Chapter Five

MOLECULAR BIOLOGY AND BIOCHEMISTRY OF INDUCED INSECT DEFENSE IN *POPULUS*

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INTRODUCTION

All plants are faced with a wide variety of potential pests and pathogens. Being long-lived and large organisms, trees in particular must be adapted to defend themselves against these threats in order to persist in the environment. Plant defense is thought to be a driving force in the evolution of diverse secondary metabolites, and many phytochemicals with strong anti-herbivore or anti-microbial activities have been identified.[1] The characterization of many anti-herbivore proteins and their corresponding genes by using the tools of biochemistry and molecular biology has contributed to our understanding of plant defense, in particular as many of these genes are upregulated by herbivore stress. Within the last decade, the rise of plant genomics has accelerated the rate of gene discovery dramatically; it now permits the rapid identification of specific defense-related genes, as well as the analysis of genome-wide patterns of gene expression in response to herbivory.

The objective of this review is to provide an overview of our knowledge of the induced anti-herbivore defenses in *Populus*, a model tree for plant molecular biology and genomics. Unlike other model plants, *Populus* are often ecological keystone species in forest ecosystems, and therefore have complex interactions with symbionts, pathogens, and pests. Furthermore, the genus is found throughout the northern hemisphere and has widely distributed species with unique adaptations. These characteristics will make *Populus* a unique system for studies of plant-environment interactions, in particular herbivore defense, to which powerful genomics tools can be applied. In this chapter, recent progress in the area of induced defense of *Populus* will be reviewed with an emphasis on molecular biology approaches, and placed in the context of earlier chemical ecology studies. Most of the studies involving plant defense to date have focused on leaf defenses against folivore pests, but this will likely change in the future as *Populus* as a model system is explored in greater detail.

THE BIOLOGY OF *POPULUS*, THE MODEL TREE

The genus *Populus* includes hardwood trees commonly referred to as poplars, cottonwoods, and aspen, which are found throughout North America, Europe, and northern Asia. They are particularly dominant in the boreal forest and in the parkland zones of Canada and the American midwest.[2] Widespread North American species include *P. balsamifera* (balsam poplar), with a range from Western Alaska to northern Quebec and Newfoundland, and *P. tremuloides* (trembling or quaking aspen), which is found from Alaska to Mexico. Both are prominent species of the boreal forest of Canada. *P. trichocarpa* (black cottonwood) is found along the Pacific coast from California to Alaska. The range of *P. tremula* (European aspen)

extends across Europe into northern Asia, and *P. nigra* (black poplar) is also widely distributed and planted.[2]

Ecologically, poplars tend to be early successional species, which is reflected in their rapid growth rates, and is one reason for their widespread use in plantation forestry. Poplars tend to reproduce asexually and form clones by vegetative propagation, an ecological adaptation for competing in favorable environments. This feature also makes them useful trees for forestry and plant biology research, as a particular clone or hybrid with desirable characteristics can be replicated easily. Many species of *Populus* occupy the riparian zones along creeks, rivers, and wet areas, exemplified by the majestic *P. deltoides* (Eastern and plains cottonwoods). Drier upland environments are a habitat for many of the aspens, and some species such as the Asian *P. euphratica* can tolerate extreme drought.[2]

The genus *Populus* has been divided into six taxonomic sections based on ecological and morphological characteristics, of which sections Aigeiros (cottonwoods; *i.e.*, *P. deltoides*), Tacamahaca (balsam poplars; *i.e.*, *P. trichocarpa*), and Populus (aspens and white poplars; *i.e.*, *P. tremuloides*) are most prominent. Eckenwalder (1996)[3] conservatively defines 29 *Populus* species; the exact number is obscured by the propensity of poplars to form interspecific hybrids within taxonomic sections where ranges overlap. The relative ease with which poplars can be crossed has led to breeding programs for rapid-cycling poplars suitable for plantation forestry and shelterbelts. There is a long history of poplar breeding in Europe and North America, and interspecific hybrids between species in the same or in different sections have been generated. Prominent examples of hybrids are *P. deltoides* x *P. nigra* (*P. x canadensis*), *P. trichocarpa* x *P. deltoides* (*P. x generosa*), and *P. nigra* x *P. macimowiczii*.[4] There are also several different aspen hybrids *(P. tremula* x *P. tremuloides*, *P. tremula* x *P. alba*). Many of these are used in research programs as well as in plantations. In the north-western United States, *P. trichocarpa x deltoides* crosses are well-adapted and produce impressive gains in intensive culture.

More than 300 pest insects have been recorded on poplar in North America, and in Europe the number of pest species is greater than 500.[5,6] Nevertheless, only a few species cause significant damage. The polyphagous forest tent caterpillar (*Malacosoma disstria*) causes dramatic defoliations of large tracts of aspen forest, with regular outbreaks approximately every 10 years in the lake states of the US and the boreal forest and parkland in Canada. While seldom lethal to healthy trees, repeated defoliation leads to reduced growth and wood formation, and under poor conditions leads to increased tree mortality.[5] The large aspen tortrix (*Choristoneura conflictana*) is also a major defoliator of aspen, causing outbreaks in the northern regions of the boreal forest. The cottonwood leaf beetle (*Chrysomela scripta*) causes significant damage to cottonwood and hybrid poplar plantations throughout North America, with the exception of the west coast.[5] These defoliators clearly have the potential for reducing productivity or growth of affected trees; simulated cottonwood leaf beetle defoliation of hybrid poplar resulted in reduction of growth and biomass

of up to 33%.[7] In addition to defoliators, there are also many boring insects (*i.e.*, *Saperda calcarata*), which cause significant damage to poplar via secondary pathogen infections or wind breakage. Overall, the number of herbivores attacking *Populus* is high relative to other tree species, which may represent a trade-off with poplar's high growth rates. In general, poplar-insect interactions studied at the molecular level should prove to be a promising area of research.

The ecological importance of *Populus* in the northern hemisphere and the extremely rapid growth of poplar hybrids in plantation forestry has generated wide interest in its use for wood products, biomass, phytoremediation, and carbon sequestration.[8, 9] Furthermore, *Populus* has become the model system for scientists interested in wood formation and tree biology in general.[10] Key characteristics of poplars which make them excellent experimental plants are their rapid growth, ease of vegetative or clonal propagation, and tractability to genetic engineering.[11] Poplar was the first woody plant to be genetically transformed, and a number of poplar and aspen genotypes are readily transformable by *Agrobacterium*.[12,13] In addition, detailed molecular genetic maps for *Populus* have been generated.[14,15] These permit specific regions and loci of the genome to be linked with physiological traits such as *Melampsora* rust resistance.[15, 16]

Extensive molecular and genomic tools, such as expressed sequence tag (EST) libraries and DNA microarrays, have been developed for poplar. Since the publication of the first *Populus* EST project,[17] over 200,000 ESTs from several different *Populus* species have been deposited in public databases (http://www.ncbi.nlm.nih.gov/). Most significantly, the recent elucidation of the complete *P. trichocarpa* genome sequence will have tremendous impact on poplar and other plant biologists. This is the first completed tree genome sequence and one of only three sequenced plant genomes, and will produce a plethora of new information for *Populus.*[9,11] Whereas *Arabidopsis* will continue to be the model plant of choice for many questions of fundamental cell biology and plant physiology, the rich environmental interactions of *Populus* will make this an attractive system for ecological studies. The large number of insect herbivores that utilize *Populus* species and the diversity of phenolic phytochemicals in *Populus* has already stimulated much research on poplar insect defense by chemical ecologists.[18-20] In addition, early molecular biology research on tree defense has been carried out in hybrid poplar,[8,21] further stimulating work on these trees.[22-24] Poplar is thus poised to become a key experimental system for studies of plant-insect interactions, where phytochemical, ecological, and genomic approaches can be integrated.

