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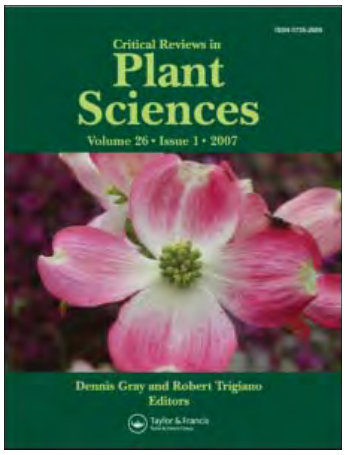

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Genomics of Secondary Metabolism in *Populus*: Interactions with Biotic and Abiotic Environments

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***Populus* trees face constant challenges from the environment during their life cycle. To ensure their survival and reproduction, *Populus* trees deploy various types of defenses, one of which is the production of a myriad of secondary metabolites. Compounds derived from the shikimate-phenylpropanoid pathway are the most abundant class of secondary metabolites synthesized in *Populus*. Among other major classes of secondary metabolites in *Populus* are terpenoids and fatty acid-derivatives. Some of the secondary metabolites made by *Populus* trees have been functionally characterized. Some others have been associated with certain biological/ecological processes, such as defense against insects and microbial pathogens or acclimation or adaptation to abiotic stresses. Functions of many *Populus* secondary metabolites remain unclear.**

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The advent of various novel genomic tools will enable us to explore in greater detail the complexity of secondary metabolism in *Populus*. Detailed data mining of the *Populus* genome sequence can unveil candidate genes of secondary metabolism. Metabolomic analysis will continue to identify new metabolites synthesized in *Populus*. Integrated genomics that combines various 'omics' tools will prove to be the most powerful approach in revealing the molecular and biochemical basis underlying the biosynthesis of secondary metabolites in *Populus*. Characterization of the biological/ecological functions of secondary metabolites as well as their biosynthesis will provide knowledge and tools for genetically engineering the production of seconday metabolites that can lead to the generation of novel, improved *Populus* varieties.

Keywords *Populus*, secondary metabolism, phytoalexin, defense priming, allelopathy, biosynthesis, genomics, metabolomics

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I. INTRODUCTION

As perennial plants, *Populus* species face constant challenges from their growing environment during their entire life cycle that can span decades. These challenges may come from the various biotic agents that are interacting with *Populus* trees, such as herbivorous insects, pathogenic fungi and bacteria, and viruses. Some of the challenges are imposed by the abiotic environment that include light and temperature stress, drought, or stresses derived from global warming parameters, such as elevated CO_2 and ozone. Like other plants, *Populus* trees possess sophisticated systems to defend themselves against biotic stresses and to acclimate or adapt to the abiotic stresses. One of such defense strategies is based on the production and accumulation of a host of low molecular weight metabolites that are called secondary metabolites.

Plant secondary metabolites, by definition, refer to those metabolic products not directly involved in primary metabolic processes. Collectively, plants make a vast array of secondary metabolites. More than 100,000 of them have been structurally identified (Wink, 1988). One important feature of plant secondary metabolism is their strain-specificity, meaning that certain metabolites are being produced by plants of certain species or families. Plants of a specific species usually make only a subset of secondary metabolites. Although collectively plants synthesize a very large number of secondary metabolites, most of these metabolites are synthesized by a few common biosynthetic pathways (Pichersky and Gang, 2000).

Despite the ongoing debate (Pichersky *et al.*, 2006) plant secondary metabolites are generally thought to be important for plant interactions with the environment, although they are not essential for plant growth and development. The production of specific metabolites in specific plant species may render unique fitness benefits to the host plants grown in a specific ecosystem. Although our understanding of the biology and biosynthesis of secondary metabolites in most plant species is still very limited, significant progress has been made in this area, especially with model plant species, benefiting from the advent of various genetic and genomics tools. Taking *Arabidopsis* as an example, novel secondary metabolites are continuing to be discovered and the genes for their biosynthesis are continuing to be identified (D'Auria and Gershenzon, 2005). Such knowledge provides novel tools for us to understand how *Arabidopsis* interacts with its environment and the roles of specific secondary metabolites.

The completion of the genome sequence of *Populus trichocarpa* (Tuskan *et al.,* 2006), and the establishment of various genomic tools, provide unprecedented opportunities for understanding how *Populus* trees interact with their environment. This review concerns the opportunities to understand the roles of secondary metabolites in *Populus*-environment interactions. We will first describe the major classes of secondary metabolites found in *Populus* and current understanding of their biosynthesis. We will then review the characterized or implicated biological/ecological processes in which specific secondary metabolites are involved. Next, we will discuss how various genomic tools can be used to advance our understanding on the types of secondary metabolites made by *Populus* trees and the genetic control of their biosynthesis. Lastly, we will elaborate on the functional elucidation of *Populus* secondary metabolites and genetic improvement of *Populus* through metabolic engineering of secondary metabolism.

II. MAJOR CLASSES OF SECONDARY METABOLITES IN *POPULUS* AND THEIR BIOSYNTHESIS

Although chemically very complex, the majority of plant secondary metabolites can be grouped into several major classes based on their chemical structure and biosynthetic origin. In this article, we focus on three biochemical pathways: the shikimate-phenylpropanoid pathway, terpenoid pathway and lipoxygenase/hydroperoxide lyase pathway.

A. Shikimate/phenylpropanoid Derivatives

Plants produce several classes of important secondary metabolites via the shikimate-phenylpropanoid pathway, including lignin, lignans, flavonoids, anthocyanin and tannins, salicylic acid, and hydroxycinnamates and their esters. These phenylpropanoid metabolites perform a variety of functions in plants including mechanical strength, pollen viability, pest deterrence, disease resistance, UV protection, and resistance to biotic and abiotic stresses (Davin and Lewis, 1992; Lewis and Davis, 1999; Croteau *et al.,* 2000; Dixon, 2001; Dixon *et al.,* 2002). Phenylpropanoids are the dominant secondary metabolites in the genus *Populus*. Lignin, as the complex aromatic bioploymer, is a principlal structual component of secondary cell wall of *Populus*. It provides the structural integrity of vascular tissues, enables water transport, and contributes to plant defense mechanisms (Boerjan *et al.,* 2003). As one branch of the phenylpropanoid pathway, lignin biosynthesis and its genetic engineering have been discussed comprehensively (Whetten and Sederoff, 1995; Anterola and Lewis, 2002; Boerjan *et al.,* 2003; Hopkins *et al.,* 2007; Vanholme *et al.,* 2008; Weng *et al.,* 2008).

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Thus, aspects of lignin biosynthesis in *Populus* will not be repeated in this review.

Besides the cell wall structural component, *Populus* trees produce a broad array of nonstructural phenylpropanoid derivatives (phenolics and polyphenolics) that exhibit a variety of effects on tree growth and development, pathogenic defense responses and host-pest interactions (Lindroth and Hwang, 1996b; Tsai *et al.,* 2006; Coleman *et al.,* 2008). Foliar phenolic glycosides and condensed tannins alone can constitute up to 35% of leaf dry weight (Lindroth and Hwang, 1996b; Tsai *et al.,* 2006). The types and abundance of phenolics and polyphenolics vary greatly among different clones, and across species and sections of *Populus*. The accumulation of these phenolic compounds also varies greatly by tissue/organ type and changes temporally and spatially. Collectively, these shikimate- phenylpropanoid derivatives are classified into four categories: salicylate-derived phenolic glycosides, hydroxycinnamates and their derivatives, flavonoids, and condensed tannins (Figure 1).

1. *Phenolic Glycosides*

Salicylate-derived phenolic glycosides are prominent foliar chemicals in *Populus*. Numerous structural forms of phenolic glycosides have been identified from different species. In *P. tremuloides*, four structurally related phenolic glycosides, salicin, salicortin, tremuloidin and tremulacin, are well characterized (Figure 1). Those phenolic glycosides act as deterrents to a variety of insect herbivores and provide protection against pathogens and abiotic stress, such as UV radiation (Lindroth and Hwang, 1996b). Some phenolics glycosides, e.g., salicin and salicortin, occur widely throughout the genus of *Populus*, but others, e.g., grandidentatin and HCH-salicortin, show a narrower taxonomic distribution (Tsai *et al.,* 2006).

The biosynthesis of these salicylate-derived phenolic glycosides in *Populus* is poorly understood. Apparently, these glycoconjugates are derived from precursors of salicylic acid (SA) biosynthesis (Pierpoint, 1994; Lee *et al.,* 1995; Dempsey *et al.,* 1999). Salicylic acid can be synthesized from chorismate-phenylalanine via cinnamate and benzoate (Pierpoint, 1994; Lee *et al.,* 1995; Pierpoint, 1997); alternatively, at least in *Arabidopsis*, salicylic acid can be synthesized in the chloroplast from isochorismate, the pathway that is known to operate in prokaryotes (Wildermuth *et al.,* 2001; Métraux, 2002).

