Poplar Extrafloral Nectaries: Two Types, Two Strategies of Indirect Defenses against Herbivores[1][C][W]

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Many plant species grow extrafloral nectaries and produce nectar to attract carnivore arthropods as defenders against herbivores. Two nectary types that evolved with *Populus trichocarpa* (*Ptr*) and *Populus tremula* × *Populus tremuloides* (*Ptt*) were studied from their ecology down to the genes and molecules. Both nectary types strongly differ in morphology, nectar composition and mode of secretion, and defense strategy. In *Ptt*, nectaries represent constitutive organs with continuous merocrine nectar flow, nectary appearance, nectar production, and flow. In contrast, *Ptr* nectaries were found to be holocrine and inducible. Neither mechanical wounding nor the application of jasmonic acid, but infestation by sucking insects, induced *Ptr* nectar secretion. Thus, nectaries of *Ptr* and *Ptt* seem to answer the same threat by the use of different mechanisms.

Plants secrete nectar to achieve two highly important mutualistic interactions with animals: pollination and indirect defense (Brandenburg et al., 2009; Heil, 2011). Floral nectar is secreted within the flowers and serves pollination. Extrafloral nectar (EFN) is secreted in general on the vegetative parts and attracts members of the third trophic level as a means of indirect defense against herbivores (Heil, 2008). In fact, EFN is one of the very few antiherbivore defense mechanisms for which an effect on plant fitness has been demonstrated unambiguously in a number of field studies (Chamberlain and Holland, 2009). Nectar secretion mechanisms have been intensively studied at the phenotypic level, and we now know that plants can adjust nectar quantity and quality to several biotic factors, such as ontogenetic stage, consumer identity, consumption rate, and, in the case of EFN, current leaf damage (Heil, 2011). However, the biochemical, physiological, and genetic mechanisms that underlie the regulation of nectar secretion remain widely unknown.

For more than 100 years, scientists have discussed two alternative mechanisms for the secretion of floral nectar. However, even less is known about EFN secretion (Escalante-Pérez and Heil, 2012). Holocrine secretion is characterized by programmed cell death that, in a one-step process, causes release of the entire cell content into exterior parts. In this case, the nectar is produced and kept within the cells until the plasma membrane is ruptured (Vesprini et al., 2008). This type of secretion has so far not been described for extrafloral nectaries. By contrast, merocrine secretion is associated with living nectar-secreting cells by prolonged large-scale exocytosis. Concerning merocrine secretion, it is debated whether the "prenectar," after uploading from the phloem, moves via an apoplastic pathway or via a vesicle-based symplastic pathway (Vassilyev, 2010; Heil, 2011, and refs. therein). For the symplastic pathway, transport via endocytosis and exocytosis, molecular transport across the plasmalemma, and transport via plasmodesmata have been considered (Fahn, 1988b; Gaffal et al., 2007; Kram and Carter, 2009; Kram et al., 2009). The merocrine type of secretion may even use eccrine secretion, which comprises carrier-based transport of individual molecules across the cell membrane, or granulocrine secretion, relying on transport of a fluid phase that seems to be governed by endoplasmic reticulum- or dictyosome-derived vesicles. The latter membrane structures subsequently fuse with the plasmalemma and finally are released into the apoplast (Dauwalder and Whaley, 1982; Sauer et al., 1994; Jürgens and Geldner, 2002). To study the mechanisms that underlie the secretion of EFN, here

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we applied physiological assays, electron microscopy, and transcriptomic analysis to gain new insight in this rather complex matter. Thereby, the special focus was on the relation between nectary type and defense.

Extrafloral nectaries of the genus *Populus* were first described by Trelease (1881). Since that time, not much attention has been paid to defense mechanisms used by different poplar species (Wooley et al., 2007). The genus *Populus* is a known host for many herbivorous insects and pathogens. One member of the genus, *Populus trichocarpa* (*Ptr*), has been sequenced, providing access to genome-wide expression studies (Tuskan et al., 2006) in general and defense genes in particular. *Populus* thus provides an excellent model tree to study biotic stress management (Arimura et al., 2004; Lawrence et al., 2006; Ralph et al., 2006).

Nectaries in general consist of three components: epidermis with or without stomata or trichomes; specialized parenchyma that produces or stores nectar; and a subnectary parenchyma composed of bigger cells more loosely packed (Stpiczyńska et al., 2005; Kaczorowski et al., 2008). Detailed studies have shown that decreases in herbivory rates associated with EFN secretion result from mechanical leaf damage, jasmonic acid (JA) production, and/or volatile emission (Heil et al., 2001; Linsenmair et al., 2001; Mathews et al., 2007; Radhika et al., 2008). Volatile organic compounds represent another indirect defense to herbivores (Arimura et al., 2000; Kessler and Baldwin, 2002; Gershenzon, 2007) and have also been demonstrated to be part of a "plant-to-plant communication" network. Volatiles can act as alarm signals for neighboring plants that yet remain undamaged (Heil and Silva Bueno, 2007; Kost and Heil, 2008). According to the conventional view, nectar is mainly composed of sugars and amino acids, originates from phloem sap, and enters the secretory cells via plasmodesmata-connected parenchyma cells (Fahn, 1988a). However, nectar might also contain substances generally not carried in the phloem sap, such as inorganic ions, proteins, lipids, organic acids, phenolics, and alkaloids (Jones, 1983). Given the enormous variability in nectar features regarding volume, concentration, and composition, the latter hypothesis appears to be a kind of oversimplification (Pacini et al., 2003; Heil, 2011).

We have used two different species of *Populus* (*Ptr* and *Populus tremula* × *Populus tremuloides* [*Ptt*]) to investigate the structure, nectar production, composition, and gene expression in their extrafloral nectaries. The results of our multidisciplinary study demonstrate that EFNs of the two poplar species studied differ in their chemical composition and are secreted via a holocrine mechanism in the first species and via merocrine secretion in the second species.

RESULTS

Two Types of Nectary Structures Harbor Unique Secretion Systems

In the two poplar species *Ptr* and *Ptt*, pairs of nectaries are located on each side of the leaf blade near the petiole (Fig. 1). *Ptt* nectaries were bigger, whereas those of *Ptr* released more nectar (Fig. 1, C and F).

Light microscopy with *Ptr* nectaries documented the structure described for many floral nectaries. They contain an epidermis in addition to a nectar and a subnectary parenchyma (Stpiczyńska et al., 2005; Wist and Davis, 2006; Kaczorowski et al., 2008; Wenzler et al., 2008; Fig. 2A). *Ptt* nectaries develop a nectar and subnectary parenchyma as well. In contrast to *Ptr*, however, the outer layer consisted of modified epidermal

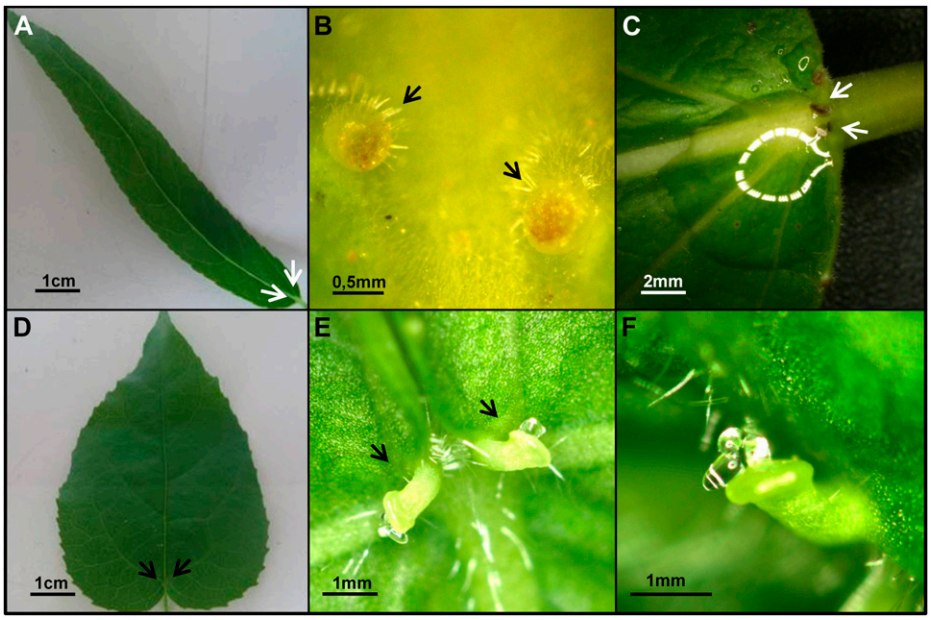

Figure 1. Positioning of poplar extrafloral nectaries. In both species, nectary pairs localize at the base of the leaf blade. A to C, *Ptr*. D to F, *Ptt*. In the leaf overviews (A and D), arrows denote nectary positions. In the leaf base enlargements, nectaries in detail (arrows in B and E), *Ptr* release of large nectar amounts (C), and large *Ptt* nectaries releasing small nectar amounts (F) are shown.

