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Populus, the New Model System for Investigating Phenylpropanoid Complexity

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Abstract: Plant secondary metabolism affects ecosystem diversity and the yield and quality of feedstocks for biomass and biofuel, through an elaborate network of pathways that share common precursors. Until recently, functional dissection of these networks has depended largely on molecular information stored in the genome of *Arabidopsis*, an annual herb. Now that the *Populus* genome sequence is available, the potential for understanding and exploiting secondary metabolism in tree species comes closer to realization. In the present overview, genomic information pointing to greatly expanded gene complexity and function of the phenylpropanoid pathway in *Populus* is summarized. Phenylpropanoid-derived flavonoid and salicylate phenolics occur in numerous functionally distinct forms, and can account for 50% of leaf biomass in *Populus* and other fast-growing tree taxa. Their potential effects on tree growth, and their documented impacts on ecosystem diversity and productivity justify molecular dissection of secondary metabolism in *Populus*. Biosynthesis of salicylate phenolics remains poorly understood. By contrast, *in silico* promoter analysis of flavonoid genes, and *in situ* flavonoid localization in *Populus* reported here, augment published gene expression data, and illustrate that intra and intercellular regulatory components dramatically affect secondary carbon partitioning in this woody perennial.

Keywords: lignin; phenolic glycosides; condensed tannins; Populus genome.

Introduction

Phenylpropanoid metabolism supplies a wide array of general as well as species-specific phenolic compounds that are central to the success of land plants and plant-based industrial applications [1]. Although traditionally classified as "secondary compounds", phenylpropanoid products are now recognized for their significant roles during plant growth, development, reproduction, adaptation, and defense. Major classes of phenylpropanoid products include *lignins* as cell wall structural components; *lignans* as defense compounds or antioxidants; *flavonoids* as pigments, signaling molecules, and protectants against biotic and abiotic stresses; and *hydroxycinnamate derivatives*, both free and conjugated, for structural and protective functions. The phenylpropanoid pathway thus offers opportunities for metabolic engineering of a range of agronomically important phenolics, affecting traits from disease resistance to fiber and wood quality, and providing the ba-

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sis for novel flavor/fragrance compounds, nutriceuticals and pharmaceuticals. Phenylpropanoid metabolism is also of prime interest in the emerging area of ecological genomics, as it underpins plant interactions with the environment. For long-lived species like trees, allocation of the phenylpropanoid pools during development and in response to the perennial environmental fluctuations represents a major fitness trait, one that may not be adequately modeled on the basis of the herbaceous annual paradigm exemplified in *Arabidopsis* or maize. Thanks to the release of the *Populus* genome sequence [2], the ecological relevance of phenylpropanoid metabolism in perennial woody species can finally be tackled at the genomics level. This overview will cover the three major phenylpropanoid pools of *Populus* (Figure 1), lignin, salicylate-derived phenolic glycosides (PGs), and flavonoid-derived condensed tannins (CTs), with a special emphasis on CTs. The readers are referred to other excellent recent reviews [3-5] for in-depth coverage.

Lignin

Lignin is the major phenolic sink in the stem, accounting for 18-25% of dry woody biomass [6]. As a structural component of the cell wall, lignins limit forage digestibility by ruminants and interfere with cellulosic-based biomass conversion for bioenergy and pulp. Positive attributes of lignins include their mineral-/protein-binding activities, which slow decomposition and release of C into the atmosphere by plant detritus [1]. Lignins can replace petroleum-based sources for use as biobased resin in the fabrication of printed wiring board for the electronics industry [7]. Lignins can also be used as filler in biodegradable plastics or package materials [8].