The proposed chorismate-phenylalanine route leading to salicylic acid concomitantly yields benzenoid metabolites that are also often found in *Populus* leaf and bud exudates (Tsai *et al.,* 2006). The formation of benzenoids and salicylic acid from cinnamate requires shortening of the side chain by C2 units. For these reactions to occur, the CoA-dependent, β-oxidative pathway and the CoA-independent, non-β-oxidative pathway, or the combination of the two have been suggested in different plant species (Jarvis *et al.,* 2000; Boatright *et al.,* 2004). So far, the route(s) to benzenoids and salicylic acid in *Populus* and the enzymes and genes involved in the biosynthetic network are still unknown. After being formed from salicylic acid or from the other biosynthetic precursors, salicin may then be benzoylated to form tremuloidin, esterified to form salicortin, or benzoylated and esterified to form tremulacin (Figure 2).

2. *Hydroxycinnamates and Their Derivatives*

Hydroxycinnamates and their derivatives constitute 2–8% of leaf dry weight in *Populus* and the other closely-related taxa, including *Salix* (willow) and *Betula* (birch) (Palo, 1984; Ikonen *et al.,* 2001; Peltonen *et al.,* 2005). A variety of hydroxycinnamates have also been identified from *Populus* bud exudate. The early investigation in chemical composition of bud exudate of different *Populus* species led to the identification of up to 60 small molecule compounds, of which, more than 70% are different types of hydroxycinnamates and the derivatives. The principal phenylpropenoic acids are ferulic acid, isoferulic acid, *p*-coumaric acid, caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, as well as their esters and aldehyde forms (Figure 1). In some *Populus* species, the bulk of exudate also contains unique glyceryl-, monoacetyl glyceryl-, diacetyl glyceryl esters, as well as prenylated hydroxycinnamates. Species-specific accumulation of hydroxycinnamates and their derivatives among *Populus* has led to their use in chemical taxonomy (Greenaway and Whatley, 1990; 1991a; 1991b).

Biosynthesis of hydroxycinnamates is closely related to the lignin biosynthetic pathway (Figure 2). Hydroxycinnamates were proposed to originate from cinnamate and a series of hydroxylation and *O*-methylation reactions at the aromatic ring generated different forms of hydroxycinnamates (Higuchi, 1981). These compounds are, in turn, converted to their corresponding hydroxycinnamoyl CoA, hydroxycinnamaldehydes and hydroxycinnamyl alcohols by the combined action of 4-coumarate:CoA ligase, (hydroxy)cinnamoyl-CoA reductase, and (hydroxyl)cinnamyl alcohol dehydrogenase, linking to lignin biosynthesis. Molecular and genetic studies have revealed that the hydroxylation and methylation do not occur at the free hydroxycinnamic acid forms (Humphreys and Chapple, 2002). In *Arabidopsis*, the production of hydroxycinnamic acids, i.e., sinapic acid and ferulic acid, was recently demonstrated invoking a novel aldehyde dehydrogenase, which catalyzes the oxidation of sinapaldehyde and coniferaldehyde to their corresponding carboxylic acids, i.e., sinapic acid and ferulic acid (Nair *et al.,* 2004). These results suggest that hydroxycinnamates are end products diverged from monolignol biosynthetic pathway rather than the intermediates for monolignol biosynthesis (Figure 2). The characterized aldehyde dehydrogenase in *Arabidopsis* plays a major role in biosynthesis of hydroxycinnamoyl esters. Similar aldehyde dehydrogenase genes are found in many other plant species, implicating a common occurrence of the routes for the biosynthesis of hydroxycinnamoyl esters (Nair *et al.,* 2004). Whether this hydroxycinnamaldehyde to acid pathway also dominates in *Populus* needs to be further investigated. When the expression of caffeoyl CoA *O*-methyltransferase was down-regulated in *Populus,* monolignol

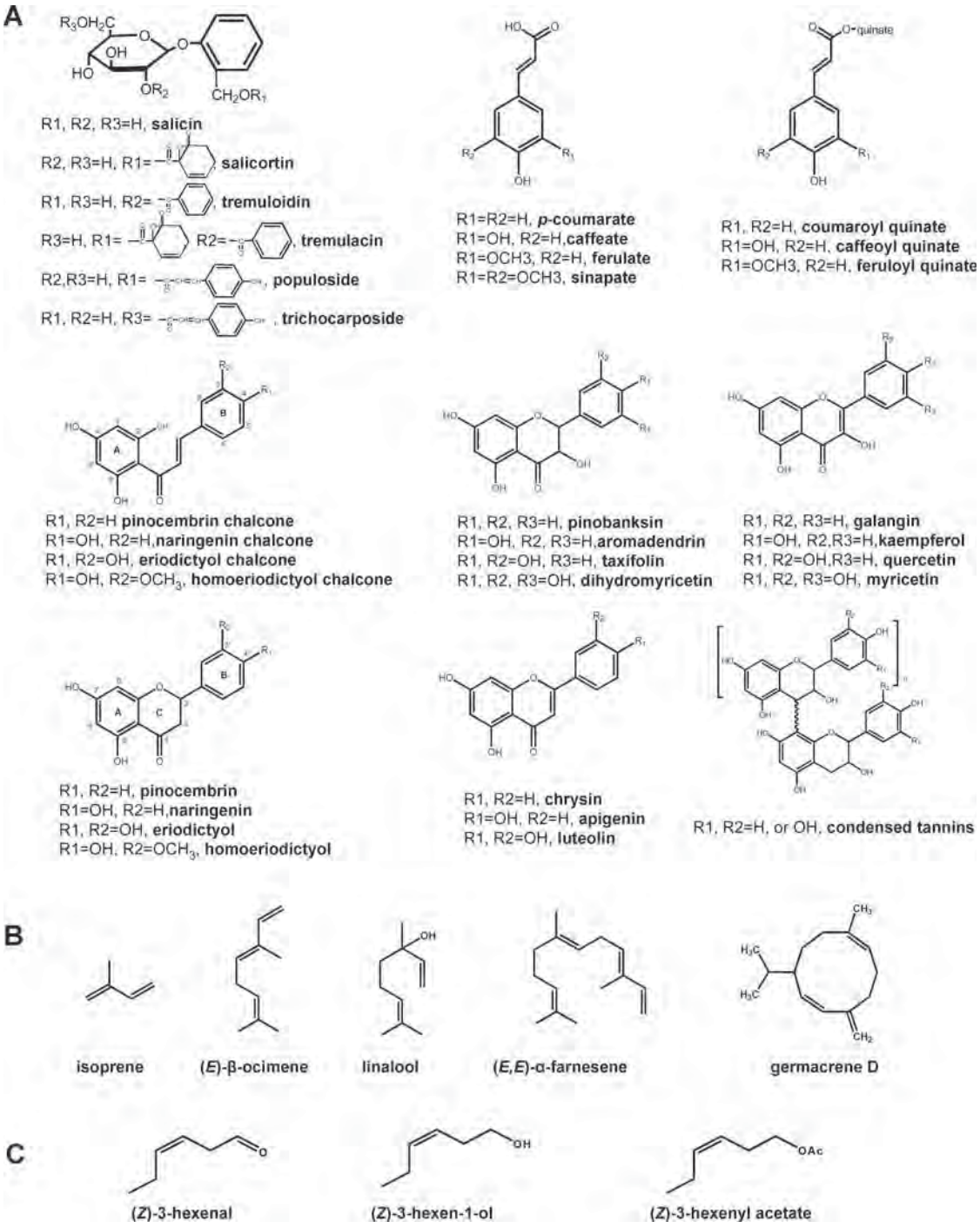

FIG. 1. Structures of representative compounds of major classes of secondary metabolites in *Populus*. A, Representative phenolics. A variety of modifications of flavonoids and hydroxycinnamates by glycosylation, methylation, and acylation are not shown; B, Representative terpenoids; C, Representative green leaf volatiles.

biosynthesis was depleted, while the novel caffeic acid 3-*O*-glucoside and sinapic acid 4-*O*-glucoside accumulated as the storage forms of hydroxycinnamates (Meyermans *et al.,* 2000), suggesting that the alternative routes to hydroxycinnamic acids, possibly even through the free acid pathway, may function in *Populus*, at least when the aldehyde hydrogenase-mediated route is blocked (Figure 2).