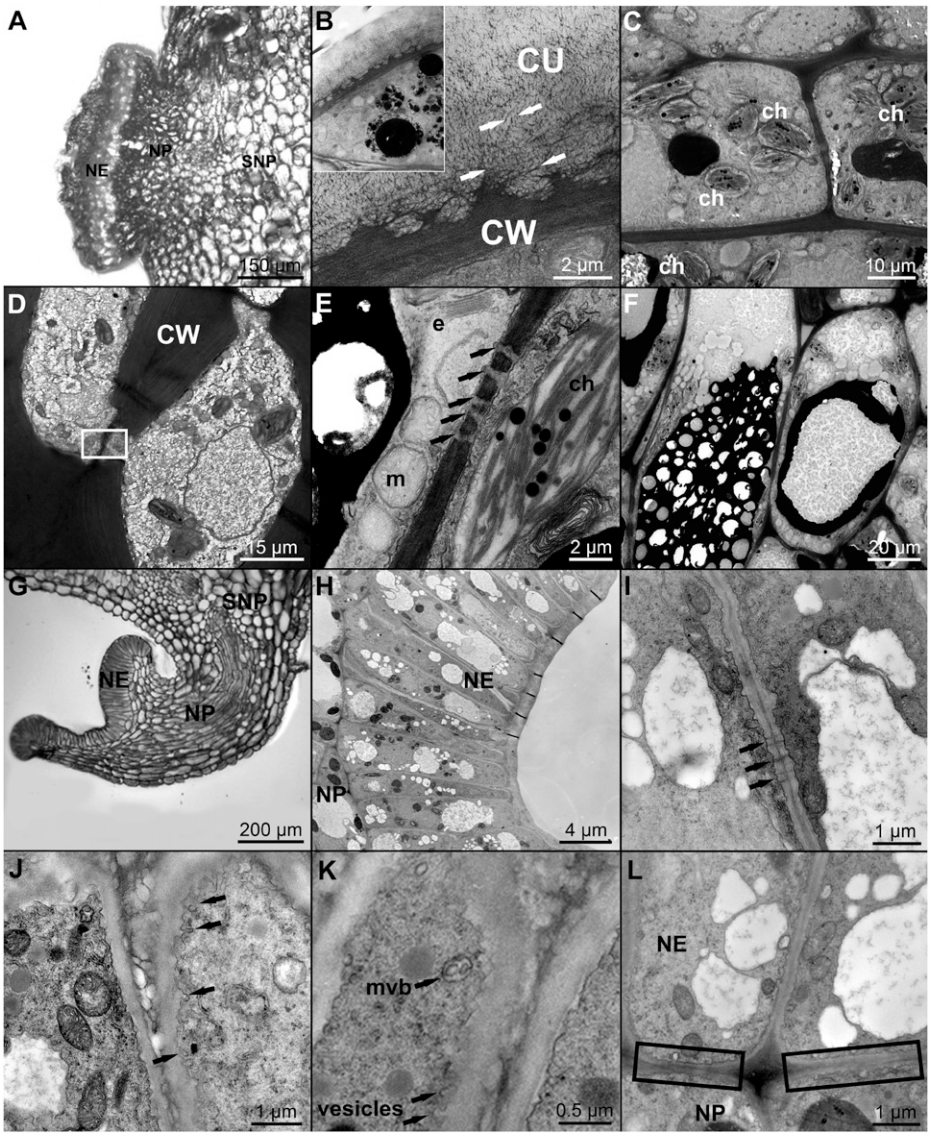

Figure 2. Anatomy of *Ptr and Ptt* nectaries. A to F, *Ptr* nectary microscopy. A, Overview. B, Detailed view of the epidermal cell wall and cuticle (inset) with microchannels marked by arrows. C and D, Overview of nectary parenchyma cells with some extant thick walls and symplastic connections (square). E and F, Plasmodesmata between cells (arrows; E) and numerous small vacuoles (F, left) and large nuclei (F, right). G to L, *Ptt* nectary microscopy. G, Overview. H, Overview of secretory cells. I, Plasmodesmata (arrows) between two secretory cells. J and K, Secreted vesicles and multivesicular bodies (arrows) occur within the epidermal cells and in the upper apoplastic space. Note the partial loosening of the cell wall. L, Symplastic connections between the secretory cells and the basolateral neighbors are absent (black rectangles). ch, Chloroplast; CU, cuticle; CW, cell wall; e, endoplasmic reticulum; m, mitochondria; mvb, multivesicular bodies; NE, nectary epidermis; NP, nectary parenchyma; SNP, subnectary parenchyma; v, vacuole.

cells (Fig. 2G). In transmission electron microscopy (TEM) analyses, we found the surface of nectary epidermal cells of *Ptr* covered by a cuticle entirely. Therein, microchannels appeared as narrow tubular interruptions in continuity with the cell wall. These fibrillar outgrowths of the outer epidermal cell walls might represent a path for passive nectar flow (Fig. 2B; Supplemental Fig. S1). In *Ptr*, the isodiametric nectary parenchyma cells (including the secretory cells) could be distinguished from ground parenchyma by the presence of a dense granular cytoplasm, rich in ribosomes, mitochondria, and chloroplasts (Fig. 2C). These specialized features reflected the high metabolic activity required for nectar production. Nectary parenchyma cells generally grow thin walls (D'Amato, 1984). In contrast to *Ptt*, these cells possessed unusually thick walls with numerous pits and associated plasmodesmata connecting the protoplasts of adjacent cells (Fig. 2, D and E). Around the symplastic connections, we could visualize numerous chloroplasts, plastids containing plastoglobuli, mitochondria along with rough endoplasmic reticulum, and dictyosomes (Golgi apparatus). The numerous vacuoles of the nectary parenchyma appeared small and surrounded by dark-stained matter (Fig. 2).

With *Ptt* nectaries, surprisingly, in TEM and light microscopy and after wax staining by Sudan III, no cuticle was found covering the secretory epidermal cells (Supplemental Fig. S2). Ultrastructural analyses of *Ptt* nectaries showed that the secretory cells (Fig. 2H), representing specialized epidermal cells, were interconnected at their lateral side by a large number of plasmodesmata (Fig. 2I). Such connections were absent between the nectary parenchyma at the basolateral side (Fig. 2L; Supplemental Fig. S1). In *Ptt*, extrafloral nectary vesicles are located in the outer apoplastic space as well as in the tip of the secretory cells (Fig. 2, J and K; Supplemental Fig. S1). As with

floral nectaries, these structures were associated with epidermal cell endocytosis in the process of reabsorption of nonconsumed nectar (Nepi and Stpiczyńska, 2007, 2008).

Ptt nectaries were treated with the membrane-staining but membrane-impermeable fluorescent dye FM4-64 (Fischer-Parton et al., 2000). The intracellular presence of dye in longitudinal sections of FM4-64 stained nectaries strongly pointed to endocytosis events (Supplemental Fig. S3). FM4-64, however, failed to cross the apoplastic space between secretory cells and parenchymal neighbors.

Stress, Nectary Development, and Activity

In spring, many emerging *Ptt* and *Ptr* leaves harbor nectaries (Fig. 3B). To test whether this phenomenon results from heritable genetics, the extrafloral nectary densities of field-grown *Ptt* trees were determined. The populations of leaves with (38%) and without (62%) nectaries were highly conserved among individual trees (Fig. 3A). Similar data were obtained with *Ptr* (Supplemental Fig. S4). These results are consistent with the general effectiveness of nectaries against herbivore attack (Heil et al., 2001, 2005; Kost and Heil, 2008). Sixty percent (*Ptr*) or 80% (*Ptt*) of leaves carrying nectaries developed no visible symptoms of herbivore attack, 35% (*Ptr*) or 15% (*Ptt*) were slightly damaged, and only 2% (both species) were severely affected (Fig. 3C; Supplemental Fig. S3B). Nectary-free leaves, however, exhibited a higher percentage of damage with *Ptt* and *Ptr* (Fig. 3; Supplemental Fig. S4).

Nectaries appeared with the onset of leaf emergence on both poplar species. In contrast to *Ptt*, where nectar was secreted continuously over weeks, *Ptr* nectaries released nectar within a few days only. Most of the nectaries of the latter sort died after nectar release. However, when the tree was specifically stressed after the first nectar secretion, new nectaries occurred side by side or on top of dead ones. These secondary nectaries showed the same secretion characteristics as the initial population (Fig. 4).

So, how was the secondary nectar production of *Ptr* induced? To test whether EFN production in *Ptr* results from mechanical stress, leaves of *Ptr* were wounded by puncturing the leaf blade with a needle (Fig. 5A). After this procedure, however, nectar production was not observed.

To test whether persistent wounding, a feeding fingerprint of herbivores, initiates nectar production, an automated damage procedure was applied (Fig. 5, B and C). The computer-controlled mechanical caterpillar MecWorm mimics the damage caused by herbivores in terms of the spatiotemporal pattern of leaf destruction (Mithöfer et al., 2005). Even these near-natural wounding settings did not trigger nectar production. This indicates that *Ptr* nectar production seems not to be initiated simply by wounding of leaves but requires another stimulus instead. The same result

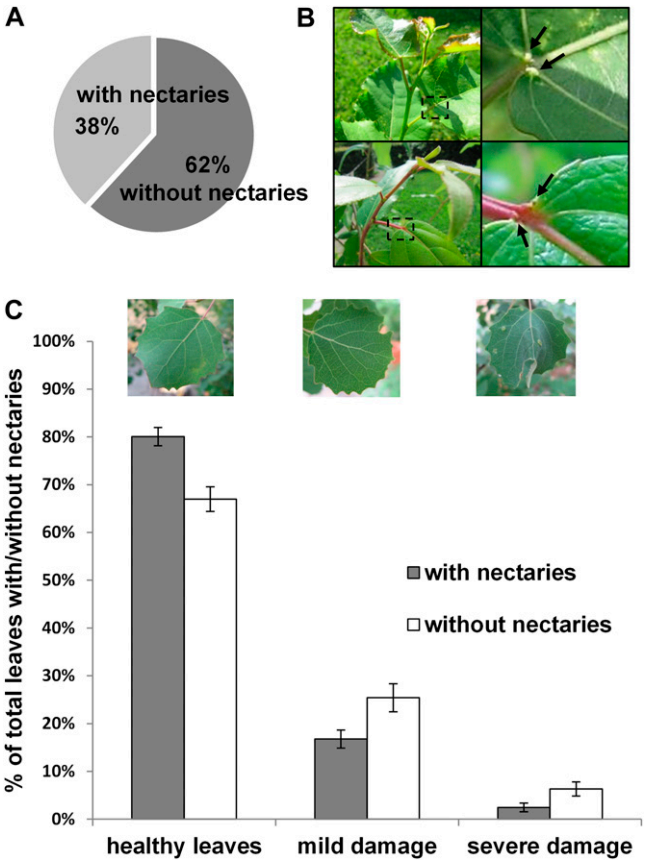

Figure 3. Nectary density and effectiveness against herbivore attack. A, Conserved percentage of *Ptt* leaves with and without nectaries. B, Both *Ptr* (bottom row) and *Ptt* (top row) leaves emerging in spring presented nectaries (arrows). C, *Ptt* extrafloral nectary effectiveness. Leaves with nectaries appeared less damaged by herbivores. Values are means ± SE; $n = 11$ branches (759 leaves). [See online article for color version of this figure.]