The branch pathways leading to the biosynthesis of monolignols (Figure 1) have been extensively characterized in trees, due to the commercial significance of lignin modification. The entire suite of putative lignin biosynthetic pathway genes identified from the *Populus* genome is listed in Table 1. All belong to multi-gene families, and only a handful of genes have been functionally characterized. However, expression and kinetic data suggest that in many cases individual gene family members have functionally distinct roles and are differentially involved in lignin and non-lignin phenolic metabolism, as exemplified for PAL [9] and 4CL [10, 11]. Although only a handful of genes have been targeted for genetic manipulation of lignin to date, both qualitative and quantitative modifications have been reported in transgenic *Populus*. Lignin structural modification has been reported in almost all cases, but a substantial increase in syringyl-to-guaiacyl lignin (S/G) ratio, a characteristic positively correlated with pulping efficiency [12], has only been achieved by over-expression of F5H [13]. Reduction of lignin content was reported following down-regulation of 4CL [13, 14], CCoAOMT [15] and CCR [3]. In addition, transgenic poplars with reduced CAD did not exhibit significant change in lignin content or S/G ratio, but there was an increase in the incorporation of both coniferyl and sinapyl aldehydes into the lignin [16, 17]. Kraft pulping of CAD-deficient transgenic poplar grown at two European field sites for 4 years showed improved pulp yields and reduced cellulose degradation compared to the control [18]. Commercial-scale application of transgenic poplars with improved lignin characteristics is expected to reduce environmental burdens associated with pulping.

Phenolic glycosides

Salicylate-derived PGs do not accumulate in *Arabidopsis* or other important herbaceous model systems, but are highly characteristic of the Salicaceae family of fast-growing woody species, including *Salix* (willows) and *Populus* [19]. The wide use of willow and poplar barks in herbal remedies can be attributed to the abundance of PGs in these species.

Salicin, the first PG identified from plants, is the pain-relief ingredient in willow extracts [reviewed in 20]. In poplars and willows, PGs serve primarily protective functions, having been associated with insect defense [21], UV-B protection [22] and drought response [23]. The putative PG precursor salicylic acid (SA) is widespread in higher plants and plays a central role in defense signal transduction. However, biosynthesis of PGs and SA remains poorly understood. It has long been thought that SA is biosynthesized from cinnamate via benzoate [24], and requires PAL. An additional, PAL-independent pathway utilizing plastidic isochorismate synthase (ICS) appears to operate in certain, but not all, SA-mediated defense responses of *Arabidopsis* [25, 26]. There are two *Arabidopsis* ICS genes, a plastid-localized AtICS1 and a cytosolic AtICS2, but neither appears to be expressed in healthy leaves, and only AtICS1 is pathogen-inducible [25]. Interestingly, the poplar genome contains a single, likely plastid-targeted ICS gene that is