3. Flavonoids and Condensed Tannins

Flavonoids are ubiquitous and a remarkably diverse group of plant secondary metabolites, serving as plant pigments,

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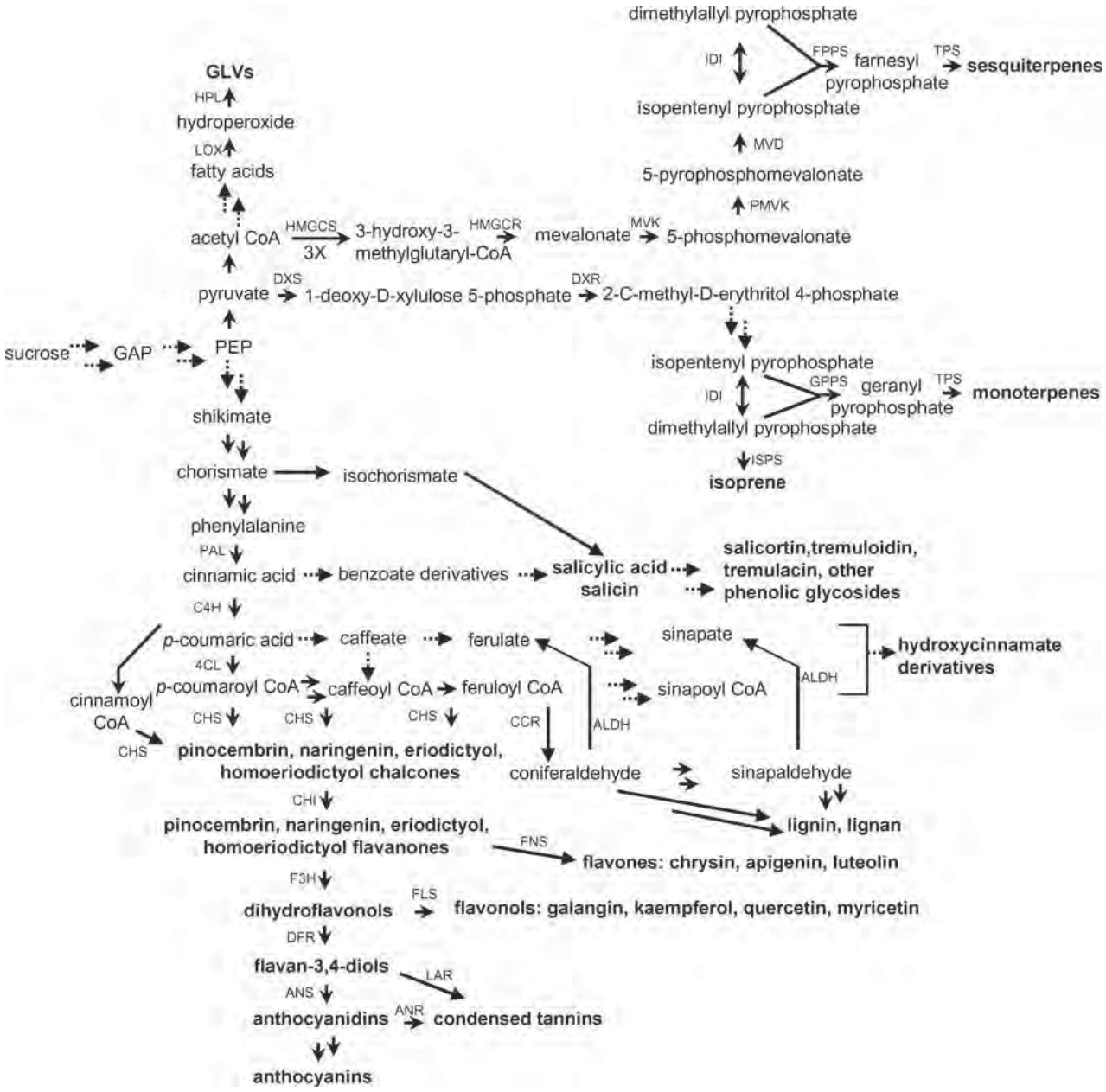

FIG. 2. Metabolic networks for production of phenolic compounds, terpenoids and green leaf volatiles (GLVs) in *Populus*. PAL, Phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanindin reductase; ANR, anthocyanidin reductase; CCR, (hydroxy)cinnamoyl CoA reductase; ALDH, aldehyde dehydrogenase; HPL, hydroperoxide lyase; LOX, lipoxygenase; HMGCS, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase; HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; MVD, mevalonate-5-pyrophosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; FPPS, farnesyl pyrophosphate synthase; TPS, terpene synthase; DXS, 1-Deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GPPS, geranyl pyrophosphate synthase; ISPS, isoprene synthase.

pollinator attractants, antioxidant and UV protective agents (Winkel-Shirley, 2001a, b). *Populus* species contain most of the major classes of flavonoids, including chalcones, dihydrochalcones, flavanones, flavones, dihydroflavonols (flavanonols), flavonols, flavan-3,4-diols, which are the precursors of anthocyanins and proanthocyanidins, and flavan-3-ols, the precursors of proanthocyanidins. The representative compounds found in *Populus* include chrysin, galagnin, pinocembrin, and quercetin derivatives (Figure 1). Most flavanones and flavonols are methylated. Methylation renders the molecule more lipophilic

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(Harborne and Mabry, 1982; Harborne, 2000). The dihydroflavonol pinobanksin in *Populus* buds is also acetylated at the 3-hydroxyl position (Greenaway *et al.,* 1992). Like hydroxycinnamate derivatives, the occurrence and concentration of flavonoids vary among *Populus* species and clones. In *P. tremuloides* buds, the concentration of flavonoids in buds amounted to 8.1% of the dry matter. In the section *Tacamahaca* (including *P. balsamifera* and *P. trichocacpa*), the bulk of the exudate is characteristically composed of dihydrochalcones, whereas these compounds are essentially missing from bud exudate of *P. deltoides* and *P. nigra* of the section *Aigeiros*, where flavanonols and phenylpropenoic acids and their esters are abundant (Greenaway *et al.,* 1989; 1991a; 1991b).

Biosynthetic pathways leading to flavonoids are well defined at both the biochemical and molecular genetic levels (Winkel-Shirley, 2001a). Many of the enzymes have been characterized in different species. The biosynthesis is initiated by chalcone synthase (CHS), a plant-specific polyketide synthase that converts 4-coumaroyl CoA into a variety of C6-C3-C6 products. 4-coumaroyl moiety contributes to the B-ring of the diphenylchroman nucleus (Winkel-Shirley, 2001a). In *Populus*, the various pinocembrin chalcone and erodictyol chalcone derivatives are probably derived from cinnamoyl CoA and caffeoyl CoA (Morreel *et al.,* 2006) (Fig. 2).

Proanthocyanidins, which are also called condensed tannins (CTs), are oligomers or polymers of flavonoid units (flavan-3-ols, also known as catechins) (Stafford, 1990; McMahon *et al.,* 2000). Condensed tannins occur in both leaves and woody tissues of *Populus*. Their concentrations in foliage are highly variable and range from 1∼18% dry weight and the accumulation of these compounds are inducible in response to insect damage (Peters and Constabel, 2002; Lindroth and Hwang 1996).

CTs share the same upstream biosynthetic pathway as the flavonoids and anthocyanin flower pigments (Figure 3). The branch point between the condensed tannin and anthocyanin pathways occurs at the level of leucoanthocyanidin, which is converted to anthocyanidin by the action of anthocyanidin synthase (ANS, also known leucoanthocyanidin dioxygenase) and to 2,3-*trans*-flavan-3-ol (catechin) by a leucoanthocyanidin reductase (LAR). In addition, an anthocyanindin reductase (ANR) functions immediately downstream of anthocyanidin synthase to convert anthocyanidins into the corresponding 2,3-*cis*-flavan-3-ols that serve as the building blocks predominant in many CTs (Xie, 2003).

B. Terpenoids

The terpenoid pathway is one of the most fundamental biochemical pathways occurring in all plants. In addition to its involvement in the production of light harvesting pigments for photosynthesis, including both carotenoids and chlorophyll (the phytol chain of chlorophyll is of terpenoid origin), the terpenoid pathway is also involved in the biosynthesis of a number of plant hormones including gibberellins, abscisic acid, cytokinins and brassinolides. Moreover, the terpenoid pathway is responsible for the production of the largest class of secondary metabolites produced in the plant kingdom. About 50,000 plant terpenoids have been structurally identified (McCaskill and Croteau, 1997).