INDUCED DEFENSE AND ITS EFFECTIVENESS FOR POPLAR PEST RESISTANCE

Plant defense mechanisms can be preformed, or primarily expressed following herbivory, that is, induced. Ecologists first coined the term "induced resistance" for the observation that previous herbivory can result in heightened resistance to subsequent attacks by the same or different herbivores. This phenomenon has been described in dozens of plant species and taxa including conifers, grasses, herbs, and deciduous trees.[25] Interestingly, most examples come from perennial plants, consistent with a predicted evolutionary strategy of investing more heavily in defense compared to annuals. Most biochemical studies on induced defense have been carried out with herbaceous plants, and the defense mechanisms that underlie this resistance typically include anti-nutritive proteins, such as protease inhibitors and oxidative enzymes that reduce the assimilation of essential amino acids or otherwise destroy nutrients.[26-30] In addition, enzymes that lead to the synthesis of toxic or anti-feedant secondary metabolites can be induced, including alkaloids, phenolics, or terpenoids.[31] Recently, the release of volatiles during induced defense has been the focus of intense investigation.[32] These can act as direct defenses to discourage oviposition by the herbivore,[33] and as an indirect defense by attracting predators and parasitoid insects.[34-36]

The overall importance of the induced defense response in protecting the plant has been elegantly shown using jasmonate signaling mutants or transgenic plants that cannot induce a defense response; these plants show a hypersusceptible phenotype.[37-39] Such increased susceptibility extends to natural conditions, and in field experiments with transgenic *Nicotiana attenuata*, Kessler *et al*. (2004) were able to show that the induced defense is effective in limiting damage by both adapted and generalist herbivores.[40]

In poplar, the direct effects of induced defenses on insect pests has been extensively investigated by Raffa and coworkers. Using both choice and no-choice insect bioassays with a series of poplar hybrids, Havill and Raffa (1999) found that saplings damaged by previous herbivory were poorer hosts for gypsy moth larvae in subsequent attacks, sustaining up to 71% less damage. These changes were induced not only by live herbivores, but also by mechanical wounding and simulated herbivory.[41] Damage-induced protection of hybrid poplar to subsequent pest attacks was also demonstrated in experiments with forest tent caterpillar and whitemarked tussock moth (*Orygia leucostigma*).[42,43] Significantly, the induced resistance is observed not only in the wounded leaf but also in unwounded leaves of the plant. This indicates a systemic, or plant-wide, response, consistent with the systemic induction of defense-related genes (see below). A systemic defense system is well documented in other model plants such as tomato.[26, 44]

Interestingly, the same poplar hybrids can show substantial differences in the degree of induced resistance when different clones are compared. Some clones had dramatic induction of resistance, whereas others showed no effect.[41,42] Such genotype- and hybrid-dependent differences in pest susceptibility have also been documented when trees were not previously exposed to damage or other inducing treatments. In common garden experiments with *P. tremuloides*, large clonal differences in forest tent caterpillar and gypsy moth palatability are also observed.[45] Strong correlations of pest resistance with high levels of phenolic glycosides and, to some extent with condensed tannins, suggest that phytochemical defenses could be key factors for host suitability, at least in preformed defense. By contrast, the mechanisms which underlie induced resistance phenomena are just now beginning to be dissected, and are a major focus for research in poplar defense. These mechanisms appear to have both phytochemical and protein components.

PHYTOCHEMICAL DEFENSE IN *POPULUS*

Phytochemicals, also called secondary metabolites, have been extensively studied in *Populus* from both chemical and ecological perspectives. Like other Salicaceae, the genus *Populus* is characterized by a diversity of phenolics; in particular, the salicin-based phenolic glycosides are found in leaves and bark of all species examined.[18] These phytochemicals consist of glucosides of salicyl alcohol, which are generally further esterified and benzoylated.[46] Tremulacin and salicortin are particularly common in *P. tremuloides,* but related glycosides are present at varying levels in other species.[18] They are potent herbivore toxins, with negative effects on forest tent caterpillar, tiger swallowtail (*Papilio glauca*), gypsy moth, and large aspen tortrix larvae feeding on *P. tremuloides*.[20] In general, high levels of phenolic glycosides correlate closely with increased larval development times and reduced pupal weights. Since salicortin and tremulacin are unstable and may degrade during herbivore feeding,[46,47] the biologically active chemicals are not definitively known. However, during leaf maceration these glycosides decompose to release 6-hydroxy-2-cyclohexenone, which subsequently gives rise to phenol or catechol.[47,48] Ruuhola *et al*. (2001) found that salicortin is entirely degraded to salicin and catechol after passage through the alkaline gut of a lepidopteran herbivore (*Operophtera brumata*).[49] Work in the authors' laboratory also demonstrated the release of catechol from salicortin at high pH. Catechol is an excellent substrate for polyphenol oxidase, an induced defense enzyme in hybrid poplar and aspen.[22,50] The enzyme-mediated activation of phenolic glycoside-derived products could thus enhance their toxic effects (see below). To date there is little evidence that the synthesis of these phenolics is induced by insect damage. Although Lindroth and Kinney (1998) found a small increase in tremulacin and salicortin following leaf damage,[51] later experiments failed to corroborate this.[52, 53] This inconsistency may be due to environmentally-induced variability in phenolic levels.

A second class of defensive phytochemicals often found in poplar and aspen at substantial levels are the proanthocyanidins, or condensed tannins (CTs) (Fig. 5.1). Unlike the phenolic glycosides that are found exclusively in the Salicaceae, CTs are widespread in the plant kingdom.[54] These flavonoid polymers consist of mostly 4,8-linked flavan-3,4-diols and flavan-3-ols, ranging in size from 1440 to over 4500 Da depending on the species.[55,56] Flavonoids are derived from the general phenylpropanoid pathway by a series of enzymes beginning with the enzyme

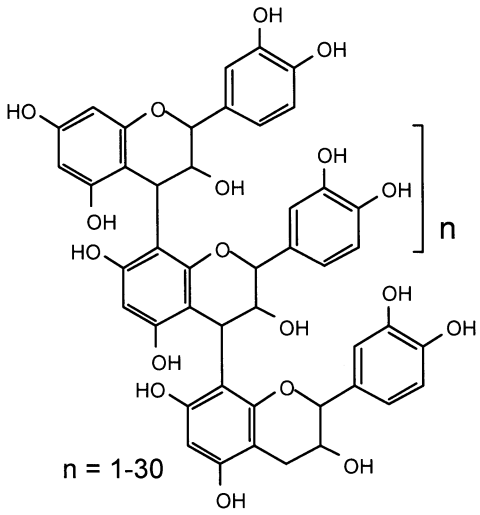
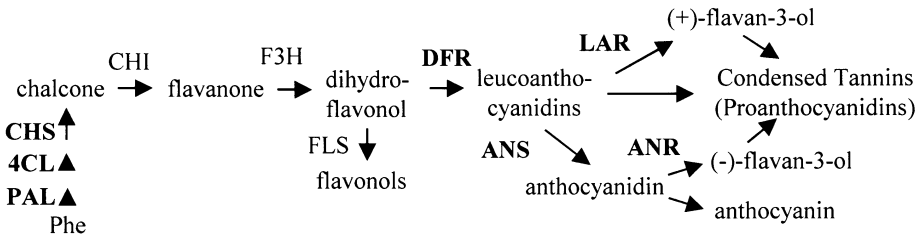


Fig. 5.1: Structure of condensed tannin. *P. tremuloides* condensed tannins are composed of an average of seven flavonoid monomers (Ayres *et a*l).[56]

chalcone synthase (Fig. 5.2), and essentially all major flavonoid enzymes have been cloned from *Arabidopsis* or other plants.[57] The final condensation steps of CT biosynthesis are not yet elucidated, however.[55] The availability of heterologous sequence information for genes involved in flavonoid synthesis has facilitated a molecular approach in the analysis of CTs in poplar defense (see below).

