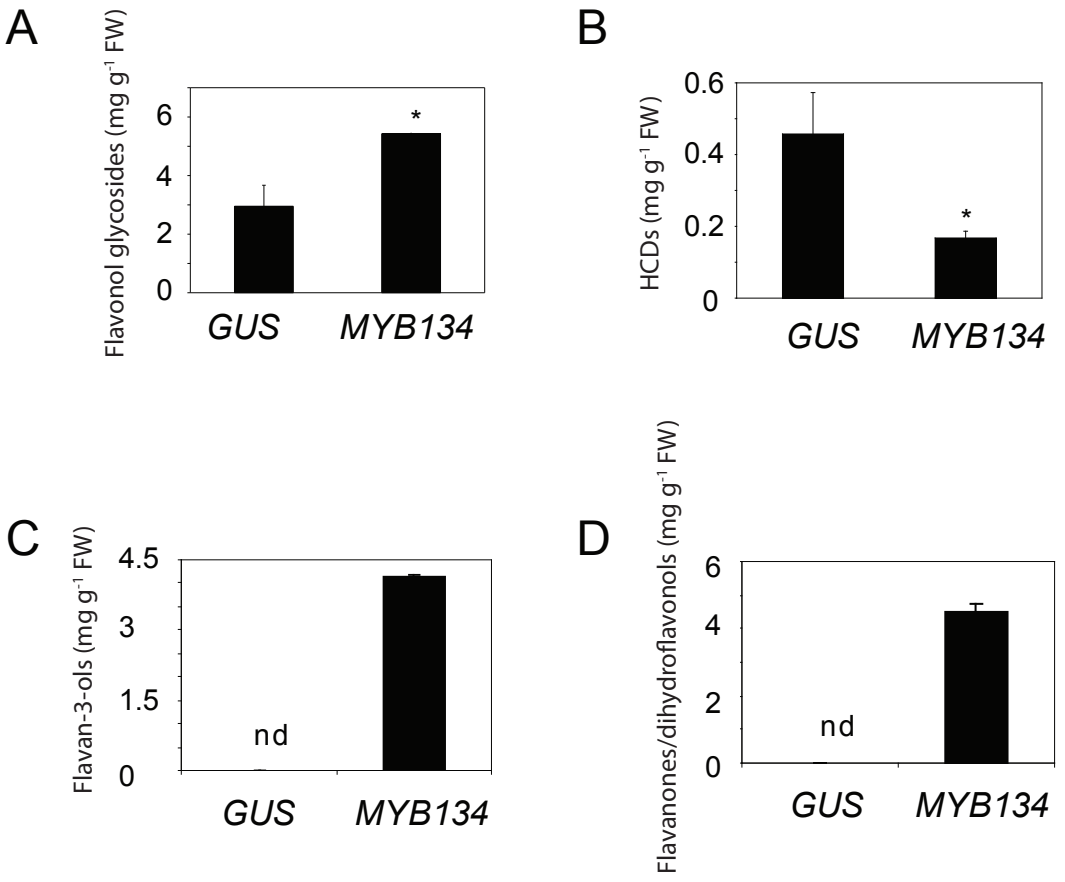


Figure 3-16. HPLC analysis of soluble phenolic metabolites in control (*GUS*) 353-38 and *MYB134*-overexpressor poplar leaves. Total flavonol glycoside (**A**), HCD (**B**), flavan-3-ols (**C**) and flavanones/dihydroflavonols (**D**) in leaves of 353-38 *GUS* controls (*GUS*) and *MYB134*-overexpressors (*MYB134*). Bars indicate means of four independently transformed lines (*GUS* lines 1-4 and *MYB134*-overexpressor lines 2-5), with error bars indicating + SE . Asterisks indicate significant differences using Student's t test (* $P<0.05$).

increased while the monohydroxylated flavonol kaempferol exhibited no increase, indicating that *MYB134* overexpression results in increased B ring 3' hydroxylation activity. *MYB134*-overexpressing plants of both genotypes were found to accumulate a significantly higher proportion of quercetin (Table 3-2).

MYB134 overexpression also resulted in a small decrease in levels of non-flavonoid HCDs (Fig. 3-16B). The changes to flavonol glycoside and HCD levels were minor in comparison to the large increase in PAs and decrease in PG levels. Anthocyanins were not detected in control or *MYB134*-overexpressor mature leaves. A number of peaks with absorption spectra and retention times corresponding to PA biosynthetic intermediates, including flavan-3-ols (catechin and epicatechin) (Fig. 3-16C) and flavanone/dihydroflavonols (Fig. 3-16D), were observed in *MYB134*-overexpressor leaf extracts but were undetectable in controls. The same pattern of reduced non-flavonoid phenylpropanoids (PGs and HCDs), and increased PAs and non-PA flavonoids (flavonol glycosides and PA biosynthetic intermediates) was also found in the 717-1-B4 *MYB134*-overexpressor lines (data not shown).

An independent HPLC analysis of 353-38 wild type and *MYB134*-overexpressor (line 3) leaf phenolic composition confirmed the large increase in PA and total phenolic concentrations and the large decrease in PG concentrations, with the nine different PGs detected all present at reduced levels in the *MYB134*-overexpressor leaves (Table 3-3). The smaller increase in flavonol glycosides was also confirmed. While total flavonol glycoside levels were found to be increased in *MYB134*-overexpressing leaves, levels of some individual flavonol glycosides were unchanged or even reduced (Table 3-3), possibly reflecting the specific increase in dihydroxylated but not monohydroxylated flavonols.

To determine PA localization in *MYB134*-overexpressing and control poplar plants, leaf, petiole, and stem sections were stained with DMACA, which reacts specifically with PAs and flavan-3-ols to form a blue chromophore (Feucht and Treutter, 1990). As expected, DMACA stained the *MYB134*-overexpressor tissues more intensely than control tissues (Fig. 3-18). In leaves of control plants, DMACA staining revealed PA accumulation primarily in the abaxial epidermis, with very light staining in the adaxial epidermis (Fig. 3-18A). In *MYB134*-overexpressor leaves, PAs were also present in both

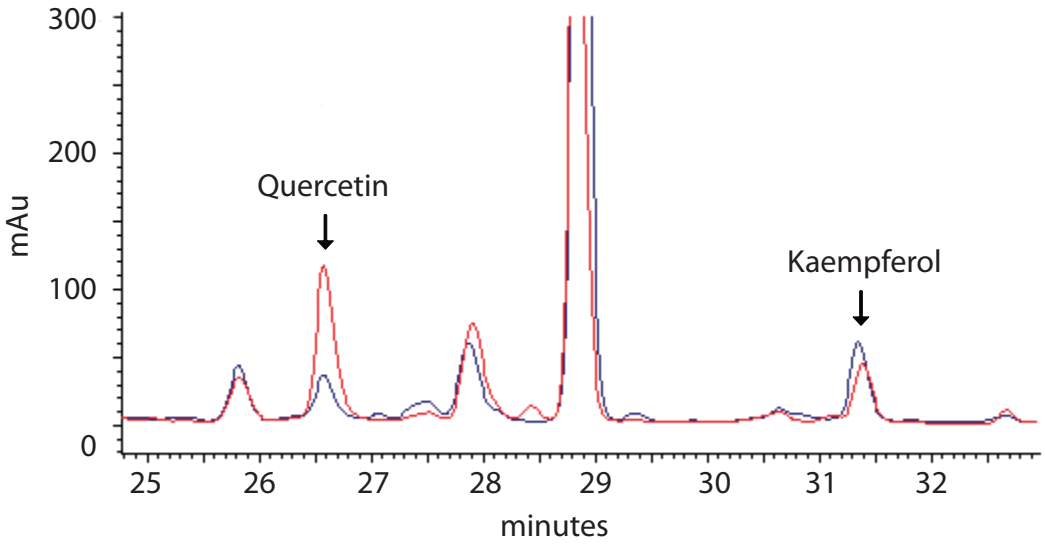


Figure 3-17. *MYB134*-overexpressing poplar accumulate elevated levels of the orthodihydroxylated flavonol quercetin but not the monohydroxylated flavonol kaempferol. Overlayed representative HPLC chromatograms (320 nm) showing quercetin and kaempferol peaks in control (dark blue) and *MYB134*-overexpressor (red) 353-38 leaves. Methanol extracts treated with heat and acid to hydrolyze glycosides for analysis of flavonol aglycones.

Table 3-2. *MYB134*-overexpressing poplar leaves accumulate a significantly higher proportion of the *ortho*-dihydroxylated flavonol quercetin than wild type. Methanol extracts treated with heat and acid to remove glycosides from quercetin and kaempferol glycosides and then total quercetin and kampferol determined using HPLC anlaysis.

Transgenic genotype	Proportion quercetin[a]	p value[b]
P. tremula x tremuloides 353-38 *GUS*	0.45 ± 0.01	
		0.0083
P. tremula x tremuloides 353-38 *MYB134*	0.68 ± 0.05	
P. tremula x alba 717-1-B4 *GUS*	0.42 ± 0.01	
		0.0001
P. tremula x alba 717-1-B4 *MYB134*	0.74 ± 0.02	

[a]proportion that quercetin constitutes of the total amount of quercetin and kaempferol (± SE); [b]Significance of difference between *MYB134*-overexpressor and control determined using Student's t test.

Table 3-3. HPLC analysis of phenolic commpounds in leaves of wild type control and *MYB134*-overexpressor (*MYB134*-OE) poplar leaves. Data contributed by Juha-Pekka Salminen (Department of Chemistry, University of Turku, Finland).

Compound	Control (mg g^{-1} DW)	*MYB134* -OE (mg g^{-1} DW)
Salicylate 1	3.39	0.12
Salicylate 2	0.47	0.40
Salicylate 3	5.28	0.15
Salicylate 4 (salicortin)	17.19	13.43
Salicylate 5	4.66	0.25
Salicylate 6	0.45	0.14
Salicylate 7 (tremulacin)	12.06	5.49
Salicylate 8	0.08	0.03
Salicylate 9	0.28	0.25
Sum of salicylates	43.9	20.27
Chlorogenic acid or derivative 1	3.64	4.60
Chlorogenic acid or derivative 2	0.00	0.00
Chlorogenic acid or derivative 3	0.27	0.26
Sum of chlorogenic acid or derivatives	3.92	4.87
Coumaroylquinic acid or derivative 1	0.23	0.16
Coumaroylquinic acid or derivative 2	0.36	0.25
Coumaroylquinic acid or derivative 3	0.52	0.64
Sum of coumaroylquinic acid or derivatives	1.11	1.05
Flavonoid glycoside 1	0.27	0.58
Flavonoid glycoside 2	0.03	0.03
Flavonoid glycoside 3	0.17	0.16
Flavonoid glycoside 4	0.04	0.07
Flavonoid glycoside 5	0.48	0.38
Flavonoid glycoside 6	0.44	1.09
Flavonoid glycoside 7	0.01	0.15
Flavonoid glycoside 8	0.24	1.08
Flavonoid glycoside 9	0.06	0.04
Sum of flavonoid glycosides	1.73	3.58
Total PAs	nd	72.35
Total phenolics	50.6	102.12

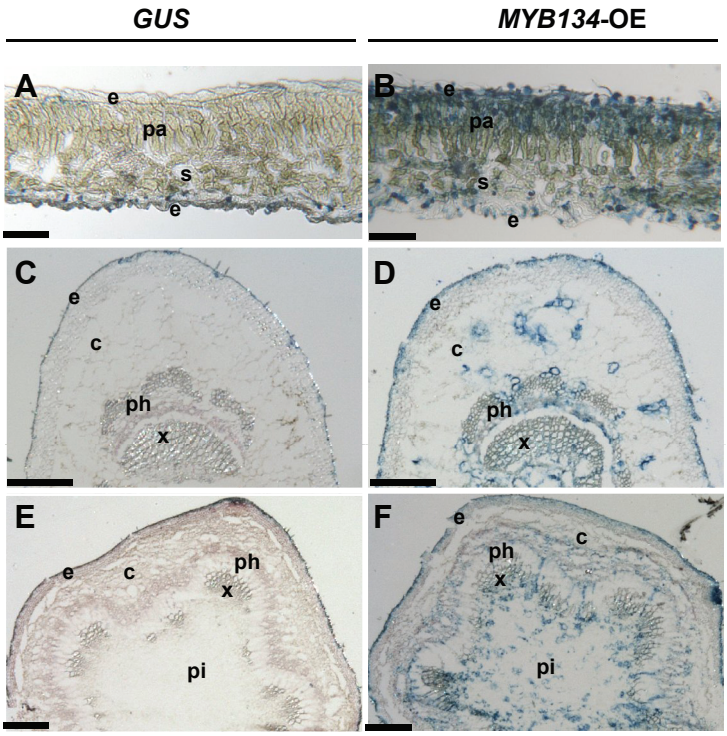


Figure 3-18. Localization of PAs in tissues of control and *MYB134*-overexpressor plants. Leaf (LPI 10), petiole (LPI 10), and stem (4th internode) cross sections stained with the PA-specific stain DMACA (blue color) (**A**, **C**, **E**: control leaf, petiole, stem, respectively; **B**, **D**, **F**: MYB134-overexpressor (353-38, MYB134-overexpressor line 4) leaf, petiole, stem, respectively). Scale bars = 200 μm (**A** and **B**) or 400 μm (**C**, **D**, **E**, and **F**). e, Epidermis; pa, palisade mesophyll; s, spongy mesophyll; c, cortex; ph, phloem; x, xylem; pi, pith. Results are representative of multiple independently transformed lines.

the adaxial and abaxial epidermal layers but in much greater concentration. DMACA staining was also very abundant in the upper layer of palisade mesophyll cells and sporadically within the spongy mesophyll (Fig. 3-18B). In petioles, staining was observed only in the epidermal cells in controls (Fig. 3-18C), while in *MYB134*-overexpressors, strong staining was observed in the epidermis with sporadic staining in the cortex and vascular tissues (Fig. 3-18D). In stem sections (4th internode), PAs were localized only to the epidermis of control plants (Fig. 3-18E), while staining was observed in the epidermis, cortex, xylem, and pith in the *MYB134*-overexpressors (Fig. 3-18F).

3.3.4 Overexpression of *MYB134* in transgenic poplar activates the PA biosynthetic pathway genes

In order to confirm that MYB134 activates genes of the PA biosynthetic pathway, phenylpropanoid and flavonoid structural gene expression was profiled in leaves of control and *MYB134*-overexpressing plants. In both the 353-38 and 717-1-B4 clones, *MYB134* overexpression was found to activate the entire phenylpropanoid pathway leading to PA production, including the flavonoid-specific general phenylpropanoid genes *PAL1* and *4CL2* (Section 1.5.4) (Fig. 3-19). Both early (*CHS1*, *CHI1*, and *F3H*) and late (*DFR1*, *ANR1*, *ANS1*, and *LAR1*) flavonoid biosynthetic genes were more highly expressed in the *MYB134*-overexpressing plants, but the level of late biosynthetic gene up-regulation was greater (Fig. 3-19). Consistent with the hypothesis that MYB134 specifically regulates PA metabolism and does not directly regulate other flavonoid pathways, the light stress- and UV-B-induced *FLS4* gene was not up-regulated by *MYB134* overexpression. Rather, *FLS4* was expressed in leaves of both control and *MYB134*-overexpressor plants at similarly low levels (Fig 3-19). The expression of *PAL2* and *cinnamoyl CoA-reductase 1* (*CCR1),* genes known to be involved in lignin production (Kao et al., 2002; Li et al., 2005), was likewise not altered in leaves of *MYB134*-overexpressors (Fig 3-19). Consistent with the suppression of PA pathway activation by a high number of T-DNA insertions in 353-38 *MYB134*-overexpressor line

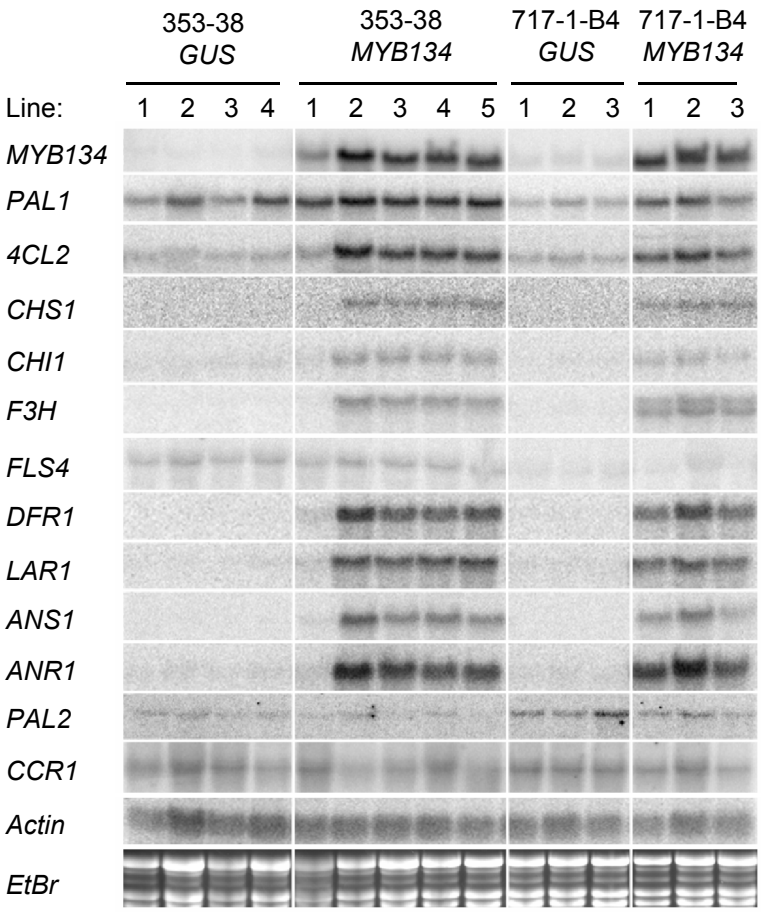


Figure 3-19. Northern analysis showing expression of phenylpropanoid and flavonoid structural genes in leaves of independently transformed *GUS* and *MYB134*-overexpressor 353-38 and 717-1-B4 hybrid poplar clones. See Fig. 1-1 legend for gene names. *CCR1*, cinnamoyl-CoA reductase 1; EtBr, ethidium bromide stained total RNA. CDS sequences used for probe synthesis, corresponding to the *P. trichocarpa* Nisqually1 gene models or GenBank accession numbers listed in Table A1-1.

1, northern analysis revealed a correspondingly lower level of transgene expression in this line (Fig. 3-19).

The possibility that MYB134 acts indirectly by activating expression of one of the other stress-responsive TT2-Related Subclade *MYB* genes, *MYB097* and *MYB183*, was investigated by analyzing the expression of these genes in the *MYB134*-overexpressor plants. Neither *MYB097* nor *MYB183* were up-regulated in *MYB134*-overexpressing plants (Fig. 3-20). To test whether these two related *MYB* genes could activate the PA biosynthetic pathway like *MYB134*, they were overexpressed under the control of the maize ubiquitin promoter in 353-38 and 717-1-B4 plants using *A. tumefaciens*- mediated transformation. Confirmed transgenic lines were grown under the same greenhouse conditions as the *MYB134*-overexpressing trees and PA levels were analyzed. Constitutive expression of neither *MYB097* nor *MYB183* resulted in altered accumulation of PAs (Fig. 3-21). Additionally, extensive HPLC profiling of phenolic metabolites showed no significant differences to flavonoids or other phenolics attributable to *MYB183*- or *MYB097*-overexpression (data not shown). Consistent with these data, expression of the PA marker genes *4CL2*, *DFR1*, and *ANR1* was not altered in *MYB097*- or *MYB183*-overexpressing plants (Fig. 3-22).

3.3.5 PA pathway promoter sequences contain putative MYB-binding sites

Activation of PA pathway genes by constitutive *MYB134* overexpression in transgenic poplar plants suggests that the MYB134 protein functions as a direct regulator of PA pathway genes. However, it is possible that this regulation is indirect, perhaps mediated by as yet unidentified factors acting downstream of MYB134. In order to determine whether the poplar flavonoid pathway genes contain putative MYB and BHLH binding sites, as has been found for numerous other species (Section 1.5.6), sequences upstream of the flavonoid biosynthetic genes were analyzed (Table 3-4). Fortunately, the *P. trichocarpa* clone Nisqually 1, for which a complete genome sequence is available (Tuskan et al., 2004), responds to wounding and UV-B irradiation with a rapid activation

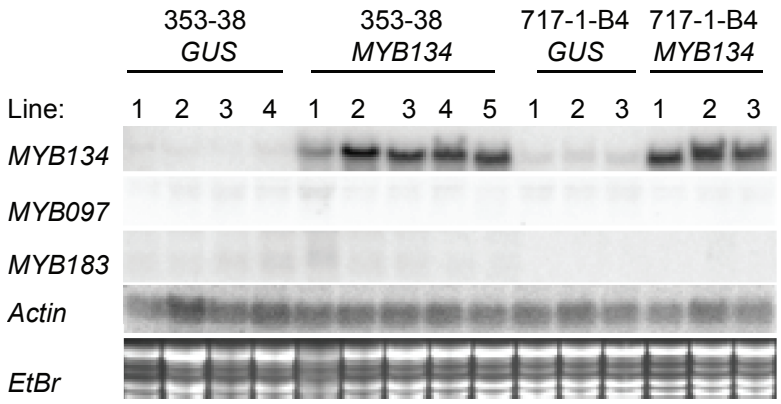


Figure 3-20. Expression of *MYB183* and *MYB097* is not altered in *MYB134*-overexpressing poplar leaves. Northern analysis of *MYB183* and *MYB097* expression in leaves of independently transformed *GUS*- and *MYB134*-overexpressor 353-38 and 717-1-B4 plants.

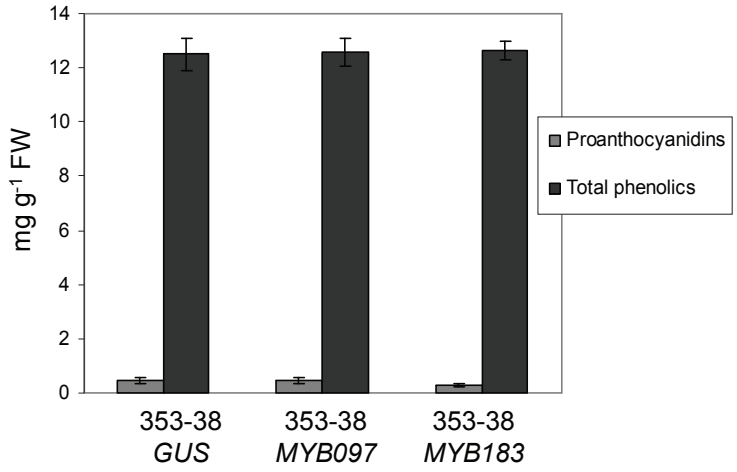


Figure 3-21. Overexpression of *MYB097* or *MYB183* under the control of the maize ubiquitin promoter does not alter total PA or soluble phenolic concentrations in transgenic 353-38 poplar leaves. For *GUS* controls, 3 independently transformed lines analyzed, with at least 6 trees per line. For *MYB097*-overexpressors, 10 independently transformed lines analyzed, with at least 3 trees per line. For *MYB183*-overexpressors, 6 independently transformed lines analyzed, with at least 4 trees per line. No significant difference seen between lines of either MYB-overexpressors and controls.

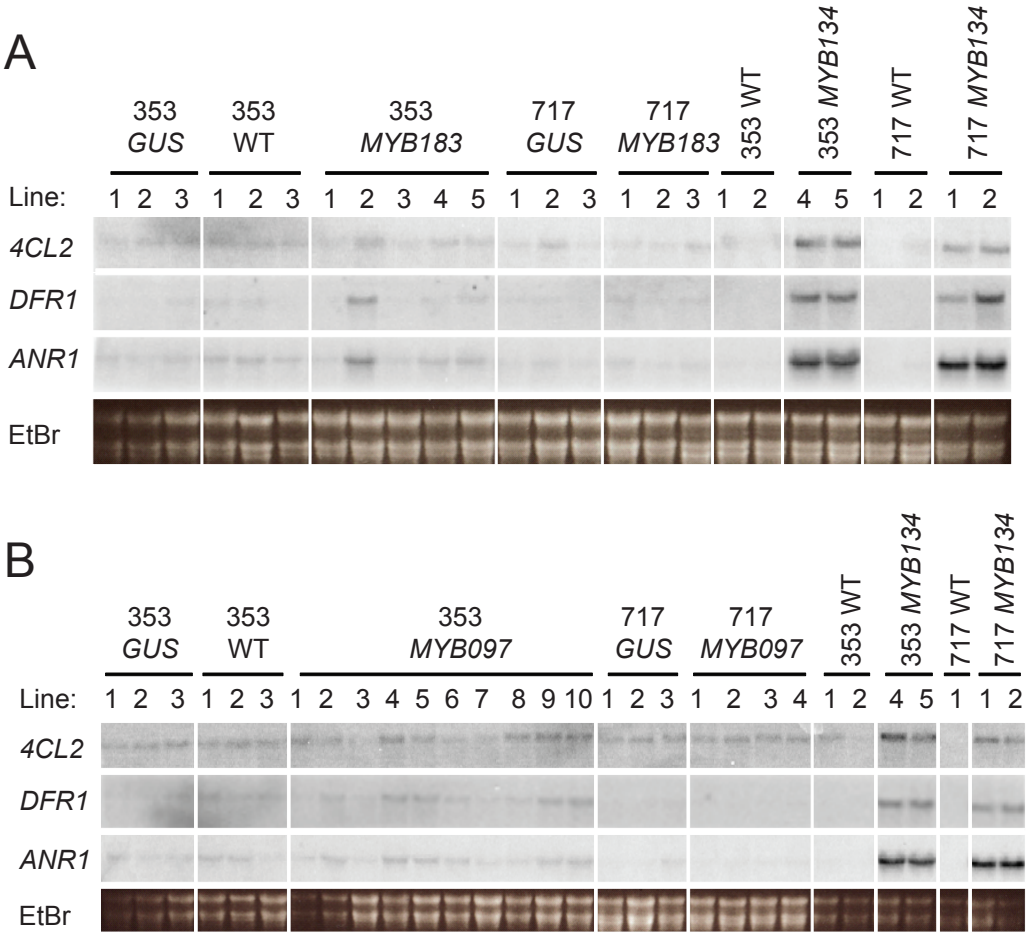


Figure 3-22. PA pathway gene expression is not affected by *MYB183*- or *MYB097*-overexpressing poplar. Expression of marker genes for early (*4CL2*) and late (*DFR1*, *ANR1*) PA pathway monitored using northern blot analysis in 353-38 (353) and 717-1B4 (717) clones transformed with Ub::MYB183 (A) and Ub::MYB097 (B) constructs, or 70S::GUS and WT controls. RNA from *MYB134*-overexpressor and corresponding control leaves probed as controls.

of PA metabolism (Section 2.3.3). As such, it is likely that the promoter sequences of the stress-induced PA pathway genes from Nisqually 1 contain binding sites for the transcription factors mediating this up-regulation, facilitating promoter analysis and identification of putative binding sites for transcription factors that mediate stress-induced up-regulation of the pathway.

For each gene shown in Table 3-4, the regions upstream of the transcription start site for 2 kb beyond 5' UTR was analyzed with the PLACE Plant *Cis*-Element Database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) using the SIGNAL SCAN program (Prestridge, 1991; Higo et al., 1998). This search revealed that the majority of these promoter sequences contain sequences matching the consensus of the c-Myb protein ('MYBCORE', consensus CNGTTR) and AC element-like sequences, including the L Box (consensus ACCWWCC), the P Box ('MYBPLANT', consensus MACCWACC), and the maize P MYB binding site ('MYBPZM', consensus CCWACC) (Section 1.5.3) (Tables 3-4 and A1-5). Putative BHLH binding sites were also numerous in flavonoid pathway genes, including the E Box ('MYCCONSENSUS', consensus CANNTG) and more specifically the G Box (CACGTG) (Tables 3-4 and A1-5). The positioning, orientation and precise sequences of each putative MYB and BHLH binding site was mapped onto sequence files for each promoter sequence, and the element sequences are reported in Table A1-5. These data suggest that regulation by MYB and BHLH proteins is likely important in mediating the activation of different branches of flavonoid metabolism in poplar. In order to better understand how MYB134 may function to regulate the PA branch pathway, the promoters of the PA pathway-specific genes *ANR1* and *ANR2* were compared to the arabidopsis *BAN* (*ANR*) promoter, which is known to mediate activation by TT2 (Fig. 3-23).