In plants there are two biochemical pathways (Figure 2) leading to the formation of terpenoids: the cytosol-localized mevalonate (MVA) pathway and the plastid-localized methylerythritolphosphate (MEP) pathway, which has also been named the 1-deoxy-D-xylulose (DOX) pathway (Tholl, 2006). Both pathways lead to the formation of the C5 precursor isopentenyl pyrophosphate (IPP), which may be converted to dimethylallyl pyrophosphate (DMAPP), the allylic isomer of IPP. Condensation of IPP and DMAPP leads to the formation of geranyl pyrophosphate (GPP) and geranylgeranyl pyrophosphate (GGPP) in plastid and farnesyl pyrophosphate (FPP) in the cytosol. The formation of monoterpenes, diterpenes and sesquiterpenes from GPP, GGPP and FPP, respectively, is catalyzed by terpene synthases.

Isoprene (Figure 1) is the simplest form of terpenoids and the most abundant biogenic volatile organic compound emitted from vegetation (Loivamäkia *et al.,* 2008). It has been estimated that 5–10% of the photosynthetically assimilated carbon is partitioned for isoprene emission (Sharkey and Yeh, 2001). *Populus* has been a good model for studying the emission as well as the biosynthesis of isoprene. Isoprene is synthesized by the action of isoprene synthase (ISPS) using DMAPP as substrate. ISPS proteins have been purified from several plants and the encoding genes isolated from several hybrid poplars: *P. alba* × *tremula* (Miller *et al.,* 2001), and *P. alba* (Sasaki *et al.,* 2005). A subcellular localization study using green florescence protein (GFP) fusion showed that ISPS is localized in plastids (Sasaki *et al.,* 2005), suggesting that the substrate of ISPS is produced by the MEP pathway. The *ISPS* gene isolated from *P. alba* (*PaIspS*) is predominantly expressed in leaves. Its expression is strongly induced by heat stress and continuous light irradiation, and is substantially decreased in the dark, suggesting that isoprene production is regulated at the transcriptional level (Sasaki *et al.,* 2005).

Two monoterpenes, (*E*)-β-ocimene and linalool (Figure 1), have been detected in *P. trichocarpa* × *deltoides* (Arimura *et al.,* 2004). The genes for their biosyntheis, however, have not been identified. Sesquiterpenes have also been detected in *Populus*. (*E*, *E*)-α-farnesene (Figure 1) was the major sesquiterpene emitted from forest tent caterpillar-damaged hybrid poplar (Arimura *et al.,* 2004). In addition to (*E*, *E*)-α-farnesene, another sesquiterpene (*E*)-β-caryophyllene was detected from gypsy moth-infested hybrid poplar (Frost *et al.,* 2007). All sesquiterpenes are synthesized by the action of sesquiterpene synthase using FPP as substrate. An important feature of terpene synthase is that it can produce multiple products from a single substrate (Chen *et al.,* 2003). A full-length sesquiterpene synthase cDNA (*PtdTPS1*) was isolated from *Populus* and functionally identified as (−)-germacrene D synthase (Arimura *et al.,* 2004). The *Populus* genome contains a large number of

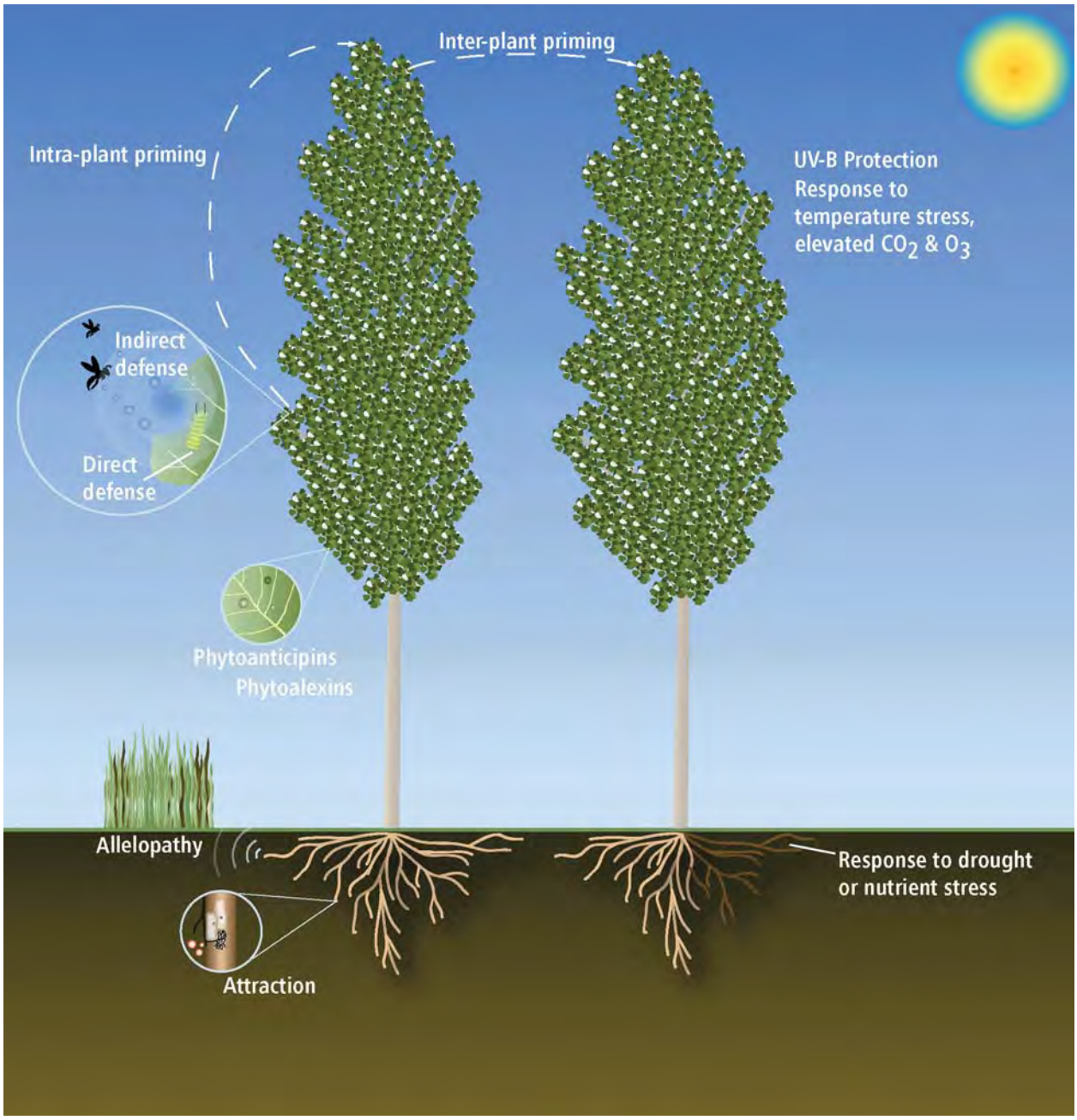

FIG. 3. Functions of secondary metabolites in *Populus*-environment interactions. The details are described in the text except for attraction, which depicts the role of certain secondary metabolties secreted from *Populus* roots to attract root-interacting microorganisms such as mycorrhizal fungi.

putative *TPS* genes (Table 1). Detailed characterization of these genes will provide rich information about the terpenoids being produced by *Populus* as well as their biological/ecological functions.

Two homoterpenes, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), are also produced by *Populus* (Arimura *et al.,* 2004; Behnke *et al.,* 2009). Previous studies suggest that DMNT is synthesized from the regular terpene alcohol, nerolidol, by fragmentation into DMNT and butanone (Degenhardt and Gershenzon, 2006). The sesquiterpene synthase gene catalyzing the formation of nerolidol has been identified in maize (Schnee *et al.,* 2002). TMTT has been suggested to be a degradation product of the diterpene alcohol (*E*,*E*)-geranyllinalool. Recently, the terpene synthase gene encoding geranyllinalool synthase was identified from *Arabidopsis* (Herde *et al.,* 2008). The *Populus* genes involved in the biosynthesis of DMNT and TMTT, however, have not been identified.