CTs can be effective anti-nutrients against insects, especially at high concentrations as can be found in *P. tremuloides* (up to 18 % DW).[20] Tannin effectiveness and mechanism of action has been debated,[58] but it clearly depends both on variations in chemical structure and plant source, as well as on the biochemical conditions in the gut of the particular insect species.[56,59,60] In *P. tremuloides*, CT levels were shown to be negatively correlated with gypsy moth and forest tent caterpillar larvae performance, although the effects were smaller than with the phenolic glycosides (C. P. Constabel and J. Spence, unpublished data).[45, 61]



Fig, 5.2. Biosynthesis of flavonoids and proanthocyanidins (condensed tannins). Enzymes in bold have been cloned from *P. tremuloides* and show induction by herbivory (Peters and Constabel, 2002[62]; R. Mellway and C. P. Constabel, unpublished data). Abbreviations are as follows: Phe, phenylalanine; PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate CoA Ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

CT synthesis is induced by herbivory of *P. tremuloides*, supporting the idea that they are important chemicals for defense.[52,62] The cloning and expression analysis of *P. tremuloides* dihydroflavonol reductase (DFR) showed that wounding and herbivory induces DFR transcripts as well as DFR activity as part of a systemic response.[62] This demonstrated that flavonoid and CT synthesis are part of the induced defense response in aspen leaves. Transcripts encoding the key phenylpropanoid and flavonoid enzymes phenylalanine ammonia lyase (PAL), 4-coumarate CoA ligase (4CL), and CHS were also induced by wounding.[62] Interestingly, specific isoforms of PAL and 4CL are associated with wound-induction and CT accumulation, whereas other isoforms appear to be responsible primarily for lignin synthesis.[53] We recently cloned two additional genes putatively encoding enzymes downstream from DFR in the CT pathway, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), by searching *Populus* EST databases with new sequence data from heterologous species.[63,64] LAR reduces flavan-3,4-diols to 2,3-*trans*-flavan-3-ols ((+)-catechin), whereas the latter is in a newly discovered pathway from anthocyanidins to 2,3-*cis*-flavan-3-ols ((-)-

epicatechin). Flavan-3-ols are required as starter units in the CT polymer, although *Arabidopsis* appears to use only the ANR-derived (-)-epicatechin.[65] Both ANR and LAR are expressed and induced by wounding in aspen leaves, suggesting both types of monomer are synthesized in *Populus* (R. Mellway and C. P. Constabel, unpublished). This is consistent with C-13 NMR analysis of the CT polymer isolated from aspen leaves, which demonstrated it contains both types of subunits.[56] Overall, the herbivore-induced pattern of expression of genes involved in CT synthesis points to a role of these chemicals in defense, and is the first step in the identification of regulatory genes modulating this pathway.

In contrast to the leaf phytochemical constituents, the study of poplar volatiles in the context of herbivore defense is only now beginning. Leaf damage by forest tent caterpillar herbivory leads to the release of E-ß-ocimene, (-)-germacrene D, and other terpenoid volatiles.[24] These emissions are substantially higher following insect damage than after mechanical wounding. Work in cotton and maize first reported that the mixture of volatiles released from wounded leaves is distinct from leaves subjected to live herbivores, and that different herbivores induce the release of different blends.[66, 67] Plant volatile bouquets are used by parasitic wasps to locate their hosts, and the release of volatiles in response to herbivory can be considered an indirect defense mechanisms. [35] In poplar, the induced emissions are preceded by increased expression of the germacrene D synthase gene, *PtdTPS1*. Therefore, volatile induction appears to be a component of the transcriptional response of poplar leaves to herbivory. Like other induced defenses, the induction of this gene has been observed in unwounded leaves of a wounded plant, indicating a systemic response.[24] It will be interesting to determine if the release of poplar volatiles from poplar influences the behavior of tent caterpillar parasitoids and predators, that is, if these induced volatiles can act as an indirect defense.

A characteristic feature of phytochemicals in natural populations is the variation in concentration and profile that is found among different individuals. Extensive work by Lindroth and others has documented such genotype-dependant variation in phenolics in *P. tremuloides* genotypes.[20,45,61,68] As mentioned above, high levels of phenolics correlate with increased pest resistance in this system. Comparisons of different *Populus* species and hybrids have also demonstrated a significant variation in levels and types of phenolic phytochemicals.[18,19] Further variability is often due to environmental conditions, as aspen plants grown with high soil nutrient availability contain reduced levels of condensed tannins[52,69,70] Such phytochemical variability is likely to have profound impacts on the effectiveness of defenses and evolution of resistance. Elucidating the genetic mechanisms for phytochemical variation in natural populations will be an important advance in understanding how defense mechanisms work in nature.

MOLECULAR ANALYSIS OF INDUCED DEFENSE

The induced defense response of plants is characterized by major changes in transcriptional patterns. By isolating herbivore or wound-inducible genes using molecular differential screening techniques, a number of defense-related genes have been isolated. Large-scale genomics and microarray studies indicate that hundreds of genes respond to herbivory, and genome-wide surveys of these responses will soon be possible in poplar and other plants (see below). How many induced genes contribute to defense is not clear, however. In this section, genes for which a defensive role has emerged or is likely will be highlighted.

Kunitz Trypsin Inhibitors

The pioneering work of Milton Gordon and coworkers first demonstrated wound-induced gene expression in hybrid poplar and led to the isolation of several wound-responsive genes by differential cDNA screening.[8,21] Genes with strong similarity to Kunitz trypsin inhibitors (TIs), chitinases, and vegetative storage protein (VSP) genes were cloned and shown to respond rapidly and systemically to damage. TIs are common defense proteins in many plants that interfere with protein digestion in insects and may have other toxic effects. Kunitz TIs are one of eight proteinase inhibitor families.[27,71] Their discovery in wounded poplar leaves was significant because it demonstrated the adaptiveness of the induced defense response. Subsequently, poplar TI-overexpressing tobacco leaves were found to reduce growth of tobacco budworm.[72]

Recent work from the author's laboratory has identified three additional hybrid poplar genes (*PtdTI3*, *PtdTI4*, *PtdTI5*), which belong to the Kunitz TI family, but show less than 50 % identity among themselves or with the original poplar TI gene.[21, 23] This sequence divergence suggested redundancy or multiplicity in defense; a preliminary scan of the completed *P. trichocarpa* genome indicates that there are over 30 distinct Kunitz TI genes (I. Major and C. P. Constabel, unpublished data). This number is similar to that estimated from potato tubers, where 21 distinct Kunitz TIs were identified.[73] We speculate that multiple protease inhibitors, perhaps with different protease specificities, have evolved to match the numerous gut proteases in insect pests.[74] The protease inhibitory activity of three TIs has been confirmed *in vitro* using recombinant proteins, and provides preliminary evidence for distinct specificities for these inhibitors (I. Major, C. Melnyk, and C. P. Constabel, unpublished data).[75] Given the observation that pests can express inhibitor-resistant proteases, it is likely that the multiplicity of poplar TIs reflects functional specialization within the TI gene family.[76-78]

The idea that Kunitz TIs have diversified in response to selective pressure by insect pests is further supported by preliminary evidence that the TI genes are

evolving rapidly. In *P. tremuloides*, Haruta *et al.*, (2001) found that native aspen genotypes contain more restriction fragment polymorphisms for two TI genes (*PtTI1/2* and *PtTI3*) than other genes.[75] Later comparative studies of Kunitz TI orthologs among *P. trichocarpa*, *P. tremula*, and related hybrids demonstrated a greater than expected sequence divergence and high ratio of non-synonymous to synonymous nucleotide changes, suggesting positive selection of these genes.[79] In the context of plant defense, such sequence divergence could indicate an evolutionary arms race with insect herbivores.

Polyphenol Oxidase

In hybrid poplar and trembling aspen, leaf damage by wounding or herbivory induces polyphenol oxidase activity (PPO; EC 1.10.3.2).[22,50,80] This enzyme can oxidize a variety of *ortho*-diphenolic compounds to their respective reactive quinones using molecular oxygen.[81] Leaf PPOs have been cloned from hybrid poplar and trembling aspen, and their wound- and herbivore-induced expression suggests a defensive function.[22,50] Quinones produced by PPO during insect feeding can lead to protein and amino acid alkylation and cross-linking, with concomitant loss of essential amino acid assimilation by leaf-eating herbivores.[82,83] In addition, reverse disproportionation of quinones to semiquinones, ultimately resulting in H_2O_2, could contribute to oxidative stress for the insects. Poplar PPO is stable and not only resists proteolysis in the gut of forest tent caterpillar, but is activated by passage through the gut, consistent with a role in defense against these insects.[84] Which substrates poplar or aspen PPO oxidizes during defense reactions is not yet known; as mentioned earlier, catechol is an excellent PPO substrate and can be produced by decomposition of the phenolic glycoside salicortin and tremulacin.[47,48,50] Other possible substrates include caffeic acid derivatives or catechins, common phytochemicals that have been reported from *Populus*.[18,19]

Three poplar PPO gene families have been described to date, and members of at least two of these families show induction by wounding and expression in different plant organs.[84] To test if the inducible poplar PPO is important in defense against lepidopteran insects, the hybrid poplar *PtdPPO1* cDNA was overexpressed in hybrid aspen. Feeding on PPO-overexpressing leaves reduced forest tent caterpillar performance relative to control leaves; however the effect was visible only with older egg masses that gave rise to less vigorous larvae.[84] Nevertheless, this direct proof of the anti-herbivore effects of PPO is the first such demonstration for a poplar defense gene in transgenic *Populus*, and is a first step in the functional analysis of defense genes in poplar defense.