was obtained when the MecWorm-damaged tissue was additionally treated with the oral secretion of the Mediterranean climbing cutworm (*Spodoptera littoralis*). Finally, caterpillars of *S. littoralis*, *Spodoptera exigua*, and *Lymantria dispar*, which are polyphagous insects and thus feed on poplar leaves, were placed on *Ptr* (Fig. 5D). Again, neither nectary nor nectar production was observed within 48 h of caterpillar feeding. These results clearly showed that herbivore-evoked leaf damage is not causing *Ptr* nectar secretion. But when intact plants of *Ptr* were subjected to mealy bugs (Hemiptera: Pseudicoccidae), which, in contrast to caterpillars, feed on phloem sap, nectar secretion set in (Fig. 5, E and F). At the beginning of the experiment, less than 10% of the leaves showed nectaries without nectar. Four days after infestation, 42% of all leaves were equipped with nectaries, and about 50% of them produced nectar (data not shown). In most cases, the secondary nectar production occurred upon attack by sucking insects (Fig. 5, E–H). Thus, *Ptr* nectar formation seemed to be confined to specific types of herbivores.

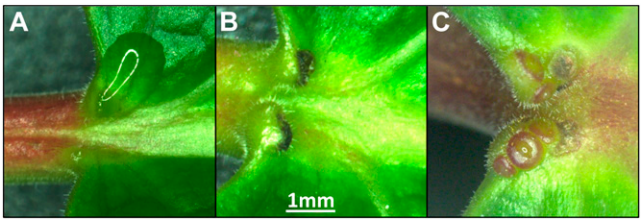

Figure 4. *Ptr* requires new nectaries for repeated nectar secretion. A, First holocrine nectar secretion. B, Nectaries died after holocrine secretion. C, Secondary nectar-secreting nectaries on top of dead ones.

Volatiles Are Not Involved in *Ptr* Nectary Induction

Volatile organic compounds (VOCs) in plants take part in indirect defense to overcome herbivore attack. They attract predatory arthropods and/or repel herbivores (Arimura et al., 2000; De Moraes et al., 2001; Kessler and Baldwin, 2002; Gershenzon, 2007). Moreover, green leaf volatiles released from lima bean (*Phaseolus lunatus*) leaves after herbivore damage are able to induce EFN flow in undamaged neighboring plants (Heil et al., 2008). Herbivory or even mechanical leaf damage elevates endogenous levels of JA, stimulating volatile biosynthesis. As a consequence, externally added JA also triggered volatile emission from plants (Boland et al., 1995) or induced nectar flow from extrafloral nectaries of *Macaranga tanarius* (Heil et al., 2001). Mechanical damage was obviously not sufficient to induce *Ptr* secondary nectar release. Thus, the role of volatiles in EFN secretion of this poplar species was examined. To trigger the VOC emission of *Ptr* by jasmonates or herbivory-related stressors, poplar leaves were continuously damaged by the MecWorm or by *S. littoralis*, *S. exigua*, and *L. dispar* caterpillars. After mechanical damage, herbivory, and application of JA or coronalon, which acts as a mimic of jasmonoyl-Ile (Schüler et al., 2001; Svoboda and Boland, 2010), VOCs were released, but no nectaries were formed and no nectar flow was stimulated. The emitted volatiles comprised (*E*)-β-ocimene, 4,8-dimethyl-1,3,7-nonatriene, farnesene, nerolidol, and 4,8,12-trimethyltridec-1,3,7,11-tetraene (Danner et al., 2011). The compounds $C_{10}H_{16}O$ and $C_{10}H_{14}$ result from catalytic oxidation of the originally emitted (*E*)-β-ocimene by the active carbon trap used for volatile collection (Sonwa et al., 2007). Irrespective of the ocimene artifacts, the compounds shown in Figure 6 are characteristic for induced volatiles observed after induction with jasmonates or by herbivory. Thus, we concluded that in *Ptr*, volatiles were induced as expected, but nectar production was not VOC dependent.

Nectar Composition Feeds Back on a Visitor's Attraction

Nectar is a complex mixture of metabolites. The sweet nectar blend is dominated by Suc, Glc, and Fru. Nectar with high Suc content attracts generalists, whereas nectars with higher hexose contents are preferred by specialists (Heil et al., 2005). The amounts of Suc, Fru, and Glc in poplar EFN appeared relatively constant in the different samples from the same species but differed remarkably between species (Supplemental Fig. S5). In *Ptt*, the ratios between Glc, Fru, and Suc were equal, whereas, in contrast, the percentage of Suc in *Ptr* nectar was rather low. Thus, EFNs of *Ptt* likely attract generalists such as honeybees, wasps, and parasitic wasps, whereas those of *Ptr* attract more specialized visitors, such as ants (Supplemental Fig. S6; Steidle and van Loon, 2003; Heil et al., 2005).

Amino acids are the second most common class of solutes in nectar, and their composition is important for nectar taste (Baker and Baker, 1983). Insects possess receptors that enable them to sense amino acids (Shiraishi

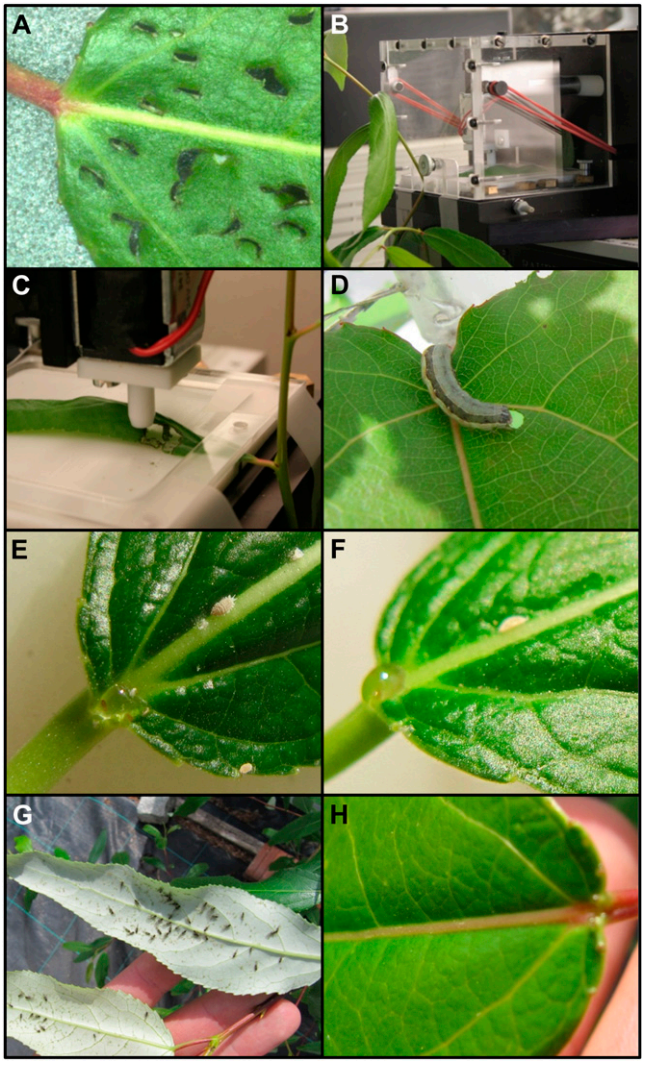

Figure 5. Induction of *Ptr* nectar release by sucking insects. A, Mechanical damage performed by a needle. B, MecWorm setup for mimicking caterpillar-caused mechanical leaf damage. C, Continuous leaf wounding by MecWorm. D, *S. exigua* feeding on *Ptr* leaves. E and F, Mealy bugs on a leaf blade with ongoing nectar secretion. G, Aphids on a leaf bottom side. H, Top side of the leaf shown in G with ongoing nectar secretion.