Table 3-4. MYB and BHLH domain protein consensus binding sites in 2 kb promoter regions of selected phenylpropanoid and flavonoid genes. Promoter regions from Nisqually 1 genome sequence (Tuskan et al. 2004) analyzed using the PLACE plant cis-element database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) with the SIGNAL SCAN program (Prestridge, 1991; Higo et al., 1998). Annotated elements include, bHLH consensus sites (MYCCONSENSUSAT, CANNTG), c-Myb consensus sites (MYBCORE, consensus CNGTTR) and AC elements and element-like sequences including MYBZMP (consensus CCWACC), L Box (consensus ACCWWCC), and P Box (MYBPLANT, consensus MACCWAMC).

	MYB				BHLH	
Gene	CNGTTR	L Box (ACCWWCC)	P Box (MACCWAMC)	MYBPZM (CCWACC)	G Box (CACGTG)	E Box (CANNTG)
PAL1	✓	✓	✓	✓	✓	✓
PAL2	✓	✓	✓	✓	✓	✓
PAL3	✓	✓	✓	✓	×	✓
4CL1	✓	✓	✓	✓	✓	✓
4CL2	✓	✓	✓	✓	×	✓
C4H2	✓	✓	✓	✓	×	✓
CHS1	✓	✓	✓	✓	✓	✓
CHS2	✓	✓	✓	✓	✓	✓
CHS3	✓	✓	✓	✓	✓	✓
CHS4	✓	✓	✓	✓	✓	✓
CHS5	✓	×	×	×	✓	✓
CHS6	✓	✓	✓	×	✓	✓
CHI1	✓	✓	✓	✓	✓	✓
F3H	✓	✓	✓	✓	×	✓
F3'H	✓	✓	✓	✓	✓	✓
F3'5'H1	✓	✓	×	✓	✓	✓
FLS1	✓	✓	✓	✓	×	✓
FLS2	✓	×	×	×	✓	✓
FLS3	✓	✓	✓	✓	✓	✓
FLS4	×	✓	×	✓	×	✓
DFR1	✓	✓	×	✓	×	✓
DFR2	✓	✓	✓	✓	✓	✓
ANR1	✓	✓	✓	✓	✓	✓
ANR2	✓	✓	✓	✓	×	✓
LAR1	✓	✓	✓	✓	×	✓
LAR2	✓	✓	✓	✓	×	✓
LAR3	✓	✓	✓	×	×	✓
ANS1	✓	×	×	×	✓	✓
ANS2	✓	×	×	✓	✓	✓

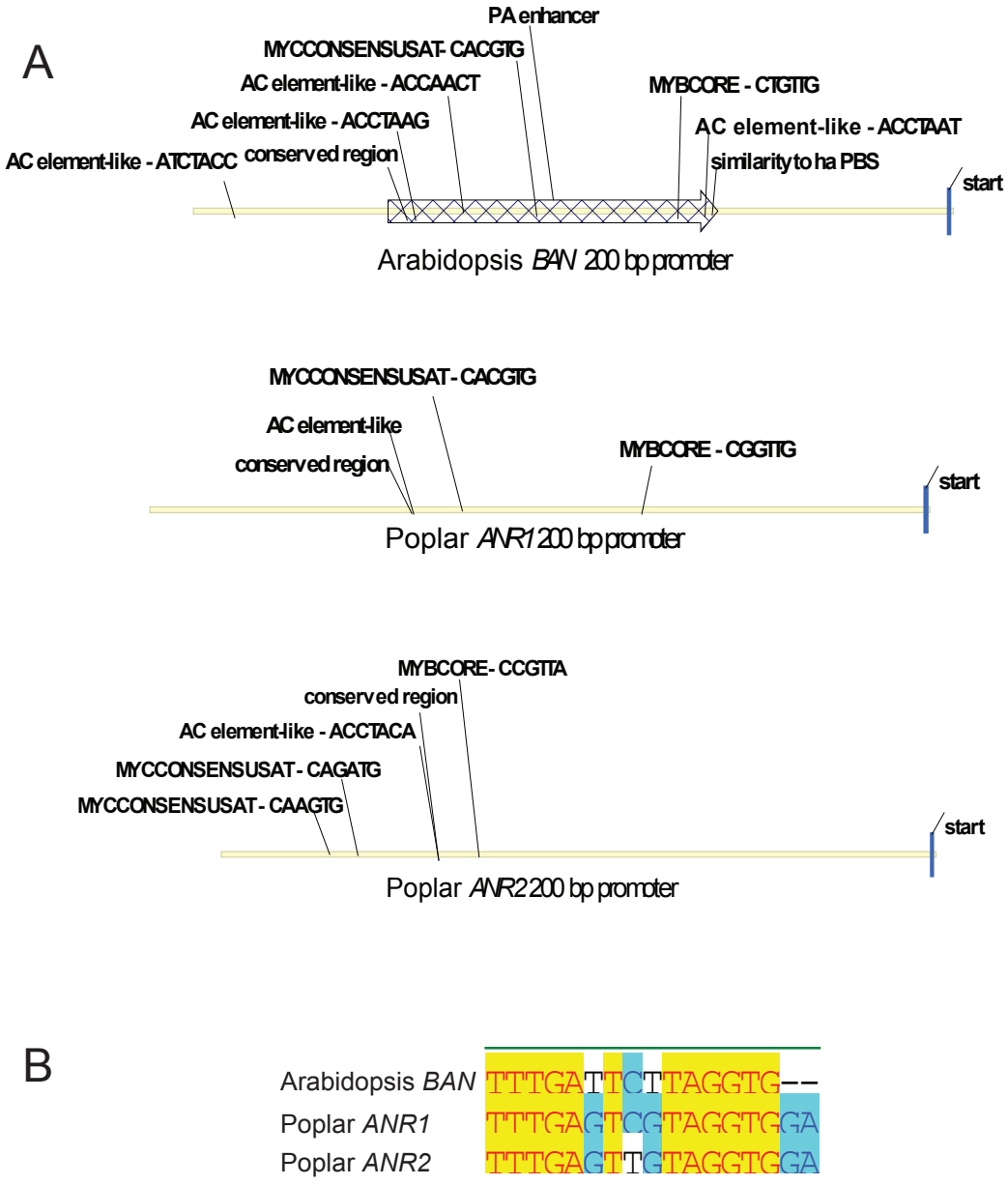
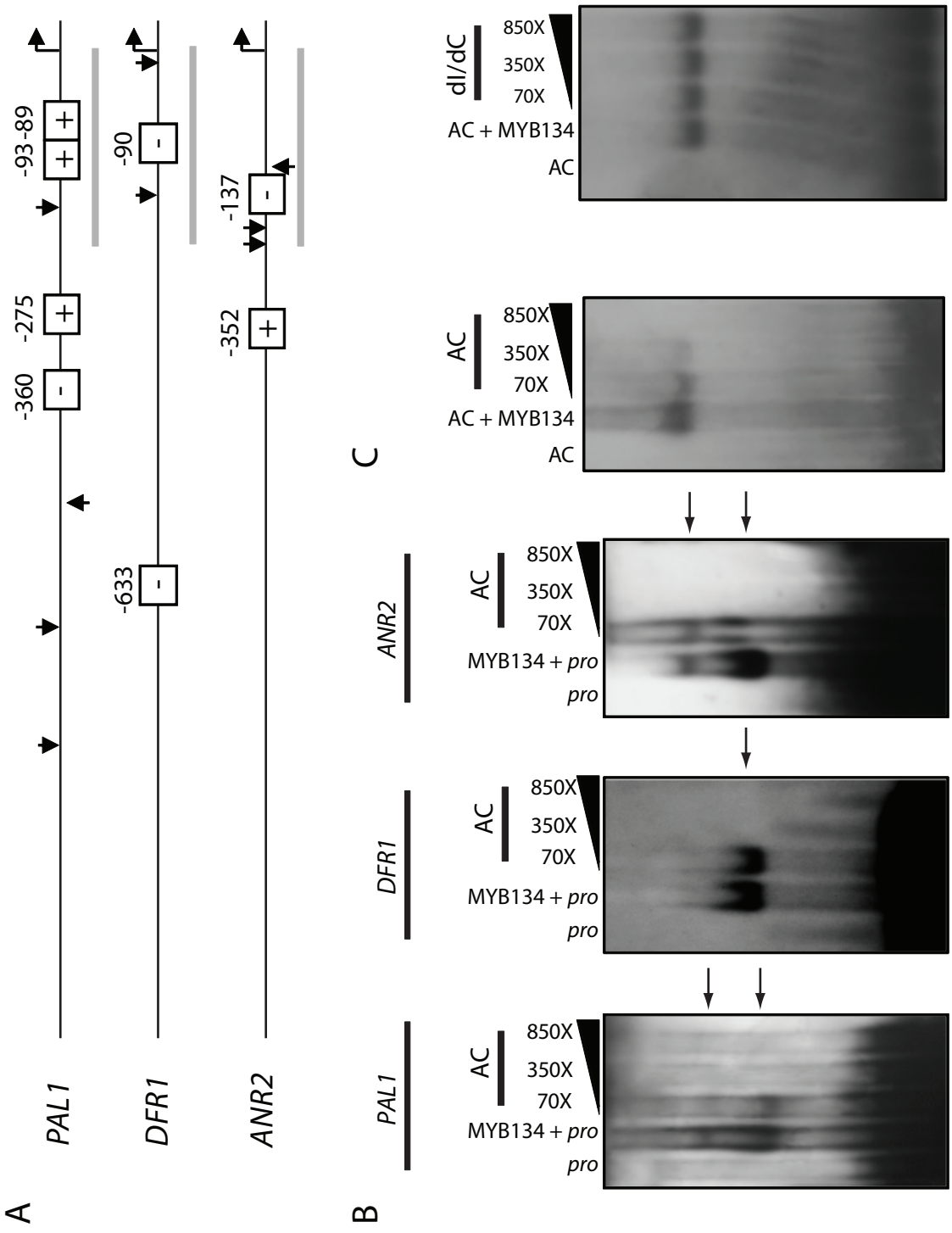


Figure 3-23. Comparison of the -200 bp promoter regions of the arabidopsis and poplar *ANR* genes. **A**. The minimal arabidopsis *BAN* (*ANR*) promoter defined by Debeaujon et al. (2003), termed the PA enhancer, contains possible MYB and BHLH binding sites, including a c-Myb consensus site (`MYBCORE`, CTGTTG), several AC element-like sequences, and a putative BHLH binding site (`MYCCONSENSUS`, CACGTG). These are compared to the poplar *ANR1* and *ANR2* -200 bp promoter sequences, which contain similar c-Myb consensus sequences, putative BHLH binding sites, and AC element-like sequences. **B**. Conserved region in poplar and arabidopsis ANR promoters identified by multiple sequence alignment. Alignment and visual inspection of the -200 bp promoter sequences shown in **A** revealed the presence of a conserved region that contains an AC element-like sequence (reversed consensus CACCTA[C/A]A[C/A]TCAAA).

As described in Section 1.5.3, the region of the promoter of the arabidopsis *BAN* gene required for TT2-mediated expression was identified by deletion analysis and termed the "PA enhancer" by Debeaujon et al. (2003). This sequence region contains possible MYB and BHLH binding sites, including a c-Myb consensus site, several AC element-like sequences, and a G Box BHLH binding site. TT2 was shown to activate expression of a reporter gene fused to this minimal *BAN* promoter, although the precise sequence(s) within the PA enhancer bound by TT2 was not defined. Comparison of 200 bp upstream of the arabidopsis *BAN* translational start site, a region containing the PA enhancer, with the corresponding regions of the poplar *ANR1* and *ANR2* genes revealed the presence of similar sequence motifs within all three sequences. The -200 bp promoter regions of the poplar *ANR* genes each contain a c-Myb consensus site, at least one BHLH consensus sites, and an AC element-like sequence (Fig. 3-23A). Alignment of these sequences revealed the presence of a larger region shared by all three sequences containing within it an AC element-like sequence (CACCTA[C/A]A[C/A]TCAAA) (Fig. 3-23A and B).

To determine whether MYB134 can directly bind to putative target gene promoters containing AC element-like sequences, three 180 bp promoter fragments containing putative MYB binding sites and corresponding to putative MYB134 target genes were cloned for protein-DNA binding assays. These target genes were chosen to

Figure 3-24. MYB134 binds to the promoters of putative downstream target genes. **A**. Schematic representation of 1000 bp of 5′ non-coding sequence for three putative MYB134 downstream target genes. +/- indicate the orientation of AC element-like motifs relative to the sense coding strand; numbers indicate the position of these motifs relative to the putative transcriptional start. Arrowheads above each line indicate BHLH consensus sites (CANNTG) while arrowheads below indicate c-Myb consensus sites (CNGTTR). Light grey horizontal lines under the sequences correspond to the location of the DNA sequence used as the binding target in the electrophoretic mobility shift assay (EMSA) shown in (**B**). **B**. MYB134 binding to 5′ non-coding sequences of the three putative target genes as determined by EMSA. Recombinant MYB134 bound to all three 5′-non coding sequences, as determined by a gel shift of the probe (arrows), which could be outcompeted with increasing quantities of unlabelled DNA corresponding to an AC element motif (AC: 5′-attgttcttcctggggtgaccgtccACCTAACgctaaaagccgtcgcgggataagcctgtctg-3′). **C**. MYB134 binding to the AC element-containing sequence as determined by EMSA. Binding of recombinant MYB134 to radiolabelled AC can be outcompeted by cold competitor AC (left panel), but not by the non-specific competitor, poly-dIdC. EMSA assays conducted by M. B. Prouse and M. M. Campbell (University of Toronto).


A
PAL1
DFR1
ANR2
-93
-89
-275
-360
-90
-633
-137
-352
B
C
dI/dC
AC
AC + MYB134
MYB134 + pro
pro
850X
350X
70X

represent general phenylpropanoid/early PA metabolism (*PAL1*), late flavonoid metabolism (*DFR1*), and the PA-specific branch of flavonoid metabolism (*ANR2*). Candidate MYB134 binding sites in the regulatory regions of these genes were identified by visual examination of the upstream genomic sequence and comparison to characterized phenylpropanoid promoters as well as with a search of the PLACE database. The promoter regions of the target genes all contain AC element-like motifs (Fig. 3-24A). As mentioned above, the 180 bp *ANR2* promoter region analyzed also contains a motif matching the consensus sequence bound by the vertebrate c-Myb. Inspection of these representative promoter sequences also revealed the presence of BHLH consensus sites in close proximity to the putative MYB binding sites for all three sequences (Fig 3-24A). The upstream region of poplar *PAL1* contains two overlapping AC element sequences identical to the high affinity P binding site (ACCTACCAACC) identified in the maize *A1* (*DFR*) promoter sequence that is bound by maize MYB proteins C1 and P (Section 1.5.6).

Consistent with the hypothesis that PA biosynthetic pathway genes function as downstream targets of MYB134, electrophoretic mobility shift assays (EMSAs) revealed that recombinant MYB134 protein bound to these promoter fragments (Fig. 3-24B). Two shifted bands were observed for the *PAL1* and *ANR2* 180 bp probes, while only one was seen with the *DFR1* probe (Fig. 3-24B). It is possible that the MYB134 protein binds to both of the overlapping AC elements in the *PAL1* promoter, and both the AC element-like sequence and the c-Myb binding site in the *ANR2* promoter. A sequence containing a canonical AC element was an effective competitor and eliminated MYB134 binding (Fig. 3-24B), and recombinant MYB134 also bound to this element in a specific manner (Fig. 3-24C). Thus, MYB134 appears to bind to the gene regulatory regions of putative target genes in an AC element-dependent fashion.

In order to verify that there were no additional motifs common to the three 180 bp promoter fragments bound by MYB134 that were missed by visual scanning and SIGNAL SCAN search using the PLACE database, the three sequences were analyzed using the Constrained Search for Motifs in DNA Sequences (COSMO) program (Bembom et al., 2009). This program searches a set of unaligned DNA sequences for

A

Sequence	Site identified
DFR1	T G C G T G G T A G G C C G
PAL1	T T A C C T A C C A A C C T
ANR2	T G A G T T G T A G G T G G

B

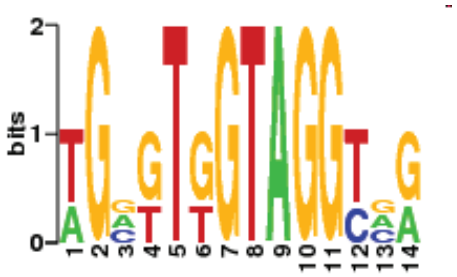


Figure 3-25. Identification of putative *cis*-elements common to poplar *DFR1*, *PAL1*, and *ANR2* 180 bp promoter fragments bound by recombinant MYB134 protein. An AC element-like motif was the only conserved motif identified using the Constrained Search for Motifs in DNA Sequences (COSMO) program in the three 180 bp promoter sequences analyzed. Individual sequences are shown in **A**, while the consensus sequence is shown in **B**.

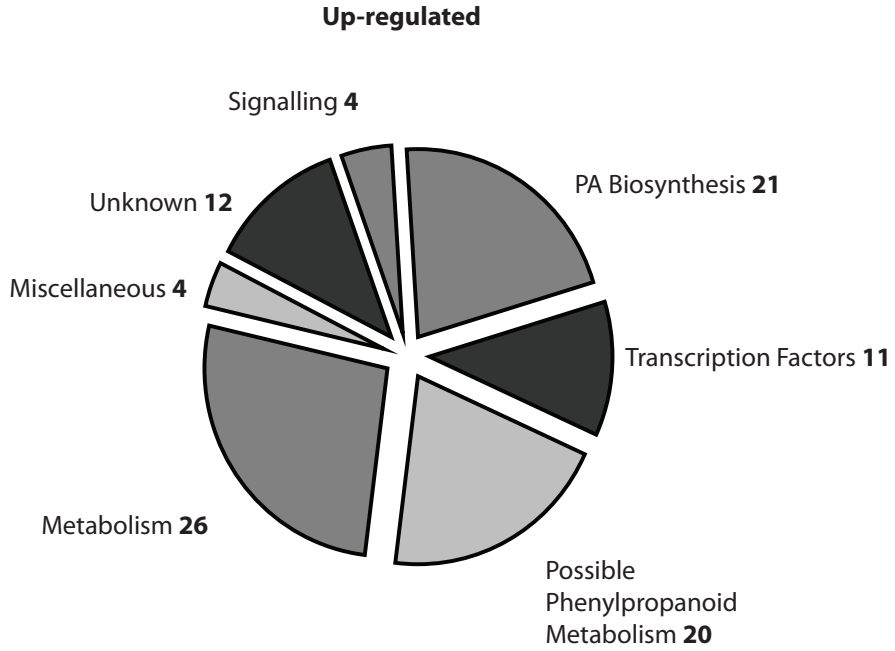
shared motifs that may represent transcription factor binding sites (Bembom et al., 2009). Only one conserved motif shared between the three sequences was identified by the COSMO program (Fig. 3-25A and B). This motif corresponds to the AC element-like sequences found in these three promoter fragments, and contains the core consensus [A/G]CCTAC[C/A] (Fig. 3-25B).

3.3.6 Global analysis of gene expression in *MYB134*-overexpressing poplar

3.2.6.1 Genes deregulated by constitutive expression of *MYB134* in Poplar

Overexpression of transcription factor genes followed by large scale transcriptome profiling is a useful strategy for identifying genes with novel biosynthetic or regulatory functions in flavonoid metabolism (Section 1.6.4). Overexpression of poplar MYB134 was shown using northern analysis to activate genes of the PA biosynthetic pathway, including general phenylpropanoid genes such as *PAL* and *4CL*, early flavonoid genes such as *CHS* and *F3H*, and PA-specific flavonoid genes such as *ANR* and *LAR* (Section 3.3.4). Several biosynthetic genes that are involved in other branches of phenylpropanoid metabolism, such as lignin and flavonol production, were not activated by *MYB134* overexpression, indicating that the transcription factor specifically activates the PA branch of flavonoid metabolism. In order to identify additional targets of MYB134 regulation, RNA samples from leaves of *MYB134*-overexpressing and control poplar plants were analyzed using microarray analysis with the Affymetrix GeneChip® Poplar Genome Array. One hundred and seventy-three probesets were differentially expressed between *MYB134*-ovexpressing and control plants with a twofold or greater difference in gene expression ($P \leq 0.01$) (Appendix A, Table A1-4). One hundred and thirty-eight probesets were up-regulated, representing 98 different gene models when cross-referenced with the annotated *P. trichocarpa* Nisqually 1 genome sequence (*P. trichocarpa* genome sequence v1.1 protein ID numbers will be used in the following data) (Tuskan et al., 2006). There were also 34 down-regulated probesets, corresponding to 26 different gene models. The *MYB134* transgene itself was

A



B

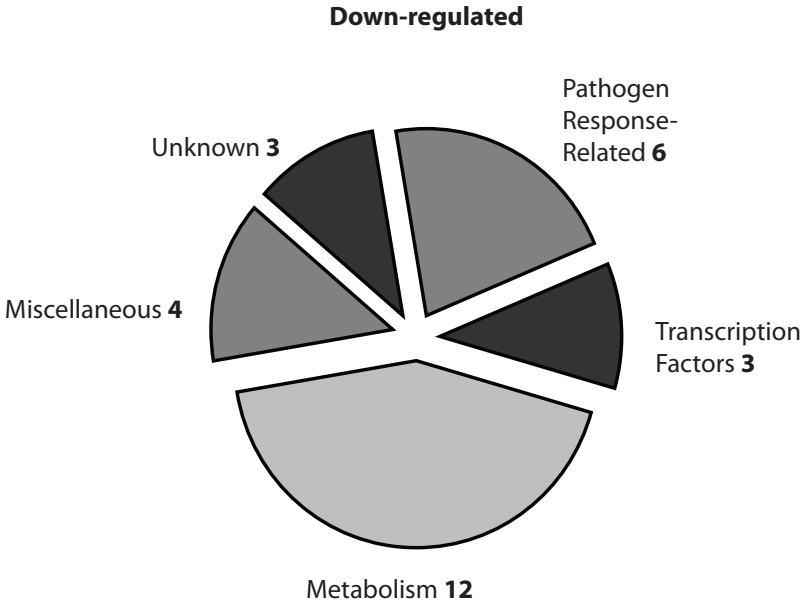


Figure 3-26. Categorization of genes up-regulated (**A**) and down-regulated (**B**) by overexpression of *MYB134*, determined using Affymetrix GeneChip® Poplar Genome Array analysis. Numbers of deregulated genes in each category given after category label.

found to be up-regulated 7.97-fold. For summary purposes, a rough categorization of up- and down-regulated genes is given in Fig. 3-26.

3.3.6.2 Overexpression of MYB134 results in a strong, specific activation of PA metabolism

Of the up-regulated genes, 21 correspond to known PA biosynthetic pathway genes, representing over one fifth of the genes induced by *MYB134* overexpression, with every PA pathway step with the exception of *PAL* represented by at least one gene family member (Table 3-5, Fig. 3-27). One of four *C4H* genes in poplar, *C4H2*, was up-regulated, as was *4CL4*, the closest homologue to the *P. tremuloides 4CL2* gene that is specifically involved in PA metabolism (Section 1.4.5), and one of 6 *4CL* genes in the *P. trichocarpa* Nisqually 1 genome. Four of the six *CHS* genes in the *P. trichocarpa* genome were up-regulated (Table 3-5). Poplar possesses only one gene encoding each of *CHI*, *F3H*, and *F3'H*, and all were up-regulated in *MYB134*-overexpressing poplar (Table 3-5). The *F3'5'H1* gene, one of two *F3'5'H* genes in poplar was also up-regulated (Table 3-5).

In general, the late PA pathway genes exhibited greater level of up-regulation in the *MYB134*-overexpressing plants than did the early flavonoid biosynthetic genes, with *C4H2*, *4CL2*, and *CHI*, but none of the later genes encoding PA biosynthetic enzymes, up-regulated less than 10-fold (Table 3-5, Figure 3-27). Both poplar *DFR* genes, *DFR1* and *DFR2*, were strongly up-regulated, as was *ANS2*, one of two *ANS* genes in poplar (Table 3-5). *ANR* and *LAR*, encoding the proteins that mediate entry of flavonoid metabolites into the PA branch pathway, are represented by two and three genes, respectively; all gene family members were strongly up-regulated (Table 3-5). The level of up-regulation of the *ANR1* and *ANR2* genes was similar (28-fold and 26-fold,

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Table 3-5. Significantly up-regulated probesets representing PA pathway and associated structural genes in *MYB134*-overexpresing poplar leaves, determined using Affymetrix GeneChip® Poplar Genome Array analysis. Fold change relative to wild type control are given.

Affymetrix probeset ID	Protein ID	TAIR (Blastx)	Annotation†	Fold change	P-value
Ptp.6632.1.S1_at	665925	AT2G30490.1	cinnamate 4-hydroxylase 2 (C4H2)*	3.04	1.11E-04
PtpAffx.12056.3.S1_a_at	665396	AT1G65060.1	4-coumarate-CoA ligase 4 (4CL4)*	3.40	2.30E-07
Ptp.6711.1.S1_s_at	572875	AT5G13930.1	naringenin-chalcone synthase 1 (CHS1)*	6.10	1.07E-05
PtpAffx.7896.3.S1_a_at	814871	AT5G13930.1	naringenin-chalcone synthase 2 (CHS2)*	26.52	1.09E-07
PtpAffx.7896.2.S1_at	554827	AT5G13930.1	naringenin-chalcone synthase 4 (CHS4)*	41.13	1.88E-11
PtpAffx.7896.4.A1_a_at	554829	AT5G13930.1	naringenin-chalcone synthase 6 (CHS6)*	93.77	3.53E-09
PtpAffx.4850.1.A1_s_at	724846	AT3G55120.1	chalcone isomerase 1 (CHI1)*	4.92	1.50E-07
Ptp.1512.1.S1_s_at	836090	AT5G05270.2	chalcone isomerase-like 1 (CHIL1)*	4.65	1.66E-08
Ptp.323.1.S1_s_at	836585	AT3G51240.1	flavanone 3-hydroxylase (F3H)*	14.08	2.11E-07
PtpAffx.142603.1.A1_s_at	823742	AT5G07990.1	flavonoid 3' hydroxylase (F3'H)*	27.06	1.41E-08
PtpAffx.83404.1.A1_at	804404	AT5G07990.1	flavonoid 3', 5' hydroxylase 1 (F3'H1)*	10.20	1.32E-08
PtpAffx.37082.1.A1_at	710083	AT5G42800.1	dihydroflavonol reductase 1 (DFR1)*	39.54	1.01E-09
PtpAffx.25553.1.A1_at	206010	AT5G42800.1	dihydroflavonol reductase 2 (DFR2)*	16.57	3.02E-08
Ptp.6057.1.S1_at	180639	AT4G22880.2	anthocyanidin synthase 2 (ANS2)*	30.75	6.57E-06
PtpAffx.5092.1.A1_at	831060	AT1G61720.1	anthocyanidin reductase 1 (ANR1)*	28.38	1.02E-10
PtpAffx.5092.2.S1_a_at	834000	AT1G61720.1	anthocyanidin reductase 2 (ANR2)*	26.08	6.18E-09
PtpAffx.6065.2.S1_at	656768	AT1G75280.1	leucoanthocyanidin reductase 1 (LAR1)*	38.84	5.78E-11
PtpAffx.6065.3.A1_a_at	658944	AT1G75290.1	leucoanthocyanidin reductase 2 (LAR2)*	20.61	2.03E-08
PtpAffx.18705.2.A1_a_at	835080	AT1G75290.1	leucoanthocyanidin reductase 3 (LAR3)*	60.60	2.60E-11
PtpAffx.224485.1.S1_s_at	761186	AT3G59030.1	TT12 (TRANSPARENT TESTA 12)**	23.98	1.60E-08
PtpAffx.94822.1.A1_at	816155	AT3G59030.1	TT12 (TRANSPARENT TESTA 12)**	6.80	9.86E-09
PtpAffx.211115.1.S1_at	242061	AT3G03620.1	MATE efflux family protein**	2.09	6.71E-06
Ptp.8030.1.S1_at	740558	AT5G48810.1	ATB5-B (Cytochrome b5 B)**	48.05	5.83E-09
PtpAffx.205491.1.S1_at	559146	AT1G36160.1	acetyl-CoA carboxylase 1 (ACC1)**	5.18	5.48E-09
PtpAffx.1150.1.A1_s_at	830215	AT1G36160.1	acetyl-CoA carboxylase 1 (ACC1)**	2.49	3.76E-05

† Manually annotated gene names from the *P. trichocarpa* Nisqually 1 genome sequence are indicated by (*), while TAIR blastx top hit annotations are indicated by (**).