Chitinases

Genes with similarity to chitinases were among the first wound-induced defense genes from *Populus* to be characterized. Two distinct chitinase genes named *win6* and *win8*, sharing approximately 50% amino acid sequence identity, are strongly upregulated in hybrid poplar leaves following wounding.[21, 85] Leaf chitinase activity increases following wounding, and transgenic tobacco expressing *win6* also show enhanced chitinase activity.[86] The *win6* and *win8* chitinases are unusual in having close resemblance to class 1B basic chitinases from other species, yet having an acidic pI. Other class 1B chitinases are also wound-induced, unlike the typical acidic chitinases.[87] EST sequencing of a wound-induced cDNA library identified two other chitinase-like genes, at least one of which is wound-induced.[23] Chitinases have been shown to act as pathogen defenses, and their wound-induction may prevent wound site ingress of opportunistic pathogens. In addition, it is possible that they also act directly against insect herbivores. The abundance of chitinase transcripts in the EST library and their strong induction on macroarrays supports this idea.[23] A possible site of action of anti-herbivore chitinase activity might be the peritrophic membrane, which functions in protecting the underlying cells from digestive enzymes.[88] Microbial and insect-derived chitinases have been shown to be detrimental to herbivorous insects.[89, 90]

Other Induced Genes and Proteins

Several other known defense-related genes have been found to be upregulated by wounding. Lipoxygenase and hydroperoxide lyase are rapidly induced, most plausibly for biosynthesis of jasmonate and other octadecanoid signals.[23] Genes involved in phenylpropanoid and lignin biosynthesis, such as phenylalanine ammonia lyase, cinnamoyl Co-A reductase, are also upregulated (I. Major and C. P. Constabel, unpublished), possibly contributing to leaf toughening via lignin-like depositions.[91] Some of these genes may also contribute to the accumulation of other phenolic phytochemicals such as the condensed tannins. The strong induction of cytochrome P450 genes, which often encode hydroxylases, may also be related to secondary metabolism.

Other wound- or herbivore-induced transcripts encode proteins previously identified as vegetative storage proteins. For example, Davis *et al*. (1991) identified a strongly inducible cDNA (*win4*) with high similarity to a bark storage protein.[92] This so-called vegetative storage protein (VSP) is also expressed at low levels in young, growing shoots, and is often found in association with vascular tissue.[93] Another highly inducible gene in poplar leaves encodes an acid phosphatase, which in *Arabidopsis* and soybean acts as a VSP.[94] How these function in defense is unclear, but it may be related to nitrogen remobilization as part of a plant-wide defense strategy similar to senescence.

IMPACT OF GENOMICS ON STUDIES OF *POPULUS* DEFENSE

Since the first report of a poplar expressed sequence tag (EST) project,[17] *Populus* genomics has grown rapidly, and there are now several high-throughput sequencing projects in Europe and North America.[9] EST collections have provided a crucial resource for the cloning of genes and gene families, and are the basis for the construction of current poplar microarrays. Poplar arrays permit the simultaneous analysis of gene expression of thousands of genes, and have been used to document the complexity of gene expression underlying developmental processes such as wood formation and resource allocation,[95, 96] as well as environmental adaptation and stress responses.[23,91,97,98] Poplar fully entered the genomics era with the complete elucidation of the nucleotide sequence of the *P. trichocarpa* genome (released in September 2004). Many new resources will soon be available, including arrays for monitoring gene expression of essentially the entire poplar genome. These will allow for faster discovery of genes important for developmental and adaptive processes.

EST Libraries as a Resource for Poplar Genomics

With well over 200,000 ESTs from *Populus* species in the public domain, poplar EST databases are useful in many experiments. First, they provide immediate access to whole sets of genes of interest for specific tissues and organs. Second, ESTs from several *Populus* species are available, which permits comparisons of orthologous genes that can provide insight into molecular evolution of genes. As described earlier, comparative analyses of ESTs encoding Kunitz TIs from several different *Populus* species and hybrids suggest these genes are subject to positive selection and evolving rapidly, perhaps due to herbivore pressure. Third, the EST collections can be used to generate tissue-specific expression profiles, or "digital northerns." Since the ESTs are derived from separate cDNA libraries constructed from a range of tissues and experimental treatments, the abundance of a given transcript in the library provides an indication of its level of expression in that tissue.[99] This approach has been effectively used to study lipid metabolism, and can be used to obtain preliminary gene expression data on any gene.[100] The relative representation of genes within an EST library can also be used to obtain a snapshot of gene expression, for example during the herbivore defense response of hybrid poplar.[23] In this study, the inducible chitinases and trypsin inhibitors were found to be among the ten most abundant predicted gene products in the EST library, together with many photosynthesis-related and primary metabolism genes.[23] This result suggests that wound stress has had a significant impact on the leaf transcriptome as a whole.

Transcript Profiling of Poplar Induced Herbivore Defense

Global changes in gene expression in response to wounding and insect herbivory have been studied using cDNA macro- or micro-arrays. In addition to providing insight into signal transduction and global patterns of gene expression, these approaches are identifying many new wound- and herbivore-responsive genes. Macroarrays constructed from a small EST set of 569 genes were used in the authors' laboratory to profile gene expression in *P. trichocarpa x P. deltoides* (TD) hybrid poplar. Mechanical wounding and treatment with the defense hormone methyl jasmonate significantly upregulated 107 and 163 genes after 24 h, respectively, including the defense genes discussed previously. An experiment using a much larger array (approximately 10,000 genes) found 947 significantly upregulated transcripts 14 days after wounding TD hybrid poplar leaves by abrasion.[91] While it is difficult to directly compare array experiments carried out under different conditions and using partial genome arrays, the proportions of induced genes are similar to reports from *Arabidopsis*. Cheong *et al.* (2002) identified 657 wound-responsive transcripts on arrays with 8200 genes (8%),[101] and Schenk *et al.* (2000) found that approximately 9% of genes on a 2375 gene array were upregulated by methyl jasmonate.[102]

Such array studies produce interesting lists of genes, but it is not always easy to establish their biological significance or roles. As more studies of plant defenses employ microarrays, broad comparisons between experiments and plant species should help to narrow the search for major defense genes. For example, there are common features of the induced defense response in poplar and *Arabidopsis,* including expression of jasmonate biosynthesis genes, trypsin inhibitors (though from different families), chitinases, and cytochrome P450 enzymes. Interestingly, Smith *et al*. (2004) identified a number of genes encoding cysteine proteases, which were also highly represented in a wounded leaf EST library.[23, 91] Cysteine proteases were recently found to have direct toxicity to insects.[103,104] Wound- or herbivore-induced phenylpropanoid gene expression in *Populus* (see below) appears to be a common feature of the defense response and is seen in *Arabidopsis*, *Nicotiana attenuata*, and other species.[101,105] Other wound-induced poplar genes have not been described from other plants in a defense context; these include genes encoding apyrase, lipases, invertase, and predicted proteins without any known functions (I. Major and C. P. Constabel, unpublished data).[23] How these might function in plant defense is being investigated further, but their strong response to herbivore stress suggests a direct involvement. Many of the 14-day wound-induced genes identified in poplar by Smith *et al.* (2004) are metabolism-related, but those transcripts that encode gene products related to photosynthesis and protein synthesis typically decreased in response to wounding.[91] The repression of photosynthesis-related genes is presumably indicative of resource reallocation for defense and has also been described in *N. attenuata*.[105,106]

DNA array experiments are clearly powerful tools that are beginning to have an impact in studies of poplar defense. Whole genome oligonucleotide-based arrays will soon be available, which will increase the volume of gene expression data further. We can expect an unprecedented expansion of our knowledge of poplar defense at the level of transcription. Controlled cross-species experiments will provide comparisons of global gene expression, and determine how variable induced defense is among species. Genomics tools will also be crucial in the drive to understand the molecular basis for variation in pest resistance among poplar genotypes.