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TABLE 1
Gene families of secondary metabolism in *Populus*

Gene family	Number of homologs[a]	References/sources
Phenylalanine ammonia lyase	5	Tsai *et al.,* 2006; Tuskan *et al.,* 2006
Cinnamate 4-hydroxylase	3	Tsai *et al.,* 2006; Tuskan *et al.,* 2006
4-coumarate:CoA ligase	5	Tsai *et al.,* 2006; Tuskan *et al.,* 2006
Hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyltransferase	7	Tsai *et al.,* 2006; Tuskan *et al.,* 2006
Coumaroyl shikimate 3′-hydroxylase	3	Tsai *et al.,* 2006
Caffeoyl CoA 3-O-methyltransferase	6	Tsai *et al.,* 2006
Caffeic acid 3-O-methyltransferase	9	Tsai *et al.,* 2006
Cinnamoyl CoA reductase	7	Tuskan *et al.,* 2006
Cinnamyl alcohol dehydrogenase	1	Tuskan *et al.,* 2006
Chalcone synthase	6	Tsai *et al.,* 2006
Chalcone isomerase	1	Tsai *et al.,* 2006
Flavonoid 3′-hydroxylase	1	Tsai *et al.,* 2006
Flavonoid 3′,5′-hydroxylase	2	Tsai *et al.,* 2006
Flavanone 3-hydroxylase	1	Tsai *et al.,* 2006
Flavone synthase II	2	Tsai *et al.,* 2006
Flavonol synthase	4	Tsai *et al.,* 2006
Dihydroflavonol reductase	2	Tsai *et al.,* 2006
Anthocyanidin synthase	2	Tsai *et al.,* 2006
Anthocyanidin reductase	2	Tsai *et al.,* 2006
Leucoanthocyanidin reductase	3	Tsai *et al.,* 2006
Terpene synthase	64	Chen, unpublished
SABATH methyltransferase	33	Chen, unpublished
Methylester esterase	30	Zhao et al., 2009
BAHD acyltransferase	94	Liu, unpublished
P450	312	Nelson, 2006

[a]It should be noted that some members of certain families, such as the terpene synthase, are involved in primary metabolism.

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C. Fatty-acid Derivatives

Fatty acids are substrates for the production of a wide assortment of metabolites in plants, some of which are secondary metabolites. Best representatives of this group are a group of compounds collectively called "green leaf volatiles" (GLVs), which include C6 aldehydes, alcohols, and their esters (Figure 1) (Matsui, 2005). GLVs determine the distinctive scent that is produced when leaves are physically injured (Hatanaka, 1993). Five GLVs, including (*Z*)-3-hexenylacetate, (*Z*)-3-hexenol, (*E*)-2-hexenal, (*Z*)-3-hexenal and 1-pentenol, were reported to be emitted from ozone-exposed *Populus* × *canescens* (Behnke *et al.,* 2009). (*Z*)-3-hexenylacetate has also been detected from insect-damaged *Populus* plants (Frost *et al.,* 2007).

The formation of GLVs is relatively well understood (Figure 2). The first level of regulation of GLV formation occurs at the step of lipid-hydrolysis, which is often triggered by tissue disruption. Lipid hydrolysis provides free fatty acids to the lipoxygenase pathway (Matsui, 2006). In the lipoxygenase pathway, lipoxygenase (LOX) catalyzes the dioxygenation of polyunsaturated fatty acids, such as linoleate and linolenate to yield hydroperoxides (Porta and Rocha-Sosa, 2002). Two lipoxygenase genes, *PdLOX1* and *PdLOX2*, were cloned from *P. deltoides* cv. 'Lux' (I-69/55) (Cheng *et al.,* 2006). Recombinant PdLOX1 and PdLOX2 proteins showed predominant 13-LOX activity. Gene expression analysis showed that both *PdLOX1* and *PdLOX2* expression were up-regulated by fungal pathogen, mechanical damage and exposure to methyl jasmonate that mimics insect feeding. The results suggest that *PdLOX1* and *PdLOX2* play an important role in *Populus* resistance to biotic and abiotic stresses, including the formation of GLVs (Cheng *et al.,* 2006). The hydroperoxides formed through LOX action may serve as substrates for allene oxide synthase that leads to the formation of jasmonate, one important signaling molecule for plant defenses (Stintzi *et al.,* 2001). Alternatively, hydroperoxides can be further metabolized via the hydroperoxide lyase (HPL) pathway to produce volatile aldehydes and alcohols. In *Populus*, an *HPL* gene was found to be induced by both wounding and insect herbivory (Lawrence

et al., 2006). GLVs formed upon wounding are released rapidly from plant surfaces. In *Arabidopsis* plants, (*Z*)-3-hexenylacetate emission peaked after 5 min and declined to pre-damage levels after 90 min of mechanical damage (D'Auria *et al.,* 2007).

The formation of esters is catalyzed by acyltransferases that belong to the BAHD family (D'Auria et al, 2002). The *BAHD* gene for the biosynthesis of (*Z*)-3-hexenylacetate in *Arabidopsis* has been identified, which encodes acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase (CHAT) (D'Auria *et al.,* 2007). Similar BAHD enzymes are responsible for the production of (*Z*)-3-hexenylacetate in *Populus* (F. Chen, unpublished).

III. ROLES OF SECONDARY METABOLITES IN *POPULUS* DEFENSCES AGAINST ENVIRONMENTAL STRESSES

A. Secondary Metabolites in *Populus* Defense against Herbivores

In their life cycle, *Populus* trees may encounter various types of insect herbivores, including leaf feeders (such as the forest tent caterpillar), boring insects (such as *Saperda calcarata*), and sucking insects (such as the meadow spittlebug). These insect herbivores could cause extensive reduction in growth and productivity of *Populus* trees. In defense against insect herbivory, one strategy that *Populus* trees use is the production and accumulation of a myriad of secondary metabolites (Figure 3).

Salicortin and tremulacin, the two phenolic glycosides commonly found in *Populus*, are potential herbivore toxins. They have been demonstrated to have strong negative effects on herbivore growth and development (Hwang and Lindroth 1997; Hemming and Lindroth 1995; Hwang and Lindroth 1998; Osier and Lindroth 2001, 2004) and fecundity (Osier *et al.,* 2000). A number of studies have found that developmentally-based variation of plant secondary metabolites can significantly influence herbivore behavior and performance (Kearsley and Whitham, 1989; Swihart and Bryant, 2001; Lawrence *et al.,* 2003). In general, high concentrations of phenolic glycosides result in increased development time and reduced weights of herbivores (Osier and Lindroth, 2006).

The effect of condensed tannins on the herbivore resistance has been debated. Some studies indicated that condensed tannins had no apparent anti-herbivore activity (Lindroth and Hwang 1996; Ayres *et al.,* 1997; Schweitzer *et al.,* 2008). However, an induced accumulation of condensed tannins in *Populus* has been found after damage by herbivores (Osier and Lindroth, 2001). The study of condensed tannin synthesis provides evidence that they are important in defense against herbivores. A dihydroflavonol reductase (DFR) cDNA, which is involved in condensed tannin synthesis, was isolated from trembling aspen (Peters and Constabel, 2002). Both the expression of *DFR* and the concentrations of condensed tannin were significantly induced by insect treatment, suggesting that the induction of condensed tannins may be important for defense against herbivores in *Populus* (Peters and Constabel, 2002).

Upon insect herbivory, in addition to the accumulation of metabolites in tissues, some metabolites may be released as volatiles from damaged *Populus* plants. These include monoterpenes, sesquiterpenes, homoterpenes and green leaf volatiles (Behnke *et al.,* 2009). In many plants, herbivore-induced plant volatiles have been shown to act as direct defense because of the toxicity of the metabolites and/or indirect defense by attracting the natural enemies of the attacking insects (Yuan *et al.,* 2008). Insect-induced volatiles in *Populus* have been suggested to have similar functions (Arimura *et al.,* 2004), which, however, still needs to be verified in bioassays.

Secondary metabolites are also important for *Populus* defense against other herbivores. For example, *P. balsamifera* uses different types of secondary metabolites in different parts of the plant as antifeedants in defenses against snowshoe hare rabbits (*Lepus americanus*) (Reichardt *et al.,* 1990). Cineol, benzyl alcohol, and (+)-bisabolol have been related to defense in buds. 6-Hydroxycyclohexenone (6-HCH) and salicaldehyde has been related to the defense in internodes. It was observed that the concentration of 6-HCH can be supplemented by the hydrolysis of phenol glycosides when plant tissue is disrupted, suggesting that the chemical defense of *Populus* against *L. americanus* is dynamic (Reichardt *et al.,* 1990).

B. Secondary Metabolites in *Populus* Defense against Microbial Pathogens

Many diseases of *Populus* are the result of infection by microbial pathogens, such as fungi (e.g., *Melampsora* species), bacteria (e.g., *Xanthomonas popular*), and viruses (e.g., *Populus* mosaic virus) (Ostry and McNabb, 1985). Foliar diseases may result in premature leaf drop, loss of biomass, and even mortality in *Populus* plantations (Newcombe *et al.,* 1994). Many secondary metabolites, when accumulated are toxic to microbial pathogens, and therefore can act as defenses. Depending on the production mechanism, defensive plant secondary metabolites can be grouped into two categories: phytoanticipins, which are constitutively synthesized and accumulated, and phytoalexins, which are induced upon pathogen infection (Van Etten *et al.,* 1994).