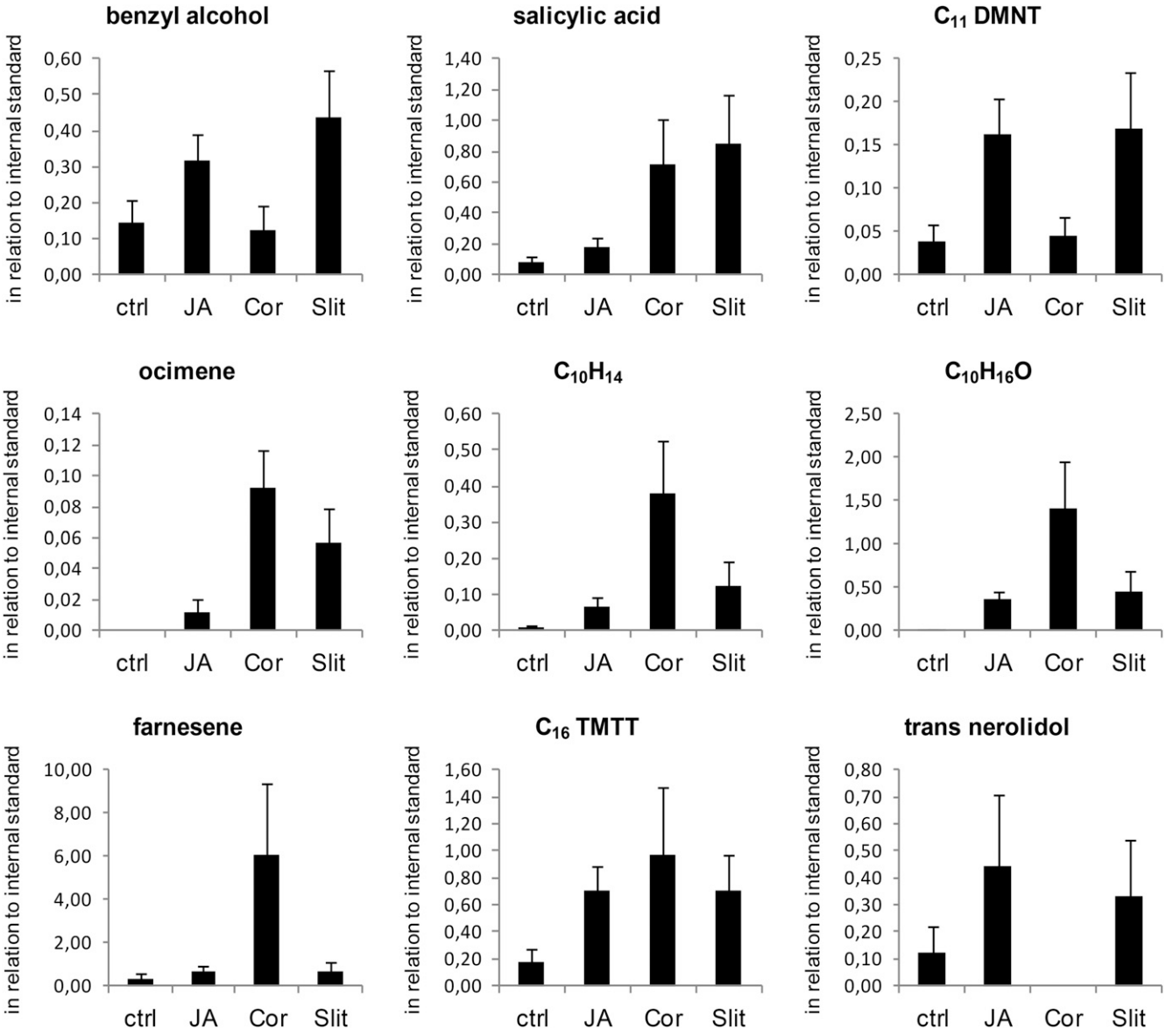

Figure 6. Volatiles emitted by *Ptt* after treatment with chemical elicitors and feeding larvae of *S. littoralis*. Control (crtl), $n = 11$; JA (1 mM), $n = 13$; coronalon (Cor; 0.1 mM), $n = 9$; *S. littoralis* (Slit), $n = 15$. *n*-Bromodecane was used as an internal standard; $C_{10}H_{14}$ and $C_{10}H_{16}O$ are oxidation products of ocimene. DMNT, 4,8-Dimethyl-1,3,7-nonatriene; TMTT, 4,8,12-trimethyltridec-1,3,7,11-tetraene.

and Kuwabara, 1970). Interestingly, amino acids responsible for sweet taste, like Phe, Trp, and Val, were well represented in both *Ptt* and *Ptr* nectar samples but low or absent in leaves (Fig. 7; Supplemental Fig. S7). Pro, which has a "salty taste," was only found in nectar samples but not in leaf extracts (Fig. 7; Supplemental Fig. S7). In *Ptt*, Phe and Gln dominated, whereas in *Ptr*, Asn, His, and Tyr were most abundant.

Nectary-Specific Genes

To identify the genes encoding defense proteins and to gain a deeper insight into the *Ptt* nectary transcriptome, poplar DNA microarrays (Affymetrix) were hybridized with RNA obtained from extrafloral nectaries or nectary-free leaf sections. With extrafloral nectaries, only the apical fraction of the organ harboring the nectar-producing cells was sampled (Fig. 8, inset). mRNA samples of nectaries with secretory cells enriched and leaf sections without nectaries were analyzed. The 500 most differentially expressed genes (Supplemental Table S1) were considered for further analysis. Among them in nectaries, 365 (73%) appeared up-regulated and 135 (27%) appeared down-regulated. Array data were validated by quantitative real-time PCR (qPCR) with a set of randomly selected transcripts and a set of further analyzed transcripts (see below; Supplemental Table S2). For an unbiased view

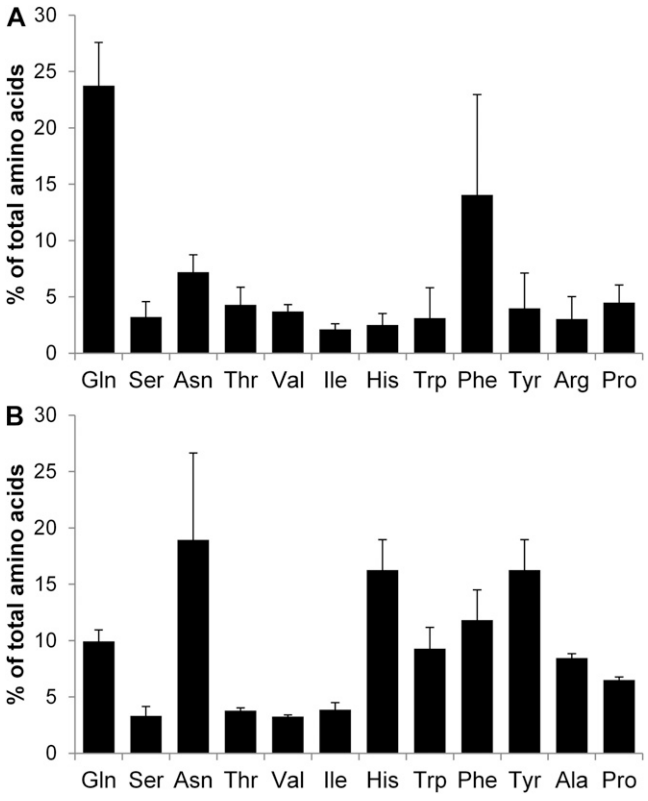

Figure 7. Most abundant amino acids in poplar EFN. A, *Ptt*. B, *Ptr*. Of the aromatic amino acids, particularly the sweet Phe and Trp were present in higher quantities. Pro was not detectable in leaf extracts of both species. Values are means ± SD; $n = 4$.

of the impact of differential expression on nectar biology, gene functions were analyzed by using MapMan (Usadel et al., 2009; Fig. 8). Among the 27 different gene clusters found, nine (signaling, stress, transport, development, and carbohydrate, lipid, hormone, cell wall, and secondary metabolism) were considered as important for nectary development. Interestingly, in the population of the 102 genes related to these clusters, about 90% appeared up-regulated in nectaries. The strong induction of metabolic activity clusters in this organ was already predicted by light microscopy and TEM (Fig. 2). Genes related to hormone action and metabolism, lipids (vesicle transport), as well as sugar metabolism and the cell wall appeared up-regulated, in many cases to very high levels.

Exocytosis-Associated Genes

Microscopic inspection provided evidence for granulocrine secretion only with *Ptt* and not with *Ptr* nectaries. Recycling of lipids and proteins is characteristic for this kind of secretion and a prerequisite for ongoing nectar flow. Secretory vesicles thus appeared to be very prominent in secretory cells from *Ptt* nectaries (Fig. 2). Accordingly, in the full array data set, we could identify at least 21 genes related to exocytosis (Table I). Among them, five were remarkably higher expressed in the nectaries (2- to 7.9-fold) and eight were slightly induced (1.5- to 1.9-fold). In this population, seven nectary genes were linked to multivesicular body formation. Most of them belong to the SNARE superfamily. One family member, PEP12 syntaxin, was found 4.6-fold enriched in nectary cells. Six genes encoded proteins of the trans-Golgi network (TGN), such as the RabA (four), RabE, and VHA-a1 class. We identified a number of genes that, according to Zárský et al. (2009), seemed to be involved in vesicle or TGN-to-plasma membrane carrier formation. Among them, three genes appeared remarkably up-regulated. The latter species of *Ptt* nectary-expressed genes belong to the SEC14, Ala-3, and dynamin classes. SEC14 encodes a phosphatidylinositol transfer protein and Ala-3 a flippase. Both are essential for vesicle budding from the Golgi complex (Sha et al., 1998; Litvak et al., 2005; Poulsen et al., 2008), whereas dynamins seem to be required for membrane scission (Bashkirov et al., 2008).

These granulocrine secretion-related transcripts were then monitored by qPCR in samples of *Ptr*. With this poplar species, none of the genes appeared to be induced in nectaries (Table II), supporting the hypothesis of different secretion mechanisms used by *Ptt* and *Ptr* extrafloral nectaries.

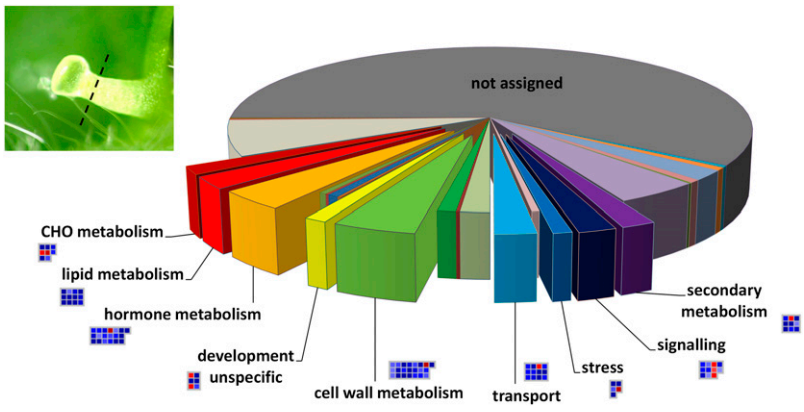

Figure 8. Clustering of *Ptt* differently expressed genes (extrafloral nectaries versus leaves). The 500 highest differentially expressed genes were imported into MapMan 3.1.1 and classified accordingly. The inset shows the *Ptt* nectary, where only the upper third (line) was used for RNA isolation. In the pie chart, most of the genes were not annotated (not assigned). Clusters related to nectary function are highlighted, and MapMan rasters are included. The boxes near the gene clusters represent the genes of that particular category (blue squares = up-regulated, red squares = down-regulated). Note that genes in these clusters are almost all up-regulated. CHO, Carbohydrate.