DEFENSE SIGNALING IN *POPULUS*

How cellular signaling pathways activate the defense response is a major question for poplar and other model plants.[29,30,107] As has been established for other plants, a central component in defense signaling in poplar appears to jasmonic acid and related compounds, collectively known as jasmonates. These are fatty acid derived plant hormones that act as both developmental and stress signals.[108] Treatment of poplar and aspen with methyl jasmonate has a strong effect on inducing defense gene expression (PPO and TIs) and resistance to gypsy moth larvae.[22,41,50,75] Conversely, wounding of poplar leaves induces key enzymes of jasmonate biosynthesis such as lipoxygenase and allene oxide cyclase, suggesting involvement of jasmonate biosynthesis as an amplification of a primary defense signal.[23] Other components of herbivore defense signaling pathways of poplar remain unknown, but this will likely change rapidly as the poplar genome database is mined for genes with candidate signaling functions. In *Arabidopsis* the wound-induced transcriptome includes a large number of transcription factor- and signaling protein-encoding genes that may modulate the induced response;[101] the wealth of information available from this model plant will facilitate rapid progress in poplar.

Poplar induced defense has features that could make it a valuable system for studies of whole-plant signaling. Parsons *et al.* (1989) first demonstrated that hybrid poplar herbivore defense is systemic, and that wounding of lower leaves induces gene expression in the upper, unwounded leaves.[21] Subsequent work demonstrated that the strongest systemic induction occurred in orthostichous leaves that have direct vascular connections.[109] The wound signal appears to be transmitted from source to sink leaves, and is dependent on photoassimilate transport in phloem, since it can be disrupted by shading of the source leaf.[109] Arnold and Schultz (2002) reported that wounding and jasmonic acid increase the sink strength of young poplar leaves, and that the concomitant increase of imported carbohydrates in these leaves is used for the synthesis of condensed tannins.[110] It thus appears that source-sink relations and wound signaling are related, and that signaling correlates with sink import of sucrose. However, in *P. deltoides*, leaf resistance to folivore beetles could be induced both above and below the damaged leaves.[111] Likewise in *N. attenuata*, wound signal transmission was reported from both sink and source leaves.[112] In our

laboratory, wounding of poplar leaves was found to cause increased TI gene expression in roots, thus suggesting that signal movement does occur from shoots to roots (I. Major and C. P. Constabel, unpublished data). The systemic wound signal in poplar has not been identified, but in tomato, recent evidence suggests that it is a jasmonate-related compound that acts in concert with a short 18 amino acid peptide called systemin.[44,113] While jasmonates are universally found in the plant kingdom, systemin-like peptides have only been found in the Solanaceae.

Many defense responses can be triggered by mechanical damage as well as by live insect herbivores. Other responses, for example volatile production, require insect-specific cues.[114] The analysis of volatiles from maize infested with different leaf-eating pests suggests that there are pest species-specific effects, even when these feed on the same host plant.[67] The effects of live caterpillars can be mimicked with insect regurgitant from feeding caterpillars, which has led to the purification of insect elicitors from insect saliva and regurgitant. A fatty acid conjugate known as volicitin was first isolated from beet army worm regurgitant, and identified as N-(17-hydroxylinolenoyl)-L-glutamine.[115] Similar fatty acid conjugates that stimulate plant defense reactions have been purified from saliva in other leaf-eating insects.[29,116] Regurgitant can activate many wound-induced defenses in the absence of significant mechanical damage, and can variously modulate the wound response.[39, 116, 117]

The ability of herbivorous insect regurtitant to elicit plant defense responses have also been observed in poplar. Havill and Raffa (1999) first showed for poplar that gypsy moth and tent caterpillar larvae regurgitant stimulates induced resistance to subsequent pest attack, much like mechanical wounding or MeJa treatment.[41] At the level of gene expression, macroarray experiments have demonstrated that forest tent caterpillar regurgitant to leaves is a strong inducer of all the major wound-induced defense genes, even in the absence of significant leaf damage (I. Major and C. P. Constabel, unpublished data).

SUMMARY

Populus species are susceptible to many insect pests. The induced pest defense of various *Populus* species or hybrids has been studied at the phytochemical, molecular, and genomic levels. This response involves rapid changes in gene expression, which has provided insight into many defense processes. Induced defenses include flavonoid gene expression and condensed tannin accumulation, release of terpenoid and other volatiles, and synthesis of trypsin inhibitors and enzymes such as chitinase and polyphenol oxidase. Induced defense mechanisms thus include both protein-based and phytochemical defense mechanisms. Transgenic studies have demonstrated that induced defense proteins, such as trypsin inhibitors and polyphenol oxidase, can have a negative effect on leaf-eating insects. In the future, the availability of genomics tools will greatly facilitate the identification of the complete herbivore-induced suite of genes, but a major challenge will be to

determine the function of individual genes in defense. Comparing herbivore-induced gene suites of poplar to *Arabidopsis* and other plants will help to define general defense responses and conserved signaling processes, and comparisons of responses to different herbivore guilds will be also be instructive. In addition, genomics approaches should lead to rapid progress in our understanding of defense signaling, such as identifying transcription factors that regulate sets of induced genes and signal transduction cascades.

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REFERENCES

1. HARBORNE, J.B., Introduction to Ecological Biochemistry. 4th ed., Academic Press, London. 1993, 318 p.
2. DICKMAN, D.I., An overview of the genus *Populus*, *in:* Poplar Culture in North America (J.G. Isebrands, D.I. Dickmann, J.E. Eckenwalder, J. Richardson, eds.), NRC Research Press, Ottawa. 2001, pp. 1-42
3. ECKENWALDER, J.E., Systematics and evolution of *Populus*, *in:* Biology of *Populus* and Its Implications For Management and Conservation (H.D. Bradshaw, R.F. Stettler, P.E. Heilman, T.M. Hinckley, eds.), NRC Research Press, Ottawa. 1996, pp. 7-32
4. ECKENWALDER, J.E., Descriptions of clonal characteristics, *in:* Poplar Culture in North America (J.G. Isebrands, D.I. Dickmann, J.E. Eckenwalder, J. Richardson, eds.), NRC Research Press, Ottawa. 2001, pp. 331-382
5. MATTSON, W.J., HART, E.A., VOLNEY, W.J.A., Insect pests of *Populus*: coping with the inevitable, *in:* Poplar Culture in North America (J.G. Isebrands D.I. Dickmann, J.E. Eckenwalder, J. Richardson, eds.), NRC Research Press, Ottawa. 2001, pp. 219-248
6. YVES, W.G.H., WONG, H.R., Tree and Shrub Insects of the Prairie Provinces.Canadian Forest Service Information Report NOR-X-292., Edmonton. 1988.
7. REICHENBACKER, R.R., SCHULTZ, R.C., HART, E.R., Artificial defoliation effect on *Populus* growth, biomass production, and total nonstructural carbohydrate concentration, *Environ. Entomol.*, 1996, **25**, 632-642.
8. BRADSHAW, H.D., PARSONS, T.J., GORDON, M.P., Wound-responsive gene expression in poplars, *For. Ecol. Manag.*, 1991, **43**, 211-224.
9. TUSKAN, G.A., DIFAZIO, S.P., TEICHMANN, T., Poplar genomics is getting popular: The impact of the poplar genome project on tree research, *Plant Biol.*, 2004, **6**, 2-4.