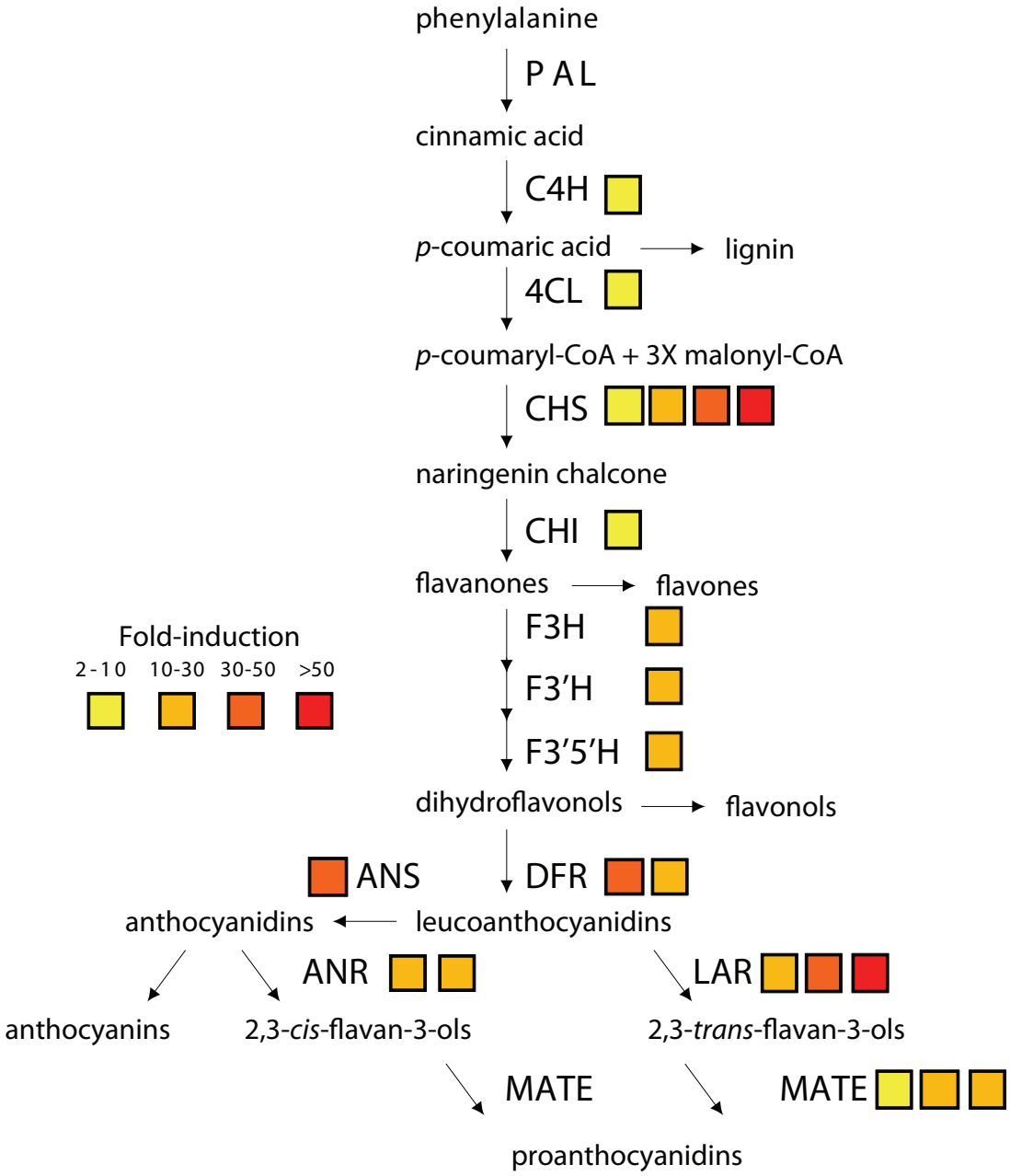

Figure 3-27. Activation of the flavonoid and PA pathway showing genes required for PA biosynthesis in *MYB134*-overexpressing poplar. Coloured boxes show fold-induction in *MYB134*-overexpressing poplar leaves over wild type controls, determined with Affymetrix GeneChip® Poplar Genome Array analysis. Where more than one gene family member is up-regulated, fold-change is shown for each member using a separate box.

respectively). Of the three *LAR* genes up-regulated by *MYB134*-overexpression, *LAR3* was more strongly induced (61-fold) than *LAR1* and *LAR2* (Table 3-5). In addition to the PA-specific genes *LAR* and *ANR*, there were also three up-regulated genes (761186, 816155, and 242061) encoding putative MATE transporters (Section 1.2.2). Of the three MATE transporter genes up-regulated in *MYB134*-overexpressing plants, 761186 and 816155 share 87% predicted amino acid sequence identity with each other and 72% identity with the arabidopsis TT12 protein, while 242061 is divergent (39% identity). 761186 and 816155 are the closest TT12 homologues in the *P. trichocarpa* genome, while 242061 is less closely related (Fig. 3-28). The former two are also the most strongly up-regulated of the three (24- and 6.8-fold, respectively), while 242061 is induced only 2.09-fold. No genes encoding functionally characterized proteins involved in any other branch of flavonoid or phenylpropanoid metabolism were up-regulated by ectopic expression of *MYB134* in poplar (Table A1-4).

In addition to the core PA pathway genes, several genes encoding proteins that are likely associated with PA production were also activated (Table 3-5). These include two genes encoding homologues of arabidopsis *acetyl-coenzyme A carboxylase 1* (ACC1; 559146 and 830215), which catalyzes the formation of malonyl-CoA (Section 1.2.1). Additionally, a *CHI*-like gene, *CHIL2* (836090), was up-regulated 6.45-fold, and a cytochrome b5 B gene (740558) with high similarity to the petunia *difF* and grapevine CytoB5 genes that are required for F3'H and F3'5'H activity (Section 1.2.1) was up-regulated up to 163-fold (Table 3-5).

3.3.6.3 Putative novel PA biosynthetic genes activated in *MYB134* overexpressing poplar

A number of genes with possible functions related to phenylpropanoid metabolism, but not known to be part of PA biosynthesis or any other phenylpropanoid pathway, were significantly up-regulated in leaves *MYB134*-overexpressing plants (Table 3-6). Eight glucosyltransferase genes were up-regulated by *MYB134*-overexpression (584786, 555562, 555559, 778252, 258388, 575952, and 590098) (Table 3-6). Also of interest are five genes that are annotated as *cinnamoyl-CoA reductase (CCR)-related*

Table 3-6. Significantly up-regulated probesets representing genes with possible functions related to PA or phenylpropanoid metabolism in *MYB134*-overexpressing poplar leaves, determined using Affymetrix GeneChip® Poplar Genome Array analysis. Fold change relative to wild type control are given.

Affymetrix probeset ID	Protein ID	TAIR (Blastx)	Annotation†	Fold change	P-value
PtpAffx.7740.2.A1_a_at	825925	AT2G23910.1	cinnamoyl-CoA reductase-related**	8.76	4.24E-08
PtpAffx.161181.1.S1_at	832029	AT4G30470.1	cinnamoyl-CoA reductase-related**	45.07	9.29E-09
PtpAffx.204062.1.S1_at	199038	AT5G14700.1	cinnamoyl-CoA reductase-related**	2.73	3.11E-06
PtpAffx.215434.1.S1_s_at	743671	AT5G14700.1	cinnamoyl-CoA reductase-related**	2.73	1.03E-04
PtpAffx.30128.1.S1_at	590025	AT2G23910.1	cinnamoyl-CoA reductase-related**	5.58	3.45E-06
PtpAffx.162989.1.S1_at	557092	AT5G17050.1	UDP-glucoronosyl/UDP-glucosyl transferase**	19.52	4.53E-09
Ptp.3138.2.A1_a_at	584786	AT5G17050.1	UDP-glucoronosyl/UDP-glucosyl transferase**	45.81	3.18E-09
PtpAffx.224381.1.S1_at	555562	AT3G16520.3	UDP-glucoronosyl/UDP-glucosyl transferase**	3.12	8.07E-06
PtpAffx.204049.1.S1_s_at	555559	AT3G16520.3	UDP-glucoronosyl/UDP-glucosyl**	2.23	1.14E-04
Ptp.4362.1.S1_at	778252	AT1G22360.2	UDP-glycosyltransferase **	2.54	2.14E-06
Ptp.5131.1.S1_at	258388	AT1G22360.1	UDP-glucoronosyl/UDP-glucosyl transferase**	2.34	4.80E-05
PtpAffx.157532.1.S1_at	575952	AT3G21760.1	UDP-glucoronosyl/UDP-glucosyl transferase**	2.22	3.96E-06
PtpAffx.220100.1.S1_at	590098	AT4G34135.1	UDP-glucosyltransferase UGT73B2**	2.53	2.68E-06
PtpAffx.225544.1.S1_s_at	576112	AT2G40890.1	CYP98A23*	3.33	5.95E-08
Ptp.5940.1.S1_at	591136	AT2G40890.1	CYP98A25*	2.62	8.22E-07
Ptp.4458.1.S1_s_at	835035	AT3G62760.1	glutathione S-transferase (class phi 13)**	7.81	8.94E-09
PtpAffx.160113.1.A1_s_at	708301	AT1G55320.1	ligase: acyl activating enzyme 18**	10.23	6.46E-08

† Manually annotated gene names from the *P. trichocarpa* Nisqually 1 genome sequence are indicated by (*), while TAIR blastx top hit annotations are indicated by (**).

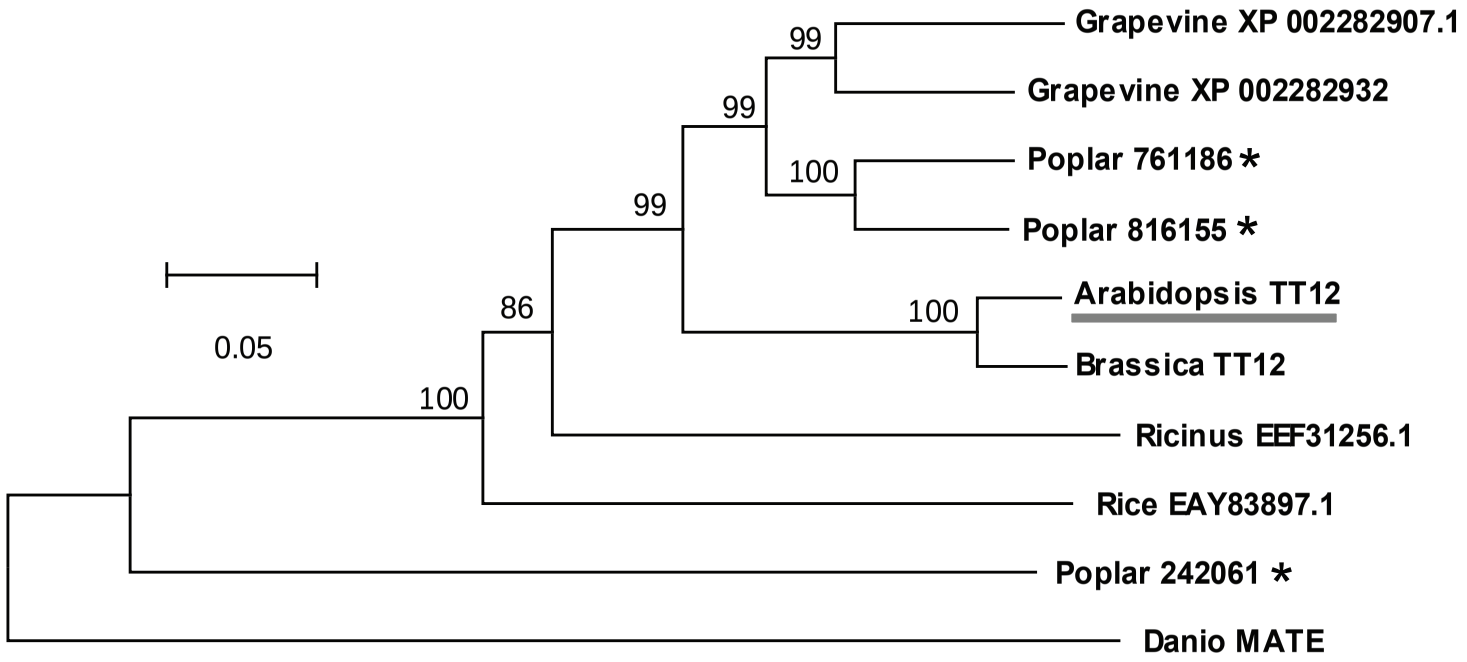

Figure 3-28. Phylogenetic tree showing relationship of poplar MATE proteins up-regulated in *MYB134*-overexpressing poplar, indicated by *, to arabidopsis TT12 and other MATE proteins. The arabidopsis TT12 protein is underlined in grey. Sequences of characterized and predicted MATE proteins aligned using ClustalW and NJ tree constructed using Mega 3.1 (Kumar et al., 2004) with p-distance model, 1000 bootstrap replicates. *Danio* MATE protein was used as an outgroup. *Populus trichocarpa* NIsqually 1 protein IDs given for the three MYB134-regulated poplar MATE genes, and GenBank accession numbers given for *Vitis*, *Oryza*, and *Ricinus* predicted proteins. Genbank accession nos. for the others as follows: *Brassica* TT12, ACJ36213; arabidopsis TT12, NP_191462; *Danio* MATE protein, A1L1P9.

(825925, 832029, 199038, 743671, and 590025). The proteins encoded by these genes are quite different from functionally characterized CCRs and are also distinct from the poplar *CCR-like* gene family (Fig. 3-29) (Hamberger et al., 2007). Several of the *MYB134*-activated *CCR-related* genes share high sequence similarity with grapevine *CCR-related* genes identified in a grape tissue expression profiling analysis as exhibiting differential expression between tissue types (Grimplet et al., 2007). Among these, two were also found by Terrier et al (2008) to be up-regulated by overexpression of the PA regulatory MYBs MYBPA1 and MYBPA2 (Section 1.6.4). Other up-regulated genes of interest include those encoding cytochrome P450 enzymes similar to cinnamate 3-hydroxylase (C3H) proteins, a Phi type GST, and an acyl activating enzyme (Table 3-6).

3.3.6.4 MYB134 overexpression up-regulates homologues of conserved PA regulatory genes

A number of genes were up-regulated by *MYB134*-overexpression that exhibit high sequence similarity to known regulators of PA metabolism in other species (Table 3-7). These include a gene (205424) encoding a BHLH protein with high similarity to arabidopsis TT8. 205424 is one of the two most closely related genes to arabidopsis TT8 in the *P. trichocarpa* genome (Fig. 3-30). Two genes encoding WDR proteins, 561472 and 576561, were up-regulated (5.22- and 4.62-fold, respectively) in the *MYB134*-overexpressing leaves (Table 3-7). However, these predicted proteins are not the most closely related poplar genes to arabidopsis *TTG1* (Fig. 3-31). Also among the putative

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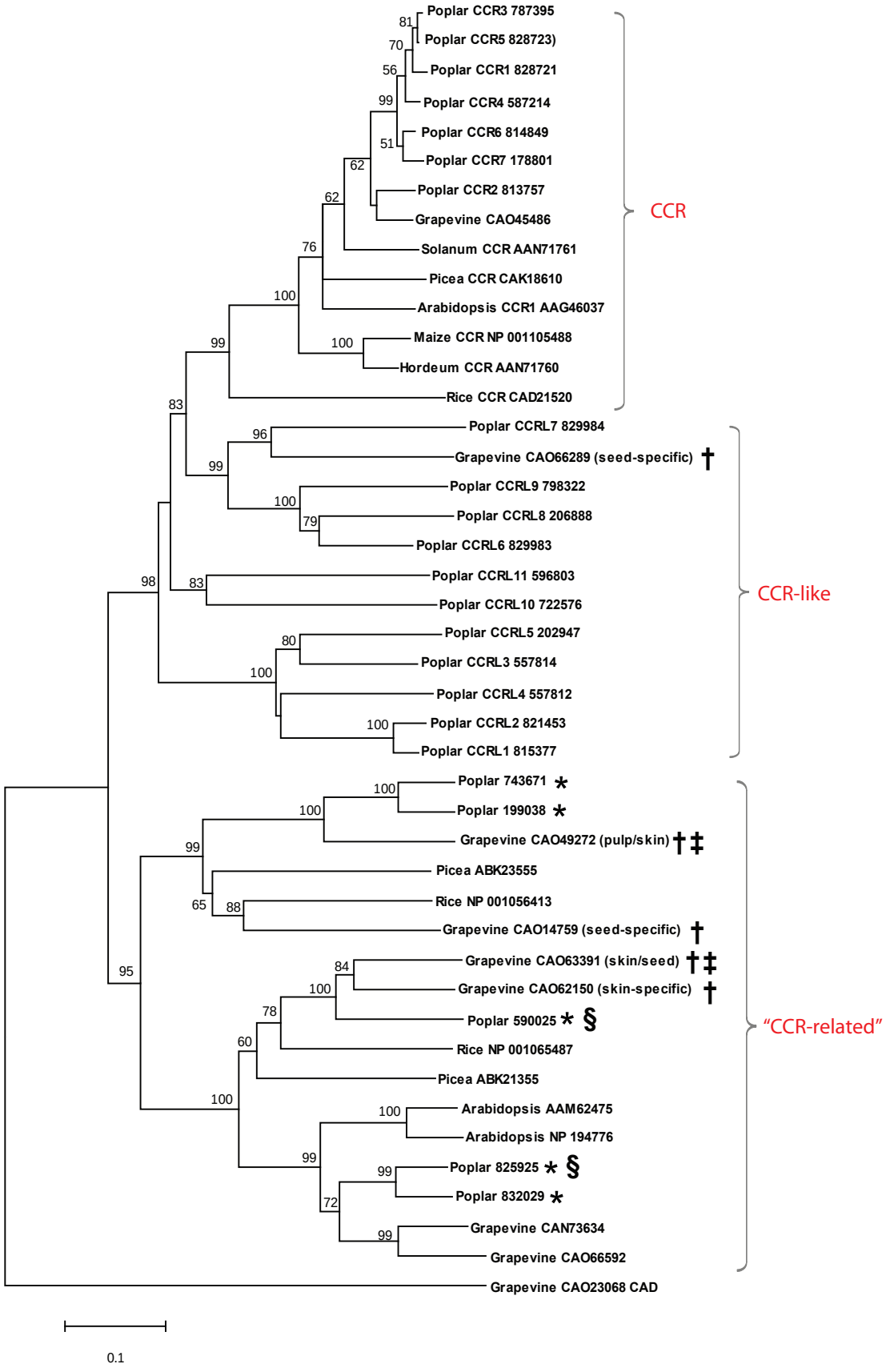
Figure 3-29. Phylogenetic tree of predicted CCR, CCR-like, and CCR-related proteins from poplar and a several other species. Poplar CCR-related genes up-regulated by *MYB134* overexpression are indicated by (*), while proteins encoded by grapevine CCR-related genes found to be expressed in specific grape berry tissue by Grimplet et al. (2007) indicated by (†). Two of the latter were also found to be up-regulated by overexpression of both MYBPA1 and MYBPA2 by Terrier et al. (2008) and these are indicated by (‡). Predicted proteins corresponding to genes co-activated with the PA pathway in poplar leaves in the analysis of Miranda et al. (2007) are indicated by (§). Sequences of characterized and predicted CCR, CCR-like, and CCR-related proteins aligned using ClustalW and NJ tree constructed using Mega 3.1 (Kumar et al., 2004) with p-distance model, 1000 bootstrap replicates. Grapevine CAD protein used as an outgroup. *P. trichocarpa* NIsqually 1 protein IDs shown for poplar proteins, and GenBank accession numbers shown for other species.


Poplar CCR3 787395
Poplar CCR5 828723)
Poplar CCR1 828721
Poplar CCR4 587214
Poplar CCR6 814849
Poplar CCR7 178801
Poplar CCR2 813757
Grapevine CAO45486
Solanum CCR AAN71761
Picea CCR CAK18610
Arabidopsis CCR1 AAG46037
Maize CCR NP 001105488
Hordeum CCR AAN71760
Rice CCR CAD21520
CCR
Poplar CCRL7 829984
Grapevine CAO66289 (seed-specific) †
Poplar CCRL9 798322
Poplar CCRL8 206888
Poplar CCRL6 829983
Poplar CCRL11 596803
Poplar CCRL10 722576
Poplar CCRL5 202947
Poplar CCRL3 557814
Poplar CCRL4 557812
Poplar CCRL2 821453
Poplar CCRL1 815377
CCR-like
Poplar 743671 *
Poplar 199038 *
Grapevine CAO49272 (pulp/skin) †‡
Picea ABK23555
Rice NP 001056413
Grapevine CAO14759 (seed-specific) †
Grapevine CAO63391 (skin/seed) †‡
Grapevine CAO62150 (skin-specific) †
Poplar 590025 * §
Rice NP 001065487
Picea ABK21355
Arabidopsis AAM62475
Arabidopsis NP 194776
Poplar 825925 * §
Poplar 832029 *
Grapevine CAN73634
Grapevine CAO66592
"CCR-related"
Grapevine CAO23068 CAD
0.1

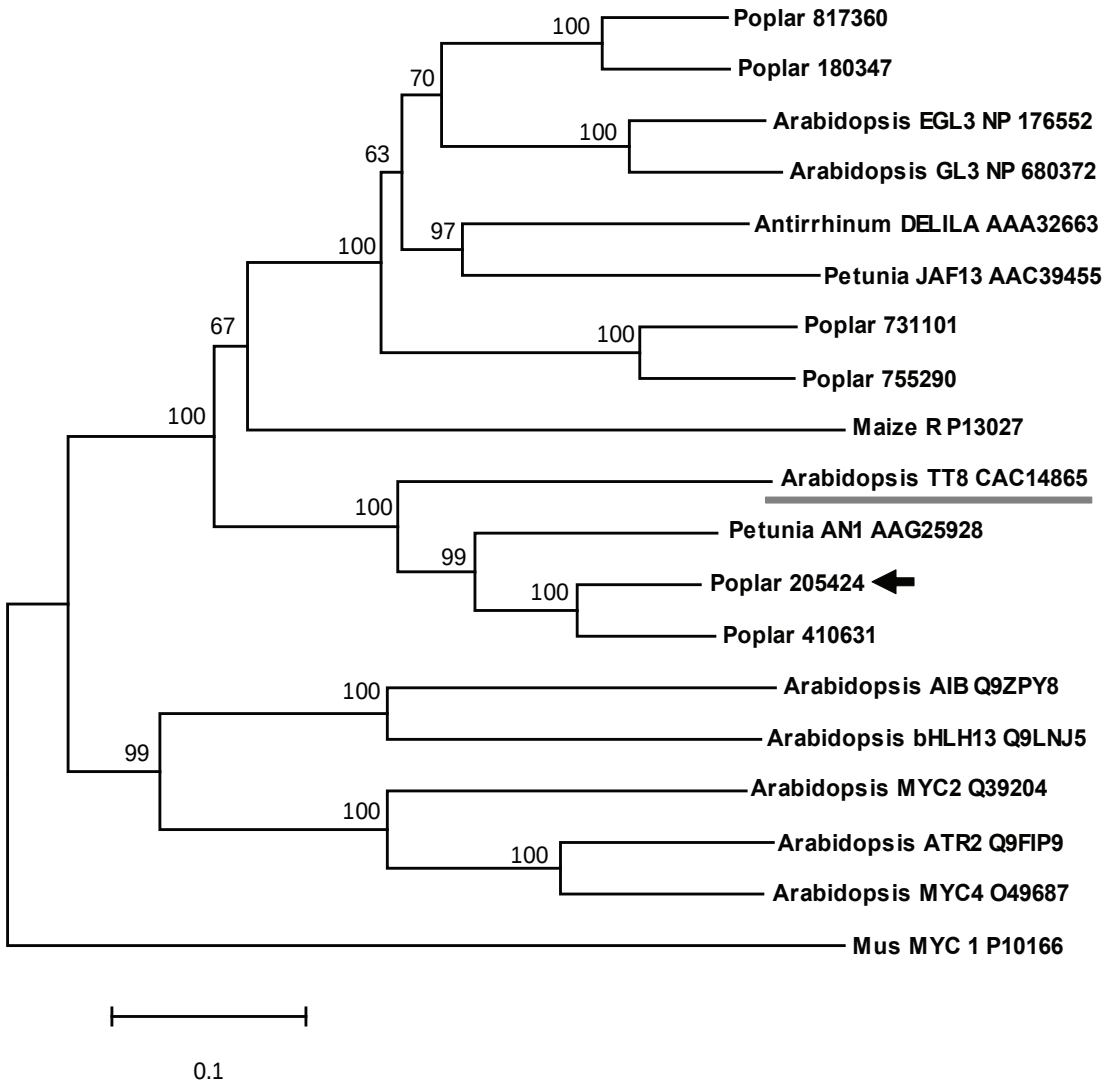


Figure 3-30. Phylogenetic tree showing relationship of poplar BHLH domain protein 205424 (arrow) to other BHLH proteins. Sequences of 205424 and BHLH domain proteins including arabidopsis TT8 (underlined in grey) and other predicted poplar BHLH proteins aligned using ClustalW and NJ tree constructed using Mega 3.1 (Kumar et al., 2004) with p-distance model, 1000 bootstrap replicates. Only bootstrap values higher than 50 are shown. *Mus* MYC1 included as an outgrtoup. *P. trichocarpa* Nisqually 1 protein IDs given for predicted poplar BHLH proteins. Genbank accession numbers given for the others.

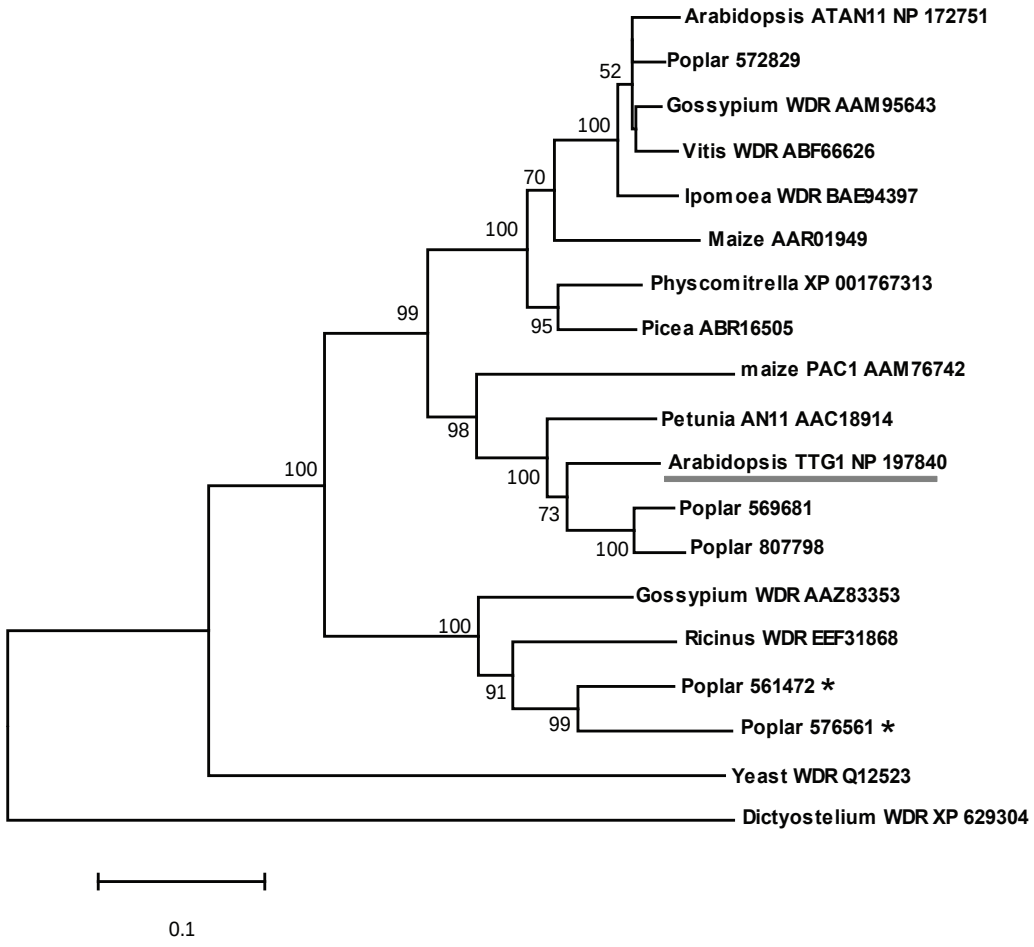


Figure 3-31. Phylogenetic tree of poplar WDR proteins up-regulated in *MYB134*-overexpressing poplar (indicated by (*)) and other WDR proteins. Sequences aligned using ClustalW and NJ tree constructed using Mega 3.1 (Kumar et al., 2004) with p-distance model, 1000 bootstrap replicates. Only bootstrap values higher than 50 are shown. *P. trichocarpa* Nisqually 1 protein IDs are given for predicted poplar WDR proteins and GenBank accession numbers are shown for known and predicted WDR proteins from other species. Arabidopsis TTG1 is underlined.