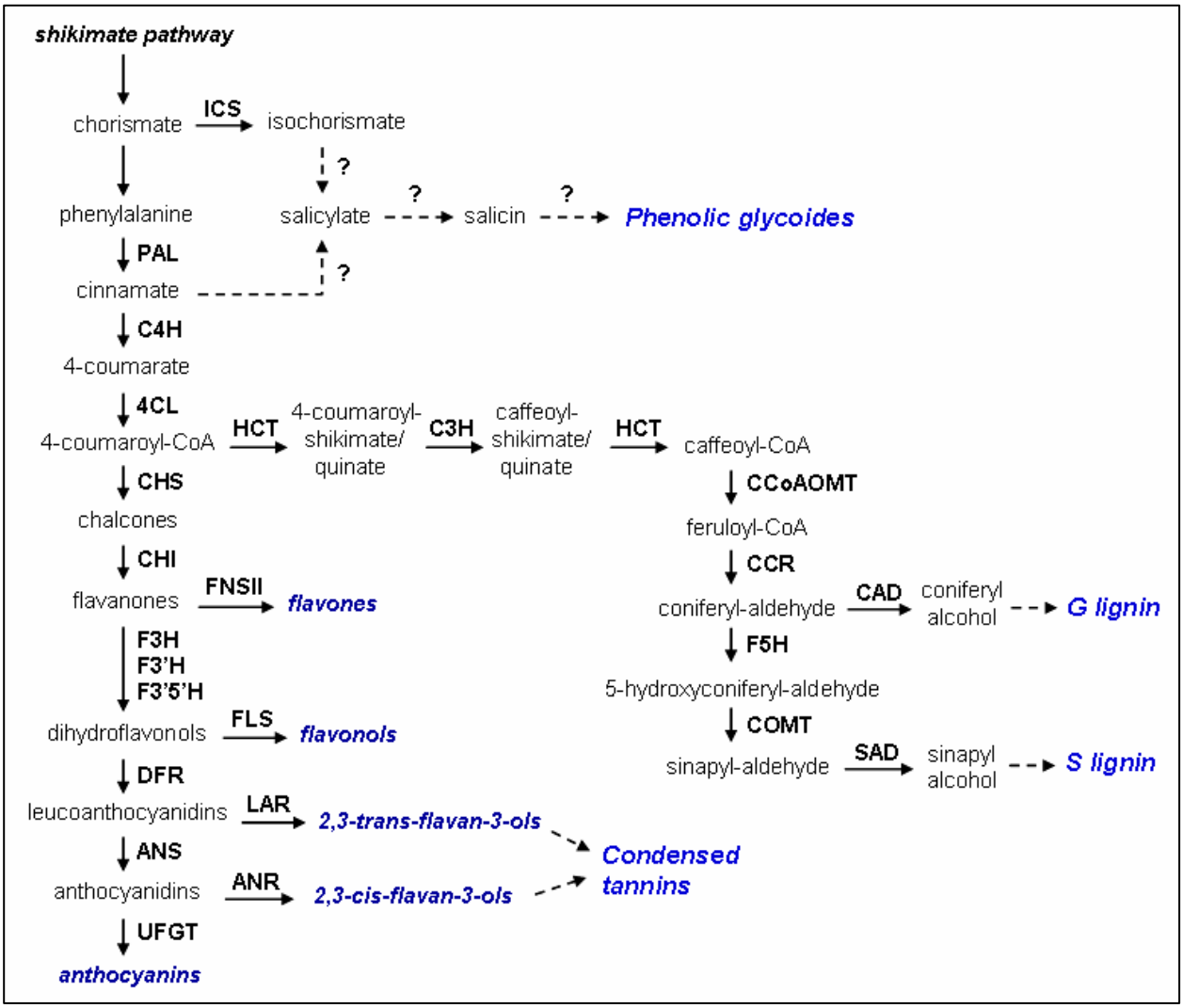

Figure 1. Biosynthetic pathways of major phenylpropanoid end products, lignins, phenolic glycosides and condensed tannins in *Populus*. Enzyme abbreviations are listed in Table 1. Enzymatic steps that are not yet identified are shown as dashed arrows