10. WULLSCHLEGER, S.D., JANSSON, S., TAYLOR, G., Genomics and forest biology: *Populus* emerges as the perennial favorite, *Plant Cell*, 2002, **14**, 2651-2655.
11. BRUNNER, A.M., BUSOV, V.B., STRAUSS, S.H., Poplar genome sequence: Functional genomics in an ecologically dominant plant species, *Trends Plant Sci.*, 2004, **9**, 49-56.
12. PENA, L., SEGUIN, A., Recent advances in the genetic transformation of trees, *Trends Biotechnol.*, 2001, **19**, 500-506.
13. HAN, K.H., MEILAN, R., MA, C., STRAUSS, S.H., An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus Populus), *Plant Cell Rep.*, 2000, **19**, 315-320.
14. TAYLOR, G., *Populus*: *Arabidopsis* for forestry. Do we need a model tree?, *Ann. Bot.*, 2002, **90**, 681-689.
15. YIN, T.M., DIFAZIO, S.P., GUNTER, L.E., JAWDY, S.S., BOERJAN, W., TUSKAN, G.A., Genetic and physical mapping of *Melampsora* rust resistance genes in *Populus* and characterization of linkage disequilibrium and flanking genomic sequence, *New Phytol.*, 2004, **164**, 95-105.
16. VILLAR, M., LEFEVRE, F., BRADSHAW, H.D., DUCROS, E.T., Molecular genetics of rust resistance in poplars (*Melampsora larici-populina* Kleb Populus sp) by bulked segregant analysis in a 2x2 factorial mating design, *Genetics*, 1996, **143**, 531-536.
17. STERKY, F., REGAN, S., KARLSSON, J., HERTZBERG, M., ROHDE, A., HOLMBERG, A., AMINI, B., BHALERAO, R., LARSSON, M., VILLARROEL, R., VAN MONTAGU, M., SANDBERG, G., OLSSON, O., TEERI, T.T., BOERJAN, W., GUSTAFSSON, P., UHLEN, M., SUNDBERG, B., LUNDEBERG, J., Gene discovery in the wood-forming tissues of poplar: Analysis of 5,692 expressed sequence tags, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 13330-13335.
18. PALO, R.T., Distribution of birch (*Betula* spp), willow (*Salix* spp), and poplar (*Populus* spp) secondary metabolites and their potential role as chemical defense against herbivores, *J. Chem. Ecol.*, 1984, **10**, 499-520.
19. JULKUNEN-TIITTO, R., A chemotaxonomic survey of phenolics in leaves of northern Salicaceae species, *Phytochemistry*, 1986, **25**, 663-667.
20. LINDROTH, R.L., HWANG, S.-Y., Diversity, redundancy, and multiplicity in chemical defense systems of aspen, *in:* Phytochemical Diversity and Redundancy in Ecological Interactions (J.A. Saunders J.T. Romeo, P. Barbosa, eds.), Plenum, New York. 1996, pp. 25-56
21. PARSONS, T.J., BRADSHAW, H.D., GORDON, M.P., Systemic accumulation of specific messenger RNAs in response to wounding in poplar trees, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 7895-7899.
22. CONSTABEL, C.P., YIP, L., PATTON, J.J., CHRISTOPHER, M.E., Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory, *Plant Physiol.*, 2000, **124**, 285-295.

23. CHRISTOPHER, M.E., MIRANDA, M., MAJOR, I.T., CONSTABEL, C.P., Gene expression profiling of systemically wound-induced defenses in hybrid poplar, *Planta*, 2004, **219**, 936-947.
24. ARIMURA, G., HUBER, D.P.W., BOHLMANN, J., Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa* x *deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1, *Plant J.*, 2004, **37**, 603-616.
25. KARBAN, R., BALDWIN, I.T., eds., Induced Responses to Herbivory, University of Chicago Press, Chicago. 1997, 319 p.
26. BERGEY, D.R., HOI, G.A., RYAN, C.A., Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 12053-12058.
27. RYAN, C.A., Protease inhibitors in plants - genes for improving defenses against insects and pathogens, *Annu. Rev. Phytopath.*, 1990, **28**, 425-449.
28. DUFFEY, S.S., STOUT, M.J., Antinutritive and toxic components of plant defense against insects, *Arch. Insect Biochem. Physiol.*, 1996, **32**, 3-37.
29. KESSLER, A., BALDWIN, I.T., Plant responses to insect herbivory: The emerging molecular analysis, *Ann. Rev. Plant Biol.*, 2002, **53**, 299-328.
30. WALLING, L.L., The myriad plant responses to herbivores, *J. Plant Growth Regul.*, 2000, **19**, 195-216.
31. CONSTABEL, C.P., A survey of herbivore-inducible defensive proteins and phytochemicals, *in:* Induced Plant Defenses Against Herbivores and Pathogens (A.A. Agrawal, Bent, E., Tuzun, S., eds.), APS Press, St. Paul. 1999, pp. 137-166
32. DUDAREVA, N., PICHERSKY, E., GERSHENZON, J., Biochemistry of plant volatiles, *Plant Physiol.*, 2004, **135**, 1893-1902.
33. KESSLER, A., BALDWIN, I.T., Defensive function of herbivore-induced plant volatile emissions in nature, *Science*, 2001, **291**, 2141-2144.
34. TAKABAYASHI, J., DICKE, M., Plant-carnivore mutualism through herbivore-induced carnivore attractants, *Trends Plant Sci.*, 1996, **1**, 109-113.
35. PARE, P.W., TUMLINSON, J.H., Plant volatiles as a defense against insect herbivores, *Plant Physiol.*, 1999, **121**, 325-331.
36. PICHERSKY, E., GERSHENZON, J., The formation and function of plant volatiles: perfumes for pollinator attraction and defense, *Curr. Op. Plant Biol.*, 2002, **5**, 237-243.
37. MCCONN, M., CREELMAN, R.A., BELL, E., MULLET, J.E., BROWSE, J., Jasmonate is essential for insect defense in *Arabidopsis*, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 5473-5477.
38. LI, C.Y., WILLIAMS, M.M., LOH, Y.T., LEE, G.I., HOWE, G.A., Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway, *Plant Physiol.*, 2002, **130**, 494-503.
39. HALITSCHKE, R., BALDWIN, I.T., Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*, *Plant J.*, 2003, **36**, 794-807.

40. KESSLER, A., HALITSCHKE, R., BALDWIN, I.T., Silencing the jasmonate cascade: Induced plant defenses and insect populations, *Science*, 2004, **305**, 665-668.
41. HAVILL, N.P., RAFFA, K.F., Effects of elicitation treatment and genotypic variation on induced resistance in *Populus*: impacts on gypsy moth (Lepidoptera : Lymantriidae) development and feeding behavior, *Oecologia*, 1999, **120**, 295-303.
42. ROBISON, D.J., RAFFA, K.F., Effects of constitutive and inducible traits of hybrid poplars on forest tent caterpillar feeding and population ecology, *Forest Sci.*, 1997, **43**, 252-267.
43. GLYNN, C., HERMS, D.A., EGAWA, M., HANSEN, R., MATTSON, W.J., Effects of nutrient availability on biomass allocation as well as constitutive and rapid induced herbivore resistance in poplar, *Oikos*, 2003, **101**, 385-397.
44. RYAN, C.A., PEARCE, G., Systemins: A functionally defined family of peptide signal that regulate defensive genes in Solanaceae species, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 14577-14580.
45. HWANG, S.Y., LINDROTH, R.L., Clonal variation in foliar chemistry of aspen: Effects on gypsy moths and forest tent caterpillars, *Oecologia*, 1997, **111**, 99-108.
46. PIERPOINT, W.S., Salicylic acid and its derivatives in plants: medicines, metabolites and messenger molecules, *Adv. Bot. Res.*, 1994, **20**, 164-235.
47. RUUHOLA, T., JULKUNEN-TIITTO, R., VAINIOTALO, P., *In vitro* degradation of willow salicylates, *J. Chem. Ecol.*, 2003, **29**, 1083-1097.
48. CLAUSEN, T.P., REICHARDT, P.B., BRYANT, J.P., WERNER, R.A., POST, K., FRISBY, K., Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores, *J. Chem. Ecol.*, 1989, **15**, 2335-2346.
49. RUUHOLA, T., TIKKANEN, O.P., TAHVANAINEN, J., Differences in host use efficiency of larvae of a generalist moth, *Operophtera brumata* on three chemically divergent *Salix* species, *J. Chem. Ecol.*, 2001, **27**, 1595-1615.
50. HARUTA, M., PEDERSEN, J.A., CONSTABEL, C.P., Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates, *Physiol. Plant.*, 2001, **112**, 552-558.
51. LINDROTH, R.L., KINNEY, K.K., Consequences of enriched atmospheric CO_2 and defoliation for foliar chemistry and gypsy moth performance, *J. Chem. Ecol.,* 1998, **24**, 1677-1695.
52. OSIER, T.L., LINDROTH, R.L., Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance, *J. Chem. Ecol.,* 2001, **27**, 1289-1313.
53. KAO, Y.Y., HARDING, S.A., TSAI, C.J., Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen, *Plant Physiol.*, 2002, **130**, 796-807.
54. PORTER, L.J., HRSTICH, L.N., CHAN, B.G., The conversion of procyanidins and prodelphinidins to cyanidin and delphidin, *Phytochemistry*, 1986, **25**, 223-230.
55. MARLES, M.A.S., RAY, H., GRUBER, M.Y., New perspectives on proanthocyanidin biochemistry and molecular regulation, *Phytochemistry*, 2003, **64**, 367-383.