Table 3-7. Significantly up-regulated probesets representing transcription factor genes in *MYB134*-overexpresing poplar leaves, determined using Affymetrix GeneChip® Poplar Genome Array analysis. Fold change relative to wild type control are given.

Affymetrix probeset ID	Protein ID	TAIR (Blastx)	Annotation†	Fold change	P-value
PtpAffx.30659.1.A1_at	755418	AT3G13540.1	MYB115*	35.30	6.23E-12
PtpAffx.224878.1.S1_at	774521	AT4G09460.1	MYB201*	3.21	1.42E-06
PtpAffx.162546.1.A1_at	554534	AT2G47460.1	MYB153*	2.49	5.11E-05
PtpAffx.224602.1.S1_at	766132	AT1G22640.1	MYB194*	7.96	1.05E-08
PtpAffx.224650.1.S1_s_at	566512	AT4G38620.1	MYB165*	3.55	7.53E-08
PtpAffx.137746.1.S1_at	714142	AT1G22640.1	MYB182*	3.53	4.79E-08
PtpAffx.31264.2.S1_a_at	674550	AT5G53200.1	MYB179*	2.59	2.04E-05
PtpAffx.213439.1.S1_at	576561	AT1G12910.1	ANTHOCYANIN11**	4.62	2.00E-95
PtpAffx.127289.1.A1_at	561472	AT1G12910.1	ANTHOCYANIN11**	5.22	3.81E-07
PtpAffx.205684.1.S1_at	205424	AT4G09820.1	TRANSPARENT TESTA 8 (TT8)**	3.99	3.06E-08
Ptp.1110.1.A1_s_at	817110	AT1G79840.1	GLABRA 2 (GL2)**	2.09	4.95E-05

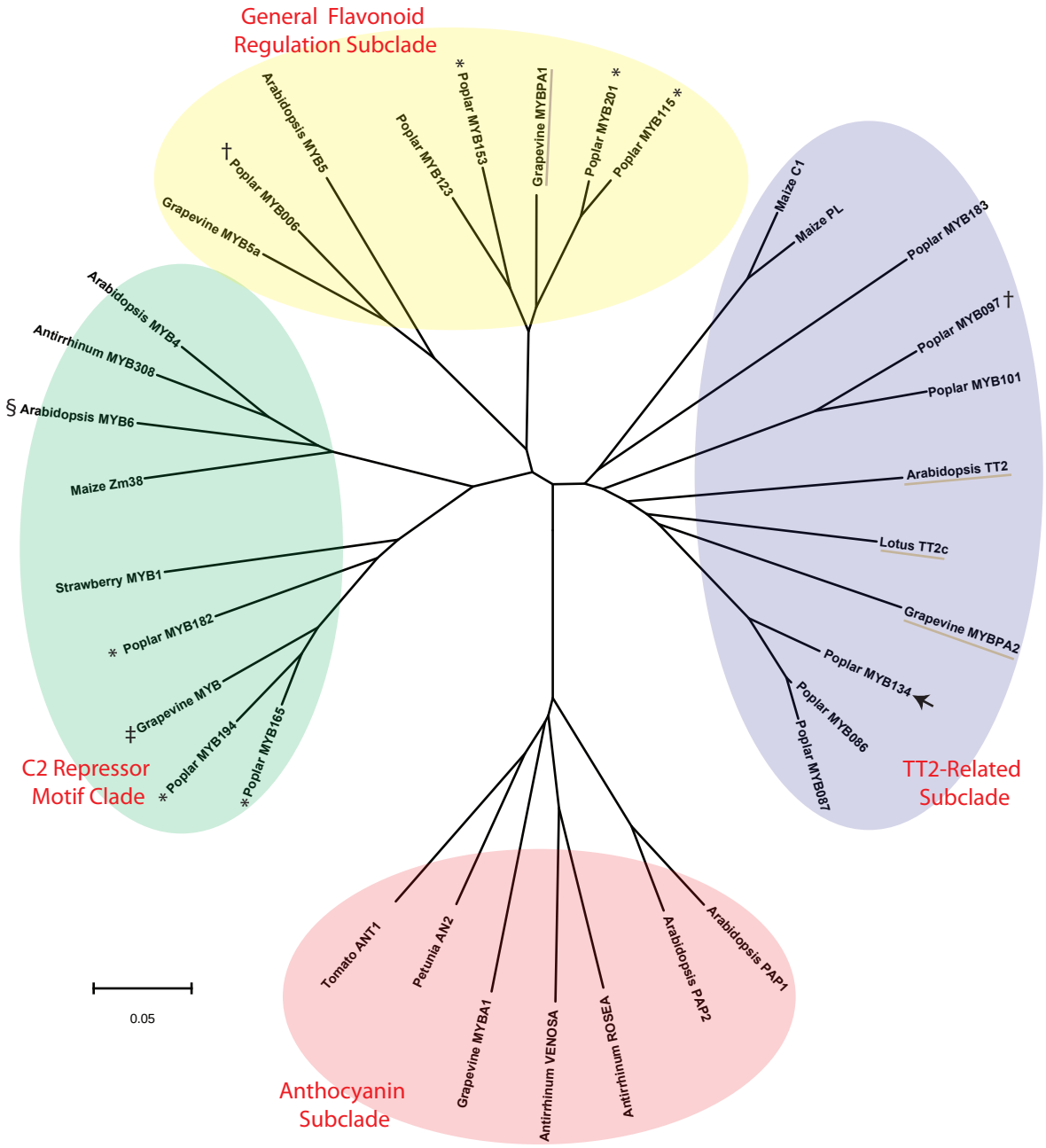
† Manually annotated gene names from the *P. trichocarpa* Nisqually 1 genome sequence are indicated by (*), while TAIR blastx top hit annotations are indicated by (**).

regulators of PA metabolism up-regulated in *MYB134*-overexpressing poplar were three genes, *MYB115* (755418), *MYB153* (554534), and *MYB201* (774521), that are members of the General Flavonoid Regulation Subclade and share sequence similarity to the grapevine PA regulator MYBPA1 (Table 3-7, Fig. 3-32).

3.3.6.5 Additional MYB genes up-regulated in *MYB134* overexpressing poplar leaves

A number of up-regulated probesets represent MYB genes that are not homologous to known activators of PA metabolism. Three up-regulated MYB genes, *MYB194* (766132), *MYB165* (566512), and *MYB182* (714142) (Table 3-7), are members of the C2 Repressor Motif Clade (Fig. 3-32). The predicted MYB194, MYB165, and MYB182 proteins share the C1- and C2-motifs characteristic of repressors of this subgroup. Strawberry FaMYB1 is the only characterized member of this group that specifically regulates flavonoid metabolism (Aharoni et al., 2001), and it is also the most closely related to the three MYB134-activated poplar C2 Repressor Motif Clade genes.

Figure 3-32. Phylogenetic tree showing relationships of MYB134-up-regulated poplar *R2R3 MYB* genes with selected known and putative flavonoid regulatory R2R3 MYB domain proteins and other species. Sequences aligned using ClustalW and NJ tree constructed using Mega 3.1 (Kumar et al., 2004) with p-distance model, 1000 bootstrap replicates. Clade and Subclade designations of Matus et al. (2008) are shown in red. Poplar MYB134 is indicated with the black arrow. Functionally characterized specific regulators of PA metabolism are underlined. Poplar genes up-regulated by MYB134-overexpression are indicated by (*), while those down-regulated by MYB134-overexpression are indicated by (†). The grapevine C2 Repressor Motif Clade MYB gene activated by ectopic expression of the grapevine PA regulators MYBPA1 and MYBPA2 (Terrier et al., 2009) is indicated by (‡), while arabidopsis MYB6, which is up-regulated during high light and low temperature-induced repression of the anthocyanin pathway in PAP1-overexpressing plants (Rowan et al., 2009) is indicated by (§). *P. trichocarpa* Nisqually 1 protein IDs (for poplar genes) or GenBank accession numbers (for others) are as follows: Arabidopsis PAP1 (Q9FE25); Arabidopsis PAP2 (Q9ZTC3); Grapevine MYBPA2 (ACK56131); Lotus TT2c (BAG12895); Poplar MYB134 (819461); Poplar MYB086 (668844); Poplar MYB087 (578002); Arabidopsis TT2 (Q9FJA2); Poplar MYB101 (586326); Poplar MYB097 (745315); Poplar MYB183 (717084); Maize PL (AAN12277); Maize C1 (P10290); Poplar MYB115 (410086); Poplar MYB201 (774521); Grapevine MYBPA1 (CAJ90831); Poplar MYB153 (554534); Poplar MYB123 (554533); Arabidopsis MYB5 (Q38850); Poplar MYB006 (639636); Grapevine MYB5a (AY555190); Arabidopsis MYB4 (Q9SZP1); Antirrhinum MYB308 (P81393); Arabidopsis MYB6 (Q38851); Maize Zm38 (P20025); Strawberry MYB1 (AAK84064); Poplar MYB182 (648122); Grapevine MYB (CAO42256);Poplar MYB194 (766132); Poplar MYB165 (566512); Tomato ANT1 (AAQ55181); Petunia AN2 (AAF66727); Grapevine MYBA1 (ABD72953); Antirrhinum VENOSA (ABB83828); Antirrhinum ROSEA (ABB83826).


General Flavonoid
Regulation Subclade
Arabidopsis MYB5
* Poplar MYB153
Poplar MYB123
Grapevine MYBPA1
Poplar MYB201 *
Poplar MYB115 *
† Poplar MYB006
Grapevine MYB5a
Maize C1
Maize PL
Poplar MYB183
Poplar MYB097 †
Poplar MYB101
Arabidopsis MYB4
Antirrhinum MYB308
§ Arabidopsis MYB6
Maize Zm38
Arabidopsis TT2
Lotus TT2c
Grapevine MYBPA2
Poplar MYB134
Poplar MYB086
Poplar MYB087
Strawberry MYB1
* Poplar MYB182
‡ Grapevine MYB
* Poplar MYB194
* Poplar MYB165
C2 Repressor
Motif Clade
TT2-Related
Subclade
Tomato ANT1
Petunia AN2
Grapevine MYBA1
Antirrhinum VENOSA
Antirrhinum ROSEA
Arabidopsis PAP2
Arabidopsis PAP1
0.05
Anthocyanin
Subclade

A single-repeat R3 MYB gene, *MYB179* (674550), was also up-regulated by overexpression of *MYB134* (Table 3-7). This gene is most similar to the arabidopsis R3 MYB gene TRY (74% amino acid identity), a member of the family of R3 proteins that negatively regulate the WER/GL1-GL3/EGL3-TTG1 interaction (Section 1.5.1). Also of interest is the up-regulation of a gene (817110) with high sequence similarity to arabidopsis *GL2*. This gene encodes a HD-Zip homeodomain protein involved in trichome cell fate and is a direct target of the MYB-BHLH-WDR complex composed of TTG1, the MYB protein GL1, and the BHLH proteins GL3 and EGL3, (Section 1.5.1).

3.3.6.6 MYB134 overexpression results in down-regulation of pathogen response-related genes and several flavonoid regulatory genes

Among the MYB134-down-regulated genes are a group of possible pathogen response-related genes including those encoding two chitinases (831333 and 233978), a thaumatin-like protein (180318), a PPO (276236), a dirigent-related protein (208234), and a pathogenesis-related 4 protein (PR4, 823674) (Table 3-8). Also of interest among the down-regulated genes are two MYB genes, *MYB097* (785462), *MYB006* (177847), that share sequence similarity with flavonoid regulatory MYB genes of the TT2-Related and General Flavonoid Regulation Subclades, respectively (Fig 3-32), as well as an R3 MYB, *MYB166* (568212), most similar to arabidopsis ETC1. Additionally, a UFGT gene (811849) sharing sequence similarity to characterized anthocyanidin 3-O-glucosyltransferases was down-regulated (Table 3-8).

3.3.6.7 Stress-responsive expression of MYB134-activated MATE and UFGT genes

Expression of two genes up-regulated in *MYB134*-overxpressing leaves, encoding putative MATE (816155) and UFGT (557092) proteins, was profiled in stress-treated poplar leaves (Figs. 3-34 and 3-35). Both genes were found to be co-activated with PA pathway genes following mechanical wounding, HL-exposure, and *M. medusae* infection

Table 3-8. Table 3-8. Selected significantly down-regulated probesets in *MYB134*-overexpresing poplar leaves, determined using Affymetrix GeneChip® Poplar Genome Array analysis. Fold change relative to wild type control are given.

Affymetrix probeset ID	Protein ID	TAIR (Blastx)	Annotation†	Fold change	P-value
Pathogen response-related					
PtpAffx.77318.2.S1_at	831333	AT3G12500.1	ATHCHIB (BASIC CHITINASE); chitinase**	0.17	6.13E-05
PtpAffx.249.47.A1_s_at	180318	AT4G11650.1	ATOSM34 (OSMOTIN 34)**	0.34	8.80E-05
Ptp.2463.1.S1_s_at	823674	AT3G04720.1	PR4 (PATHOGENESIS-RELATED 4)**	0.26	1.78E-04
PtpAffx.64603.1.S1_at	233978	AT5G24090.1	acidic endochitinase (CHIB1)**	0.42	8.66E-06
PtpAffx.25920.1.S1_a_at	208234	AT1G58170.1	disease resistance-responsive protein-related**	0.49	1.51E-04
Ptp.2155.1.S1_at	276236	AT1G04210.1	Polyphenol oxidase 276236*	0.35	7.67E-06
Regulatory					
PtpAffx.60092.1.S1_at	785462	AT2G01180.1	MYB097*	0.35	3.70E-05
Ptp.1229.1.S1_at	568212	AT1G01380.1	MYB166*	0.39	1.02E-05
PtpAffx.153725.1.A1_at	177847	AT3G13540.1	MYB006*	0.44	1.55E-04
Metabolism					
Ptp.4093.1.S1_at	665543	AT5G17540.1	transferase family protein **	0.27	1.09E-05
PtpAffx.6696.2.S1_at	571376	AT5G17540.1	transferase family protein**	0.28	1.37E-04
PtpAffx.67540.1.A1_s_at	811849	AT5G54010.1	glycosyltransferase family protein**	0.28	4.49E-07
PtpAffx.225012.1.S1_at	575957	AT1G07250.1	UDP-glucoronosyl/UDP-glucosyl transferase**	0.40	5.39E-05
Ptp.1094.1.S1_at	732175	AT3G59010.1	pectinesterase family protein**	0.42	1.45E-04
Ptp.1677.1.A1_at	596084	AT3G16850.1	glycoside hydrolase family 28**	0.46	1.01E-04
PtpAffx.2286.3.S1_a_at	569153	AT1G17180.1	Arabidopsis thaliana Glutathione S-transferase (class tau) 25**	0.43	7.81E-05
PtpAffx.249.26.A1_s_at	205001	AT1G71695.1	peroxidase 12 (PER12)**	0.46	1.19E-05
Ptp.4381.1.S1_s_at	645827	AT5G07990.1	CYP92A20v1*	0.34	3.49E-05
PtpAffx.216301.1.S1_s_at	582552	AT1G55740.1	ARABIDOPSIS THALIANA SEED IMBIBITION 1 (ATSIP1)**	0.49	9.00E-05
Ptp.4073.1.S1_s_at	824161	AT4G02290.1	hydrolase, hydrolyzing O-glycosyl compounds glycosyl hydrolase family 9 protein**	0.50	1.25E-04

† Manually annotated gene names from the *P. trichocarpa* Nisqually 1 genome sequence are indicated by (*), while TAIR blastx top hit annotations are indicated by (**).

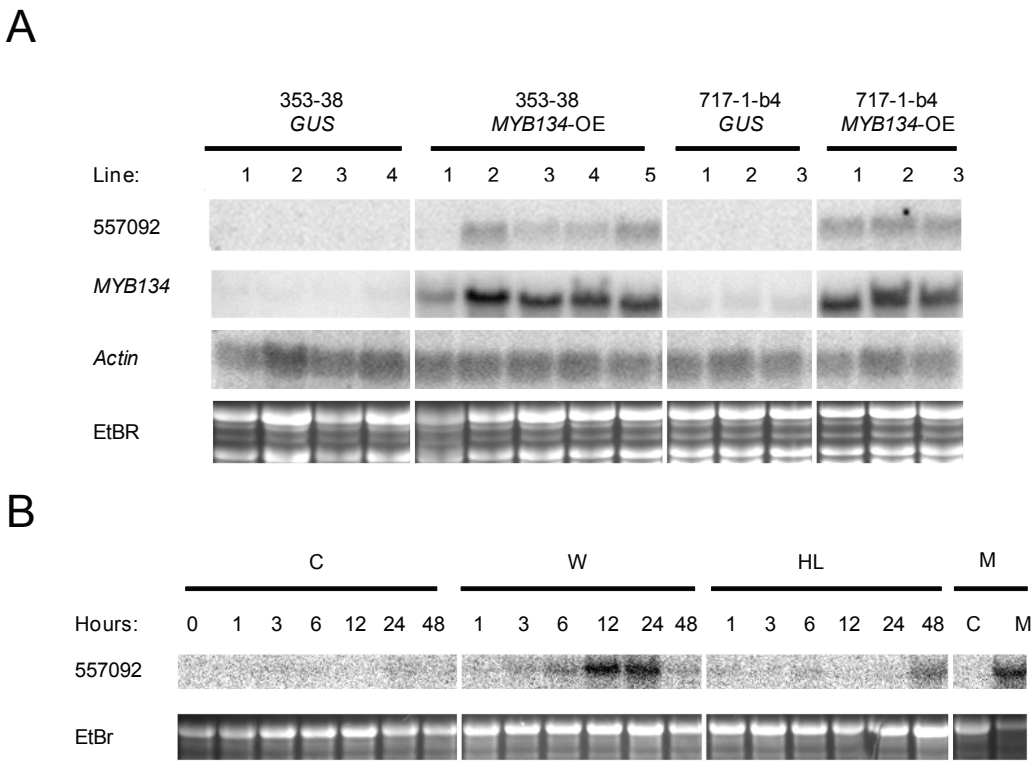


Figure 3-33. Northern analysis of the poplar 557092 (UFGT) gene. **A**. 557092 expression in control and *MYB134*-overexpressing poplar. **B**. 557092 expression in control (C), wounded (W), high light (HL)-exposed, and *M. medusae*-infected (M) poplar. EtBr, ethidium bromide stained total RNA.

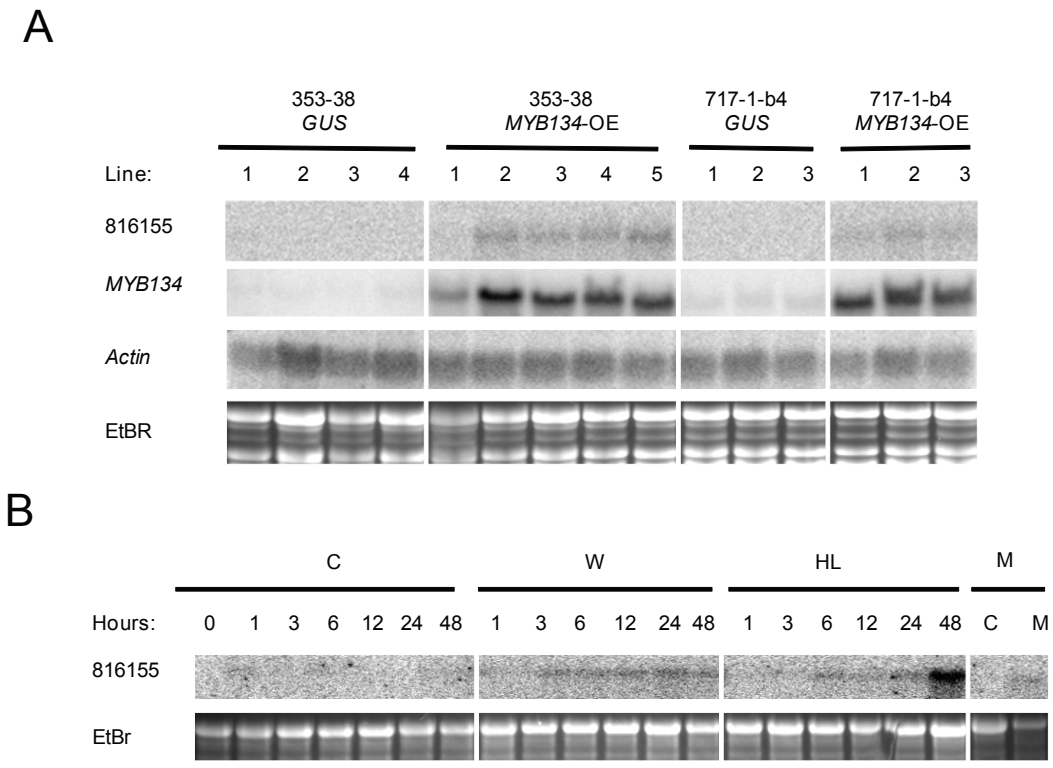


Figure 3-34. Northern analysis of the poplar 816155 (MATE transporter) gene. **A**. 816155 expression in control and *MYB134*-overexpressing poplar. **B**. 557092 expression in control (C), wounded (W), high light (HL)-exposed, and *M. medusae*-infected (M) poplar. EtBr, ethidium bromide stained total RNA.

(6 dpi), although the stress-induced expression of 816155 was quite weak in wounded and *M. medusae*-infected leaves.

3.3.6.8 Analysis of the promoter regions of genes up-regulated in *PtMYB134*-overexpressing poplar

The 2 kb promoter regions of selected genes up-regulated by MYB134 overexpression were analyzed as described in section 3.3.5. Many of these genes were found to contain c-Myb, AC element, and BHLH consensus sites (Tables 3-9 and A1-5). In a number of cases, putative MYB and BHLH binding sites were in close proximity, indicating that some of these genes might be directly regulated by MYB-BHLH-WDR complexes (data not shown). In other cases, the promoters of MYB134-up-regulated genes did not contain AC element like sequences, indicating that regulation may not be direct in some cases (Tables 3-9 and A1-5).

3.3.7 Biological effects of *MYB134* overexpression in poplar

The stress-responsive expression analyses presented in Chapter 2 indicate that the biological functions of PAs may be broader than defence against insect herbivores. Manipulation of phenolic metabolism using PA regulators such as MYB134 might represent an opportunity to directly test potential biological functions *in planta*. The experiments reported in this and the following section represent initial steps from which hypotheses can be developed to drive future research, but are not intended to evaluate the biological functions of PAs in plants.

PGs are considered potent poplar anti-herbivore compounds (Section 1.3.2). It is possible that the large reduction in PG concentrations in *MYB134*-overexpressor leaf tissue could result in compromised defence against insect herbivores. Supporting this is the observation that a chance outbreak of the greenhouse pest, thrips (order Thysanoptera, unidentified species), resulted in a marked difference in damage to leaves of *MYB134*-overexpressors and GUS control trees (Fig. 3-35). Small holes made by feeding thrips in

Table 3-9. MYB and BHLH domain protein consensus binding sites in 2 kb promoter regions of selected genes up-regulated in MYB134-overexpressing poplar leaves. Annotated elements include, BHLH consensus sites (MYCCONSENSUSAT, CANNTG), c-Myb consensus sites (MYBCORE, consensus CNGTTR) and AC element-like sequences including MYBZMP (consensus CCWACC), L Box (consensus ACCWWCC), and P Box (MYBPLANT, consensus MACCWAMC). Check mark indicates presence of element, x indicates absence.