Table I. *Exocytosis-associated genes in Ptt nectaries*

Public ID[a]	Arabidopsis Genome Initiative Code[b]	Annotation[c]	Fold Change	Log_2 of Fold Change	Adjusted *P*	Type[d]	Class[e]
DN496417	At3g46060	GTP-binding protein ara-3	1.4	0.5	3.4E-02	RabE/Rab8 (1)	TGN
BU893135	At5g59840	GTP-binding protein ara-3	2.2	1.1	1.3E-03	RabE/Rab8 (2)	TGN
CV241725	At1g53050	Ser/Thr kinase activity	1.7	0.8	1.4E-02	RabA	TGN
CF120135	At4g39080	Vacuolar proton ATPase subunit	1.9	1.0	3.6E-03	VHA-a1	TGN
XP_002327686	At2g18250	Pantetheine-phosphate adenylyltransferase	0.5	−0.9	1.2E-03	SNARE (1)	TGN/MVB
CK093245	At1g08560	Syntaxin SYP111 (KNOLLE)	1.3	0.4	1.1E-01	SNARE (2)	TGN/MVB
CK092620	At5g46860	Syntaxin SYP22 (VAM3)	4.0	2.0	1.6E-04	PEP12 (1)	MVB
CF936851	At5g46860	Syntaxin SYP22 (VAM3)	1.8	0.8	3.6E-03	PEP12 (2)	MVB
CV277800	At4g19640	GTP-binding protein	1.1	0.1	7.9E-01	RabF (1)	MVB
CV240653	At5g45130	Ras-related GTP-binding protein RHA1	1.1	0.1	5.2E-01	RabF (3)	MVB
CX178052	At5g45130	Ras-related GTP-binding protein RHA1	1.5	0.6	7.2E-02	RabF (2)	MVB
BP932273	At5g06140	Sorting nexin-like protein	0.7	−0.5	1.8E-02	SNX1	RE
CV275482	At3g62290	ADP-ribosylation factor-like protein	2.0	1.0	4.3E-03	Arf1 (1)	VF
CK088579	At3g62290	ADP-ribosylation factor-like protein	1.7	0.8	1.7E-03	Arf1 (2)	VF
BU824735	At1g60500	Dynamin family protein, GTP-binding	7.9	3.0	5.0E-05	Dynamin (1)	VF
CK090501	At4g33650	Dynamin-like protein ADL2	1.9	0.9	8.7E-03	Dynamin (2)	VF
XP_002314626	At1g59820	Chromaffin granule ATPase II homolog	1.1	0.1	6.2E-01	Ala-3 (1)	VF
XP_002339708	At1g59820	Chromaffin granule ATPase II homolog	1.8	0.8	5.1E-03	Ala-3 (2)	VF
BU810775	At1g17500	P-type ATPase	2.2	1.1	1.4E-03	Ala-3 (3)	VF
CV278073	At1g32580	Plastid protein	1.0	0.1	8.9E-01	DAG	VF
XP_002306120	At4g39170	SEC14-like protein	1.9	0.9	1.1E-03	SEC14	VF

[a]Accession number of the corresponding sequence at www.ncbi.nlm.nih.gov/guide/ received from http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar with Affymetrix Probe Set identifiers. [b]Arabidopsis Genome Initiative code of the nearest Arabidopsis homolog according to [a]. [c]Annotation according to [b]. [d]Type of protein. Abbreviations are according to Zárský et al. (2009). [e]Class of protein. MVB, Multivesicular body; RE, recycling endosome; VF, vesicle formation.

Cell Wall

Among the cell wall cluster, transcripts for esterases and lyases, enzymes involved in pectin metabolism, were found to be increased (Table III). Within the same category, Leu-rich repeat proteins were elevated and might have a function in plant defense mechanisms, protein-protein recognition, and exocytosis (Kobe and Deisenhofer, 1995). Transcript levels with genes encoding enzymes engaged in UDP-sugar metabolism point to increased carbohydrate synthesis for cell wall formation (Gibeaut and Carpita, 1994).

Hormones/Defense

Within the hormone cluster, six genes appeared to be associated with auxin signaling (Table IV). This group contained genes coding for pin-formed proteins (PIN) that have been associated with auxin distribution, cell division, cell expansion, and polar growth (Blilou et al., 2005; Petrásek et al., 2006). Linked to vascular differentiation, three brassinosteroid metabolism genes were found to be up-regulated in nectaries. Within the hormone cluster, two genes associated with ethylene, two associated with JA, and five associated with salicylic acid were induced. Interestingly, these phytohormones represent key players in the response to wounding as inflicted by herbivores and pathogens (Li et al., 2001; Kachroo and Kachroo, 2007; Turner, 2007). In this context, it should be noted that 84 of the 500 most differentially expressed genes are related to biotic stress (Supplemental Fig. S8).

Sugar Metabolism and Transport

Nectar production demands high amounts of sugars, very likely provided by the phloem (Table V). Because *Ptt* nectar-secreting cells are apoplastically separated from the parenchyma and phloem cells (Fig. 2), sugars might be processed and supplied by the subjacent cells. Thus, besides sugar metabolic enzymes, sugar transporters are required for unloading from the source cells and loading into the *Ptt* nectar-secreting cells. Therefore, the full microarray data set was analyzed for transcripts involved in sugar metabolism and transport. In *Ptt* nectaries, a cell wall and a neutral invertase were up-regulated 10- and 6-fold, respectively. Among the sugar transporters expressed in *Ptt* nectaries, 16 hexose transporters appeared to be

Table II. *qPCR monitoring of exocytosis-associated transcripts in Ptr nectaries*

Comparison with microarray data from *Ptt* (Table I).

Type[a]	Class[b]	Fold Change, Microarray Data for *Ptt*	Fold Change, qPCR Data for *Ptr*
RabE/Rab8 (1)	TGN	1.4	0.3
RabE/Rab8 (2)	TGN	2.2	0.6
RabA	TGN	1.7	0.5
VHA-a1	TGN	1.9	0.9
SNARE (1)	TGN/MVB	0.5	1.4
SNARE (2)	TGN/MVB	1.3	0.2
PEP12 (1)	MVB	4.0	0.2
PEP12 (2)	MVB	1.8	0.7
RabF (1)	MVB	1.1	0.6
RabF (2)	MVB	1.5	0.9
RabF (3)	MVB	1.1	0.7
SNX1	RE	0.7	0.6
Arf1 (1)	VF	2.0	0.5
Arf1 (2)	VF	1.7	0.5
Dynamin (1)	VF	7.9	0.6
Dynamin (2)	VF	1.9	0.7
Ala-3 (1)	VF	1.1	0.3
Ala-3 (2)	VF	1.8	0.4
Ala-3 (3)	VF	2.2	0.3
DAG	VF	1.0	0.6
SEC14	VF	1.9	1.2

[a]Type of protein. Abbreviations are according to Zárský et al. (2009). [b]Class of protein. MVB, Multivesicular body; RE, recycling endosome; VF, vesicle formation.

induced (2- to 4-fold), but only two Suc transporters of the suc3 type were present and not induced. In addition, a Suc synthase (SUS3) was induced by a factor of 5. Furthermore, the analysis of the released nectar blend revealed almost equal amounts of Glc, Fru, and Suc (Supplemental Fig. S5). From these data, one might speculate that (1) Suc released from the phloem is mainly cleaved by invertases within the extracellular space, and (2) the secretory cells predominantly import hexose via the monosaccharide transporters, of which a part is reconverted to Suc by SUS3. Interestingly, further sugar metabolism-related enzymes like raffinose synthase, myoinositol oxygenase, and α-glucosidase, which play an important role in cell wall formation and thus reflect high cell division activity in the nectaries of *Ptt*, were highly up-regulated.

DISCUSSION

Here, we provide evidence that two tree species of the same genera might have evolved different mechanisms to control herbivory. *Ptt* and *Ptr* secrete EFN from young leaves. *Ptt* nectaries operate long-term merocrine nectar release. In contrast, *Ptr* grows new nectaries for each holocrine nectar secretion event. Thereby, the production of new nectaries on demand in *Ptr* is associated with short-term but massive holocrine nectar release. The nectar composition in both nectary types is rich in tastes that are known to attract different kinds of visitors, including bodyguards.

Nectary Morphology and Function

Extrafloral nectary, development, morphology, and nectar secretion represent highly correlated items. It has been demonstrated that the gland morphology type determines the dose and velocity of nectar flow. The kinetics and nectar flavor furthermore depend on the vascular supply of basic nectar compositions, very likely specified by factors produced as defense in the secretory cell on demand (Díaz-Castelazo et al., 2005).

Ptr nectaries are characterized by cells covered by a remarkably thick wall that probably impeded the free flow of nectar. Numerous plasmodesmata between cells in the complex likely provide for symplastic transfer of nectar between neighboring cells. Secretory vesicles were not observed in this nectary type. Instead, large numbers of mitochondria were found. This feature may point to the active transport of nectar across the plasma membrane. Microchannels in the nectary cuticle represent a potential low-resistance pathway for nectar secretion and were previously described in floral nectaries of *Platanthera chlorantha* (Orchidaceae), *Abutilon* sp., and *Helleborus foetidus* (Ranunculaceae; Kronestedt et al., 1986; Stpiczyńska et al., 2004; Koteeva, 2005). Nectaries from *Ptr*, after the release of large volumes of nectar, eventually died. The facts that (1) short-term secretion with *Ptr* nectaries results in cell death and this organ type (2) does not express granulocrine secretion-related transcripts point to a self-destructing holocrine secretion. This mode of secretion may thus be similar to floral nectaries of *H. foetidus* or *Digitalis purpurea* (Gaffal et al., 2007; Vesprini et al., 2008).