56. AYRES, M.P., CLAUSEN, T.P., MACLEAN, S.F., REDMAN, A.M., REICHARDT, P.B., Diversity of structure and antiherbivore activity in condensed tannins, *Ecology*, 1997, **78**, 1696-1712.
57. WINKEL-SHIRLEY, B., Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology, *Plant Physiol.*, 2001, **126**, 485-493.
58. HAGERMAN, A.E., BUTLER, L.G., Tannins and lignins, *in:* Herbivores: Their Interaction with Secondary Metabolites (G.A. Rosenthal, M.R. Berenbaum, eds.), Academic Press, San Diego. 1991, pp. 355-387
59. BARBEHENN, R.V., MARTIN, M.M., Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera) - roles of the peritrophic envelope and midgut oxidation, *J. Chem. Ecol.*, 1994, **20**, 1985-2001.
60. APPEL, H.M., Phenolics in ecological interactions - the importance of oxidation, *J. Chem. Ecol.*, 1993, **19**, 1521-1552.
61. HEMMING, J.D.C., LINDROTH, R.L., Intraspecific variation in aspen phytochemistry - effects on performance of gypsy moths and forest tent caterpillars, *Oecologia*, 1995, **103**, 79-88.
62. PETERS, D.J., CONSTABEL, C.P., Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (Populus tremuloides), *Plant J.*, 2002, **32**, 701-712.
63. TANNER, G.J., FRANCKI, K.T., ABRAHAMS, S., WATSON, J.M., LARKIN, P.J., ASHTON, A.R., Proanthocyanidin biosynthesis in plants - Purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA, *J. Biol. Chem.*, 2003, **278**, 31647-31656.
64. XIE, D.Y., SHARMA, S.B., PAIVA, N.L., FERREIRA, D., DIXON, R.A., Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis, *Science*, 2003, **299**, 396-399.
65. ABRAHAMS, S., LEE, E., WALKER, A.R., TANNER, G.J., LARKIN, P.J., ASHTON, A.R., The Arabidopsis TDS4 gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development, *Plant J.*, 2003, **35**, 624-636.
66. TURLINGS, T.C.J., LOUGHRIN, J.H., MCCALL, P.J., ROSE, U.S.R., LEWIS, W.J., TUMLINSON, J.H., How caterpillar-damaged plants protect themselves by attracting parasitic wasps, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 4169-4174.
67. DE MORAES, C.M., LEWIS, W.J., PARE, P.W., ALBORN, H.T., TUMLINSON, J.H., Herbivore-infested plants selectively attract parasitoids, *Nature*, 1998, **393**, 570-573.
68. CHILCOTE, C.A., WITTER, J.A., MONTGOMERY, M.E., STOYENOFF, J.L., Intraclonal and interclonal variation in gypsy moth larval performance on bigtooth and trembling aspen, *Can. J. For. Res.,* 1992, **22**, 1676-1683.
69. HEMMING, J.D.C., LINDROTH, R.L., Effects of light and nutrient availability on aspen: Growth, phytochemistry, and insect performance, *J. Chem. Ecol.*, 1999, **25**, 1687-1714.
70. LINDROTH, R.L., OSIER, T.L., BARNHILL, H.R.H., WOOD, S.A., Effects of genotype and nutrient availability on phytochemistry of trembling aspen (Populus

tremuloides Michx.) during leaf senescence, *Biochem. System. Ecol.*, 2002, **30**, 297-307.

71. RICHARDSON, M., Seed storage proteins: the enzyme inhibitors, *Meth. Plant Biochem.*, 1991, **5**, 259-305.
72. LAWRENCE, S.D., NOVAK, N.G., A rapid method for the production and characterization of recombinant insecticidal proteins in plants, *Molec. Breed.*, 2001, **8**, 139-146.
73. HEIBGES, A., GLACZINSKI, H., BALLVORA, A., SALAMINI, F., GEBHARDT, C., Structural diversity and organization of three gene families for Kunitz-type enzyme inhibitors from potato tubers (Solanum tuberosum L.), *Molec. Gen. Genom.*, 2003, **269**, 526-534.
74. HEGEDUS, D., BALDWIN, D., O'GRADY, M., BRAUN, L., GLEDDIE, S., SHARPE, A., LYDIATE, D., ERLANDSON, M., Midgut proteases from *Mamestra configurata* (Lepidoptera: Noctuidae) larvae: Characterization, cDNA cloning, and expressed sequence tag analysis, *Arch. Insect Biochem. Physiol.,* 2003, **53**, 30-47.
75. HARUTA, M., MAJOR, I.T., CHRISTOPHER, M.E., PATTON, J.J., CONSTABEL, C.P., A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory, *Plant Molec. Biol.*, 2001, **46**, 347-359.
76. BROADWAY, R.M., Dietary proteinase inhibitors alter complement of midgut proteases, *Arch.Insect Biochem.Physiol.*, 1996, **32**, 39-53.
77. JONGSMA, M.A., BAKKER, P.L., PETERS, J., BOSCH, D., STIEKEMA, W.J., Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition, *Proc. Natl. Acad. Sci. USA,* 1995, **92**, 8041-8045.
78. BOWN, D.P., WILKINSON, H.S., GATEHOUSE, J.A., Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors, *Physiol. Entomol.*, 2004, **29**, 278-290.
79. MIRANDA, M., CHRISTOPHER, M.E., CONSTABEL, C.P., The variable nature of herbivore defense: evidence for a rapidly diverging Kunitz trypsin inhibitor gene in *Populus*, *in:* Plant Adaptation: Molecular Genetics and Ecology (Q.C.B. Cronk, J. Whitton, R.H. Ree, I.E.P. Taylor, eds.), NRC Press, Ottawa. 2004, pp. 153-158
80. CONSTABEL, C.P., RYAN, C.A., A survey of wound- and methyl jasmonate-induced leaf polyphenol oxidase in crop plants, *Phytochemistry*, 1998, **47**, 507-511.
81. CONSTABEL, C.P., BERGEY, D.R., RYAN, C.A., Polyphenol oxidase as a component of the inducible defense response of tomato against herbivores, *in:* Phytochemical Diversity and Redundancy in Ecological Interactions (J.T. Romeo, J.A. Saunders, P. Barbosa, eds.), Plenum Press, New York. 1996, pp. 231-252
82. FELTON, G.W., DONATO, K., DELVECCHIO, R.J., DUFFEY, S.S., Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores, *J. Chem. Ecol.*, 1989, **15**, 2667-2694.