	MYB				BHLH	
Gene	CNGTTR	L Box (ACCWWCC)	P Box (MACCWAMC)	MYBPZM (CCWACC)	G Box (CACGTG)	E Box (CANNTG)
MATE (816155)	✓	✓	×	✓	✓	✓
MATE (202461)	✓	✓	✓	×	✓	✓
MATE (761186)	✓	✓	✓	✓	✓	✓
CHIL2 (836090)	✓	✓	×	✓	✓	✓
Cytochrome B5b (740558)	✓	✓	✓	✓	✓	✓
ACC1 (559146)	✓	✓	✓	✓	×	✓
ACC1(830215)	✓	✓	✓	✓	✓	✓
GT (557092)	✓	✓	×	✓	✓	✓
CCR-related (832029)	✓	✓	✓	✓	✓	✓
CCR-related (825925)	✓	✓	✓	×	×	✓
CCR-related (743671)	✓	×	×	×	✓	✓
CCR-related (199038)	✓	×	×	✓	✓	✓
CCR-related (590025)	×	✓	✓	✓	×	✓
CYP98A23 C3H2 (576112)	✓	✓	×	✓	✓	✓
CYP98A25 (591136)	✓	×	×	×	×	✓
MYB194 (766132)	✓	✓	✓	×	×	✓
BHLH (205424)	✓	✓	✓	✓	×	✓
PtMYB115 (755418)	✓	×	×	×	×	✓

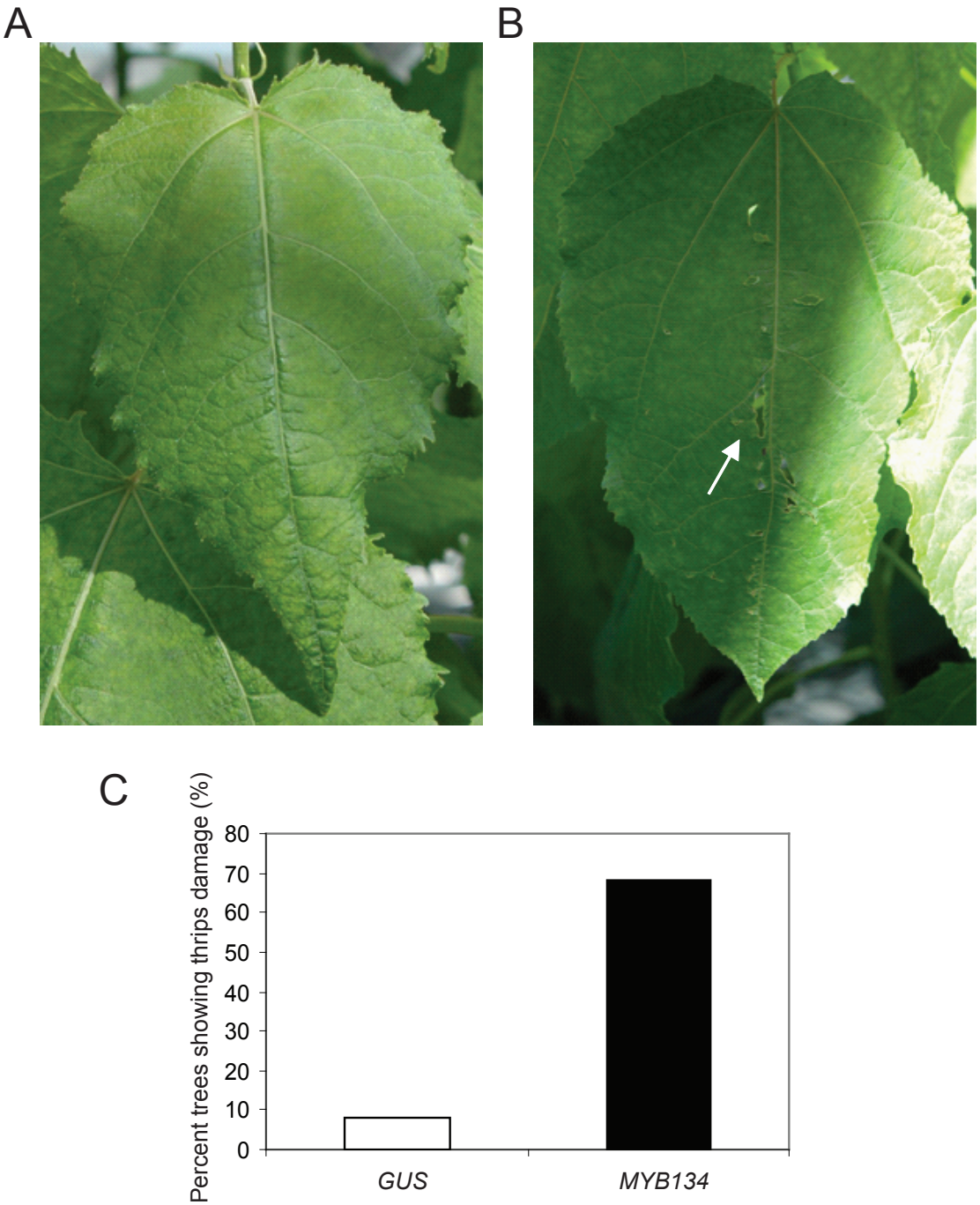


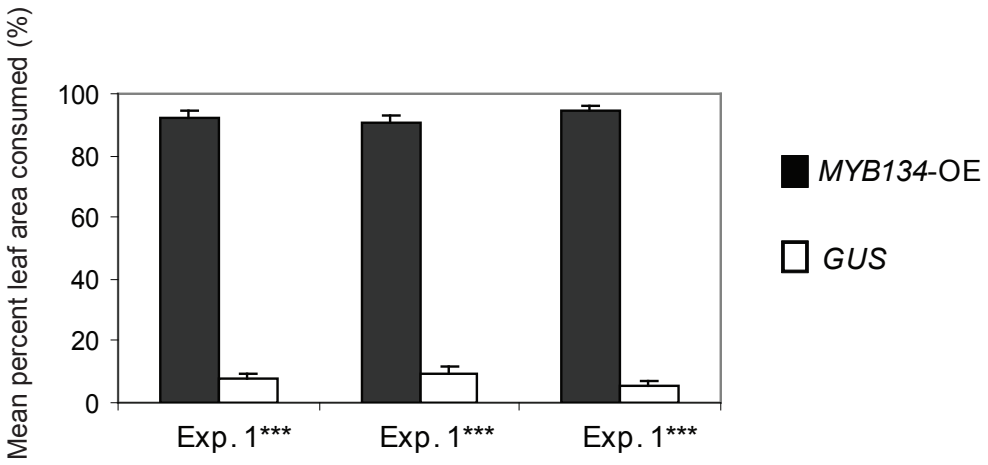
Figure 3-35. *MYB134*-overexpressor 353-38 trees exhibit distinct damage phenotype after greenhouse thrips outbreak. **A**. Photograph of typical *GUS* control plant after thrips outbreak. **B**. Photograph of typical damaged *MYB134*-overexpressor leaf after thrip outbreak showing leaf damage (white arrow). **C**. Graph showing percentage of trees with leaves showing signs of thrips damage phenotype after infestation, determined by visual inpection of plants (*GUS*, n = 24 trees; *MYB134*-overexpressor, n = 20 trees).

young leaves grew as leaves expanded to full size (Fig. 3-35B). During this outbreak, ~70% of *MYB134*-overexpressor leaves exhibited signs of thrips damage, while less than 10% of *GUS*-overexpressing control plants showed signs of damage (Fig. 3-35B). Additionally, the insect damage evident on the few control trees exhibiting signs of feeding was less severe than that observed on *MYB134*-overexpressors. This anecdotal observation indicates that these insects may prefer to feed on the *MYB134*-overexpressing leaf tissue.

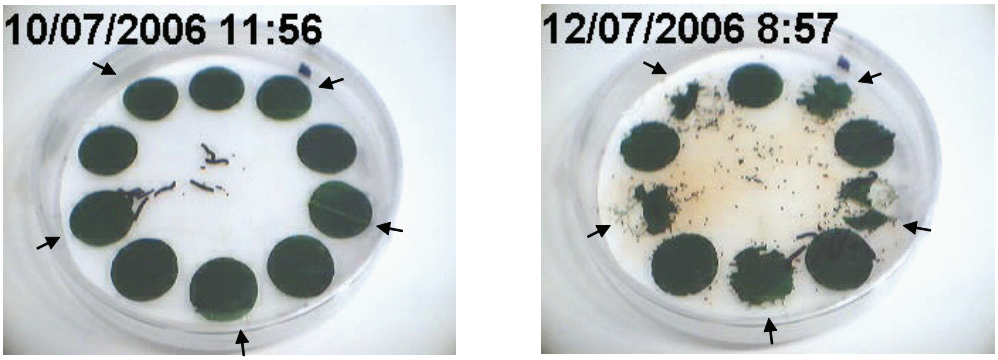
Experiments were designed to test the feeding preference and performance of an important insect herbivore of *P. tremuloides*, the FTC (Sections 1.3.1 and 1.4.2). Feeding choice assays were conducted using age-matched leaf disks from several independently transformed control and *MYB134*-overexpressor lines. First instar FTC were placed in Petri dishes containing alternately arranged control and *MYB134*-overexpressor leaf disks. In three independently replicated assays, FTC larvae consistently demonstrated a strong preference for *MYB134*-overexpressor leaf disks, and clearly avoided feeding on control tissue (Fig. 3-36A and B). In a corresponding series of "no-choice" assays, in which larvae were reared on either control or *MYB134*-overexpressor leaf disks, larvae feeding on tissue from the *MYB134*-overexpressors exhibited a significantly ($P < 0.01$) lower mortality by day 8 than those reared on control tissue (Fig. 3-36C). This reduced mortality on *MYB134*-overexpressor leaf disks is likely a reflection of a higher overall rate of feeding, as seen in the choice assays. Thus, the phytochemical alterations due to *MYB134*-overexpression improved the palatability of the leaves for FTC larvae, while the control leaf tissue used in these experiments remained relatively unpalatable.

Figure 3-36. Feeding preference and survival of FTC larvae on control and *MYB134*-overexpressor leaf tissue. **A**. FTC larvae prefer to consume *MYB134*-overexpressor leaf tissue over *GUS* control tissue in three independent experiments. Twelve dishes each containing 10 leaf discs (5 control, 5 *MYB134*-overexpressor (*MYB134*-OE), in alternating arrangement around perimeter of Petri dish), Expt. 1: 4 larvae per plate (hatched 14 days prior to expt), Expt. 2: 8 larvae per plate (hatched 8 days prior); Expt. 3: 7 larvae per plate (hatched 6-7 days prior). Experiments each run for 2 days. **B**. Photograph of FTC larval feeding choice Expt. 3 showing typical results. Arrows indicate *MYB134*-overexpressing leaf tissue. Left photograph shows typical experimental plate at the beginning of the experiment, while right photograph shows typical experimental plate after two days. **C**. Mortality of FTC larvae fed exclusively *MYB134*-overexpressor or *GUS* leaf tissue. In three independent replicates of this experiment (not shown), FTC larvae showed greater mortality feeding on *GUS* leaf tissue after 8 days ($P < 0.01$). Asterisks indicate results of Student's t test run on area eaten (**A**) or number of larvae surviving (**C**) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

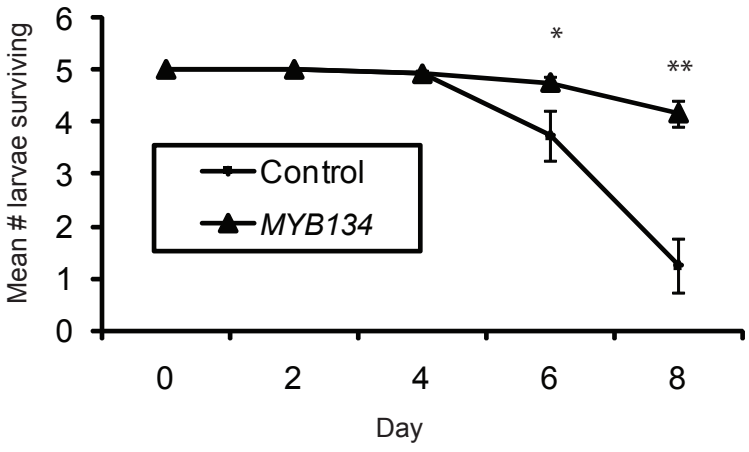
A


Mean percent leaf area consumed (%)
100
80
60
40
20
0
Exp. 1***
Exp. 1***
Exp. 1***
MYB134-OE
GUS

B


10/07/2006 11:56
12/07/2006 8:57

C


Mean # larvae surviving
6
5
4
3
2
1
0
0
2
4
6
8
Day
*
**
Control
MYB134

The observation that UV-B light rapidly activates the PA biosynthetic pathway and PA accumulation (Section 2.3.3) indicates that PAs may have a diversity of biological functions related to plant defense, including functions such as light filters or antioxidants. Although the broad alterations to phenolic metabolism in *MYB134*-overexpressing plants preclude any controlled evaluation of the UV-B-protective roles of PAs, high PA *MYB134*-overexpressing and *GUS* control plants were grown under UV-B irradiation and growth over a two week period was monitored. As reported earlier, under normal greenhouse growth conditions, *MYB134*-overxpressors exhibit no significant difference in height or other growth characteristics. However, after two weeks of elevated UV-B irradiation (the same levels used for the UV-B stress experiments), *MYB134*-overexpressors exhibited significantly greater height increase than control plants (Fig. 3-37).

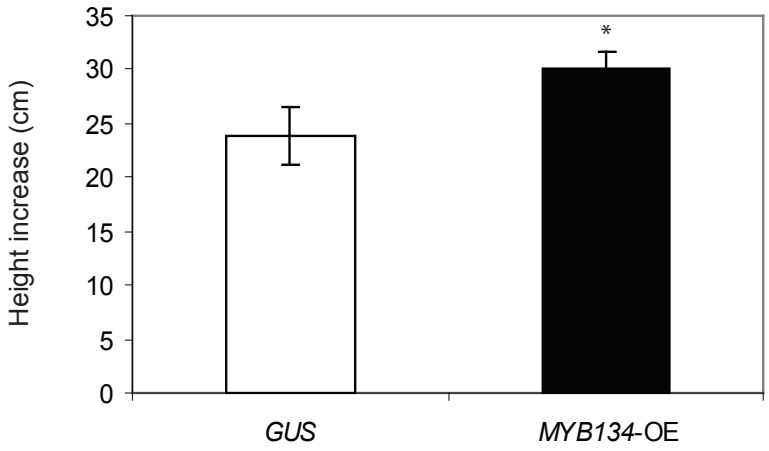


Figure 3-37. Height increase of *GUS* (control) and *MYB134*-overexpressing poplar grown under elevated UV-B light for 14 days. *MYB134*-overexpressors exhibited a small but significantly greater increase in height after the 14 day period. Asterisk indicates significant difference using Student's T test (* $P < 0.05$).

3.3.8 Constitutive expression of poplar *MYB134* activates PA metabolism in tobacco anthers

MYB regulators of flavonoid metabolism are highly conserved in plants, and there are numerous reports of MYB proteins from one species activating flavonoid biosynthetic genes when heterologously expressed in another species (Sections 1.5.2 and 1.6.3). As a first step toward investigating the possibility that MYB134 might function to regulate PA biosynthetic genes in plant species other than poplar, *MYB134* was overexpressed in tobacco (*Nicotiana tabacum*) using the same *A. tumefaciens* vector that had been used to transform poplar. Multiple *MYB134* expressing lines were produced, and tobacco plants were grown under standard greenhouse growth conditions. In order to screen for activation of the PA pathway, plants at various ages were dissected and tissues were stained with DMACA to reveal differences in PA or flavan-3-ol accumulation. Root and shoot systems of both seedlings and mature flowering plants were exhaustively dissected and stained. Seeds and flowers and associated structures were also dissected and stained. No staining was observed in any tobacco tissues with the exception of seed coat, which stained equivalently in wild type and *MYB134*-overexpressing plants, and the mature anthers of open flowers, which showed clear staining of a subset of cells in *MYB134*-overexpressors but not in wild type plants (Fig. 3-38). Normal dehiscence was observed for anthers of *MYB134*-overexpressing tobacco. No change in the anthocyanin pigmentation patterns in flowers was observed in MYB134-overexpressing tobacco (Fig. 3-39).

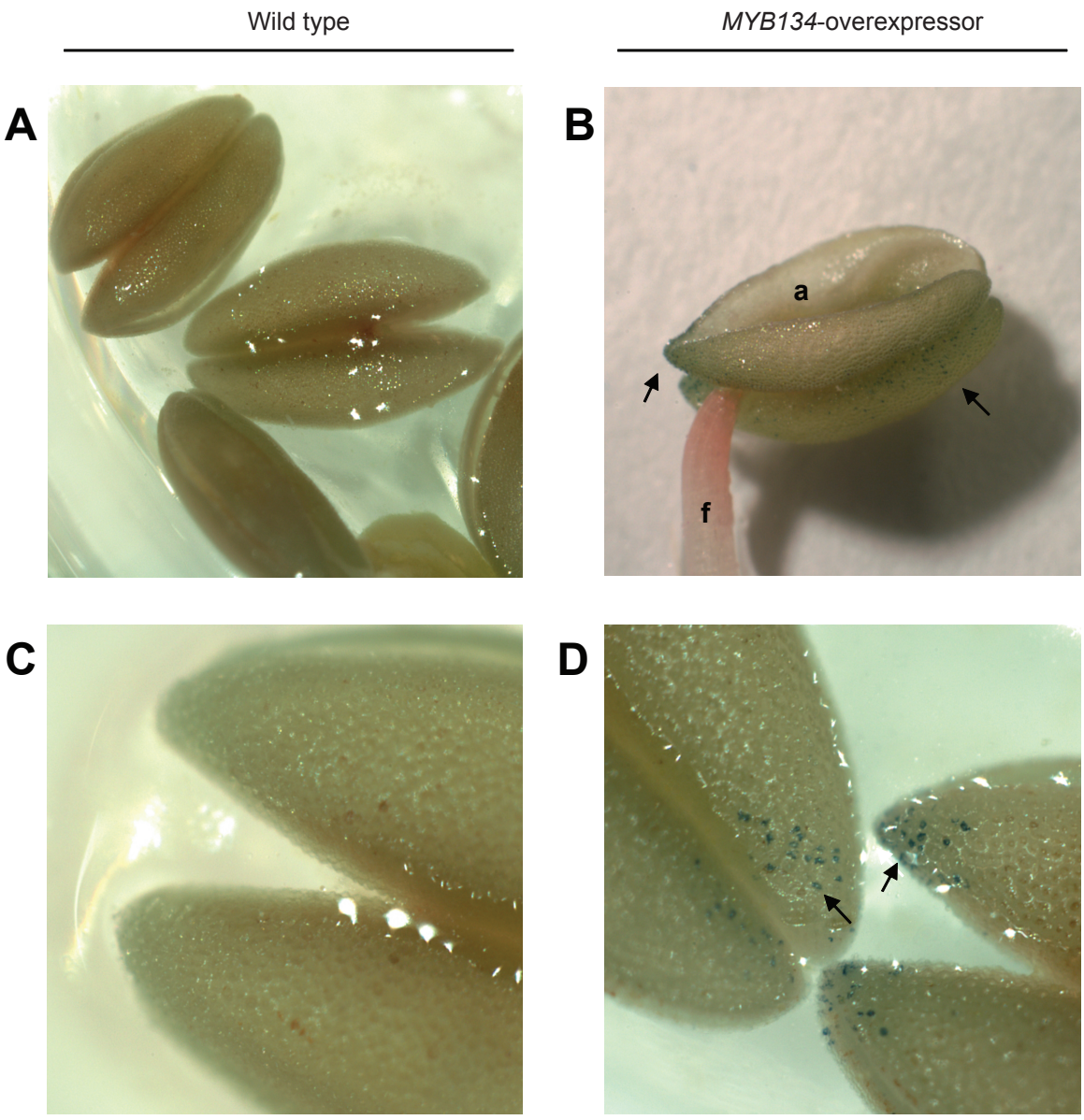


Figure 3-38. DMACA staining of control and *MYB134*-overexpressing tobacco anthers. Blue staining indicates flavan-3-ols or PA accumulation. Results typical of multiple independently transformed lines. No staining was observed in any wild type anthers. **A**. Representative wild type anthers stained with DMACA. **B**. MYB134-overexpressing tobacco anther stained with DMACA showing sporadic staining (arrows); a, anther; f, filament. **C**. Magnified view of wild type tobacco anther stained with DMACA. **D**. Magnified view of *MYB134*-overexpressor anther stained with DMACA showing PA accumulating cells (arrows) near filament attachment site.

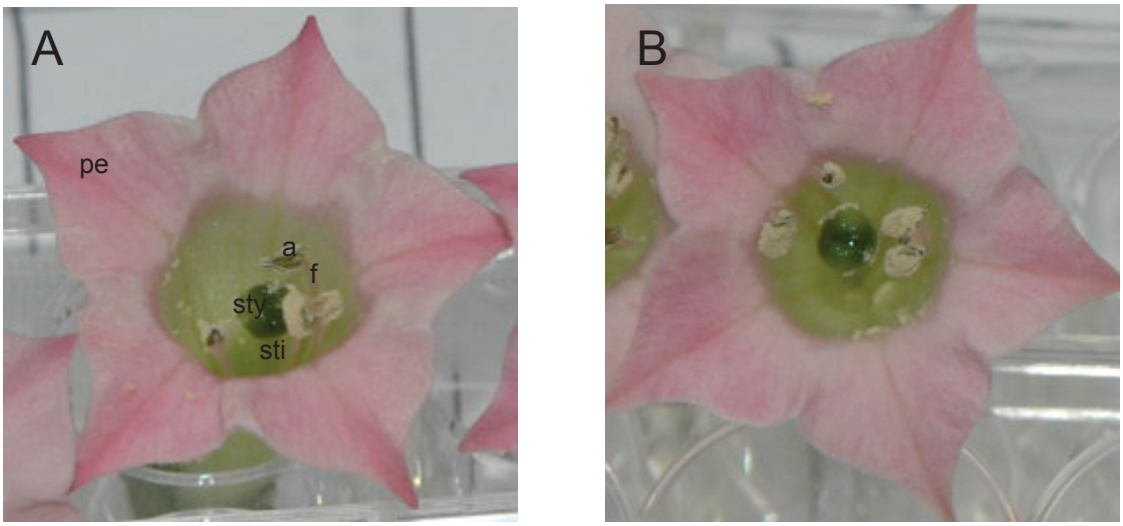


Figure 3-39. Tobacco flower from control (**A**) and MYB134-overexpressor (**B**) plant showing normal anthocyanin pigmentation and dehiscence. Sti, stigma; sty, style; a, anther; f, filament; pe, petal. No pigmentation of morphological difference was observed bewteen *MYB134*-overexpressors and controls in any flower parts. Results typical of numerous independently transformed lines.

3.4 DISCUSSION

3.4.1 Identification of MYB134, a stress-induced regulator of PA metabolism in poplar

PAs are important molecules for plant adaptation to the environment and for human health, and understanding and manipulating the regulatory systems controlling PA biosynthesis may have important applications. Here, evidence is presented to indicate that MYB134 may play a role in the regulation of PA synthesis in poplar leaves, the first example of such a regulator implicated in stress-induction of PAs in vegetative tissues. Several lines of evidence indicate that MYB134 is a poplar PA regulator. First, of all the predicted R2R3 MYB genes in the *P. trichocarpa* genome, *MYB134* exhibits the highest sequence similarity to *TT2*, the arabidopsis *R2R3 MYB* gene required for PA synthesis in the seed coat endothelial cells. Second, transcript profiling following several stresses that

induce the PA biosynthetic pathway demonstrated that unlike other *TT2*-like poplar *MYB* genes, *MYB134* was consistently co-induced with the PA biosynthetic pathway genes including the PA-specific genes *LAR1* and *ANR1*. Third, overexpression of *MYB134* in transgenic poplar behind a strong constitutive promoter led to a large plant-wide accumulation of PAs. This was the result of the activation of the full PA biosynthetic pathway, including general phenylpropanoid genes, early and late flavonoid genes, and PA-specific genes. Fourth, EMSA experiments show that MYB134 binds to representative early and late PA pathway gene regulatory regions containing putative MYB binding sites. Lastly, heterologous overexpression of poplar *MYB134* in tobacco resulted in PA or flavan-3-ol accumulation in a subset of mature anther cells, indicating that MYB134 is competent to activate PA biosynthetic gene expression in some tissues of other plant species under some conditions.

These data indicate that MYB134 functions specifically in regulating PA biosynthesis and is unlikely to be involved in activating other flavonoid branches. *MYB134* transcripts were not co-induced with *FLS4* and the other early flavonoid biosynthetic genes by elevated light and UV-B stress. Similarly, the absence of *FLS4* up-regulation in wounded and *M. medusae*-infected leaves, in which *MYB134* expression is induced, as well as the lack of *FLS4* up-regulation in the *MYB134*-overexpressor plants, indicates that it is not a target of MYB134 regulation. In addition, in *MYB134*-overexpressing poplar, transcript levels of the lignin-specific *PAL2* (Kao et al., 2002) and *CCR1* genes (Li et al., 2005) were unaffected. Global gene expression profiling showed no up-regulation of any characterized genes encoding enzymes involved in other branch pathways of phenylpropanoid or flavonoid metabolism (Table A1-4). When the data are considered as a whole, including the stress-responsive expression profiling, promoter binding, and the specificity of phenylpropanoid gene activation in *MYB134*-overexpressing plants, together with the functional conservation of flavonoid regulatory MYB proteins across species (Section 1.5), the role of MYB134 as a stress-responsive regulator of PA biosynthetic genes is supported.

For the PA-specific *LAR* and *ANR* families, all members are up-regulated by ectopic expression of *MYB134*. The strong, specific activation of these genes supports the hypothesis that MYB134 is a specific regulator of this pathway and that some of the up-

regulated genes identified in the expression profiling analysis may represent novel PA biosynthetic or regulatory functions. This is supported by the identification of poplar *TT12* homologues as MYB134 targets. The strong up-regulation of two close *TT12* homologues indicates that, as with arabidopsis, MATE proteins are likely involved in PA precursor transport in poplar (Section 1.2.2). Consistent with a role for one of these genes in stress-induced PA metabolism in poplar, the *MATE* gene represented by *P. trichocarpa* protein ID 816155 was activated by PA-inducing stresses such as mechanical wounding, high light exposure, and *M. medusae* infection (Fig. 3-34).

Some of the general phenylpropanoid and flavonoid biosynthetic multi-gene families, such as the *PAL*, *4CL*, *C4H*, *CHS*, *F3'5'H*, and *ANS* families, show differential activation by MYB134, with only some members up-regulated or some members more strongly up-regulated (Table 3-5). Interestingly, a comparison of the MYB134-up-regulated flavonoid and PA pathway genes (Table 3-5) with the systemic wounding-induced expression data published by Tsai et al. (2006a) and the *M. medusae*-infection (6 dpi) PA pathway up-regulation (Miranda et al., 2007) reveals that many of the same gene family members are most strongly up-regulated in all three datasets. For example, *PAL1*, *4CL2*, *ANR1*, *LAR3*, and *ANS2* are the most strongly up-regulated members of their respective gene families in all three studies. This correspondence between the gene family members up-regulated by PA-inducing stresses and those up-regulated by *MYB134* overexpression supports a function for MYB134 as a regulator of stress-induced PA metabolism.

In general, *MYB134* overexpression results in a differential degree of activation of early and late PA biosynthetic genes (Figs. 3-19 and 3-27). This indicates that MYB134 is like other WDR-dependent flavonoid regulatory MYB genes in that it is a stronger activator of late flavonoid genes (Gonzalez et al., 2008). However, the strong activation of general phenylpropanoid genes might indicate that either MYB134 targets a wider set of genes than arabidopsis TT2 or that other factors also play a role in regulating PA biosynthetic genes. It is possible that regulation of the full pathway *in planta* involves additional MYB or other regulatory proteins (see Section 3.4.5). The other two stress-induced poplar TT2-Related Subclade genes (*MYB183* and *MYB097*) were not up-regulated in the *MYB134*-overexpressors, indicating that they are not required for

pathway activation by MYB134. Additionally, overexpression of *MYB183* and *MYB097* in transgenic poplar does not result in activation of the full PA biosynthetic pathway, or the PA pathway genes *4CL2*, *DFR1*, and *ANR1* (Fig. 3-22). Since many of the flavonoid enzymes in poplar are encoded by small gene families, it is possible that these other stress-induced MYB proteins regulate more restricted sets of biochemically distinct flavonoid structural gene family members. Such fine regulation mediated by a group of closely related but functionally distinct transcription factors would permit poplar to tailor biologically important features of PA chemistry to specific stress conditions (e.g., B ring hydroxylation). Analogously, the closely related members of the anthocyanin regulatory MYB family in snapdragon have distinct target gene specificities; in this case, differences in their expression control the anthocyanin pigmentation patterns observed in different snapdragon species (Schwinn et al., 2006).

Alternatively, MYB097 and MYB183 may function in negative feedback regulation of the PA pathway following stress conditions. The predicted MYB097 and MYB183 proteins do not share the conserved C-terminal Vx_2IRTKA[IL]RC[SN] motif found in poplar MYB134, arabidopsis TT2, lotus TT2 and a number of other TT2-Related Subclade proteins. It is possible that this motif may be essential for function, perhaps mediating interaction with unknown proteins involved in transcriptional activation of target genes. MYB097 and MYB183 may bind to and occupy DNA elements recognized by MYB134 or interact with BHLH cofactor proteins, but not function to activate gene expression. Such a function would fit the stress-induced up-regulation of the *MYB097* and *MYB183* genes, permitting fine modulation of PA pathway gene expression under different stress conditions through expression of a pool of positive and negative regulators that interact with the same cofactor proteins and DNA elements. It is interesting to note that microarray analysis revealed that the *MYB097* gene was down-regulated in *MYB134*-overexpressing plants (Table 3-8).

The accumulation of DMACA-reacting compounds in anther cells of *MYB134*-overexpressing tobacco indicates that like other flavonoid regulatory MYBs, MYB134 exhibits functional activity when expressed heterologously. Heterologous expression of PA and flavonoid regulatory and biosynthetic genes in tobacco has been found to affect flower pigmentation and PA production in petals. For example, Xie et al. (2003)

overexpressed the *M. truncatula BAN* (*ANR)* gene in tobacco and found that the flowers lost their normal pink coloration due to anthocyanin accumulation, and stained positively for PAs with DMACA. Heterologous expression in tobacco of the grapevine *MYB5b* gene, encoding a MYB protein that appears to function in developing grape as an activator of multiple branches of flavonoid metabolism including PA and anthocyanin synthesis (Section 1.5.3), resulted in enhanced anthocyanin accumulation in flower petals, anthers, and filaments, and PA accumulation in petal tips and anthers. Interestingly, the pattern of DMACA staining in *MYB5b*-overexpresing tobacco anthers was very similar to that observed in poplar *MYB134*-overexpressing tobacco, with staining near the site of filament attachment as well as toward the distal end of the anther (Fig. 3-40B; Deluc et al. 2008). The PA accumulation found in grapevine *MYB5b*-overexpressing tobacco anthers was found to correspond to an up-regulation of the flavonoid genes *CHS*, *CHI*, *F3H*, *DFR*, and *ANS* (Deluc et al., 2008), and it is likely that the poplar MYB134 protein similarly activated flavonoid biosynthetic genes in tobacco leading to the observed PA accumulation.