Nectaries from *Ptt*, in contrast, developed just one layer of brush border-like large secretory cells (Fig. 2). Epithelia-like nectary cells like these have not yet been discovered in any other plant nectary type. The absence of symplastic connections between this outer cell layer and the nectary parenchyma, in order to secrete nectar, requires an apoplastic loading via parenchyma cells located inside the nectary. Metabolic continuity between the secretory cells is facilitated by a large number of plasmodesmata in their periclinal cell walls. The abundance of numerous secretory vesicles within and outside of the secretory cells and the up-regulated *Ptt* nectary lipid metabolism gene clusters (Fig. 8) point to a granulocrine nectary type. In this context, one should mention that transcriptome analysis of Arabidopsis (*Arabidopsis thaliana*) floral nectaries also suggested a granulocrine secretion mechanism (Kram and Carter, 2009; Kram et al., 2009). Nectar production is an expensive investment for the plant. In order to save energy, floral nectaries of some plants are able to reabsorb unconsumed nectar (Nepi and Stpiczyńska, 2008). A similar situation exists in *Ptt* extrafloral nectaries. The lack of a cuticle and the distribution of membrane FM dye would support nectar reabsorption

Table III. *Differentially expressed cell wall metabolism-associated genes in Ptt nectaries*

Public ID[a]	Arabidopsis Genome Initiative Code[b]	Annotation[c]	Fold Change	Log_2 of Fold Change	Adjusted *P*
BP929963	At5g25610	RD22 (responsive to desiccation22)	22.9	4.5	5.4E-05
CV268088	At3g43270	Pectinesterase family protein	15.2	3.9	2.6E-05
CV281908	At3g19320	Leu-rich repeat family protein	10.0	3.3	6.6E-05
CV230945	At3g61490	Polygalacturonase (pectinase) family protein	10.0	3.3	4.4E-05
CX176311	At3g61490	Polygalacturonase (pectinase) family protein	8.7	3.1	5.5E-05
CN519614	At3g23730	Endo-xyloglucan transferase, putative	8.5	3.1	2.6E-05
DN502989	At3g19320	Leu-rich repeat family protein	8.0	3.0	7.1E-05
CV281908	At3g19320	Leu-rich repeat family protein	7.5	2.9	7.8E-05
CF227942	At4g24780	Pectate lyase family protein	6.6	2.7	2.9E-05
BU893810	At1g69530	ATEXPA1 (Arabidopsis expansin A1)	6.3	2.7	3.7E-05
BU811740	At3g14310	ATPME3 (Arabidopsis pectin methylesterase3)	5.3	2.4	8.2E-05
DN486441	At3g14310	ATPME3 (Arabidopsis pectin methylesterase3)	4.9	2.3	3.3E-05
CX659087	At3g08900	RGP3 (reversibly glycosylated polypeptide3)	4.9	2.3	5.8E-05
CN520892	At3g23730	Endo-xyloglucan transferase, putative	4.9	2.3	8.2E-05
AI165969	At4g24780	Pectate lyase family protein	4.2	2.1	5.1E-05
XP_002324379	At4g31590	ATCSLC05 (cellulose synthase-like C5)	4.2	2.1	5.4E-05
CA927911	At3g14310	ATPME3 (Arabidopsis pectin methylesterase3)	4.0	2.0	8.2E-05
XP_002326320	At2g26440	Pectinesterase family protein	3.9	2.0	8.2E-05
CV244899	At1g08200	AXS2 (UDP-D-apiose/UDP-D-Xyl synthase2)	3.8	1.9	5.8E-05
CV227643	At5g15490	UDP-Glc-6-dehydrogenase, putative	3.6	1.9	8.3E-05
CV230306	At2g27860	AXS1 (UDP-D-apiose/UDP-D-Xyl synthase1)	3.5	1.8	8.2E-05
XP_002314796	At1g23200	Pectinesterase family protein	0.1	−3.0	2.6E-05

[a]Accession number of the corresponding sequence at www.ncbi.nlm.nih.gov/guide/ received from http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar with Affymetrix Probe Set identifiers. [b]Arabidopsis Genome Initiative code of the nearest Arabidopsis homolog according to [a]. [c]Annotation according to [b].

via endocytosis of cells in the outer layer of the secretory organ. Here, bulk secretion seems to be mediated by multivesicular bodies (Fig. 2), components of the plant exocytosis system (Foresti et al., 2008; Foresti and Denecke, 2008; Robinson et al., 2008; Zárský et al., 2009). Accordingly microarray studies with *Ptt* nectaries identified an enrichment of transcripts involved in granulocrine secretion. In *Ptr* nectaries, however, the same genes appeared not to be induced. Moreover, *Ptr* nectaries run dry after a few days. Another nectar-release cycle, therefore, depends on the production of a new set of holocrine-secreting nectaries. Our findings with the

Table IV. *Differentially expressed hormone metabolism-associated genes in Ptt nectaries*

Public ID[a]	Arabidopsis Genome Initiative Code[b]	Annotation[c]	Fold Change	Log_2 of Fold Change	Adjusted *P*	hor[d]
XP_002312924	At1g29430	Auxin-responsive family protein	3.4	1.8	6.5E-05	aux
XP_002316954	At5g54510	DFL1/GH3.6 (dwarf in light1)	15.7	4.0	3.3E-05	aux
XP_002319398	At5g54510	DFL1/GH3.6 (dwarf in light1)	10.7	3.4	4.9E-05	aux
XP_002306218	At1g29510	SAUR68 (small auxin up-regulated68)	5.2	2.4	3.9E-05	aux
CV237921	At1g77690	Amino acid permease, putative	5.1	2.3	8.2E-05	aux
XP_002336658	At5g16530	PIN5 (pin-formed5); auxin:hydrogen symporter	6.7	2.8	4.4E-05	aux
CK092098	At1g75080	BZR1 (brassinazole-resistant1)	4.2	2.1	8.4E-05	bra
CA823181	At3g19820	DWF1 (diminuto1); catalytic	6.3	2.7	5.5E-05	bra
AJ778035	At3g19820	DWF1 (diminuto1); catalytic	4.8	2.3	5.6E-05	bra
XP_002300561	At4g20880	Ethylene-regulated nuclear protein (ERT2)	5.2	2.4	7.4E-05	eth
CX184416	At2g40940	ERS1 (ethylene response sensor1); receptor	4.1	2.0	6.4E-05	eth
DN490862	At1g06620	2-Oxoglutarate-dependent dioxygenase, putative	0.1	−3.5	2.6E-05	eth
BP933461	At5g42650	AOS (allene oxide synthase)	5.0	2.3	8.2E-05	JA
CV262113	At1g55020	LOX1 (lipoxygenase1)	3.5	1.8	7.4E-05	JA
BU825949	At4g36470	BSMT1; *S*-adenosyl-Met-dependent methyltransferase	40.0	5.3	2.3E-05	SA
CV263317	At4g36470	BSMT1; *S*-adenosyl-Met-dependent methyltransferase	30.7	4.9	3.4E-05	SA
XP_002335942	At5g38020	BSMT1; *S*-adenosyl-Met-dependent methyltransferase	22.7	4.5	4.3E-05	SA
XP_002334262	At1g19640	JMT (jasmonic acid carboxyl methyltransferase)	25.2	4.7	6.9E-05	SA
CA929119	At1g68040	Carboxyl methyltransferase family protein	15.9	4.0	2.6E-05	SA

[a]Accession number of the corresponding sequence at www.ncbi.nlm.nih.gov/guide/ received from http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar with Affymetrix Probe Set identifiers. [b]Arabidopsis Genome Initiative code of the nearest Arabidopsis homolog according to [a]. [c]Annotation according to [b]. [d]Related to the hormone auxin (aux), brassinosteroids (bra), ethylene (eth), JA, or salicylic acid (SA).

Table V. *Sugar metabolism- and transport-associated genes in Ptt nectaries*

Public ID[a]	Arabidopsis Genome Initiative Code[b]	Annotation[c]	Fold Change	Log_2 of Fold Change	Adjusted *P*
Major carbohydrate metabolism					
BU879414	At3g52600	ATCWINV2, CWIN4, cell wall invertase	10.1	3.3	2.8E-04
CV260807	At3g05820	Alkaline/neutral invertase H	6.2	2.6	8.2E-05
BP928698	At4g02280	ATSUS3, Suc synthase3	4.8	2.3	3.7E-05
Minor carbohydrate metabolism					
BP933945	At2g19800	Myoinositol oxygenase2	75.5	6.2	2.8E-05
XP_002330589	At1g55740	Raffinose synthase1	21.0	4.4	4.1E-05
CV271186	At5g08370	α-Galactosidase2	4.2	2.1	6.4E-05
Sugar transport					
CV267500	At5g16150	GLT1, Glc transporter1	3.9	2.0	1.7E-04
CV265114	At2g43330	ATINT1, inositol transporter1	3.4	1.8	1.5E-04
CA823118	At1g73220	ATOCT1, organic cation/carnitine transporter1	2.8	1.5	2.0E-04
XP_002313809	At3g18830	ATPLT5, polyol/monosaccharide transporter5	2.7	1.4	4.3E-04
CV237054	At5g17010	Sugar transporter family protein	2.5	1.3	2.2E-03
CB307011	At5g26340	STP13, sugar transport protein13	2.5	1.3	2.6E-03
XP_002310808	At1g75220	Putative sugar/inositol transport protein	2.4	1.2	2.8E-04
XP_002308798	At4g16480	ATINT4, inositol transporter4	2.3	1.2	2.6E-03
CK107634	At5g17010	Sugar transporter family protein	2.2	1.1	1.6E-03
CV241535	At4g35300	TMT2, tonoplast monosaccharide transporter2	2.2	1.1	7.4E-03
CV272388	At5g41760	Nucleotide-sugar transporter family protein	2.2	1.1	9.3E-04
CA928662	At3g59360	ATUTR6, UDP-Gal transporter6	2.1	1.1	2.4E-03
CV279380	At5g26340	STP13, sugar transport protein13	2.1	1.1	8.9E-04
BU866894	At5g59250	Sugar transporter family protein	2.0	1.0	2.2E-03
XP_002329696	At1g73220	ATOCT1, organic cation/carnitine transporter1	2.0	1.0	2.8E-03
XP_002324427	At1g67300	Hexose transporter, putative	2.0	1.0	4.6E-03
XP_002315798	At2g02860	ATSUC3, Suc transporter3	1.5	0.6	4.6E-03
XP_002311596	At2g02860	ATSUC3, Suc transporter3	1.5	0.6	9.7E-03

[a]Accession number of the corresponding sequence at www.ncbi.nlm.nih.gov/guide/ received from http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar with Affymetrix Probe Set identifiers. [b]Arabidopsis Genome Initiative code of the nearest Arabidopsis homolog according to [a]. [c]Annotation according to [b].

tree model *Populus* thus seem to support the notion that *Ptt* and *Ptr* operate two different nectary types and secretion mechanisms.