83. FELTON, G.W., DONATO, K.K., BROADWAY, R.M., DUFFEY, S.S., Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, S*podoptera exigua*, *J. Insect Physiol.*, 1992, **38**, 277-285.
84. WANG, J., CONSTABEL, C.P., Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*), *Planta*, 2004, **220**, 87-96.
85. DAVIS, J.M., CLARKE, H.R.G., BRADSHAW, H.D., GORDON, M.P., *Populus* chitinase genes - structure, organization, and similarity of translated sequences to herbaceous plant chitinases, *Plant Molec. Biol*,, 1991, **17**, 631-639.
86. CLARKE, H.R.G., LAWRENCE, S.D., FLASKERUD, J., KORHNAK, T.E., GORDON, M.P., DAVIS, J.M., Chitinase accumulates systemically in wounded poplar trees, *Physiol. Plant.*, 1998, **103**, 154-161.
87. COLLINGE, D.B., KRAGH, K.M., MIKKELSEN, J.D., NIELSEN, K.K., RASMUSSEN, U., VAD, K., Plant chitinases, *Plant J.*, 1993, **3**, 31-40.
88. BARBEHENN, R.V., Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals, *Arch Insect Biochem Physiol.*, 2001, **47**, 86-99.
89. BROADWAY, R.M., GONGORA, C., KAIN, W.C., SANDERSON, J.P., MONROY, J.A., BENNETT, K.C., WARNER, J.B., HOFFMANN, M.P., Novel chitinolytic enzymes with biological activity against herbivorous insects, *J. Chem. Ecol.,* 1998, **24**, 985-998.
90. DING, X.F., GOPALAKRISHNAN, B., JOHNSON, L.B., WHITE, F.F., WANG, X.R., MORGAN, T.D., KRAMER, K.J., MUTHUKRISHNAN, S., Insect resistance of transgenic tobacco expressing an insect chitinase gene, *Transgen. Res.,* 1998, **7**, 77-84.
91. SMITH, C.M., RODRIGUEZ-BUEY, M., KARLSSON, J., CAMPBELL, M.M., The response of the poplar transcriptome to wounding and subsequent infection by a viral pathogen, *New Phytol.*, 2004, **164**, 123-136.
92. DAVIS, J.M., EGELKROUT, E.E., COLEMAN, G.D., CHEN, T.H.H., HAISSIG, B.E., RIEMENSCHNEIDER, D.E., GORDON, M.P., A family of wound-induced genes in *Populus* shares common features with genes encoding vegetative storage proteins, *Plant Molec. Biol.*, 1993, **23**, 135-143.
93. LAWRENCE, S.D., GREENWOOD, J.S., KORHNAK, T.E., DAVIS, J.M., A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar, *Planta*, 1997, **203**, 237-244.
94. BERGER, S., BELL, E., SADKA, A., MULLET, J.E., Arabidopsis thaliana Atvsp is homologous to soybean Vspa and Vspb, genes encoding vegetative storage protein acid phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars, light and phosphate, *Plant Molec. Biol.*, 1995, **27**, 933-942.
95. HERTZBERG, M., ASPEBORG, H., SCHRADER, J., ANDERSSON, A., ERLANDSSON, R., BLOMQVIST, K., BHALERAO, R., UHLEN, M., TEERI, T.T., LUNDEBERG, J., SUNDBERG, B., NILSSON, P., SANDBERG, G., A transcriptional roadmap to wood formation, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 14732-14737.

96. COOKE, J.E.K., BROWN, K.A., WU, R., DAVIS, J.M., Gene expression associated with N-induced shifts in resource allocation in poplar, *Plant Cell Env.*, 2003, **26**, 757-770.
97. KOHLER, A., DELARUELLE C., MARTIN, D., ENCELOT N., MARTIN F., The poplar root transcriptome: analysis of 7000 expressed sequence tages, *FEBS Lett.*, 2003, **542**, 37-41.
98. ANDERSSON, A., KESKITALO, J., SJODIN, A., BHALERAO, R., STERKY, F., WISSEL, K., TANDRE, K., ASPEBORG, H., MOYLE, R., OHMIYA, Y., BRUNNER, A., GUSTAFSSON, P., KARLSSON, J., LUNDEBERG, J., NILSSON, O., SANDBERG, G., STRAUSS, S., SUNDBERG, B., UHLEN, M., JANSSON, S., NILSSON, P., A transcriptional timetable of autumn senescence, *Genome Biol.*, 2004, **5**.
99. STERKY, F., BHALERAO, R.R., UNNEBERG, P., SEGERMAN, B., NILSSON, P., BRUNNER, A.M., CHARBONNEL-CAMPAA, L., LINDVALL, J.J., TANDRE, K., STRAUSS, S.H., SUNDBERG, B., GUSTAFSSON, P., UHLEN, M., BHALERAO, R.P., NILSSON, O., SANDBERG, G., KARLSSON, J., LUNDEBERG, J., JANSSON, S., A *Populus* EST resource for plant functional genomics, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 13951-13956.
100. MEKHEDOV, S., DE ILARDUYA, O.M., OHLROGGE, J., Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis, *Plant Physiol*, 2000, **122**, 389-401.
101. CHEONG, Y.H., CHANG, H.S., GUPTA, R., WANG, X., ZHU, T., LUAN, S., Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis, *Plant Physiol*, 2002, **129**, 661-677.
102. SCHENK, P.M., KAZAN, K., WILSON, I., ANDERSON, J.P., RICHMOND, T., SOMERVILLE, S.C., MANNERS, J.M., Coordinated plant defense responses in Arabidopsis revealed by microarray analysis, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 11655-11660.
103. PECHAN, T., COHEN, A., WILLIAMS, W.P., LUTHE, D.S., Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 13319-13323.
104. KONNO, K., HIRAYAMA, C., NAKAMURA, M., TATEISHI, K., TAMURA, Y., HATTORI, M., KOHNO, K., Papain protects papaya trees from herbivorous insects: Role of cysteine proteases in latex, *Plant J.*, 2004, **37**, 370-378.
105. VOELCKEL, C., BALDWIN, I.T., Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations, *Plant J.*, 2004, **38**, 650-663.
106. HERMSMEIER, D., SCHITTKO, U., BALDWIN, I.T., Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs, *Plant Physiol.*, 2001, **125**, 683-700.
107. GATEHOUSE, J.A., Plant resistance towards insect herbivores: A dynamic interaction, *New Phytol.*, 2002, **156**, 145-169.

108. CREELMAN, R.A., MULLET, J.E., Biosynthesis and action of jasmonates in plants, *Annu. Rev. Plant Physiol. Plant Molec. Biol.*, 1997, **48**, 355-381.
109. DAVIS, J.M., GORDON, M.P., SMIT, B.A., Assimilate movement dictates remote sites of wound-induced gene expression in poplar leaves, *Proc. Natl. Acad. Sci. USA.*, 1991, **88**, 2393-2396.
110. ARNOLD, T.M., SCHULTZ, J.C., Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*, *Oecologia*, 2002, **130**, 585-593.
111. JONES, C.G., HOPPER, R.F., COLEMAN, J.S., KRISCHNIK, V.A., Control of systemically induced herbivore resistance by plant vascular architecture, *Oecologia*, 1983, **93**, 452-456.
112. SCHITTKO, U., BALDWIN, I.T., Constraints to herbivore-induced systemic responses: Bidirectional signaling along orthostichies in *Nicotiana attenuata*, *J. Chem. Ecol.*, 2003, **29**, 763-770.
113. LI, C.Y., LIU, G.H., XU, C.C., LEE, G.I., BAUER, P., LING, H.Q., GANAL, M.W., HOWE, G.A., The tomato Suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression, *Plant Cell*, 2003, **15**, 1646-1661.
114. DICKE, M., HILKER, M., Induced plant defences: From molecular biology to evolutionary ecology, *Basic Appl. Ecol.*, 2003, **4**, 3-14.
115. ALBORN, T., TURLINGS, T.C.J., JONES, T.H., STENHAGEN, G., LOUGHRIN, J.H., TUMLINSON, J.H., An elicitor of plant volatiles from beet armyworm oral secretion, *Science*, 1997, **276**, 945-949.
116. HALITSCHKE, R., SCHITTKO, U., POHNERT, G., BOLAND, W., BALDWIN, I.T., Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses, *Plant Physiol.*, 2001, **125**, 711-717.
117. SCHITTKO, U., HERMSMEIER, D., BALDWIN, I.T., Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. II. Accumulation of plant mRNAs in response to insect-derived cues, *Plant Physiol.*, 2001, **125**, 701-710.