Pyrocatechol, benzoic acid and salicylic acid isolated from the bark of *P. tremuloides* were shown to inhibit the growth of *Hypoxylon pruinatum* (Hubbes, 1962; 1969). Benzoic acid and catechol were also identified from extracts of aspen leaves. When added to a synthetic medium, these compounds had a strong inhibitory effect on the growth of different kinds of fungal pathogens (Olsen, 1971). These compounds function as phytoanticipins because they are found to be constitutively present in the host tissues and are active before fungal attack (Flores and Hubbes, 1979). Other compounds are produced upon pathogenic infection. When inoculated by *H. mammatum* and *Alternaria* spp., *P. tremuloides* plants produced novel

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secondary metabolites, including phenolic glycosides, which were inhibitory to spore germination of these two fungi (Flores and Hubbes, 1979; 1980).

Condensed tannins are also involved in *Populus* defense against microorganisms. Tannins may act as resistant chemicals against bacterial and fungal pathogens or enhance structural strength to exposed tissues (Tegelberg and Julkunen-Tiitto, 2001). When *P. trichocarpa* × *deltoides* was infected by *M. medusae* leaf rust, genes encoding enzymes required for condensed tannin synthesis were dramatically up-regulated. Phytochemical analysis confirmed that, late in the infection, condensed tannin levels increased in infected leaves (Miranda *et al.,* 2007). A similar defense role may be conferred by the lignin pathway. While some intermediates of lignin, monolignols and lignan may have a direct effect on the performance of infecting pathogens, lignification, which is a major response at the infection site, may provide a structural defense (Moerschbacher *et al.,* 1990; Northcote *et al.,* 1989).

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C. *Populus* Secondary Metabolites in Intra- and Inter-plant Interactions

Allelopathy is a mechanism of competition between plants. Plants synthesize chemicals and release them, often through roots, into their environment. These chemicals may inhibit the growth of neighboring plants. *Populus* are known to have allelopathic properties. For example, when wheat plants are grown under *Populus* plantations, their yield was significnaly lower than crops grown in open condition (Singh *et al.,* 1993). Allelopathy is often attributed to secondary metabolites produced by plants, such as flavonoids (Bais *et al.,* 2004). Although the exact identities of the responsible allelopathic compounds remain to be identified, the aqueous extracts of *Populus* leaves were found to significantly influence the germination of certain wheat varieties (Nandal and Dhillon, 2005).

Secondary metabolites are also involved in plant defense priming, which is defined as a physiological process by which a plant prepares itself for future attacks by herbivores and pathogens (Frost *et al.,* 2008). When attacked by insects or pathogens, primed plants display stronger activation of defense responses. Priming may occur at two levels: intra-plant and inter-plant. When cultured parsley cells were treated with salicylic acid or a benzothiadiazole, the inducers of systemic resistance, it was found that one set of defence-related genes were directly activated and another set of defense genes including the phenylalanine ammonia lyase gene were primed for stronger elicitation (Conrath *et al.,* 2006). This is one example of intra-plant priming. Tobacco plants, when infected by *Pseudomonas syringae*, emitted methyl salicylate, which can prime the defense of nearby plants (Shulaev and Raskin, 1997). This is one example of inter-plant priming involving pathogens. In *Populus*, the green leaf volatile (*Z*)-3-Hexenylacetate has been shown to have intra-plant priming function (Frost *et al.,* 2008). (*Z*)-3-Hexenylacetate induced by herbivory can prime the production of jasmonic acid and linolenic acid, the expression of genes that mediate oxylipin signaling and direct defense, and the production of terpenoid volatiles in the leaves of hybrid poplar (Frost *et al.,* 2008). The link between lipid-based signaling and the induction of a primed state that involves secondary metabolism has recently been established in Arabidopsis, where azelaic acid was demonstrated to the mobile signal for the priming of plant systemic immunity (Jung *et al.,* 2009).

D. *Populus* Secondary Metabolites in UV-B Protection

In the last several decades, the increase in solar ultraviolet-B (UV-B) radiation caused by the depletion of stratospheric ozone has been well documented (McKenzie *et al.,* 1999; Ziemke *et al.,* 2000). Although UV-B comprises only a small fraction of the solar spectrum, it has a profound photobiological effect on plants because UV-B radiation can degrade and cause conformational changes in proteins, nucleic acids, and other macromolecules (Caldwell, 1981). UV-B radiation often results in a series of growth and physiological changes on plants, e.g., reduction in height, biomass accumulation and leaf area, damage to photosystem II, and reduction in photosynthetic rate (Correia *et al.,* 1999; Heinrich *et al.,*1999; Bassman *et al.,* 2003; Feng *et al.,* 2003; Yang *et al.,* 2005). The production and accumulation of secondary metabolites are important for plant resistance to UV-B stress.

A number of studies demonstrated that the enhancement of UV-B radiation leads to the accumulation of phenolics in *Populus* (Sullivan *et al.,* 2003; Warren *et al.,* 2003; Sullivan, 2005). These phenolics function as UV-absorbing compounds to reduce the damage by excessive UV-B radiation. In *Populus*, the predominant absorbing compounds were glycosylated flavonols, hydroxycinnamates and chlorogenic acid (Sullivan *et al.,* 2003). In response to the UV-B radiation, these compounds accumulated at the epidermal layer, suggesting they may function as epidermal attenuation of UV-B in *Populus*. The alteration of the production of various types of secondary metabolites, such as flavonoids and lignin, in plants regulated by UV-B radiation may have other important physiological and ecological consequences. This may lead to changes in plant morphology and defenses to insects and pathogens.

E. Secondary Metabolites in Responses of *Populus* to Elevated CO_2 and Ozone

Global CO_2 and tropospheric O_3 concentrations are projected to continue to increase over the coming decades at an unprecedented pace (IPCC, 2001). Forest trees are major source of biogenic volatile organic compound emissions. It is reported that elevated levels of CO_2 and ozone significantly affect tree physiology, growth and secondary metabolite emission (Ceulemans *et al.,* 1999; Makino and Mae, 1999; Krupa *et al.,* 2001; Karnosky *et al.,* 2005). When exposed to ozone treatment, *Populus* trees displayed an enhanced emission of isoprene and monoterpenes (Loreto *et al.,* 2001; Loreto and Fares, 2007).

Isoprene and monoterpenes have been demonstrated to function as powerful antioxidants reducing oxidative damage in plants. Although the mechanism of this protection is still not clear, some studies proposed that isoprene may stabilize the cell, particularly chloroplast thylakoid membranes (Loreto *et al.,* 2001).

In addition, the levels of condensed tannin and phenolic glycosides were also influenced by CO_2 and ozone. The influence of CO_2 on tannin levels shows genotype-dependence. For some genotypes, tannin levels increased at elevated CO_2. Whereas in other genotypes, tannin levels were nonresponsive to higher levels of CO_2 (Mansfield *et al.,* 1999). In contrast, ozone increased the concentration of condensed tannins (Holton *et al.,* 2003). With respect to phenolic glycosides, elevated CO_2 did not affect salicortin, but increased tremulacin levels, whereas ozone reduced both salicortin and tremulacin levels (Kopper and Lindroth, 2003). The enhanced accumulation of phenolics induced by ozone may serve as antioxidants (Nogués *et al.,* 2008). Due to the defense functions of condensed tannins and phenolic glycosides on herbivores and microorganisms, elevated CO_2 and ozone may impact the plant-herbivore and plant-microbe interactions (Kopper and Lindroth, 2003). Some reports suggest that *Populus*-herbivore-parasitoid interactions may also be affected by the alteration of CO_2 and ozone levels (Holton *et al.,* 2003).

F. Secondary Metabolites in Responses of *Populus* to Temperature and Drought Stresses

Most plants need to cope with extreme temperatures at some point in their life cycle. Metabolic changes play important roles in plant acclimation or adaptation to temperature stresses. Isoprene, the compound involved in plant resistance in ozone, has also been suggested to function in the protection of leaf physiological processes against high temperature stress. Using a transgenic approach, Behnke *et al.,* (2007) showed that *Populus* clones that do not emit isoprene have reduced rates of net assimilation and photosynthetic electron transport during heat stress, providing direct evidence that isoprene emission is linked to the thermotolerance of photosynthesis.