Appearance of Nectaries and Nectar

In wild cotton (*Gossypium thurberi*), the size and density of extrafloral nectaries are heritable (Rudgers and Strauss, 2004). One report focusing on *P. tremuloides* ecotypes with extrafloral nectary induction has suggested a genetic component (Wooley et al., 2007). This study showed that nectaries were generally more abundant on younger than on older leaves and decreased with tree age. Our finding that 4-year-old *Ptt* trees expressed nectaries on about 40% of the leaves is well in agreement with data that were found with 4- to 5-year-old trees (Wooley et al., 2007). However, we cannot exclude that the number of *Ptt* nectaries might increase under massive herbivore attack, as predicted by Wooley et al. (2007). In spring, nectaries appeared on most emerging leaves of both species, and they seem to protect *Ptt* as well as *Ptr* leaves from herbivore attack (Fig. 3; Supplemental Fig. S4). Secondary nectar release from *Ptr* was observed in response to the invasion of phloem-feeding insects. Poplar species are known to have several direct defense mechanisms against herbivores, like the production of condensed tannins, phenolic glycosides, and salicortin (Hwang and Lindroth, 1998; Osier et al., 2000; Donaldson and Lindroth, 2007). Direct and indirect defenses are costly. The inducible expression of secondary nectaries with *Ptr* might thus be a strategy to avoid interference of the different defense systems. Recent studies indicate that continuous mechanical tissue damage is sufficient to trigger JA production and subsequent volatile emission (Mithöfer et al., 2005). In addition to wounding, chemical elicitors present in insect saliva also may play an important role in the extrafloral nectary response (Radhika et al., 2010). Upon wounding of *Ptr*, *a* typical JA or herbivory-linked VOC spectrum was emitted, but new nectaries or the release of nectar from existing nectaries were not observed. These findings clearly indicate that neither jasmonates nor induced volatiles

trigger *Ptr* nectar production, as was reported previously in lima beans (Heil et al., 2008). Herbivore-induced *Ptr* VOCs, however, may be used directly by carnivorous arthropods as a cue for host localization or as a plant-plant signaling mechanism, as proposed (Heil et al., 2008). This also implies that the mealy bug-induced nectar production and nectary formation is linked to currently unknown signaling pathways.

Nectar Blend

Gross chemical properties of nectars tend to be similar in plants attracting the same animals. The EFN of myrmecophyte *Acacia* contains just Glc and Fru. It has been shown that the addition of Suc significantly changed the attractiveness of this nectar (Heil et al., 2005). Cell wall invertases (CWIN) are important determinants of sink strength and, thus, phloem sugar unloading and the suppression of reloading (Roitsch, 1999). Recently, CWIN4 was described as the first cell wall invertase that is required for nectar production in Arabidopsis (Kram et al., 2009; Ruhlmann et al., 2010). In the nectary transcriptome of this model plant, CWIN4-associated sugar synthases appeared increased. Similarly, *Ptt* nectaries up-regulate genes encoding invertases, hexose transporters, and sugar synthases. In addition, an invertase sharing high homologies with CWIN2 as well as CWIN4 was found 10-fold up-regulated. In agreement with the aforementioned Arabidopsis studies, our data give rise to the hypothesis that with *Ptt* nectaries, sugar is mainly imported and further processed by secretory cells as monosaccharide. The fact that *Ptt* nectar contains equal amounts of Fru, Glc, and Suc strengthens this theory. Nectar of *Ptt* might thus be considered as tasty for generalists, which appeared to be reflected by the diversity of visitors described for *P. tremula* (Wooley et al., 2007). *Ptr*, in contrast, seems to be suited for specialized visitors. Thus, insights into the kind of visitors might already be gained by the nectar blend. Analyzing the nature of the visitors as a function of nectary type, nectar composition, and bodyguards represents a future goal. Thereby, the question will be addressed whether poplar plants can shape the blend of the nectar in an insect-dependent manner.

In this respect, amino acids represent tasty nectar components (Baker and Baker, 1973, 1983). The EFNs from *Ptt* and *Ptr* were rich in "sweet" taste amino acids like Phe, which is the most abundant amino acid in nectars of bee-pollinated plants and also is known to serve as a bee phagostimulant (Inouye and Waller, 1984; Petanidou, 2007). Pro has the unique ability to stimulate the insect's salt cell, which results in enhanced feeding behavior (Hansen et al., 1998; Wacht et al., 2000). We found Pro in EFN of both poplar species but not in leaf extracts. It is by far the most abundant amino acid in honeybee hemolymphe, important for egg laying (Hrassnigg et al., 2003), and regulates the conversion of nectar into honey (Davies, 1978). Therefore, the composition of *Ptt* nectar seemed suitable to support the honeybee lifestyle. It has been shown that honeybees, parasitic flies (Tachinidae), parasitic wasps (Ichneumonidae), and bees are common visitors of all poplar nectaries (Supplemental Fig. S6; Trelease, 1881; Wooley et al., 2007). Flying honeybees and wasps produce similar air disturbances that stimulate sensory hairs of many caterpillars. As a result, caterpillars stop moving or drop off the plant. Thereby, the feeding intensity of the herbivores is reduced (Tautz, 1977; Tautz and Markl, 1978; Tautz and Rostas, 2008). Attracting honeybees and wasps with tasty nectar, therefore, might be an effective strategy to reduce poplar leaf damage by herbivore infestation.

CONCLUSION

The morphology of *Ptt* nectaries revealed granulocrine secretion via multivesicular bodies, whereas *Ptr* showed typical multilayer secretory cells with a structure similar to floral nectaries. *Ptt* continuously secretes nectar from long-living nectaries, and excess nectar is reabsorbed via endocytosis. Thus, membrane trafficking is frequent and might explain why only one distinct layer of secretory cells is present in these nectaries. Both *Ptt* and *Ptr* trees seem to protect their delicate first leaves in spring against herbivores by nectar production. In contrast to continuously nectar-secreting *Ptt*, *Ptr*, only in the case of special insect attack, produces secondary nectar by extrafloral nectaries. The emerging *Ptr* nectar seems to attract rather specialist visitors, maybe depending on the particular herbivore. *Ptt*, in contrast, provides nectar for generalists according to unspecific nectar production.

These different defense strategies require varying secretion systems, which we confirmed by transcript and metabolite compositions, as well as the morphology and physiology of nectaries and nectar of both poplar species.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Populus tremula × *Populus tremuloides* plants (clone T89) and *Populus trichocarpa* (clone 93-968) were field grown in soil under natural conditions in the Botanical Garden of Wuerzburg or cultivated under long-day conditions in climate chambers (16 h of light [22°C]/8 h of darkness [17°C]; TLD 58 W/840 Super 80 [Philips] and 58 W L58/77 [Osram] lamps). Field-grown trees used for effectiveness tests and the visitor determinations were about 3 to 4 years old and 15 to 20 feet high. All other experiments were performed using cultivated trees of about 4 to 5 feet in height. These plants were watered twice a week and fertilized frequently.

Light Microscopy

Nectaries from *Ptr* or *Ptt* were harvested and fixed by passing through ascending grades of ethanol for 45 min each. After dehydration, nectaries were embedded in 2-hydroxyethyl methacrylate/glycol methacrylate (Agar Scientific) according to the manufacturer's advice. Sections of extrafloral nectaries (20 μm) were cut with a c-profile 16-cm knife (Leica) in a Leica RM2165 microtome and heat fixed to microscope slides. Specimens were stained with

toluidine blue, mounted in immersion oil under coverslips, and examined with a VHX-100k digital microscope (Keyence Corp.).

Nectary Appearance and Effectiveness

Nectary appearance and leaf damage were quantified using 11 randomly chosen branches (1,257 leaves in total) of three different trees (*Ptt*) or six branches (327 leaves in total) of two different trees. Damage was classified as severe (more than 75% damage of the leaf surface), mild (less than 75%), or healthy (no damage).

TEM

Small sections of leaf tissue (1–2 mm) were cut with a razor blade and immediately immersed for 4 h in fixation medium containing 1% (w/v) formaldehyde, 1 mM EGTA, 50 mM cacodylate buffer, and 5% glutaraldehyde. Subsequently, the tissue was postfixed with 2% (w/v) osmium tetroxide overnight at room temperature, stained with 3% (w/v) uranyl acetate in 20% ethanol for 1 h, dehydrated in a graded series of ethanol, and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin sections with a thickness of 70 to 80 nm were cut with a diamond knife on an ultramicrotome (Ultratome Nova; LKB), transferred onto formvar-coated copper grids, and stained for 10 min with lead citrate. Sections were examined using a Zeiss EM 10c transmission electron microscope at 80 kV.