Table 1. List of phenylpropanoid pathway genes in *Populus*

Protein name	JGI Gene Model	Locus	Protein name	JGI Gene Model	Locus
Phenylalanine ammonia-lyase			***Cinnamoyl-CoA reductase***		
PAL1	estExt_Genewise1_v1.C_280661	scaffold_28:2031644-2035061	CCR1	fgenesh4_pg.C_scaffold_208000034	scaf-fold_208:275317-27778
PAL2	estExt_fgenesh4_pg.C_LG_VIII0293	LG_VIII:1885833-1890028	CCR2	estExt_fgenesh4_kg.C_LG_III0056	LG_III:16013635-16017348
PAL3	grail3.0004045401	LG_XVI:7316776-7319927	CCR3	gw1.208.126.1	scaf-fold_208:329845-33231
PAL4	estExt_fgenesh4_pg.C_LG_X2023	LG_X:19171449-19174898	CCR4	gw1.208.109.1	scaf-fold_208:292935-29538
PAL5	gw1.X.2713.1	LG_X:19181547-19184719	CCR5	estExt_fgenesh4_pg.C_2080041	scaf-fold_208:343600-34630
Cinnamate 4-hydroxylase			CCR6	estExt_fgenesh4_pg.C_LG_I0389	LG_I:3184141-3186481
C4H1	estExt_fgenesh4_pg.C_LG_XIII0519	LG_XIII:12820991-12825303	***Cinnamyl alcohol dehydrogenase***		
C4H2	grail3.0094002901	LG_XIX:10989662-10992697	CAD	estExt_Genewise1_v1.C_LG_IX2359	LG_IX:4268866-4271137
C4HL1	gw1.164.158.1	scaffold_164:432424-434020	SAD	grail3.0004034803	LG_XVI:5830946-5834884
4-Coumarate-CoA ligase			***Chalcone synthase***		
4CL1	grail3.0100002702	LG_I:1432116-1435302	CHS1	eugene3.00140920	LG_XIV:7151030-7153182
4CL2	grail3.0099003002	LG_XIX:4083532-4087345	CHS2	estExt_fgenesh4_pg.C_LG_I0449	LG_I:3683042-3684777
4CL3	estExt_fgenesh4_pg.C_1210004	scaffold_121:49867-56929	CHS3	estExt_fgenesh4_pg.C_LG_I0450	LG_I:3690127-3692694
4CL4	gw1.XVIII.2818.1	LG_XVIII:9666118-9671112	CHS4	eugene3.00031460	LG_III:15665340-15667449
4CL5	fgenesh4_pg.C_LG_III001773	LG_III:17994254-17998436	CHS5	eugene3.00031461	LG_III:15672197-15673829
Hydroxycinnamoyl-CoA quinate/shikimate hydroxycinnamoyltransferase			CHS6	eugene3.00031462	LG_III:15678905-15680833
HCT1	fgenesh4_pg.C_LG_III001559	LG_III:16193170-16196683	***Chalcone isomerase***		
HCT2	estExt_fgenesh4_pm.C_LG_XVIII0344	LG_XVIII:10668161-10672154	CHI1	estExt_Genewise1_v1.C_LG_X2396	LG_X:18468933-18471655
HCT3	estExt_fgenesh4_pg.C_LG_XVIII0910	LG_XVIII:10642781-10645134	***Flavanone 3-hydroxylase***		
HCT4	eugene3.00180947	LG_XVIII:10631699-10633511	F3H	gw1.57.31.1	scaf-fold 57:716253-717841
HCT5	fgenesh4_pg.C_scaffold_133000007	scaffold_133:86412-88323	***Flavonoid 3'-hydroxylase***		
HCT6	eugene3.02080010	scaffold_208:113566-117563	F3'H	estExt_fgenesh4_pg.C_LG_XIII0337	LG_XIII:6197800-6200404
HCT7	eugene3.18780002	scaffold_1878:6898-8809	***Flavonoid 3'5'-hydroxylase***		
4-Coumarate 3-hydroxylase			F3'5'H1	eugene3.00090961	LG_IX:6110882-6112723
C3H1	eugene3.36160002	scaffold_3616:2997-5408	F3'5'H2	eugene3.00011827	LG_I:19972937-19975122
C3H2	eugene3.00160247	LG_XVI:1538875-1542646	***Flavone synthase***		
C3H3	fgenesh4_pg.C_LG_VI000268	LG_VI:1979652-1982315	FNSII1	estExt_fgenesh1_pg_v1.C_LG_XIII0255	LG_XIII:1794033-1795947
Ferulate 5-hydroxylase			FNSII2	eugene3.00700209	scaf-fold 70:1407665-14101
F5H1	estExt_fgenesh4_pm.C_570058	scaffold_57:1035361-1038589	***Flavonol synthase***		
F5H2	eugene3.00071182	LG_VII:11484639-11486746	FLS1	grail3.0191001301	LG_XIX:123233-126067
F5HL1	eugene3.00090440	LG_IX:2644256-2646866	FLS2	eugene3.00020803	LG_II:6082658-6084346
Caffeic acid O-methyltransferase			FLS3	eugene3.01350040	scaf-fold 135:427496-43062
COMT1	estExt_fgenesh4_pm.C_LG_XII0129	LG_XII:3089139-3092252	FLS4	estExt_fgenesh4_pg.C_1350039	scaf-fold 135:441620-44432
COMT2	estExt_fgenesh4_pg.C_LG_XV0035	LG_XV:255739-258237	***Dihydroflavonol 4-reductase***		
COMT3	fgenesh4_pg.C_LG_XIV000481	LG_XIV:4314177-4316619	DFR1	estExt_Genewise1_v1.C_LG_II0799	LG_II:2174492-2176520
COMT4	estExt_Genewise1_v1.C_LG_XIV1942	LG_XIV:4327267-4329146	DFR2	gw1.V.1407.1	LG_V:15923736-15925503
COMT5	eugene3.00021675	LG_II:14132201-14134094	***Anthocyanidin synthase***		
COMT6	fgenesh4_pm.C_LG_II000840	LG_II:14167706-14169393	ANS1	grail3.0018022801	LG_III:11400392-11401847
COMT7	eugene3.00012911	LG_I:33700503-33702138	ANS2	eugene3.00010988	LG_I:8507517-8509264
COMT8	gw1.XVI.3248.1	LG_XVI:9185925-9187398	***Anthocyanidin reductase***		
COMT9	fgenesh4_pm.C_LG_XI000417	LG_XI:14176611-14178062	ANR1	estExt_fgenesh4_pm.C_LG_IV0055	LG_IV:1671017-1673302
Caffeoyl-CoA O-methyltransferase			ANR2	estExt_fgenesh4_pm.C_LG_XI0107	LG_XI:3895226-3897581
CCoAOMT1	grail3.0001059501	LG_IX:4059145-4060914	***Leucoanthocyanidin reductase***		
CCoAOMT2	estExt_fgenesh4_pm.C_LG_I1023	LG_I:26412640-26415499	LAR1	grail3.0010045601	LG_VIII:7398478-7400397