Plants, especially perennial species, need to deal with seasonal variations in water availability. Some plants tolerate water shortages by maintaining or accumulating high concentrations of primary metabolites including sugars, amino acids, and organic acids (Gebre *et al.,* 1998). Secondary metabolites have also been implicated in plant resistance to drought stress. In *Populus*, for instance, increased levels of phenolic glycosides had been observed in response to drought stress (Hale *et al.,* 2005). The increased concentration of phenolic glycosides may be the key contributing factor. Given that *Populus* represents fast-growing trees when nutrient and water availability are high (Tuskan, 1998), and that rapid growth under such conditions can deplete primary metabolites, the maintenance of stable concentrations of secondary metabolites may ensure that leaf osmotic potentials do not increase to high levels that would render plants more susceptible to rapidly-developing drought. The maintenance of low baseline osmotic potential among *Populus* clones is correlated with productivity under field conditions (Tschaplinski *et al.,* 2006).

IV. GENOMICS-BASED INVESTIGATION OF SECONDARY METABOLITE BIOSYNTHESIS IN *POPULUS*

Despite the many examples of the involvement of secondary metabolites in *Populus*-environment interactions, much more awaits to be learned about *Populus* secondary metabolism. We do not yet have a full catalog of secondary metabolites that are synthesized by *Populus*. The molecular basis underlying the biosynthesis of secondary metabolites in *Populus* is little understood. In the pre-genomics era, the investigation of plant secondary metabolite biosynthesis was conducted in a reductionist fashion, namely as "one pathway, one enzyme, one gene" at a time. The advances in molecular biology and genomics have led to a paradigm shift, enabling high-throughput characterization of genes of secondary metabolism. In particular, the availability of the *Populus* genome sequence as well as the development of various novel genomic tools will provide novel strategies for understanding the chemistry, biosynthesis and biological roles of secondary metabolites in *Populus*. In this section, we will discuss several of these genomic strategies, sometimes using examples from other plant species that have been better characterized than *Populus*.

A. Metabolomics

Metabolomics, or metabolite (metabolic) profiling, offers tremendous potential to discover novel genes and assign function to those genes. Metabolic profiling determines the consequences of a targeted change in gene activity and has the potential to provide information on gene function and its affects the complex biochemical network (Trethewey, 2001). The release of the *Populus* genome sequence has resulted in the need to ascribe function to the large number of unidentified genes to establish precise relationships between gene structure and phenotypic variation. Changes in gross phenotypes (e.g., branching, flowering time, growth rate) are the integrated result of changes in the pattern of many interacting genes. In many cases, these complex networks can only be deconvoluted through detailed analysis of changes in metabolites, the precise endpoints of complex biochemical pathways. Stated explicitly, metabolomics is the unbiased, relative quantification of the broad array of cellular metabolites, and their fluxes. Successful deployment of metabolite profiling requires the development of rapid, reliable and efficient assays for detecting phenotypes that are metabolic variants within natural or mutated populations and to ensure the phenotype is of interest in follow-up tests (Nadeau and Frankel, 2000).

Assays need to be developed which will allow the detection of as many metabolites as possible and preferably at

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high-throughput rates. Although the desire is to have a single analysis that captures all metabolites in a short time, there is, as yet, no single "silver bullet" analysis that will be appropriate for all metabolites with a high degree of sensitivity and resolution. The varying chemical characteristics of the different classes of compounds necessitate several analyses. A number of analytical approaches are currently available that can image a large number of metabolites, but they need to address the problems of co-eluting interference and be able to accurately identify as many of the peaks as possible.

Broad spectrum metabolite profiling by analyzing thermally-stable trimethylsilyl (TMS)-derivatized metabolites by gas chromatography-mass spectrometry (GC-MS) remains the gold standard, however, NMR-based metabolomics is gaining in popularity, and protocols are being developed for both capillary electrophoresis-MS and liquid chromatography-MS metabolomics. Typically, GC-MS analyses capture soluble carbohydrates, glycosides, sugar alcohols, sugar phosphates, organic acids, amino acids, free fatty acids and hydroxylated secondary carbon compounds (e.g., phenolic compounds) and aminated compounds that are small molecules (<1000 Da). Once the data deconvolution criteria (retention time index and key m/z fragments) are established for the large numbers of metabolite signatures, these mass criteria coupled with narrow retention time windows for each metabolite allow the identification of the metabolites that are present. Selected ion traces of the key m/z fragments are used to separate co-eluting peaks and permit accurate relative quantification.

Coupling metabolic profiling with analysis of mutant and transgenic lines is a key approach that is particularly promising. Metabolomics can be used to differentiate phenotypes and genotypes at the metabolite level that may or may not produce visible phenotypes (Sumner *et al.,* 2002). Although a number of studies are now emerging that are using such an approach, few studies have focused on secondary metabolites outside of the lignin biosynthetic pathway. Differences between wild-type *P. tremula* × *alba* and two transgenic lines with modified lignin monomer composition, were interrogated using metabolic profiling (Robinson, 2005). Analysis of metabolite abundance by GC-MS, coupled with principal components analysis (PCA), successfully differentiated between lines that had distinct phenotypes, whether samples were taken from the cambial zone or non-lignifying suspension tissue cultures. Interestingly, the GC-MS analysis detected relatively few phenolic metabolites in cambial extracts, although a single metabolite associated with the differentiation between lines was directly related to the phenylpropanoid pathway or other down-stream aspects of lignin biosynthesis. Carbohydrates, which have only an indirect relationship with the modified lignin monomer composition, featured strongly in the line-differentiating aspects of the statistical analysis. These findings demonstrate that metabolic traits can be dissected reliably and accurately by metabolomic analyses, enabling the discrimination of individual genotypes of the same tree species that exhibit marked differences in industrially-relevant wood traits (Robinson, 2005).

In another example, the effects of the over-expression of the *Acetobacter xylinum* UDP-glucose pyrophosphorylase (UGPase) under the control of the tandem repeat cauliflower mosaic virus promoter (2×35S) on plant metabolism and growth were investigated in hybrid *P. alba* × *grandidentata*. Transgenic 2×35S::UGPase *Populus* expressed impaired growth rates, significant increases in soluble sugars, starch and cellulose contents, and concurrent decreases in lignin content that favored syringyl moieties. Metabolite analysis revealed that the transgenic clone had a 270-fold increase in the salicylic acid 2-$O-\beta$-D-glucoside, a compound typically associated with the stress response, suggesting an increase in defense metabolites (Coleman, 2007).

Morse *et al.,* (2007) used metabolic profiling to investigate the molecular phenotypes of transgenic *P. tremula* × *alba* hybrids expressing the *nahG* transgene, a bacterial gene encoding salicylate hydroxylase that converts salicylic acid to catechol. Although neither salicylate nor catechol concentrations were altered in mutant lines vs the nontransformed control, the transgenic lines with the *nahG* insertion had a greatly depleted concentration of salicylic acid glucoside (5–9%) and elevated concentration of catechol glucoside (3.5–5.2x). The metabolomic analyses led to the identification of a metabolic grid immediately surrounding salicylic acid biosynthesis that indicated a complex compensatory mechanism operates to maintain consistent levels of salicylate and catechol, the substrate and product, respectively, of the *nahG* enzyme. Not only were higher-order salicylates, such as salicin, salicortin and tremuloidin, reduced in some of the transgenic lines, but so were a large number of phenolic glycosides and hydroxycinnamoyl-quinate esters. Although the transgenic lines were not visibly different from the controls, metabolomic analyses indicated an extensive impact on secondary metabolism of the transgenic clones. *Populus* species vary greatly in the presence and concentrations of such hydroxycinnamoyl-quinate esters in leaves (Tsai *et al.,* 2006) and these secondary metabolites are often greatly impacted by the manipulation of genes that are thought to even be only indirectly related to secondary metabolism through affects on shoot and root growth and alterations in carbon allocation between these organs (Busov *et al.,* 2006).