Unlike *M. truncatula BAN*- or grapevine *MYB5b*-overexpressing tobacco, the flower petals of *MYB134*-overexpressing tobacco were identical to wild type flowers in pigmentation (Fig. 3-39) and DMACA staining revealed no PA or flavan-3-ol accumulation in petals. The lack of plant-wide PA accumulation in transgenic tobacco plants may reflect the general absence of an active PA metabolism in vegetative tissues of this species. It is possible that cofactor proteins capable of functioning together with MYB134 are not present in most tobacco tissues. The arabidopsis C2 Repressor Motif Clade protein MYB32 is a predicted BHLH-WDR-dependant MYB thought to regulate phenylpropanoid and flavonoid genes (Section 1.5.4), and a *myb32* mutant shows defective anther cell development, indicating that a MYB32-BHLH-WDR complex may be active in anther cells (Section 1.5.4). If a similar developmental pathway involving a MYB-BHLH-WDR complex exists in tobacco, it is possible that tobacco BHLH and WDR proteins that function with tobacco the MYB32 homologue in anther cells are capable of interacting with poplar MYB134 and grapevine MYB5a, explaining the DMACA staining of anther cells of transgenic plants.

DMACA staining of *MYB134*-overexpressing poplar revealed that PA accumulation was not uniform within tissues (Fig. 3-18). For example, in leaves, PAs consistently accumulated in the upper layer of palisade mesophyll cells and not the lower layer (Fig. 3-18B). It is likely that cell type-specific expression patterns of additional factors, such as interacting WDR or BHLH proteins, may be integral in the regulation of the PA pathway, as has been found in arabidopsis (Baudry et al., 2004). Like arabidopsis TT2, the predicted poplar MYB134, MYB097, MYB183, and MYB086 proteins contain residues in the R3 domain involved in mediating interactions with members of the BHLH transcription factor family (Fig. 3-2), and thus these MYBs likely function with BHLH partners in transcription factor complexes. The accumulation of PAs in diverse organs and cell types of *MYB134*-overexpressing plants suggests that the additional factors required to form a functional MYB-BHLH-WDR ternary transcription complex are already present or are themselves regulated by MYB134, at least in those cell types within each tissue in which PA accumulation was observed.

3.4.2 *MYB134* overexpression affects multiple branches of phenolic metabolism in poplar

In addition to the large increase in PA levels, the up-regulation of PA biosynthesis by *MYB134* overexpression resulted in other changes to the foliar phenolic metabolite composition. Concentrations of the PGs were consistently reduced in leaves of the *MYB134*-overexpressor plants, resulting in an approximately three-fold overall decrease relative to controls. PGs are the most abundant soluble phenolic compounds in leaves of the control plants under greenhouse conditions, so this reduction represents a significant shift in carbon flux. Though less abundant overall, levels of HCDs were also reduced in the *MYB134*-overexpressor leaves, while non-PA flavonoid levels were increased slightly. These differences were consistently observed in different genotypes and transformed lines.

Based on the specificity of previously characterized flavonoid regulatory R2R3 MYB factors (Section 1.5), it seems unlikely that MYB134 controls the PG and HCD

pathways directly. Because the PG biosynthetic pathway is as yet unknown, it is not currently possible to determine if transcriptional regulation is involved. Of the genes down-regulated by MYB134, two (571376 and 665543) encode putative acyltransferases (Table 3-8) that could conceivably function in a branch of phenylpropanoid metabolism such as PG synthesis. However, even if this were the case, their down-regulation may be an indirect effect of *MYB134* overexpression and PA pathway up-regulation. It is more probable that the high rate of PA synthesis is diverting metabolites from other phenolic pathways, so that the observed changes in non-PA phenylpropanoid levels are the result of competition for common phenolic precursors between the up-regulated PA pathway and other branches of phenolic metabolism. Levels of PAs and PGs have been reported to be inversely related in some cases (Section 1.4.3). For example, Holton et al. (2003) reported decreased PG levels corresponding to increased PA accumulation in aspen grown under elevated O_3, supporting the idea of a metabolic trade-off between these two major carbon pools.

The increase in flavonol glycoside levels but not other non-PA flavonoid end products such as flavones and anthocyanins is also consistent with the hypothesis that the high flux into PAs has indirect consequences on other pathways. The increased flux into the flavonoid pathway resulting from *MYB134* overexpression and the accumulation of PA intermediates should provide higher levels of substrates for branch pathways that are already active in leaves (e.g., flavonol biosynthesis). *FLS4* was detectably but equally expressed in both control and *MYB134*-overexpressor leaves (Fig. 3-19) and a number of flavonol glycosides were present in the leaves of control plants (Fig. 3-16A and Table 3-3). With an enhanced synthesis of shared PA and flavonol intermediates such as dihydroflavonols, substrate availability for FLS enzymes would be increased. This could result in increased flavonol glycoside production without the activation of *FLS4* or other flavonol-specific genes. Flavonoid branch pathways not already active in these tissues (e.g., anthocyanin of flavone biosynthesis) would be unaffected by increased substrate availability.

3.4.3 Poplar PA biosynthetic genes promoters contain putative MYB134 binding sites

It is not known precisely how different R2R3 MYB proteins mediate the activation of distinct sets of target genes including general flavonoid biosynthesis genes common to different pathways as well as branch pathway-specific genes. This regulation likely involves different DNA binding activities as well as the presence or positioning of binding sites for required cofactor proteins such as members of the BHLH class (Section 1.5). While several PA regulatory proteins have now been identified and shown to activate arabidopsis *BAN* and other PA-specific flavonoid biosynthetic genes through assays with promoter-reporter fusion constructs, the precise DNA sequences within target gene promoters that are bound by these transcription factors are yet to be elucidated. *In silico* analysis revealed that the promoter regions of the poplar flavonoid and PA biosynthetic genes contain *cis*-elements matching the consensus sequences recognized by phenylpropanoid-regulatory R2R3 MYB proteins (Table 3-4). MYB134 was shown to bind to promoter fragments containing motifs similar to the AC elements found in a wide variety of phenylpropanoid biosynthetic gene promoters (Fig. 3-24). MYB134 was also shown to bind to a DNA sequence containing a canonical AC element (ACCTAAC) (Fig. 3-24C). These results indicate that such motifs are bound by MYB134 *in vivo*, although these results do not rule out the involvement of other putative MYB binding sites such as the animal c-Myb recognition site found in the *ANR2* promoter (Fig. 3-23). Given that AC elements are widely distributed in the regulatory regions not just of PA biosynthetic genes but of genes involved in other branches of flavonoid and phenylpropanoid metabolism, interactions with cofactors such as BHLH-domain proteins that require the presence of additional binding sites likely contributes to the specific activation of different branch pathways (Section 1.5). Consistent with specific BHLH cofactor binding sites contributing to MYB134 target gene specificity, putative BHLH binding sites are present in all poplar PA pathway genes (Tables 3-4 and A1-5).

Examination of the poplar *ANR* gene promoters reveals BHLH binding sites as well as MYB consensus binding sites similar to canonical AC elements and the c-Myb binding site. Comparison of the poplar and arabidopsis *BAN* gene promoter reveals a

conserved region at the distal end of the *BAN* 'PA Enhancer'. Within this region in all three genes are elements similar to the AC element bound by maize C1. It is possible that specific activation of these PA pathway genes is mediated by regulatory factors such as TT2 and MYB134, or other interacting proteins, through binding at this site. Consistent with this hypothesis, the three PA-specific poplar LAR genes all have AC element-like sequences within regions similar to the conserved region shared between the arabidopsis and poplar *ANR* genes (Table A1-5).

Examination of the poplar 2 kb promoter sequences analyzed in this study reveals that the regulatory mechanisms controlling flavonoid biosynthesis are likely very complex, possibly involving the presence, proximity, and orientation of several classes of *cis*-element sequence (Tables 3-4 and A1-5). Presence or absence of putative MYB134 binding sites (AC elements) alone clearly does not account for the specific regulation of the PA pathway genes or the level of gene activation of family members by MYB134. MYB and BHLH binding sites are also found in genes that are not regulated by MYB134 such as PAL2 and 4CL1 (Table A1-5). Elucidating the regulatory mechanisms controlling stress-induced PA pathway regulation will require identification of MYB134-interacting proteins and analysis of target gene activation using reporter gene activation assays, promoter deletions, and site directed mutagenesis of putative binding sites. While MYB134 may be an important PA regulator, the identification of this gene is only a first step in understanding how this pathway is regulated in poplar. Together with the *in silico* promoter analysis summarized in Table A1-5, the identification of genes such as the BHLH 205424 and the General Flavonoid Regulation Subclade MYBs through expression profiling of *MYB134*-overexpressing plants establishes a foundation for future research aimed at discovering precisely how this pathway is controlled.

3.4.4 Expression profiling reveals MYB134-regulated genes that may function in PA metabolism

While *MYB134* overexpression is sufficient for activation of PA biosynthetic genes, up-regulation of predicted target genes in stably transformed plants does not in itself demonstrate a direct regulation by MYB134, and it is thus possible that this protein

activates some downstream genes indirectly *via* other factors. Data generated using stable transformation of plants with genes encoding transcription factors must be interpreted with caution, as the resulting phenotype may be confounded by indirect effects (Broun, 2004). Overexpression of a transcription factor gene can result in off-target effects and the activation of genes that are not normally directly regulated by the protein. The CaMV 35S promoter is a strong, constitutive promoter, which likely led to expression in cell types where *MYB134* is not normally expressed. The metabolic changes resulting from the massive up-regulation of the PA biosynthetic pathway in *MYB134*-overexpressing plants may also result in the deregulation of genes not directly targeted by MYB134.

Like the PA pathway genes (Table 3-4), many of the uncharacterized genes identified as MYB134-up-regulated using Affymetrix GeneChip® Poplar Genome Array microarray analysis have MYB and BHLH consensus sites within 2 kb upstream of the start of transcription (Table 3-9), suggesting that they may be directly regulated by MYB proteins. Most of these genes contain canonical AC elements or AC element-like motifs within 2 kb of the transcription start site (e.g., the *ACC* genes, and the poplar *DifF* homologue), although some do not (e.g., the cytochrome P450 gene *CYP98A25* (591136) and the General Flavonoid Regulation Subclade gene *MYB115* (755418), Table A1-5). It is possible that MYB134 binding sites are located up- or downstream of the 2 kb region analyzed or that MYB134 functions without directly binding to DNA in some cases (e.g., through interactions with other DNA-binding proteins). However, the list of MYB134-up- and down-regulated genes likely includes both direct and indirect targets of MYB134.

The up-regulated putative PA regulators (Table 3-7) together with the known PA biosynthetic genes (Table 3-5) constitute 31% of the genes up-regulated by overexpression of *MYB134*, suggesting that PA pathway activation is quite specific and that among the remaining strongly up-regulated genes there may some encoding proteins with novel structural or regulatory functions in PA metabolism (Section 1.6.4). The late steps in PA biosynthesis remain to be fully characterized (Section 1.2.2). As mentioned in Section 1.6.4, the poplar genome contains relatively large families of phenylpropanoid biosynthetic genes, with some members exhibiting considerable sequence divergence from characterized enzymes. A number of the MYB134-up-regulated probesets represent

uncharacterized genes with annotations indicating possible roles in phenylpropanoid metabolism. These include 5 genes annotated as *CCR-related* and 8 *UFGT* genes. The two cytochrome P450 genes are related to the poplar lignin gene, *p*-coumaroyl-CoA 3'-hydroxylase (C3'H) (Coleman et al, 2008), but are divergent from this and the other functionally characterized C3'H genes, suggesting they may have evolved to serve novel functions in phenylpropanoid metabolism

It was recently shown that a *M. truncatula* glucosyltransferase gene, *UGT72L1*, was up-regulated by ectopic expression of arabidopsis TT2 and that the UGT72L1 protein specifically glycosylates the PA precursor (-)-epicatechin (Sections 1.2.2 and 1.6.4). Distinct *UFGT* genes were also identified by Terrier et al. (2009) in *MYBPA1*- and *MYBPA2*-overexpressing grapevine hairy roots (Section 1.6.4). Among the *UFGT* genes up-regulated in *MYB134*-overexpressing poplar leaves, the most strongly up-regulated (584786, 45.8-fold) was also identified as co-activated with the PA biosynthetic pathway genes in response to infection of poplar leaves by the fungal pathogen *M. medusae* (Miranda et al., 2007). This gene is also responsive to stress stimuli that activate the PA biosynthetic pathway (Fig. 3-33), suggesting that, like *UGT72L1*, it may play a novel role in PA metabolism.

Genes annotated as *CCR-related* have also been identified as up-regulated following overexpression of flavonoid and PA-regulatory MYBs in several studies (Section 1.6.4). As can be seen in Fig. 3-29, these genes are quite divergent from characterized CCR genes. They are also distinct from the uncharacterized poplar *CCR-like* genes (Fig. 3-29), suggesting that despite the "CCR-related" annotation, they may be functionally quite different from CCR enzymes. While they belong to a large group of reductase enzymes that includes CCR, DFR, LAR, and ANR, no member of this CCR-related group has been functionally characterized. Two of the five MYB134-up-regulated *CCR-related* genes (825925 and 590025) were previously found to be co-activated with the PA pathway genes following *M. medusae* infection of poplar leaves (Miranda et al., 2007). Poplar 590025, which is up-regulated by *MYB134* overexpression and co-activated with the PA pathway following *M. medusae* infection, is highly similar to a grape *CCR-related gene* (GenBank accession number: CA063391) that is specifically expressed in

flavonoid-rich grape seed skin and seed (Grimplet et al., 2007) and that is up-regulated by both grape PA regulators, MYBPA1 and MYBPA2 (Terrier et al., 2009) (Fig. 3-29).

3.4.5 The regulatory system controlling stress-induced PA pathway activation in poplar leaves

Activation of genes following overexpression of *MYB134* may be the result of direct activation by a MYB134-containing regulatory complex, may be the result of up-regulation of transcriptional regulators by MYB134, or may be an artifact of high or ectopic expression of *MYB134*. As whole leaves were analyzed in this study, some of the MYB134-up-regulated genes may be targets of other MYB-BHLH-WDR regulatory complexes, such as those involved in trichome development. Arabidopsis TT2 is known to be an activator of the *TT8* gene (Section 1.5.3), and TT8 is at least partially functional redundant with the GL3 and EGL3 proteins involved other MYB-BHLH-WDR complexes (Section 1.5.1). The promoter sequence of the poplar *TT8* homologue (protein ID 205424) contains a number of canonical AC elements (Table A1-5), indicating that it may be directly regulated by MYB134 or another MYB. Activation of a poplar *GL2* homologue (Table 3-7), a regulatory target of GL1-GL3/EGL3-TTG1 in arabidopsis, may be an example of off-target gene activation. Similarly, up-regulation of an R3 MYB gene homologous to arabidopsis TRY may be the result of ectopic activation of regulators that function in multiple regulatory pathways.

Determining the direct targets of MYB134 is further complicated by the activation of the putative PA regulatory MYB genes *MYB115*, *MYB153*, and *MYB201*, homologues of the grapevine PA regulatory gene *MYBPA1*, which is known to activate both early and late PA biosynthetic genes (Bogs et al., 2007). Interestingly, ectopic expression of grapevine *MYBPA2* (a *MYB134* and *TT2* homologue) in grapevine hairy roots was similarly found to up-regulate *MYBPA1* (Terrier et al., 2009). It is possible that regulation of stress-induced PA metabolism in poplar is complex, involving R2R3 MYB proteins of both the TT2-Related Subclade and the General Flavonoid Regulation Subclade. MYB134 may regulate PA biosynthetic genes both directly and indirectly by up-regulating other transcriptional activators. In a similar fashion, the arabidopsis R2R3

MYB protein MYB42 is thought to regulate lignin biosynthesis by both activating target biosynthetic gene expression directly as well as indirectly through the activation of the R2R3 MYB factors MYB58 and MYB63 (Zhou et al., 2009).

The up-regulation of several genes encoding R2R3 MYBs of the C2 Repressor Motif Clade supports a role for these proteins in the regulation of flavonoid metabolism (Sections 1.5.4 and 1.6.4). Interestingly, ectopic expression of the grapevine PA regulators *MYBPA1* and *MYBPA2* also induced expression of a C2 Repressor Motif Clade MYB (GenBank accession number: CA042256) that is closely related to three up-regulated poplar genes (Fig. 3-32). Arabidopsis MYB6 was recently shown to be up-regulated under conditions that cause repression of anthocyanin production in normally high anthocyanin-producing *PAP1*-overexpressing plants, suggesting that it functions as a negative regulator of anthocyanin metabolism and further establishing the link between C2 Repressor Motif Clade MYBs and regulation of flavonoid metabolism (Rowan et al., 2009). Unlike arabidopsis MYB6, all 3 MYB134-up-regulated poplar C2 Repressor Motif Clade MYB genes possess the conserved $[DE]Lx_2[RK]x_3Lx_6Lx_3R$ motif involved in interaction with BHLH partners. It is possible that these genes are up-regulated by MYB134 and then function to repress other branches of flavonoid metabolism, such as the anthocyanin biosynthetic branch. Down-regulation of a possible anthocyanidin *UFGT* gene is consistent with this (Table 3-9). Alternatively, they may repress general flavonoid pathway or PA genes, constituting a negative feedback regulatory mechanism.

The down-regulation of several pathogen response-related genes by *MYB134* overexpression is interesting. Since MYB134 may be a regulator of stress-responsive PA pathway activation in poplar leaves, it is possible that this represents an example of cross-talk between stress-response pathways. Antagonistic pathogen- and herbivore-defence signalling pathways have been documented in a number of plants, possibly reflecting trade-offs in resistance to herbivore and pathogen attack (Felton and Korth, 2000; Logemann and Hahlbrock, 2002; Rojo et al., 2003).

3.4.6 The biological roles of PAs and inducible PA metabolism in poplar

Modifications to plant phenolic metabolism caused by genetic engineering have been shown to affect herbivore feeding and performance (Johnson and Dowd, 2004; Hjalten et al., 2007). For example, Hjalten et al. (2007) reported that an increase in both leaf PA and PG levels, resulting from overexpression of a gene encoding sucrose phosphate synthase (SPS) in hybrid aspen (*P. tremula* x *tremuloides*), resulted in reduced consumption of leaf tissue by a chrysomelid leaf beetle (*Phratora vitellinae*). The observation that *MYB134*-overexpressors exhibited a higher level of tissue damage during a thrips infestation suggests that despite elevated PA levels, these transgenic plants may be compromised in their defence against herbivores (Fig. 3-26). Among the phenolic changes to leaves resulting from *MYB134* overexpression was a reduction in levels of HCDs. Leiss et al. (2009) recently determined that levels of the HCDs chlorogenic acid and feruloyl quinic acid are positively correlated with resistance to thrips in chrysanthemum (*Dendranthema grandiflora*), suggesting one possible reason for the increased thrips damage to *MYB134*-overexpressor leaves. The PGs are also potent anti-herbivore compounds, and reduced PG levels may have been a contributing factor.

The bioassays with FTC, a native pest of *P. tremuloides*, showed that these larvae have a clear preference for leaf tissue from the *MYB134*-overexpressors and that larval survival was significantly enhanced on the *MYB134*-overexpressor tissue (Fig. 3-27). This could be due to the highly increased PA levels (~ 40 fold) relative to controls, the reduced PG levels (~ 3-fold), or both. A large body of research has implicated PGs as the primary anti-lepidopteran herbivore phenolic compounds in poplar, and, more specifically, PG consumption has been shown to negatively impact FTC larval development and survival (Section 1.3.2). Holton et al. (2003) reported improved FTC larval performance on *P. tremuloides* that had been grown under elevated O_3, a treatment that resulted in increased PA concentrations and a concomitant decrease in PG levels. Although the magnitude of the change in PA and PG concentrations was much lower in these plants in comparison to the *MYB134*-overexpressors, the effects of this metabolic shift on FTC performance parallel the findings reported here.

The observation that *MYB134*-overexpressors exhibited greater height increase when grown under elevated UV-B (Fig. 3-28), when taken together with the observation that the same UV-B treatment causes an up-regulation of PA metabolism and a

significant accumulation of PAs (Figs. 2-5, 2-7), indicates that the elevated PA levels in the trees may serve to protect the leaves against the damaging effects of UV-B radiation. However, PAs are UV-B-absorbing compounds and an overall increase in UV-B absorbing phenolics of any type might lead to enhanced resistance to UV-B irradiation, whether this reflects an *in planta* function or not. Additionally, the secondary alterations to phenolic metabolism in leaves of *MYB134*-overexpressing trees included an increase in total flavonol glycoside concentrations, known to be important UV-B protective molecules. As such, these experiments should not be interpreted as supporting a UV-B screening or protective function for PAs, but do indicate that investigations in that direction might be warranted.

MYB134 overexpression results in a specific increase in flavonoids with a greater level of B ring hydroxylation (Fig. 3-17, Table 3-2), mediated by activation of *F3'H* and *F3'5'H* genes (Table 3-5). The increased B ring hydroxylation of flavonols is likely a side-effect of the up-regulation of flavonoid B ring hydroxylase enzymes that are acting on substrates primarily destined for PA polymer biosynthesis (Fig. 1-1). An increase in B ring hydroxylation of PA monomers in *MYB134*-overexpressors was confirmed by a ^{13}C-NMR analysis of PAs isolated from control and *MYB134*-overexpressing leaves (Caroline Preston, Canadian Forest Service, Pacific Forestry Centre, unpublished data). The antioxidant capacity of flavonoids and PAs increases with increasing B ring hydroxylation (Section 1.4.1). Given that MYB134 overexpression results in the accumulation of PAs with increased antioxidant capacity and that many of the *MYB134*- and PA pathway-activating stresses are likely associated with increased oxidative stress in leaves (Sections 1.4.4 and 2.4.2), it could be speculated that MYB134 mediates activation of the PA pathway as a component of an oxidative stress response.

4.1 Overall conclusions and future directions

In conclusion, the data reported here show that, in poplar leaves, PA biosynthetic pathway genes are rapidly transcriptionally activated under a variety of stress conditions in addition to herbivore damage. MYB134 is identified as a possible regulator of stress-induced PA metabolism in poplar. This represents the first report of a TT2-like transcription factor that mediates stress-responsive activation of the PA pathway. MYB134 appears to be a specific activator of the PA pathway. This activation is complete, with a large accumulation of the end product. Manipulation of the PA pathway in poplar and other plants using *MYB134* could permit investigation of proposed stress-protective functions of PAs in plants. However, while strong, constitutive overexpression of a gene in all tissues is useful for investigating gene function, controlled expression of *MYB134* using tissue-specific or treatment-inducible promoters would be better suited to investigations aimed at evaluating the stress-protective functions of PAs in plants. Such an approach might permit manipulation of PA metabolism without the large alterations to other phenolic pools, thereby removing the confounding variables that obscure interpretations of the biological effects of PA pathway up-regulation reported here. Now that MYB134 is identified as a putative regulator of stress-induced PA metabolism, it would also be valuable to knock down *MYB134* expression using transformation of poplar with RNAi or antisense constructs.

When these analyses were initiated, arabidopsis TT2 was the only known PA regulatory MYB. Grapevine MYBPA1 has since been found to be a PA pathway regulator (Chapter 1), and several homologues of MYBPA1 are up-regulated in *MYB134*-overexpressing poplar. The extent to which these poplar MYBPA1 homologues contribute to the regulation of stress-induced PA metabolism is an open question. It is possible that the regulation of stress-induced PA metabolism in poplar is very complex, involving the up- and down-regulation of multiple MYB proteins of different clades, which function as both positive and negative regulators of flavonoid biosynthetic genes. Analyzing these genes through expression profiling, DNA binding and promoter activations assays, and overexpression/knockout in transgenic plants will be an important

next step in understanding PA pathway regulation in poplar. The functions of the *MYB097* and *MYB183* genes are also unclear. While they are up-regulated with the PA pathway under some stress conditions, constitutive overexpression in poplar does not result in altered PA production. It would be interesting to determine if the carboxy-terminal Vx_2IRTKA[IL]RC[SN] motif is essential for MYB134 and TT2 function by mutating the coding sequence and analyzing gene function using promoter activation assays or plant transformation. This motif may mediate interactions with other proteins involved in PA regulation, and it may be possible to identify new components of the regulatory system using this sequence in pull-down assays.

Global transcriptome profiling of *MYB134*-overexpressing poplar resulted in the identification of a number of genes that may represent novel PA biosynthetic activities. Stress-responsive expression patterns and promoter structure support regulation by a PA-regulatory MYB factor and involvement in PA metabolism for a number of the identified genes. Additional expression profiling (e.g., under PA pathway-activating stress conditions) and functional characterization of these genes through analysis of recombinant proteins (e.g., enzyme assays for putative biosynthetic proteins), gene overexpression and knockdown in poplar or arabidopsis, and analysis of mutants in homologous loci in arabidopsis, may lead to the identification of genes that play uncharacterized roles in PA metabolism. There is considerable interest in understanding and manipulating PA biochemistry (Chapter 1), and the results reported here may contribute to the discovery of new genes with conserved functions in PA metabolism and increase our understanding of this important plant secondary pathway.

PAs are an important target trait for genetic engineering and plant improvement (Section 1.6.2). While overexpression of the poplar *MYB134* gene in tobacco did not result in a detectable increase in PA accumulation in most tissues, the accumulation of PAs or flavan-3-ols in anther cells indicates that MYB134 does retain some functionality in tobacco. Tobacco may not be an optimal system for studying the metabolic engineering of PA metabolism. It would be interesting to further investigate the use of MYB134 to engineer PA metabolism in other plants, including plants of agricultural and agronomic importance.

Constitutive leaf PA levels as well as herbivore-induced increases in leaf PA concentrations can exhibit significant variation within poplar populations (Sections 1.3.2 and 1.4.3) and expression of regulatory genes may mediate such differences. A large body of literature indicates that flavonoid regulatory R2R3 MYB proteins exhibit a high functional conservation, retaining activity when heterologously expressed in distantly related plants even when overall amino acid sequence identity between homologous proteins is quite low (Section 1.5). Flavonoid accumulation patterns in plant tissues are generally attributed to the expression of these regulatory genes (Quattrocchio et al., 2006b). Studying the expression of *MYB134* and other putative poplar PA regulators identified in this study in natural poplar populations and poplar clones exhibiting phenotypic variability may increase our understanding of the molecular mechanisms mediating variation in PA chemistry in poplar. The role of MYB134 in regulating constitutive PA production in poplar was not investigated in this study, although the constitutive expression of *MYB134* in a number of tissues (Fig. 3-8) indicates that the function of the protein may not be limited to the regulation of stress-induced PA metabolism. PAs can impact the ecosystems in which poplars are dominant species, influencing what Whitham et al. (2006) have termed community and ecosystem phenotypes: the effects of genes at levels higher than the population, resulting from inter-specific, indirect genetic effects. The identification of transcriptional regulators that control the level of PA pathway activation, both constitutively and in response to environmental conditions, is therefore a first step toward linking the expression of individual genes with community and ecosystem phenotypes.

The extensive genomics resources combined with the complexity and biological importance of phenylpropanoid metabolism in poplar make it a useful system for investigating the PA pathway. Identifying transcriptional regulators of biosynthetic pathway genes is an important goal for metabolic engineering of secondary metabolism in plants, and the identification of a putative regulator of PA metabolism in poplar may permit new experimental approaches for evaluating the biological functions of PAs.

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Appendix A

Supplementary Tables, Data and Figures

This appendix includes the following supplementary material:

Tables A1-1 to **A1-3** give primer sequences used to PCR-amplify DNA sequences used for probe synthesis in northern analyses, for cloning into plant transformation vectors, or for real time PCR analysis.

Data A1-1 gives the CDS sequences of MYB134, MYB183, and MYB097, which were cloned into plant transformation vectors. Sequencing was performed by the Centre for Biomedical Research Sequencing Facility (University of Victoria). Also included are vector maps for pRD400-MYB134, pCAMBIA1305.1-MYB183, and pCAMBIA1305.1-MYB097 generated using Vector NTI Advance (Invitrogen, Carlsbad, CA).

Table A1-4 presents the list of significantly deregulated genes in MYB134-overexpressing leaves as determined by Affymetrix GeneChip® Poplar Genome Array analysis. Up-regulated genes are given first, in order of decreasing fold-induction, followed by down-regulated genes. Putative gene functions are indicated by the results of blastx against the arabidopsis genome database (TAIR, http://www.arabidopsis.org/).