Table 1. List of phenylpropanoid pathway genes in *Populus* (continued)

Protein name	JGI Gene Model	Locus	Protein name	JGI Gene Model	Locus
CCoAOMT3	estExt_fgenesh4_pm.C_1450034	scaffold_145:744229-746661	LAR2	eugene3.00101230	LG_X:12874015-12876623
CCoAOMT4	fgenesh4_pm.C_LG_X000399	LG_X:10889762-10892213	LAR3	estExt_fgenesh4_pm.C_LG_XV0077	LG_XV:2126571-2128664
CCoAOMT5	estExt_fgenesh4_pg.C_LG_VIII1209	LG_VIII:9019120-9021383			
CCoAOMT6	fgenesh4_pg.C_LG_II001689	LG_II:14407483-14409698			

detected in PG-accumulating leaves and shoots, but absent in roots [19]. In contrast to *Populus* where PG stores can become very large, e.g., up to 30% leaf dry weight in certain genotypes [27], genetic manipulation to enhance SA-based constitutive defense outlays in *Arabidopsis* resulted in dwarfing [e.g., 28]. It appears that *Populus* and *Salix* spp have evolved a mechanism for the efficient management of PG metabolism for both growth and defense, and may serve as an attractive model to understand PG and SA biosynthesis.

Condensed Tannins (CTs)

Flavonoids make up a large class of species-specific phenolic compounds, and are commonly associated with pigmentation, stress responses, defense, reproduction and symbiotic interactions [27, 28]. CTs, in particular, encompass the most structurally, and functionally complex members of the flavonoids [29] and their protein-binding properties account for their historical importance to the tanning industry. They act as deterrents to microbial, insect or animal feeding [30-32]. CTs in leaf detritus bind to organic soil constituents, slow carbon mineralization and increase soil fertility [32, 33]. CTs also bind to potentially phytotoxic forms of aluminum [34] and other metals [35], a valuable trait to be explored for phytoremediation applications. CTs are important determinants of seed nutritional properties [36] due to their powerful antioxidant activity. Foods rich in antioxidant CTs (e.g., grape, cranberry, red wine) are of particular interest for their protective roles in human health [37].