B. Genetical Metabolomics

Genetical metabolomics aims at identifying genomic loci that regulate the level of secondary metabolites of interest using comprehensive metabolomics approaches coupled to large-scale genetic marker analyses. Genetical metabolomics [metabolite profiling combined with quantitative trait locus (QTL) analysis] has been proposed as a new tool to identify loci that control metabolite abundance (Tschaplinski *et al.,* 2005). In this preliminary GC-MS based assessment, the fine root metabolite concentrations of the progeny of an interspecific backcross between (*P. trichocarpa* × *deltoides* '52–225') × *P. deltoides*

'D124' (Family 13) was subjected to QTL analysis. A number of metabolite QTL (mQTL) were identified, including a large-effect mQTL that explained >10% of the phenotypic variation in the concentration of trichocarpinene, a secondary metabolite, and its glucoside, trichocarpin. This concept was further evaluated in a case study with a number of flavonoids in *Populus* (Morreel *et al.,* 2006). Using HPLC analysis, the peak abundances of 15 closely-related flavonoids present in apical tissues were analyzed for two full-sib families, *P. deltoides* cv. S9–2 × *nigra* cv. Ghoy and *P. deltoides* cv. S9–2 × *trichocarpa* cv. V24. Correlation and QTL analysis were used to detect flux control points in flavonoid biosynthesis. Four robust mQTL, associated with rate-limiting steps in flavonoid biosynthesis, were mapped with a tentative function assigned to three of these mQTL and the corresponding candidate genes mapped. In summary, extensive biochemical characterization of unique metabolic phenotypes of both mutants and segregating progeny (backcross and F_2) can provide insight into gene function and how metabolic pathways are interconnected. In addition, elucidating these biochemical networks will provide opportunities for tailoring metabolic engineering for the overproduction of unique secondary metabolites (Tschaplinski *et al.,* 2005).

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C. Genome Data Mining

Once genome sequences become available, data mining of the sequenced genome offers a systematic approach to exhaustively characterize the biosynthetic potential of an organism. The complete genome sequence provides a novel resource for studying secondary metabolism in *Populus*. Homology-based gene identification is the most straightforward approach. Although chemically complex, the biosynthesis of secondary metabolites involves a limited number of biochemical reactions such as methylation, acetylation, cyclation, hydroxylation and glycosylation (Dudareva *et al.,* 2004). The genes encoding enzymes catalyzing these reactions are often present in families with multiple members. For instance, the *Populus* genome contains 312 P450 genes (Nelson, 2006), which are involved in very diverse biochemical processes. The structural genes regulating phenylpropanoid metabolism in *Populus* have also been previousy characterized at the whole-genome level (Tsai *et al.,* 2006). The numbers of *Populus* homologs of selected gene families with established roles in secondary metabolite biosynthesis are listed in Table 1. The large numbers of genes of secondary metabolism in the *Populus* genome imply that many secondary metabolites synthesized by *Populus* remain to be identified.

Identifying homologs of known genes could also point to the possibility that a novel metabolite is being produced. In *Arabidopsis*, for example, a predicted open reading frame with high similarity to known oxidosqualene cyclases was identified through bioinformatics, suggesting that *Arabidopsis* produces a novel terpenoid. This gene was then cloned and shown to convert 3(S)-oxidosqualene to the previously unknown triterpene alcohol (3S,13S,14R)-malabarica-8,17,21-trien-3-ol, which was named thalianol (Fazio *et al.,* 2004). Detailed analysis of the *Populus* genome sequence may lead to similar findings.

D. Integrated Genomics

In addition to metabolomics and genome data mining, various functional genomic tools currently developed for *Populus* have significantly facilitate the identification and characterization of genes of secondary metabolism. One of these functional genomic tools is large-scale transcript profiling or transcriptomics. This can be conducted using large-scale expressed sequence tag (EST) analysis, Serial Analysis of Gene Expression (SAGE), Massively Parallel Signature Sequencing (MPSS) or microarray analysis. As described in previous sections, secondary metabolites play important roles in *Populus* defense against insect herbivores. A recent genomic study used normalized and full-length cDNA libraries, ESTs, and a cDNA microarray to study forest tent caterpillar-induced defenses of hybrid poplar (*P. trichocarpa* × *deltoides*) (Ralph *et al.,* 2006). A total of 1191 genes were found to be significantly upregulated by forest tent caterpillar feeding, which include the candidate genes of secondary metabolism encoding polyphenol oxidase, isoflavone reductase and terpene synthase.

Transcriptomics, when combined with metabolomics, is more useful in identification of genes of secondary metabolism. Correlation of transcript profiling with metabolic profiling can narrow down the number of candidate genes, providing further evidence of the involvement of specific candidate genes for production of certain metabolites. This has been well demonstrated in the identification of many genes of secondary metanolism in other plant species, for example, the methyl ketone synthase from wild tomato (Fridman *et al.,* 2005). Wild tomato is known to produce a class of secondary metabolites known as methyl ketones. Metabolic profiling indicated that the compounds are synthesized in trichomes. An EST-based transcript profiling of the trichome identified a candidate gene based on its overrepresentation. Biochemical characterization verified that the gene indeed encodes methyl ketone synthase (Fridman *et al.,* 2005). Certain secondary metabolites are produced through complex biochemical pathways. The production of such metabolites may be regulated at the pathway level. Consequently, the genes encoding individual enzymes in the pathway likely show coordinated pattern of expression. Coexpression analysis can therefore be very useful in identifying genes of the same biochemical pathway (Saito *et al.,* 2008). With the accumulation of transcriptome data sets for *Populus* in the public domains, coexpression analysis for genes/pathways of secondary metabolism will be more informative.

Proteomics can also be used as a functional genomic tool in the study of plant secondary metabolism (Jacobs *et al.,* 2000). For example, proteomics was used to study the biosynthetic pathway of isoflavonoid-derived phytoalexins in *Phaseolus vulgaris*. Phenylalanine ammonia-lyase (PAL) and chalcone

synthase (CHS) are key enzymes in the biosynthesis of these phytoalexins. An increase in the synthesis of PAL and CHS in cell suspension cultures of *P. vulgaris* treated with elicitors coincides with the onset of phytoalexin accumulation (Bell *et al.,* 1986). Proteomics has also been used to study the responses of *P. euphratica* to heat stress (Ferreira *et al.,* 2006). Many proteins involved in metabolism were identified. Proteomics is especially useful when the regulation of metabolite production occurs at the translational level.

V. CONCLUSIONS

Populus trees produce a large number of secondary metabolites that are potentially involved in mediating a complex mixture of facultative and obligate interactions with biotic agents from symbioses to pathogenicity (Singer *et al.,* 2003). In addition, many of these secondary metabolites may be involved in tolerance of *Populus* to abiotic stresses. It is foreseeable that secondary metabolites synthesized by *Populus* are going to be continually identified. Elucidation of the biological/ecological roles of individual secondary metabolites in *Populus*, however, is going to be a daunting task. Identification of the genes for their biosynthesis provides genetic tools for such investigations. Function of specific secondary metabolites in *Populus* can be elucidated using a transgenic approach. After the genes for the production of specific secondary metabolites are identified, the expression of these genes can be altered through overexpression or downexpression via RNAi or gene silencing. The biological/ecological functions of individual genes and their products can then be assessed using transgenesis. In contrast to stable transformation that is usually difficult and time-consuming, virus induced gene silencing (VIGS) provides a rapid method for functional evaluation. The recent construction of a gene-silencing vector based on *Populus* mosaic virus offers the opportunity to start investigating VIGS approaches in *Populus* (Naylor *et al.,* 2005), including characterization of genes of secondary metabolism.

Thorough understanding of secondary metabolite biosynthesis and their biological roles will provide novel knowledge and tools for genetic improvement of various traits. It is expected that *Populus* will have an increased utilization as a bioenergy feedstock. Many *Populus* cultivars are susceptible to pathogens (Ostry and McNabb, 1985). Because *Populus* plantations are complex ecosystems providing essential habitats for diverse organisms, vast application of pesticides may not be feasible (Bohlmann *et al.,* 2004). It is therefore important to develop resistant *Populus* cultivars. In addition, if *Populus* trees are going to be widely used as a bioenergy feedstock, they need to be able to grow in various environments where the trees may experience extreme weather conditions. Resistance to drought and temperature stress will be especially important. Again, secondary metabolism presents an important target for genetic improvement of *Populus* resistance to such abiotic stresses. In using *Populus* as a feedstock, one important obstacle is cell wall recalcitrance, of which lignin is the major factor. Reducing lignin content has been demonstrated to be a useful strategy to increase the yields of saccharification (Chen and Dixon, 2007). It is therefore important to fully understand the biosynthesis of lignin and its regulation. Equally important, we need to understand how the alteration of lignin content and composition will affect *Populus*-environment interactions, which in turn can impact *Populus* productivity.

Variations in secondary metabolite profiles among different genotypes of *Populus* have been observed (Lindroth *et al.,* 2002). It will also be interesting to determine whether there are correlations between specific chemical profiles with specific environmental factors associated with specific ecosystems, which may provide novel insights into the evolution of secondary metabolism. In addition, a comprehensive understanding of biosynthesis and function of secondary metabolites in *Populus* (Jansson and Douglas, 2007) will provide important references for our understanding of secondary metabolism in other tree species, which may lead to accelerated genetic improvement of trees.

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