Table A1-5 presents the sequences of putative MYB and BHLH binding sites within the 2 kb promoter sequences of putative MYB134 target genes, corresponding to the data summarized in Tables 3-4 and 3-9.

Table A1-1. Primer sequences used to amplify transcript sequences for use in probe synthesis for northern analysis.

Gene	Identifier	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
PAL1	AF480620[a]	TGGAATTCTGTCAGGACTCATGC	CGTTGAAGCCCATTGTCAGG
4CL2	AF041049[a]	CATCTTTCGCTCAAAATTACC	AAAGCTCATCATCGTCATCG
CHS1	572875[b]	ATGGCACCGTCGATTGAGGA	TTATATGCTTATAAAAATA
CHI1	724846[b]	ATGTCTCCTGCAGTGCCTCTCTCC	TCAGTTCCCATTTTCATTA
F3H	836585[b]	ATGGCCCCTTCTACCCTCACA	TCAAGCAAGGATCTCCTCA
FLS4	828087[b]	ATGGAGTTTGATAGAGTTCA	CTACTGGGGAAGCTTATTG
ANS1	646527[b]	ATGATGGTGACTTCATCAT	TTAATTAGCTTTTCTGGGT
ANR1	831060[b]	ATGGCATCCCAGTTGACCAAAA	GAAGCTTATTCAGCAATCCTT
LAR1	656768[b]	CTGTTTCAGCAGCTTCCCC	CGGAGCTCGCAAAATCATCGA
LAR3	835080[b]	ATGAATGGTCATTCTCCAAATGC	CATGCTGTAATAAATAAAGC
CCR1	AF217958[a]	GTTGATGCTTCATCACTTTCAGG	ATTTTCACAGACTCTTCTGC

[a]Genbank Accession number; [b]JGI *Populus trichocarpa v1.1* protein ID

Table A1-2. Primer sequences used to amplify MYB sequences for use in the cloning of plant transformation vectors.

Gene	Identifier[b]	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
MYB134	819461	CGGCCGGTTGTGGAAGTGCGCGTGTGTGG	CCGCGGTCAATTAAGACATTCTACAAGTG
MYB097	583166	CGGCCGATACACACTATCAATAATAAAAGCC	CCGCGGTTCAAACTCGAGAACTATTAGCC
MYB183	717084	CGGCCGTTGAGCTTTTGAGGAATATAGAG	CCGCGGTTCTAATTGGAATTCTTATGTTC

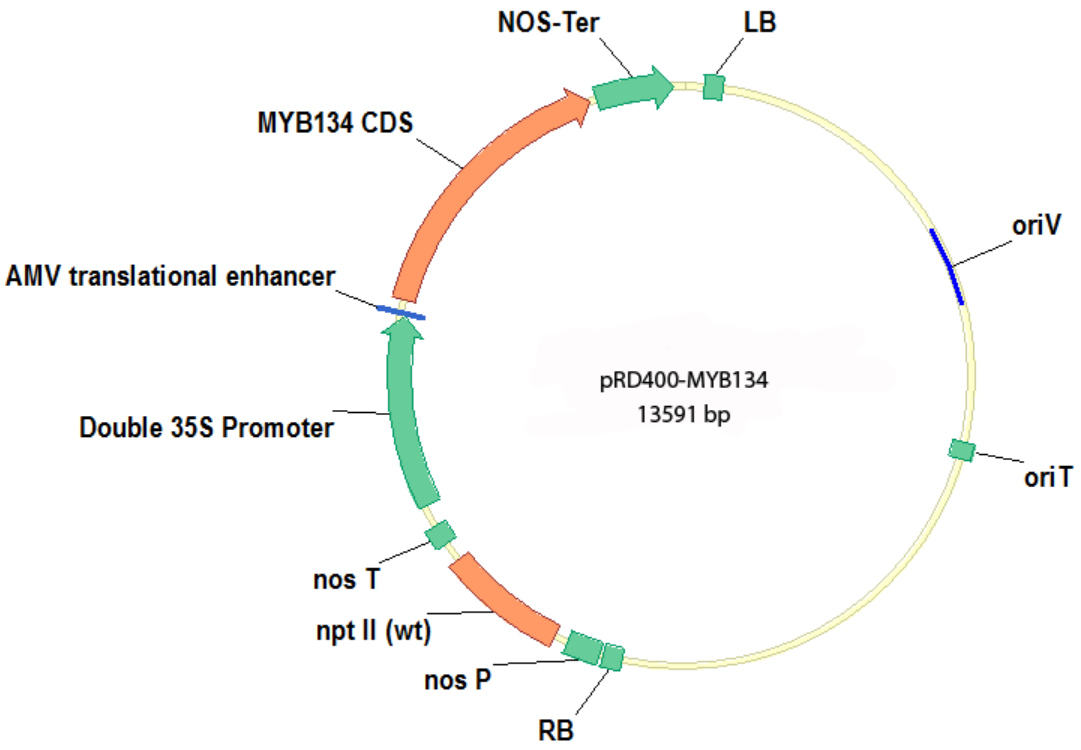
[b]**JGI *Populus trichocarpa v1.1* protein ID**

Table A1-3. Primer sequences used for real-time PCR analysis of poplar MYB genes.

Gene	Identifier	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
MYB097	583166[b]	GAAAATGAATCATCAAGCAAGG	GCAATGACATGAAATCAAAACC
MYB134	819461[b]	ATTCCCAGAGCTTTAAAAAAC	ACCTCCATCACTCGGTTGAAAC
MYB183	717084[b]	TTCCTTCACCACCAGTCTTGG	TTCTGCAGCATAGCCTCGG
MYB086	668844[b]	AAAGCTCTCAAAGAAAATC	GTCTCCGTCTATATCATTT
ACTIN	BU823250[a]	TCTCTAGATATCTCCCTCGTCTCC	AACACGCAGCTCATTGTAGAAAGTG

[a]**Genbank Accession number;** [b]**JGI *Populus trichocarpa v1.1* protein ID**

Supplemental Data A1-1. Vector maps for the plant transformation vectors, pRD400-MYB134, pCAMBIA1305.1-MYB183, and pCAMBIA1305.1-MYB097, as well as CDS sequences of poplar MYB134, MYB183, and MYB097. Vector maps generated using Vector NTI Advance (Invitrogen, Carlsbad, CA).



Poplar *MYB134* Complete CDS:

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GAGTTTCAACCGAGTGATGGAGGTACACTAATTGACAATGATTGTGACAAGAATCTCTCCATT
GGTTCTTACCATTCCTTAGCCTTATCTGATGACCTAATGTTCAAGGATTGGGCCCTGAATCGTT
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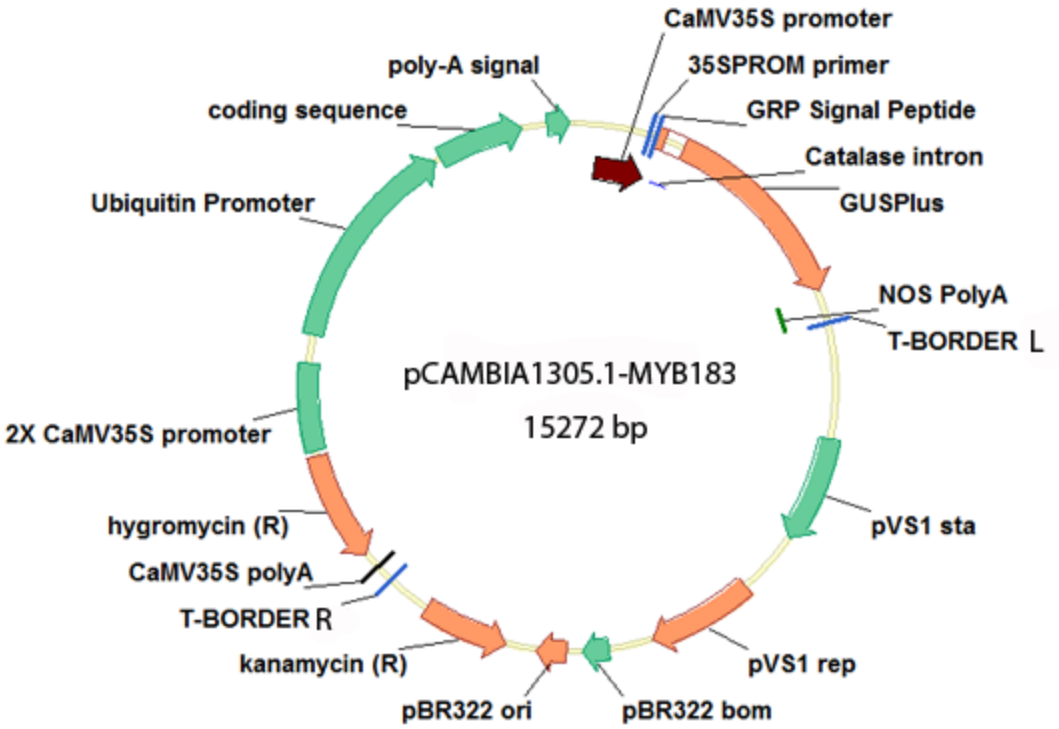
GGCCTGAGATGAGACATTGA

Continued...

Supplemental Data A1-1, continued.



Poplar *MYB134* Complete CDS:

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TAAAAGAGGAAACATTAGCCCAGATGAAGAAGATCTCATTATTAGGCTTCACAAGCTCTTAG
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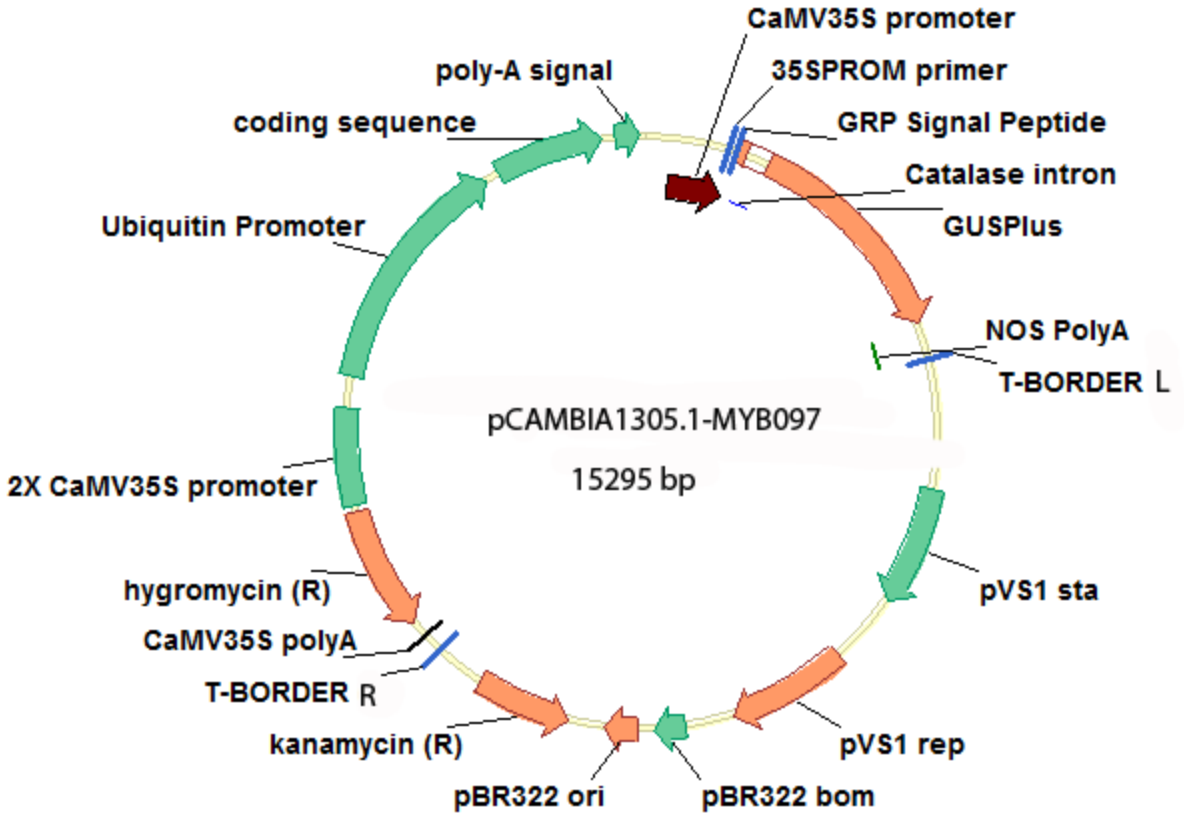
Continued...

Supplemental Data A1-1, continued.



Poplar *MYB097* Complete CDS:

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CACACATCATGAAAATGAATCATCAAGCAAGGGGTTAGCATCTGGGGATAATGACAATTTGTC
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Table A1-4. All significantly deregulated probesets from Affymetrix GeneChip® Poplar Genome Array microarray analysis of *MYB134*-overexpressor leaves (fold change relative to wild type control). Table is ranked in order of decreasing fold change for up-regulated then down-regulated genes.

Affy Probeset	AGI accession (TAIR Blastx)	Putative Function (TAIR Blastx)	Score (TAIR Blastx)	E-value (TAIR Blastx)	Fold change	P-value
Up-regulated probesets						
Ptp.8030.1.S1_s_at	AT5G48810.1	ATB5-B (Cytochrome b5 B)	108	2.00E-24	**163.2825**	3.53E-08
PtpAffx.7896.4.A1_a_at	AT5G13930.1	CHS (CHALCONE SYNTHASE); naringenin-chalcone synthase	671	0	**93.76929**	3.53E-09
PtpAffx.18705.2.A1_a_at	AT1G75290.1	isoflavone reductase, putative	247	8.00E-66	**60.59533**	2.60E-11
Ptp.8030.1.S1_at	AT5G48810.1	ATB5-B (Cytochrome b5 B)	108	2.00E-24	**48.04867**	5.83E-09
Ptp.3138.2.A1_a_at	AT5G17050.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	358	9.00E-99	**45.80518**	3.18E-09
PtpAffx.161181.1.S1_at	AT4G30470.1	cinnamoyl-CoA reductase-related	399	1.00E-111	**45.06795**	9.29E-09
PtpAffx.7896.2.S1_at	AT5G13930.1	CHS (CHALCONE SYNTHASE); naringenin-chalcone synthase	329	1.00E-90	**41.1308**	1.88E-11
PtpAffx.37082.1.A1_at	AT5G42800.1	DFR (DIHYDROFLAVONOL 4-REDUCTASE); dihydrokaempferol 4-reductase	509	1.00E-144	**39.54028**	1.01E-09
PtpAffx.6065.2.S1_at	AT1G75280.1	isoflavone reductase, putative	234	6.00E-62	**38.84089**	5.78E-11
PtpAffx.30659.1.A1_at	AT3G13540.1	ATMYB5 (myb domain protein 5); DNA binding / transcription factor	213	4.00E-56	**35.30179**	6.23E-12
PtpAffx.120325.1.S1_s_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	696	0	**31.5149**	4.44E-08
Ptp.6057.1.S1_at	AT4G22880.2	LDOX (TANNIN DEFICIENT SEED 4)	523	1.00E-149	**30.7466**	6.57E-06
PtpAffx.5092.1.A1_at	AT1G61720.1	BAN (BANYULS)	448	1.00E-126	**28.37731**	1.02E-10
PtpAffx.142603.1.A1_s_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	696	0	**27.05721**	1.41E-08
PtpAffx.7896.3.S1_a_at	AT5G13930.1	CHS (CHALCONE SYNTHASE); naringenin-chalcone synthase	670	0	**26.52301**	1.09E-07
Ptp.4863.1.S1_s_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	696	0	**26.5219**	5.64E-09
PtpAffx.5092.2.S1_a_at	AT1G61720.1	BAN (BANYULS)	429	1.00E-120	**26.07658**	6.18E-09
PtpAffx.224485.1.S1_s_at	AT3G59030.1	TT12 (TRANSPARENT TESTA 12); antiporter/ transporter	680	0	**23.98349**	1.60E-08
Ptp.1080.1.S1_s_at	AT1G75290.1	isoflavone reductase, putative	234	1.00E-61	**21.70258**	8.35E-08
PtpAffx.6065.3.A1_a_at	AT1G75290.1	isoflavone reductase, putative	234	1.00E-61	**20.60694**	2.03E-08
PtpAffx.162989.1.S1_at	AT5G17050.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	358	9.00E-99	**19.52091**	4.53E-09

Continued...

Table A1-4, continued.

PtpAffx.224252.1.S1_at	AT3G13540.1	ATMYB5 (myb domain protein 5); DNA binding / transcription factor	213	4.00E-56	**18.92459**	2.45E-08
Ptp.1080.1.S1_at	AT1G75290.1	isoflavone reductase, putative	234	1.00E-61	**18.58204**	2.72E-08
PtpAffx.25553.1.A1_at	AT5G42800.1	DFR (DIHYDROFLAVONOL 4-REDUCTASE); dihydrokaempferol 4-reductase	508	1.00E-144	**16.56918**	3.02E-08
Ptp.323.1.S1_s_at	AT3G51240.1	F3H (TRANSPARENT TESTA 6); naringenin 3-dioxygenase	573	1.00E-164	**14.08211**	2.11E-07
Ptp.6753.1.S1_s_at	AT4G24380.1	hydrolase, acting on ester bonds	186	9.00E-48	**10.65858**	8.80E-07
PtpAffx.160113.1.A1_s_at	AT1G55320.1	ligase	875	0	**10.2984**	6.46E-08
PtpAffx.83404.1.A1_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	494	1.00E-140	**10.19621**	1.32E-08
PtpAffx.7740.2.A1_a_at	AT2G23910.1	cinnamoyl-CoA reductase-related	421	1.00E-118	**8.764517**	4.24E-08
Ptp.6057.1.S1_s_at	AT4G22880.2	LDOX (TANNIN DEFICIENT SEED 4)	523	1.00E-149	**8.497026**	5.76E-07
PtpAffx.8131.6.A1_a_at	AT3G63460.2	EMB2221 (EMBRYO DEFECTIVE 2221)	30.4	0.99	**8.147173**	1.36E-08
PtpAffx.222060.1.S1_at	AT5G59950.3	RNA and export factor-binding protein, putative	32.3	0.16	**8.005913**	7.65E-07
PtpAffx.8131.4.A1_a_at	AT2G16720.1	MYB7 (myb domain protein 7); DNA binding / transcription factor	189	4.00E-49	**7.970179**	2.93E-08
PtpAffx.224602.1.S1_at	AT1G22640.1	MYB3 (myb domain protein 3); DNA binding / transcription factor	218	2.00E-57	**7.959251**	1.05E-08
Ptp.4458.1.S1_s_at	AT3G62760.1	ATGSTF13 (Arabidopsis thaliana Glutathione S-transferase (class phi) 13); glutathione transferase	298	5.00E-81	**7.807163**	8.94E-09
Ptp.5716.1.S1_at	AT1G34580.1	monosaccharide transporter, putative	670	0	**7.806562**	6.36E-09
PtpAffx.249.83.A1_x_at	AT3G10810.1	zinc finger (C3HC4-type RING finger) family protein	30	0.86	**7.356721**	1.01E-05
PtpAffx.94822.1.A1_at	AT3G59030.1	TT12 (TRANSPARENT TESTA 12); antiporter/ transporter	713	0	**6.795036**	9.86E-09
PtpAffx.54821.1.A1_at	AT4G24380.1	hydrolase, acting on ester bonds	186	9.00E-48	**6.690092**	7.40E-07
PtpAffx.133554.1.A1_s_at	AT1G36160.1	ACC1 (ACETYL-COENZYME A CARBOXYLASE 1); acetyl-CoA carboxylase	3619	0	**6.662597**	1.95E-09
PtpAffx.156161.1.A1_at	AT5G47470.1	nodulin MtN21 family protein	402	1.00E-112	**6.490875**	2.97E-08
PtpAffx.86545.1.S1_at	AT5G48670.1	AGL80/FEM111 (AGAMOUS-LIKE80); DNA binding / transcription factor	28.9	1.1	**6.242573**	2.88E-07
Ptp.6711.1.S1_s_at	AT5G13930.1	CHS (CHALCONE SYNTHASE); naringenin-chalcone synthase	655	0	**6.098758**	1.07E-05
PtpAffx.157838.2.S1_at	AT5G06640.1	proline-rich extensin-like family protein	33.5	0.11	**5.818017**	1.09E-08
PtpAffx.30128.1.S1_at	AT2G23910.1	cinnamoyl-CoA reductase-related	259	3.00E-69	**5.577246**	3.45E-06
PtpAffx.249.102.A1_x_at	AT3G10810.1	zinc finger (C3HC4-type RING finger) family protein	30	0.86	**5.553599**	2.36E-06
PtpAffx.224746.1.S1_s_at	AT1G09850.1	XBCP3 (XYLEM BARK CYSTEINE PEPTIDASE 3); cysteine-type peptidase	368	1.00E-102	**5.42057**	4.17E-08
PtpAffx.127289.1.A1_at	AT1G12910.1	ATAN11 (ANTHOCYANIN11); nucleotide binding	389	1.00E-108	**5.219675**	3.81E-07
Ptp.2833.1.S1_at	AT3G13010.1	hAT dimerisation domain-containing protein	28.9	0.73	**5.208285**	5.40E-09
PtpAffx.205491.1.S1_at	AT1G36160.1	ACC1 (ACETYL-COENZYME A CARBOXYLASE 1); acetyl-CoA	3619	0	**5.18456**	5.48E-09

Continued...

Table A1-4, continued.

		carboxylase				
PtpAffx.4850.1.A1_s_at	AT3G55120.1	TT5 (TRANSPARENT TESTA 5); chalcone isomerase	297	8.00E-81	**4.916252**	1.50E-07
Ptp.1512.1.S1_s_at	AT5G05270.2	chalcone-flavanone isomerase family protein	274	5.00E-74	**4.647331**	1.66E-08
PtpAffx.213439.1.S1_at	AT1G12910.1	ATAN11 (ANTHOCYANIN11); nucleotide binding	346	2.00E-95	**4.619583**	5.54E-08
PtpAffx.160901.1.S1_s_at	AT1G09850.1	XBCP3 (XYLEM BARK CYSTEINE PEPTIDASE 3); cysteine-type peptidase	371	1.00E-103	**4.199139**	7.42E-06
PtpAffx.205684.1.S1_at	AT4G09820.1	TT8 (TRANSPARENT TESTA 8); DNA binding / transcription factor	502	1.00E-142	**3.98721**	3.06E-08
PtpAffx.65824.1.A1_at	AT5G56350.1	pyruvate kinase, putative	870	0	**3.930059**	6.03E-07
PtpAffx.19596.1.S1_at	AT1G60990.2	similar to aminomethyltransferase, putative [Arabidopsis thaliana] (TAIR:AT1G11860.2); similar to COG0354: Predicted aminomethyltransferase related to GcvT [Nostoc punctiforme PCC 73102] (GB:ZP_00106552.1); contains InterPro domain Glycine cleavage T protein (aminomethyl transferase); (InterPro:IPR006222)	32	0.26	**3.924715**	3.93E-07
PtpAffx.33535.2.A1_at	AT2G16430.2	PAP10; acid phosphatase/ protein serine/threonine phosphatase	710	0	**3.833496**	1.18E-07
PtpAffx.5685.1.S1_x_at	AT1G40115.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G09900.1)	28.1	2.2	**3.644127**	5.30E-08
PtpAffx.224650.1.S1_s_at	AT4G38620.1	MYB4 (myb domain protein 4); transcription factor	248	2.00E-66	**3.551604**	7.53E-08
PtpAffx.256.2.S1_at	AT4G09820.1	TT8 (TRANSPARENT TESTA 8); DNA binding / transcription factor	502	1.00E-142	**3.536714**	3.18E-08
PtpAffx.137746.1.S1_at	AT1G22640.1	MYB3 (myb domain protein 3); DNA binding / transcription factor	224	1.00E-59	**3.533906**	4.79E-08
Ptp.8024.1.S1_at	AT4G09820.1	TT8 (TRANSPARENT TESTA 8); DNA binding / transcription factor	103	6.00E-23	**3.51615**	1.96E-05
PtpAffx.200035.1.S1_at	AT1G55320.1	ligase	875	0	**3.402119**	6.70E-07
PtpAffx.12056.3.S1_a_at	AT1G65060.1	4CL3 (4-coumarate:CoA ligase 3); 4-coumarate-CoA ligase	756	0	**3.400402**	2.30E-07
PtpAffx.30743.1.A1_a_at	AT3G06760.1	similar to HRB1 (HYPERSENSITIVE TO RED AND BLUE) [Arabidopsis thaliana] (TAIR:AT5G49230.1); similar to fiber protein Fb2 [Gossypium barbadense] (GB:AAN77145.1); contains InterPro domain Drought induced 19; (InterPro:IPR008598)	207	1.00E-53	**3.343861**	6.27E-07
PtpAffx.225544.1.S1_s_at	AT2G40890.1	CYP98A3 (cytochrome P450, family 98, subfamily A, polypeptide 3); p-coumarate 3-hydroxylase	785	0	**3.332724**	5.95E-08
PtpAffx.30743.5.A1_at	AT2G45430.1	DNA-binding protein-related	27.7	5.8	**3.260588**	2.46E-05
PtpAffx.224878.1.S1_at	AT4G09460.1	ATMYB6 (myb domain protein 6, myb domain protein 8); DNA binding / transcription factor	209	1.00E-54	**3.206161**	1.42E-06
PtpAffx.5685.1.S1_a_at	AT1G40115.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G09900.1)	28.1	2.2	**3.169091**	4.97E-08
PtpAffx.47237.2.A1_a_at	AT3G47110.1	leucine-rich repeat transmembrane protein kinase, putative	30.4	0.74	**3.162288**	2.98E-07
PtpAffx.200755.1.S1_s_at	AT1G07380.1	ceramidase family protein	437	1.00E-123	**3.159078**	1.75E-05

Continued...

Table A1-4, continued.

PtpAffx.224381.1.S1_at	AT3G16520.3	UDP-glucoronosyl/UDP-glucosyl transferase family protein	512	1.00E-145	**3.122538**	8.07E-06
PtpAffx.157838.2.S1_a_at	AT5G06640.1	proline-rich extensin-like family protein	33.5	0.11	**3.066728**	2.35E-07
PtpAffx.47186.1.S1_at	AT3G52520.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G06280.3)	45.8	2.00E-05	**3.045628**	3.95E-07
PtpAffx.157838.1.S1_at	AT2G39760.1	ATBPM3; protein binding	28.9	4.4	**3.045317**	1.54E-05
PtpAffx.116734.1.A1_at	AT5G48657.2	defense protein-related	28.9	0.73	**3.042292**	0.000125998
Ptp.6632.1.S1_at	AT2G30490.1	ATC4H (CINNAMATE-4-HYDROXYLASE)	271	3.00E-73	**3.040395**	0.000111067
PtpAffx.83148.2.S1_s_at	AT5G49460.1	ACLB-2 (ATP-citrate lyase B-2)	1111	0	**2.984255**	6.61E-05
PtpAffx.211755.1.S1_at	AT4G02370.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G02816.1); similar to Os05g0362300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001055314.1); similar to Protein of unknown function, DUF538 [Oryza sativa (japonica cultivar-group)] (GB:AAX95409.1)	166	5.00E-42	**2.964537**	7.16E-07
Ptp.7328.1.A1_at	AT1G02816.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G02370.1); similar to Os05g0362300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001055314.1); similar to Protein of unknown function, DUF538 [Oryza sativa (japonica cultivar-group)] (GB:AAX95409.1)	206	6.00E-54	**2.944385**	1.35E-07
PtpAffx.206509.1.S1_at	AT2G24270.1	ALDH11A3 (Aldehyde dehydrogenase 11A3); 3-chloroallyl aldehyde dehydrogenase/ glyceraldehyde-3-phosphate dehydrogenase (NADP+)	923	0	**2.842202**	4.87E-07
Ptp.3325.3.A1_at	AT3G13540.1	ATMYB5 (myb domain protein 5); DNA binding / transcription factor	159	3.00E-39	**2.791553**	1.84E-06
PtpAffx.204062.1.S1_at	AT5G14700.1	cinnamoyl-CoA reductase-related	203	1.00E-52	**2.734055**	3.11E-06
PtpAffx.215434.1.S1_s_at	AT5G14700.1	cinnamoyl-CoA reductase-related	227	1.00E-59	**2.733339**	0.000102655
PtpAffx.28572.2.S1_at	AT5G56350.1	pyruvate kinase, putative	870	0	**2.648535**	6.81E-07
Ptp.2332.2.A1_a_at	AT1G60810.1	ACLA-2 (ATP-citrate lyase A-2)	707	0	**2.634274**	2.32E-06
Ptp.5940.1.S1_at	AT2G40890.1	CYP98A3 (cytochrome P450, family 98, subfamily A, polypeptide 3); p-coumarate 3-hydroxylase	759	0	**2.619854**	8.22E-07
PtpAffx.31264.2.S1_a_at	AT5G53200.1	TRY (TRIPTYCHON); DNA binding / transcription factor	106	7.00E-24	**2.587292**	2.04E-05
PtpAffx.6692.7.S1_s_at	AT5G01450.1	protein binding / zinc ion binding	134	1.00E-31	**2.550969**	9.66E-07
PtpAffx.224381.1.S1_s_at	AT3G16520.3	UDP-glucoronosyl/UDP-glucosyl transferase family protein	506	1.00E-143	**2.546381**	2.06E-05
Ptp.4362.1.S1_at	AT1G22360.2	UDP-glycosyltransferase	281	3.00E-76	**2.544274**	2.14E-06
PtpAffx.220100.1.S1_at	AT4G34135.1	UGT73B2; UDP-glucosyltransferase/ UDP-glycosyltransferase/ flavonol 3-O-glucosyltransferase	90.9	1.00E-18	**2.52905**	2.68E-06
PtpAffx.39332.1.A1_s_at	AT1G09850.1	XBCP3 (XYLEM BARK CYSTEINE PEPTIDASE 3); cysteine-type peptidase	371	1.00E-103	**2.526891**	1.36E-06
PtpAffx.221214.1.S1_s_at	AT5G01450.1	protein binding / zinc ion binding	134	1.00E-31	**2.522584**	1.53E-06

Continued...