The flavonoid biosynthetic pathways are more complex in *Populus* than in *Arabidopsis*, both in terms of chemical diversity and gene regulation [19]. The pathway is well characterized in *Arabidopsis*, and all flavonoid biosynthetic enzymes, except flavonol synthase, are encoded by single-copy genes [28]. In contrast, a vast majority of the flavonoid pathway enzymes are encoded by gene families in *Populus* (Table 1). The only exceptions are chalcone isomerase (CHI), flavonoid 3'-hydroxylase (F3'H), and flavanone 3-hydroxylase (F3H). The expanded flavonoid gene families are consistent with substantial accumulation of flavonoid-derived CTs in vegetative tissues of *Populus*, accounting for up to 18% leaf dry weight in aspen (*P. tremuloides*) [21], and concentrations as high as 50% have been reported in cottonwood (*P. angustifolia* and hybrids) [38, 39]. *Arabidopsis*, on the other hand, produces CTs primarily in the seed coat (~1% fresh weight), with little accumulation (<0.004% fresh weight) in rosette leaves [40]. *Populus* thus offers a model system distinct from *Arabidopsis* to investigate flavonoid pathway complexity, regulation and carbon allocation during the rapid expansion of vegetative tissues including leaves, stems and roots.

As shown in Figure 1, the flavonoid biosynthetic pathway branches from phenylpropanoid metabolism by the action of chalcone synthase (CHS), whose family is particularly expanded in poplar and contains

at least six genes, several of them in tandem repeats (Table 1). In sharp contrast, enzymes involved in conversion of chalcones to flavanones and dihydroflavonols, as well as B-ring hydroxylation of flavanones and dihydroflavonols are all encoded by single-copy genes (*i.e.*, CHI, F3H and F3'H). Two flavonoid 3',5'-hydroxylase (*F3'5'H*) genes are present in the *Populus* genome, but exhaustive RT-PCR amplification from a wide range of genotypes and tissues yielded no product for *F3'5'H2* (unpublished), suggesting that F3'5'H may also be encoded by a single functional gene (*i.e., F3'5'H1*). Subsequent synthesis of the CT precursor, 2,3-*cis*-flavan-3-ols, requires dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR), whereas formation of the other CT precursor, 2,3-*trans*-flavan-3-ols is mediated by DFR and leucoanthocyanidin reductase (LAR, Figure 1). Interestingly, all four gene families contain two paralogous members derived from genome-wide duplication events [2]. However, the LAR family is unique in that it contains an additional member (LAR3) that is phylogenetically distinct from the LAR1 and LAR2 paralogs [19]. Arabidopsis lacks *LAR* and does not accumulate 2,3-*trans*-flavan-3-ols [41], but both *cis* and *trans* starter units for CTs are present in *Populus* and other tree species with large metabolic commitments to CTs [42]. Based on gene family size and expression data [19], the *CHS* and *LAR* families may play important roles in modulating CT biosynthesis, structure and functional diversity in *Populus*.

Flavonols are among the most widespread flavonoids in plants, and have been associated with a range of physiological activities, including UV-protection, signaling, male sterility and auxin transport regulation [5]. In *Arabidopsis*, they represent the only flavonoid compounds detected in vegetative tissues [43]. Flavonols are synthesized from dihydroflavonols by flavonol synthase (FLS), represented in *Arabidopsis* by six genes, only one of which has been functionally characterized [44]. *Populus* contains four *FLS* genes, three of which are expressed in leaves [19]. Unlike many other flavonoid biosynthetic genes, however, *FLSs* are not wound-inducible in *Populus* [19], suggesting a role for FLS in flavonoid partitioning. The structurally related flavones are also prevalent in higher plants, but are conspicuously absent in the Brassicaceae, including *Arabidopsis* [45]. Accordingly, flavone synthase (FNS) genes are absent in the *Arabidopsis* genome. Flavones are detected in bud exudes of *Populus* species [46, 47], consistent with the identification of 5 genes encoding FNSII of the Cytochrome P450 family 93B in the *Populus* genome.