Quantification of Nectar Sugars and Amino Acids

For nectar sugar quantification, different amounts of nectar were diluted in HPLC water (Sigma) to a final volume of 1 mL, boiled for 5 min, and centrifuged (10 min at 14,000*g*). The supernatant was treated with Serdolit MB1 (Serva; 10 mg 100 μL^{-1} sample), and the sugar concentration was measured using a pulse electrochemical detector (Dionex 4500i). Amino acid quantity and quality were measured using an amino acid analyzer (Biochrom 20 Plus).

Induction of EFN

The experiment was conducted under natural conditions with about 1-year-old soil-grown *Ptr*. The four youngest fully expanded leaves of each plant were induced, either by puncturing 100 times with a needle (1 mm diameter) or by cutting the leaf tip (about 10% of the total leaf area). Plants were observed using a Keyence VHX-100k digital microscope. Every 15 min, photographs were taken. For "herbivory induction," caterpillars (*Spodoptera littoralis*, *Spodoptera exigua*, and *Lymantria dispar*) were placed on *Ptr* leaves.

Collection and Analysis of Volatiles

The collection of volatiles emitted from leaves of the test plants damaged by caterpillars or MecWorm or treated with chemical elicitors (JA or coronalon) was achieved using charcoal traps. Elicitor-treated or insect-damaged areas of the plants were enclosed in polyethylene terephthalate (PET) foil bags that were tightly closed at both ends to guarantee the accumulation of volatiles in the enclosed volume and to avoid contamination with volatiles from the potting soil. One end of the PET bags was connected for 24 h to a volatile collection device (Kunert et al., 2009), and the emitted volatiles were trapped on charcoal filters (1.5 mg; CLSA Filter; Gränicher & Quartero) by air circulation maintained by a circulation pump. The trapped volatiles (24, 48, or 72 h) were desorbed from the filters using 2 × 20 μL of dichloromethane containing 1-bromodecane (50 $\mu g\ mL^{-1}$) as an internal standard. An aliquot (1 μL) of the stock solution was analyzed on a Finnigan Trace gas chromatography-mass spectrometry device equipped with an EC-5 column (15 m × 0.25 mm × 0.25 μm; Alltech). Helium at a flow rate of 1.5 mL min^{-1} served as a carrier gas. The gas chromatography injector, transfer line, and ion source were set at 220°C, 280°C, and 280°C, respectively. Volatiles were separated under programmed conditions using a temperature profile from 40°C (2 min) at 10°C min^{-1} to 200°C and at 30°C min^{-1} to 280°C. The split ratio of the stock solution was 1:10, and the resulting split flow was 15 mL min^{-1} (10 times the column gas flow of 1.5 mL min^{-1}). Authentic standards were used for the identification of compounds.

Induction of Volatile Biosynthesis with JA or Coronalon

Leaves of the test plants were sprayed with aqueous solutions of JA (1 and 0.5 mM) or coronalon (0.1 mM) until runoff. After drying (1 h), the pretreated plants were enclosed in PET foil bags, and volatile collection was achieved over 24 h as described. If the volatile collection was extended to 48 h, the plant leaves were sprayed and dried a second time after 24 h.

Volatile Induction by Herbivorous Insects

Larvae of *S. littoralis* (Lepidoptera, Noctuidae) were used as herbivores for volatile induction. For feeding experiments, third-instar larvae were used. Five larvae were placed on a plant, and the test plant was enclosed in a PET foil bag. Volatiles were then collected as described.

Continuous Mechanical Damage of Plant Leaves using MecWorm

Individual leaves of the intact test plant were continuously damaged by the robotic MecWorm system (Mithöfer et al., 2005) over a period of 24 h, resulting in 333 mm^2 of damaged area using four punches per minute. Volatiles were collected as described. Additional experiments were conducted by combining continuous mechanical damage with the addition of oral secretions from the larvae (1:10 dilution) to the damaged area.

RNA Isolation and Amplification for Microarrays

Total RNA of leaves was extracted using the E.Z.N.A. Plant RNA Mini Kit (Ω Bio-Tek) and nectary total RNA with the RNeasy MicroKit (Qiagen), according to the manufacturers' protocols, with minor modifications. The binding buffers were supplemented with 2% β-mercaptoethanol, 1% polyvinylpyrrolidone, and 70 mM potassium ethylxantogenate and added to 30 mg of homogenized leaf tissue or 120 homogenized nectaries, respectively. After incubation at room temperature for 30 min, the sample were briefly centrifuged before transfer to the individual homogenation columns. One microgram of total RNA was used for RNA amplification based on the BD-SMART mRNA amplification kit (BD Bioscience). The mRNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). To preamplify full-length complementary DNAs (cDNAs) prior to in vitro transcription, an additional 10-cycle PCR (95°C for 30 s, 60°C for 1 min, and 68°C for 10 min) was introduced using t7 extension and PCR primer IIA of the kit. RNA integrity and concentration were monitored using the Experion Automated Electrophoresis System (Bio-Rad) with the Experion RNA Highsense Analysis Kit according to the manufacturer's protocol.

qPCR

To remove any remaining DNA, total RNA was treated with RNase-free DNase (Fermentas) according to the manufacturer's protocol. First-strand cDNA was prepared using 2.5 μg of RNA with the Moloney murine leukemia virus reverse transcriptase kit (Promega). First-strand cDNA was 20-fold diluted for reverse transcription-PCR. qPCR was performed in a Mastercycler ep Realplex^{2}S (Eppendorf) with the ABsolute QPCR SYBR Green Capillary Mix (ABgene) in a 20-μL reaction volume. After a "hot start" (15 min at 95°C), a standard PCR program was applied: 40 times for 15 s at 95°C, 15 s at primer-specific annealing temperature, and 20 s at 72°C, followed by a dissociation curve (10 s at 95°C, followed by a temperature ramp from 60°C–95°C with an increment of 0.3°C s^{-1}). Primers used (TIB MOLBIOL) have been designed for *Ptt* or *Ptr* and were tested regarding optimal annealing temperature, specificity by dissociation curves, and gel electrophoresis (data not shown) prior to qPCR. Primers are listed in Supplemental Table S3. All quantifications were normalized to actin cDNA fragments amplified by PtACT2fwd and PtACT2rev. These fragments are homologous to the constitutively expressed Arabidopsis (*Arabidopsis thaliana*) Actin2 and Actin8 (for details, see An et al., 1996; Szyroki et al., 2001). Each transcript was quantified using individual standards. To enable the detection of contaminating genomic DNA, PCR was performed with the same RNA as template, which was used for cDNA synthesis. All kits were used according to the manufacturer's protocols.

Microarrays

Microarray analyses were conducted at the Microarray Facility, University of Tübingen. Samples of leaves and nectaries from *Ptt* field culture were analyzed. All samples were amplified using the One-Cycle Target Labeling Assay (Affymetrix) according to the manufacturer's protocol and hybridized to the Gene Chip Poplar Genome (Affymetrix). Microarrays were scanned using the GCS3000 GeneChip scanner (Affymetrix) and GCOS software version 1.4. Scanned images were subjected to visual inspection to control for hybridization artifacts and proper grind alignment. Files for quality control were generated using the program Expression Console (Affymetrix).

Biostatistical Analyses

Data preprocessing was performed using the Bioconductor software (Gentleman et al., 2004) with the statistical programming environment R (Ihaka and Gentleman, 1996). Background correction and normalization were performed using the variance stabilization method (variance stabilization normalization [vsn] and robust multichip average [rma]; Huber et al., 2002), and probe-set summaries were calculated with the medianpolish algorithm of rma (Irizarry et al., 2003). Exploratory analysis by hierarchical clustering of the arrays and correspondence analysis suggested the removal of one leaf array as an outlier, leaving a batch of five confident arrays in total. Differential expression between nectaries and leaves has been calculated using the moderated *t* statistic implemented in the eBayes function of the Limma package (Smyth, 2004), which was specifically developed for the analysis of small-sample-size experiments. By exploiting information across genes, it delivers more stable results than a conventional *t* test. The *P* values of all results were corrected for multiple testing by applying the false discovery rate from Benjamini and Hochberg (2000).

The 500 most differentially expressed genes were analyzed by MapMan 3.1.1. The Affymetrix probe set identifiers were imported and mapped with *Populus trichocarpa*/Ptrich_AFFY_09:1.0 and the pathway overview 1.0 (http://mapman.gabipd.org/web/guest). Annotation of Affymetrix probe set identifiers was done by using BarleyBase/PLEXdb (Wise et al., 2007).

Microarray data have been uploaded to the Gene Expression Omnibus. Data are accessible at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pzehnqawcmikqxw&acc=GSE23897.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Enlarged TEM images of *Ptt* and *Ptr* nectaries.

Supplemental Figure S2. *Ptt* nectaries stained with Sudan III.

Supplemental Figure S3. Fluorescence microscopy of *Ptt* nectary longitudinal sections stained with FM4-64.

Supplemental Figure S4. *Ptr* nectary density and effectiveness against herbivore attack.

Supplemental Figure S5. Sugar content in nectars of *Ptt* and *Ptr*.

Supplemental Figure S6. Visitors attracted by extrafloral *Ptt* and *Ptr* nectaries.

Supplemental Figure S7. The most abundant amino acids in leaves of *Ptt* and *Ptr*.

Supplemental Figure S8. MapMan gene clusters involved in biotic stress responses.

Supplemental Table S1. List of 500 differentially expressed genes in *Ptt* nectaries versus leaves.

Supplemental Table S2. Validation of microarray data by qPCR.

Supplemental Table S3. Primers used in qPCR.

Supplemental Protocol S1. Sudan III and FM-64 staining.

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