Table A1-4, continued.

PtpAffx.158517.1.S1_at	AT1G09430.1	ACLA-3 (ATP-citrate lyase A-3)	770	0	**2.512851**	4.37E-05
Ptp.7600.1.S1_at	AT4G12750.1	DNA binding / sequence-specific DNA binding / transcription factor	26.9	8	**2.506107**	6.77E-05
PtpAffx.13946.1.A1_at	AT4G33625.1	similar to conserved hypothetical protein [Medicago truncatula] (GB:ABD28544.1)	232	7.00E-62	**2.492587**	3.81E-07
PtpAffx.162546.1.A1_at	AT2G47460.1	MYB12 (myb domain protein 12); DNA binding / transcription factor/ transcriptional activator	209	2.00E-54	**2.491668**	5.11E-05
PtpAffx.1150.1.A1_s_at	AT1G36160.1	ACC1 (ACETYL-COENZYME A CARBOXYLASE 1); acetyl-CoA carboxylase	3663	0	**2.488185**	3.76E-05
Ptp.336.1.S1_at	AT2G30490.1	ATC4H (CINNAMATE-4-HYDROXYLASE)	795	0	**2.481498**	1.26E-05
PtpAffx.215434.1.S1_at	AT5G14700.1	cinnamoyl-CoA reductase-related	227	1.00E-59	**2.477577**	1.73E-06
Ptp.4670.1.S1_s_at	AT5G49460.1	ACLB-2 (ATP-citrate lyase B-2)	1111	0	**2.45687**	1.33E-05
PtpAffx.134162.1.A1_at	AT5G24760.1	alcohol dehydrogenase, putative	608	1.00E-174	**2.456086**	6.93E-06
Ptp.7408.1.A1_a_at	AT2G38010.2	ceramidase family protein	474	1.00E-134	**2.432498**	1.07E-06
PtpAffx.225544.1.S1_x_at	AT2G40890.1	CYP98A3 (cytochrome P450, family 98, subfamily A, polypeptide 3); p-coumarate 3-hydroxylase	759	0	**2.424882**	1.43E-06
PtpAffx.218534.1.S1_at	AT5G01450.1	protein binding / zinc ion binding	70.1	4.00E-13	**2.387301**	4.57E-06
PtpAffx.223835.1.S1_s_at	AT3G59310.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G59340.1); similar to Os05g0299500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001055130.1); similar to Os08g0540000 [Oryza sativa (japonica cultivar-group)] (GB:NP_001062380.1); similar to anthocyanin-related membrane protein 1 (Anm1)-like [Oryza sativa (japonica cultivar-group)] (GB:BAD34418.1)	276	8.00E-75	**2.368962**	1.06E-05
Ptp.5131.1.S1_at	AT1G22360.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	610	1.00E-175	**2.336465**	4.80E-05
PtpAffx.108534.1.A1_at	AT5G13930.1	CHS (CHALCONE SYNTHASE); naringenin-chalcone synthase	655	0	**2.29474**	8.49E-05
Ptp.6482.1.S1_s_at	AT1G36160.1	ACC1 (ACETYL-COENZYME A CARBOXYLASE 1); acetyl-CoA carboxylase	3663	0	**2.291337**	3.32E-06
PtpAffx.47237.3.S1_at	AT3G47110.1	leucine-rich repeat transmembrane protein kinase, putative	30.4	0.74	**2.286676**	7.24E-06
PtpAffx.148416.1.S1_s_at	AT1G08470.1	strictosidine synthase family protein	634	0	**2.26187**	0.000114775
PtpAffx.221212.1.S1_at	AT5G01450.1	protein binding / zinc ion binding	134	1.00E-31	**2.261332**	1.34E-06
PtpAffx.160133.2.A1_a_at	AT5G09360.1	LAC14 (laccase 14); copper ion binding / oxidoreductase	28.1	6.3	**2.248776**	5.70E-05
Ptp.4863.2.A1_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	315	2.00E-86	**2.246907**	6.53E-06
Ptp.7408.3.A1_at	AT1G07380.1	ceramidase family protein	314	4.00E-86	**2.234739**	1.63E-05
PtpAffx.31264.2.S1_at	AT5G53200.1	TRY (TRIPTYCHON); DNA binding / transcription factor	106	7.00E-24	**2.230327**	5.30E-05
PtpAffx.204049.1.S1_s_at	AT3G16520.3	UDP-glucoronosyl/UDP-glucosyl transferase family protein	506	1.00E-143	**2.226384**	0.00011422
PtpAffx.157532.1.S1_at	AT3G21760.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	356	2.00E-98	**2.215981**	3.96E-06

Continued...

Table A1-4, continued.

PtpAffx.158559.1.S1_at	AT1G08230.2	similar to amino acid transporter family protein [Arabidopsis thaliana] (TAIR:AT5G41800.1); similar to Os05g0586500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001056462.1); similar to Amino acid/polyamine transporter II [Medicago truncatula] (GB:ABE81500.1)	582	1.00E-166	**2.205333**	3.41E-06
PtpAffx.106733.1.A1_at	AT1G06550.1	enoyl-CoA hydratase/isomerase family protein	573	1.00E-164	**2.16464**	3.36E-06
PtpAffx.155350.1.S1_s_at	AT1G47128.1	cysteine proteinase (RD21A) / thiol protease	426	1.00E-119	**2.148938**	2.57E-05
PtpAffx.4938.1.S1_s_at	AT4G32670.1	protein binding / zinc ion binding	29.3	1.4	**2.146505**	6.51E-06
PtpAffx.163838.2.S1_a_at	AT2G01140.1	fructose-bisphosphate aldolase, putative	677	0	**2.130365**	0.000168611
PtpAffx.77835.1.S1_at	AT5G57340.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G67390.2); similar to conserved hypothetical protein [Medicago truncatula] (GB:ABE83997.1)	28.1	4.3	**2.097136**	5.33E-05
Ptp.5443.1.S1_s_at	AT1G12000.1	pyrophosphate--fructose-6-phosphate 1-phosphotransferase beta subunit, putative / pyrophosphate-dependent 6-phosphofructose-1-kinase, putative	941	0	**2.093986**	3.86E-06
PtpAffx.221492.1.S1_at	AT3G59310.1	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G59340.1); similar to Os05g0299500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001055130.1); similar to Os08g0540000 [Oryza sativa (japonica cultivar-group)] (GB:NP_001062380.1); similar to anthocyanin-related membrane protein 1 (Anm1)-like [Oryza sativa (japonica cultivar-group)] (GB:BAD34418.1)	276	8.00E-75	**2.093613**	5.25E-06
Ptp.1110.1.A1_s_at	AT1G79840.1	GL2 (GLABRA 2); DNA binding / transcription factor	900	0	**2.087409**	4.95E-05
PtpAffx.211115.1.S1_at	AT3G03620.1	MATE efflux family protein	480	1.00E-136	**2.087289**	6.71E-06
PtpAffx.134527.1.A1_at	AT1G28110.2	SCPL45; serine carboxypeptidase	392	1.00E-109	**2.086066**	1.55E-05
Ptp.2303.1.S1_at	AT1G08470.1	strictosidine synthase family protein	634	0	**2.08107**	1.04E-05
Ptp.3935.2.S1_a_at	AT5G64500.1	membrane protein-related	584	1.00E-167	**2.060216**	1.48E-05
PtpAffx.83148.1.S1_s_at	AT5G49460.1	ACLB-2 (ATP-citrate lyase B-2)	1111	0	**2.055867**	0.00013649
Ptp.1405.1.S1_at	AT2G47590.1	PHR2 (PHOTOLYASE/BLUE-LIGHT RECEPTOR 2)	545	1.00E-155	**2.05493**	5.56E-06
PtpAffx.138813.1.A1_s_at	AT1G09430.1	ACLA-3 (ATP-citrate lyase A-3)	766	0	**2.034481**	2.06E-05
PtpAffx.220057.1.S1_x_at	No hits found	No hits found	No hits found	No hits found	**2.021024**	6.08E-05
PtpAffx.51055.1.S1_at	AT5G60550.1	GRIK2 (GEMINIVIRUS REP INTERACTING KINASE 2); kinase	278	3.00E-75	**2.020855**	1.09E-05
PtpAffx.53470.1.S1_at	AT1G12050.1	fumarylacetoacetase, putative	738	0	**2.018855**	1.77E-05
PtpAffx.155350.1.S1_at	AT1G47128.1	cysteine proteinase (RD21A) / thiol protease	426	1.00E-119	**2.005884**	0.000185514
Down-regulated probesets	**AGI accession (TAIR Blastx)**	**Putative funciton (TAIR Blastx)**	**Score (TAIR**	**E-value (TAIR Blastx)**	**Fold change**	P-value

Continued...

Table A1-4, continued.

			Blastx)			
PtpAffx.18588.1.S1_at	ATCG00820.1	Encodes a 6.8-kDa protein of the small ribosomal subunit.	171	5.00E-43	**0.1696292**	2.34E-06
PtpAffx.77318.2.S1_at	AT3G12500.1	ATHCHIB (BASIC CHITINASE); chitinase	490	1.00E-139	**0.1700151**	6.13E-05
Ptp.4185.1.S1_at	AT5G49620.1	AtMYB78 (myb domain protein 78); DNA binding / transcription factor	30	1.4	**0.2409641**	3.26E-05
Ptp.2463.1.S1_s_at	AT3G04720.1	PR4 (PATHOGENESIS-RELATED 4)	306	9.00E-84	**0.2563843**	0.000177512
Ptp.4093.1.S1_at	AT5G17540.1	transferase family protein	531	1.00E-151	**0.2709191**	1.09E-05
PtpAffx.77318.3.S1_at	AT3G12500.1	ATHCHIB (BASIC CHITINASE); chitinase	490	1.00E-139	**0.2747035**	0.000103396
PtpAffx.6696.2.S1_at	AT5G17540.1	transferase family protein	529	1.00E-150	**0.2818457**	0.000137222
PtpAffx.67540.1.A1_s_at	AT5G54010.1	glycosyltransferase family protein	456	1.00E-128	**0.2843505**	4.49E-07
PtpAffx.60429.2.S1_a_at	AT2G14260.1	PIP (proline iminopeptidase); prolyl aminopeptidase	28.5	2.7	**0.3089584**	1.17E-05
PtpAffx.218021.1.S1_at	AT5G54010.1	glycosyltransferase family protein	456	1.00E-128	**0.3102446**	1.18E-06
PtpAffx.606.6.S1_a_at	AT4G22670.1	tetratricopeptide repeat (TPR)-containing protein	334	3.00E-92	**0.3362458**	6.86E-07
Ptp.4381.1.S1_s_at	AT5G07990.1	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	160	1.00E-39	**0.3390552**	3.49E-05
PtpAffx.249.47.A1_s_at	AT4G11650.1	ATOSM34 (OSMOTIN 34)	351	4.00E-97	**0.3407215**	8.80E-05
Ptp.2155.1.S1_at	AT1G04210.1	leucine-rich repeat family protein / protein kinase family protein	28.1	3.2	**0.3501214**	7.67E-06
PtpAffx.60092.1.S1_at	AT2G01180.1	ATPAP1 (PHOSPHATIDIC ACID PHOSPHATASE 1); phosphatidate phosphatase	29.6	0.79	**0.3545247**	3.70E-05
PtpAffx.216301.1.S1_at	AT1G55740.1	ATSIP1 (ARABIDOPSIS THALIANA SEED IMBIBITION 1); hydrolase, hydrolyzing O-glycosyl compounds	1183	0	**0.359709**	5.00E-05
Ptp.2463.1.S1_a_at	AT3G04720.1	PR4 (PATHOGENESIS-RELATED 4)	306	9.00E-84	**0.3652617**	0.000108637
Ptp.2155.1.S1_s_at	AT1G04210.1	leucine-rich repeat family protein / protein kinase family protein	28.1	3.2	**0.3789371**	1.31E-05
Ptp.1229.1.S1_at	AT1G01380.1	ETC1 (ENHANCER OF TRY AND CPC 1); DNA binding / transcription factor	70.5	1.00E-12	**0.3929548**	1.02E-05
PtpAffx.21205.4.S1_a_at	AT4G01350.1	DC1 domain-containing protein	29.6	0.42	**0.3941225**	2.21E-05
PtpAffx.225012.1.S1_at	AT1G07250.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	380	1.00E-105	**0.4002945**	5.39E-05
Ptp.1163.1.A1_at	AT5G54250.2	ATCNGC4 (DEFENSE, NO DEATH 2); calmodulin binding / cation channel/ cyclic nucleotide binding	26.9	2.8	**0.4122287**	0.000145404
PtpAffx.64603.1.S1_at	AT5G24090.1	acidic endochitinase (CHIB1)	353	9.00E-98	**0.4195738**	8.66E-06
Ptp.1094.1.S1_at	AT3G59010.1	pectinesterase family protein	440	1.00E-124	**0.4245334**	0.000145399
PtpAffx.2286.3.S1_a_at	AT1G17180.1	ATGSTU25 (Arabidopsis thaliana Glutathione S-transferase (class tau) 25); glutathione transferase	209	6.00E-55	**0.4334435**	7.81E-05
PtpAffx.153725.1.A1_at	AT3G13540.1	ATMYB5 (myb domain protein 5); DNA binding / transcription factor	228	5.00E-61	**0.4378649**	0.000154577
PtpAffx.249.26.A1_s_at	AT1G71695.1	peroxidase 12 (PER12) (P12) (PRXR6)	437	1.00E-123	**0.4588933**	1.19E-05

Continued...

Table A1-4, continued.

Ptp.1677.1.A1_at	AT3G16850.1	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	512	1.00E-145	**0.4635983**	0.000100802
PtpAffx.24353.1.A1_a_at	No hits found	No hits found	No hits found	No hits found	**0.4718032**	0.000150769
PtpAffx.216301.1.S1_s_at	AT1G55740.1	ATSIP1 (ARABIDOPSIS THALIANA SEED IMBIBITION 1); hydrolase, hydrolyzing O-glycosyl compounds	1183	0	**0.4854987**	9.00E-05
PtpAffx.25920.1.S1_a_at	AT1G58170.1	disease resistance-responsive protein-related / dirigent protein-related	55.8	1.00E-08	**0.4921322**	0.000150882
PtpAffx.26968.3.S1_a_at	AT5G41790.1	CIP1 (COP1-INTERACTIVE PROTEIN 1)	45.1	3.00E-05	**0.4924033**	1.06E-05
Ptp.4073.1.S1_s_at	AT4G02290.1	glycosyl hydrolase family 9 protein	786	0	**0.4971057**	0.000124914
PtpAffx.24353.2.A1_x_at	No hits found	No hits found	No hits found	No hits found	**0.497723**	0.000146199

Table A1-5. Sequences of putative MYB and BHLH protein binding sites in poplar phenylpropanoid and flavonoid gene 2 kb promoter regions as well as selected genes up-regulated by MYB134. (**) indicates gene up-regulated by PA-activating stress and/or in MYB134-overexpressing leaf tissue, while (*) indicates gene is -t MYB134-activated or associated with stress-induced PA metabolism. All sequences are from *P. trichocarpa* Nisqually 1 genome sequence.

	MYB				BHLH	
Gene	MYBCORE (CNGTTR)	L Box (ACCWWCC)	P Box (MACCWAMC)	MYBPZM (CCWACC)	G Box (CACGTG)	MYCCONSENSUS (CANNTG)
PAL1**	CTGTTA CAGTTA	ACCTACC ACCAACC (X2) ACCTAAT	CACCAACC	CCAACC	CACGTG	CACGTG CATTTG CATGTG CACATG CAATTG
PAL2*	CTGTTA (X4) CAGTTA CCGTTG	ACCAACC ACCTACA	CACCAACC	CCAACC (x2)	CACGTG (x2)	CACGTG CACTTG CAGCTG CACATG
PAL3*	CAGTTG	ACCAACC ACCTAAT	CACCAACC	CCAACC (X3) CCTACC	-	CATGTG CAACTG CATATG
4CL1 *	CCGTTA CAGTTG	ACCAACC	AACCAACC CACCAACC CACCAAAC	CCAACC	CACGTG	CACGTG CAGGTG CACCTG CAGTTG CAAATG CATATG
4CL2 **	CCGTTG CAGTTA CTGTTG CCGTTA	ACCAAAC ACCTAAC (X2) ACCTACC ACCAAAC	CACCAAAC AACCTAAC (X2) CACCTACC AACCAAAC	CCAACC CCTACC		CAAATG (X2) CATATG CATCTG CACTTG
C4H2**	CAGTTA	ACCTAAC (X2)	CACCAACC	CCAACC	-	CAATTG

Continued...

Table A1-5, continued.

	CCGTTA CTGTTG CAGTTG	ACCTTCC ACCAACC	AACCTAAC			CATTTG CAAGTG CATGTG CAGTTG
CHS1**	CTGTTA (X2) CAGTTA (X2) CAGTTG CTGTTG	ACCTACC	AACCTACC AACCAAAC* (X2)	CCTACC (X3) CCAACC (X2)	CACGTG	CATGTG CACGTG CAACTG
CHS2**	CGGTTA CAGTTA (X3) CGGTTG CTGTTA	ACCTAAC	CACCTAAC	CCAACC (X2) CCTACC	CACGTG	CACGTG
CHS3*	CCGTTG CAGTTG	ACCTACC	CACCTACC	CCAACC (X2) CCTACC	CACGTG (X2)	CACTTG CAACTG CAAATG CATATG CAGATG CATCTG CACGTG (X2) CATTTG
CHS4**	CCGTTG CTGTTG CCGTTA CAGTTG (X2) CGGTTG	ACCTAAC	AACCTAAC	CCAACC	CACGGT (X2)	CACGTG (X2) CAATTG CAACTG CAAGTG (X2) CAGTTG CAGCTG
CHS5*	CGGTTG CTGTTA CGGTTA (X2) CAGTTA CAGTTG	-	-	-	CACGTG	CAACTG (X2) CAGATG CAAGTG CATATG CAATTG

Continued...

Table A1-5, continued.

	CTGTTG					CAGCTG
CHS6**	CTGTTG CGGTTG CCGTTG (X2) CAGTTA (X2) CTGTTA CCGTTA	ACCAAAC	AACCAAAC	-	CACGTG (X2)	CACGTG (X2) CAATTG
F3H **	CGGTTA CAGTTG CGGTTG CCGTTG	ACCAAAC ACCAACC	AACCAAAC AACCAACC	CCAACC (X2)	-	CATATG CAGATG CATCTG (X2) CAAATG (X2) CAGTTG CATTTG
DFR1**	CGGTTA CCGTTA	ACCCAAC	-	CCTACC	-	CATATG CATCTG CATCTG
DFR2 **	CCGTTA (X3)	ATCTACC ACCTAAC ACCTACC (X2)	AACCTACC	CCTACC (X2)	CACGTG	CATGTG CACGTG CAATTG CAGCTG
ANR1**	CGGTTA CAGTTA CGGTTG CAGTTG	ACCTAAC ACCTACG ACCTACC ACCGACC	CACCTAAC	CCTACC	CACGTG	CATATG CAGATG CACGTG
ANR2**	CAGTTG CTGTTG CCGTTA	ACCAAAC ACCTAAC ACCAACC ACCTACA	AACCAAAC CACCTAAC AACCAACC	CCAACC (X2)	-	CAATTG CATTTG CAGATG CAAGTG CAGATG
LAR1**	CTGTTG CAGTTA (X2)	ACCAAAC ACCAACC	AACCTAAC AACCAACC	CCAACC (X4)	-	CAGCTG CACTTG

Continued...

Table A1-5, continued.

	CCGTTG CAGTTG	ACCTAAC				CATGTG CATCTG CAAATG CAACTG CAGTTG CAGATG
LAR2 **	CTGTTG CTGTTA CGGTTG CAGTTG CGGTTG	ACCAACC	CACCAACC	CCAACC	-	CATTTG (X2) CACTTG CAACTG CAGCTG CACCTG CACATG CACTTG CAGGTG
LAR3**	CTGTTA (x2) CGGTTG CAGTTA	ACCAACC ACCTAAC ACCTAAT ACCTACC ACCAACC	AACCAACC CACCTACC	CCAACC (X2) CCTACC	-	CATATG CATCTG CAAGTG
ANS1*	CTGTTA CGGTTA	-	-	-	CACGTG	CACGTG
ANS2**	CTGTTA CCGTTA	-	-	CCTACC	CACGTG	CACGTG CATATG CATCTG CAGGTG CATCTG
MATE (816155)**	CAGTTA CTGTTA	ACCAACC ACCTACC	-	CCAACC (X2) CCTACC	CACGTG	CACGTG
MATE (202461)**	CCGTTG CGGTTA	ACCAAAC	CACCAAAC	-	CACGTG	CACGTG CACATG

Continued...

Table A1-5, continued.

	CAGTTG (X2) CTGTTA					CAATTG CAGATG (X2) CACATG CATGTG CATATG CAACTG CAGATG
MATE (761186)**	CTGTTA (X3) CAGTTG	ACCAAAC	AACCAAAC	CCAACC	CACGTG	CAACTG CAGCTG CATGTG CAGGTG CACGTG
TT8 homologue (205424)**	CGGTTG CAGTTG	ACCAACC (X2) ACCTAAC	AACCAACC	CCAACC	-	CATATG CATGTG (X2) CACTTG (X2) CAAATG CATCTG
UFGT(557092)**	CAGTTG CTGTTG	ACCTACC ACCAACC	-	CCTACC (X2)	CACGTG	CACATG CAATTG CACGTG CATATG CAGCTG CAGTTG
CCR-related (832029)**	CAGTTG CCGTTA CTGTTA CTGTTG (x2)	ACCTACC ACCTAAC	AACCTACC AACCTAAC	CCAACC CCTACC	CACGTG	CAGTTG CATTTG CACATG (x2) CACGTG CATGTG
CCR-related (825925)**	CAGTTG	ACCTAAC	AACCTAAC	-	-	CAGTTG

Continued...

Table A1-5, continued.

						CACATG CAAATG/ CATATG CAGCTG CATATG CATCTG
CCR-related (743671)**	CAGTTA CTGTTG CGGTTA CCGTTA	-	-	-	CACGTG	CATGTG CACGTG CATATG CAGCTG
CCR-related (199038)**	CTGTTA (x2) CAGTTA CGGTTG CGGTTA CCGTTA	-	-	CCAACC	CAGCTG (X2)	CATTTG CAGCTG CAAGTG CAGATG CATCTG
CCR-related (590025)**	-	ACCAAAC ACCAACC	CACCAAAC AACCAACC	CCAACC		CAAATG (X2) CACCTG
MYB115 **	CGGTTA CTGTTA CCGTTG	-	-	-		CAAATG CACATG
Cytochrome B5b (740558)**	CTGTTA (X4) CTGTTG CAGTTA	ACCAACC ACCTAAC	AACCAAAC AACCTAAC CACCAAAC (X2)	CCAACC	CACGTG (x2)	CATCTG (X2) CACTTG CAGATG CATTTG CATGTG CACCTG CAGGTG CACGTG (X2)
MYB194 **	CAGTTA	ACCTAAC	AACCTAAC	-	-	CAACTG (X3)

Continued...

Table A1-5, continued.

	CAGTTG (X3) CCGTTA					CACATG CAAATG CATGTG CACATG
CYP98A23 (576112)**	CGGTTA CAGTTA	ACCTACC	-	CCAACC CCTACC	CACGTG (X2)	CAAGTG CAGGTG CATCTG CACGTG (X2)
CYP98A25 (591136)**	CCGTTG	-	-	-	-	CAAATG CATGTG/ CATGTG
ACC1 (559146)**	CCGTTG CTGTTA CTGTTG CAGTTG CTGTTA CGGTTA	ACCAAAC ACCTACC	AACCAAAC	CCAACC CCTACC	-	CACTTG CAGGTG CATGTG CAGGTG CAACTG
ACC1 (830215)**	CTGTTA (x4) CCGTTA CTGTTG CAGTTG (X2) CGGTTA	ACCTACC ACCAACC (X2) ACCAAAC	AACCTACC AACCAACC CACCAACC AACCAAAC	CCTACC (X2) CCAACC (X3)	CACGTG	CAACTG CACGTG CACCTG CACTTG CAATTG CAACTG
F3'H **	CAGTTA (X2) CTGTTG CTGTTA	ACCTACC	AACCTACC	CCTACC	CACGTG	CAAATG CACATG CATGTG CATATG (X2) CAATTG CACGTG

Continued...

Table A1-5, continued.

F3'5'H1 **	CAGTTG CTGTTA (X2) CGGTTA CAGTTA	ACCAACC		CCAACC (X2)	CACGTG (X2)	CAATTG CATGTG CAAGTG CAGTTG CATTTG CATCTG CACGTG (X2) CATGTG CACCTG CATATG
CHIL2 (836090)**	CCGTTG	ACCTACC (X2)		CCTACC (X2) CCAACC	CACGTG	CACGTG CACATG CAATTG
CHI1**	CGGTTG CAGTTA CTGTTG CCGTTG CAGTTG (X2) CTGTTA CGGTTA	ACCAAAC (X4) ACCAACC	AACCAAAC (X4) CACCAACC	CCAACC (X2)	CACGTG	CATGTG CAAGTG CACGTG CACCTG CAACTG CAACTG
FLS1 (665178)*	CAGTTA CGGTTA CTGTTA (x2)	ACCAAAC ACCTAAC	CACCAAAC CACCTAAC	CCAACC (X2)	-	CAGATG CAGGTG CACTTG CAAGTG CACATG CAATTG
FLS2 (551488)*	CAGTTA (X3) CTGTTA				CACGTG	CACGTG CACTTG CACCTG (X2) CAAATG
FLS3 (582182)*	CTGTTG (X3)	ACCAAAC (X2)	AACCAAAC (X2)	CCAACC (X2)	CACGTG	CATTTG

Continued...

Table A1-5, continued.

	CAGTTA (x2) CAGTTG CGGTTG CTGTTA	ACCAACC	AACCAACC		CACATG (x2) CATATG CAACTG CACTTG CACGTG CATTTG
FLS4 (828087)*	-	ACCTAAT		CCAACC (x2) CCTACC	CAAGTG CAAATG (X2) CATGTG (x2) CAATTG (x2) CATCTG