Regulation of CT biosynthesis

Coordinated expression of flavonoid biosynthetic pathway genes has been reported in several species, and in the case of *Populus*, is supported by *in silico* analysis of flavonoid gene promoters. An AC-rich, MYB-binding element described as L box-like (ACCWWCC) [48] or P box-like (MACCWAMC) [49] in many phenylpropanoid gene promoters is, as expected, present in the promoters of most of the *Populus* flavonoid genes (Table 2). The G-box (CACGTG), found in the promoters of ribulose 1,5-bisphosphate carboxylase/ oxygenase small subunit (rbcS) [50] and various other light-induced genes [51], including CHS [52], is also present in *Populus* flavonoid gene promoters, consistent with light-dependent regulation of flavonoid biosynthesis [5]. Another MYB-recognizing AACA motif confers endosperm-specific expression of seed storage protein, glutelin, in rice [53, 54]. The endosperm-specific activity of glutelin promoter is reminiscent of the endothelium-specific expression of the BAN promoter and proanthocyanidin-accumulation in *Arabidopsis* seed [55]. Consistent with this, the AACA motif is found in the promoters of all CT biosynthetic gene families, but under-

represented in the FLS and FNS gene promoters (Table 2). Flavonoids are known to play an important signaling role during root development and legume nodulation, processes that are intimately linked to auxin response [5]. An auxin response element (AuxRE) recognized by the auxin response factor (ARF) transcription factor family involved in auxin signaling [56] is found in the promoters of most flavonoid genes, but is poorly represented in the FLS promoters (i.e., present in only 1 of 4 FLS promoters). In contrast, an ABA responsive element (ABRE) associated with ABA-mediated dehydration or drought tolerance [57, 58], and light-regulated expression of parsley CHS [59] is ubiquitous in all *Populus* FLS promoters (Table 2). This is consistent with the significant up- regulation of *Arabidopsis* FLS1 (At5g08640), but not other flavonoid biosynthetic genes, in tran

Table 2. *In silico* analysis of putative regulatory elements in *Populus* flavonoid pathway gene promoters

***Cis* element**	**CHS**	**CHI**	**F3H**	**F3'H**	**F3'5'H**	**DFR**	**ANS**	**BAN**	**LAR**	**FLS**	**FNSII**
cis element present in most flavonoid gene promoters											
L box-like (ACCWWCC)	2/6	1/1	1/1	1/1	1/2	1/2	1/2	2/2	3/3	3/4	1/2
P box-like (MACCWAMC)	5/6	1/1	1/1	1/1	1/2	1/2	0/2	2/2	3/3	2/4	0/2
G-box (CACGTG)	6/6	1/1	0/1	1/1	2/2	1/2	2/2	1/2	0/3	2/4	0/2
cis elements underrepresented in the FLS family											
AACA motif (AACAAAC)	4/6	1/1	1/1	1/1	1/2	1/2	1/2	1/2	3/3	1/4	0/2
AuxRE (TGTCTC)	2/6	1/1	0/1	0/1	1/2	1/2	2/2	1/2	1/3	1/4	1/2
cis elements overrepresented in the FLS family											
ABRE-like (ACGTGGC)	1/6	0/1	0/1	0/1	0/2	1/2	0/2	0/2	0/3	4/4	0/2

Each data point represents the number of gene(s) containing the specific *cis* element in the 2-kb promoter(s) over the total number of genes in each family.

-sgenic plants over-expressing an ABRE-binding protein [57], and may suggest a role of flavonols in ABA signaling.

Cellular Localization of flavonoids and CTs

Intercellular transport and its gene regulation are likely to participate in CT partitioning. Based on dimethylaminocinnamaldehyde (DMACA) staining which is specific for CT starter subunit flavan-3-ols [60], stress-induced CT accumulated mostly in an abaxial layer of the spongy mesophyll (Figure 2A). The CT content of the abaxial cells of wound-induced cottonwood plants is higher than observed in abaxial cells of unstressed aspen [9]. Flavonoid epi-fluorescence in the presence of 2-aminoethyl-diphenyl borinate [61] revealed that flavonoid precursors to CT were concentrated in the, upper palisade mesophyll (Figure 2B). It appears that in expanding leaves, CTs or CT precursors are synthesized in the upper palisade cells and then exported. CT induction in roots, appears

to deplete intracellular flavonoids, and to depend more directly on flavonoid pool size than in leaves (Figure 2C-H). What limits CT induction in leaves where flavonoid intermediates are plentiful is unclear. The possible relevance of such metabolic controls to *Populus* growth is under investigation using phytochemically distinct genotypes [62].

Concluding Remarks

Because of the economic significance of *Populus* for pulp and bioenergy production, and because of its ecological importance as a keystone species in terrestrial ecosystems, advances in phenylpropanoid metabolism promise to have far-reaching impacts. Phenylpropanoid sinks are characteristic of the defense and overall fitness of *Populus* species. Their metabolic costs to biomass growth remain an area of uncertainty. Availability of the genome sequence, and the ever-growing genomics resources for *Populus* will accelerate research into mechanisms governing regulation of phenylpropanoid metabolism, resource competition and tradeoffs. For example, the dynamics of CT and PG regulation in the context of growth versus overall plant fitness await dissection at the molecular level. Advances in phytochemical regulation should elevate the potential for improved biomass quality and production through genetic selection or metabolic engineering in *Populus*.

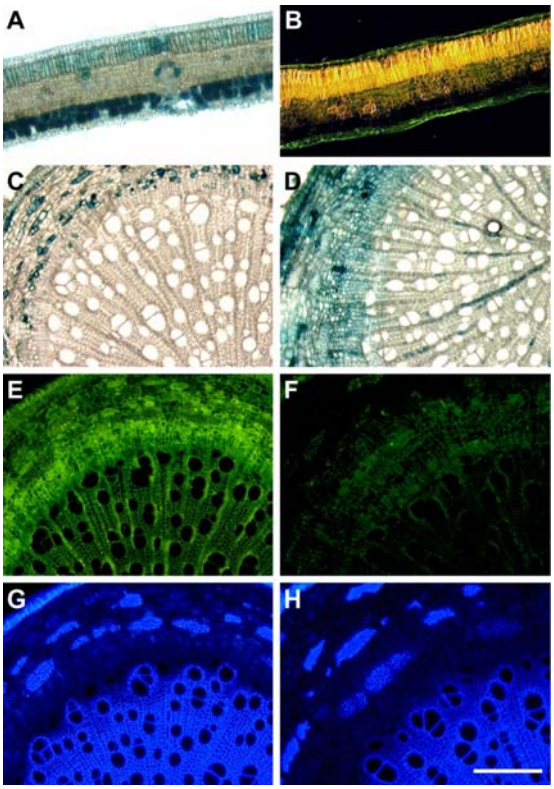

Figure 2. A-B, Cross sections of young, systemic leaves from wounded cottonwood showing (A) CT deposition (blue DMACA stain), and (B) flavonoid localization (yellow fluorescence). C-H, Cross sections of roots from cottonwood plants subjected to nitrogen replete (C, E, G) or nitrogen limiting (D, F, H) conditions for two weeks. CT accumulation shown by DMACA staining increased during limiting N (C vs. D), but flavonoid reserves decreased (E vs. F). Limiting N did not appear to affect root lignification (G vs. H). The flavonoid and lignin fluorescence images were taken using the same section

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