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Poplar extrafloral nectaries - two types, two strategies of indirect defenses against herbivores

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ABSTRACT
Many plant species grow extrafloral nectaries and produce nectar to attract carnivore arthropods as defenders against herbivores. Two nectary types evolved with *Populus trichocarpa* (*Ptr*), and *P. tremula x P. 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 holocrine and inducible. Neither mechanical wounding nor 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 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 anti-herbivore 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.

Scientists discuss since more than hundred years two alternative mechanisms for the secretion of floral nectar. Even less, however, is known about EFN secretion (Escalante-Pérez and Heil, in press). Holocrine secretion is characterized by programmed cell death that in a one-step-process causes release of the entire cell content into the 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 “pre-nectar“ - after uploading from the phloem - moves via an apoplastic pathway, or via a vesicle-based symplastic pathway (Vassilyev, 2010; Heil, 2011 and references 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 relaying
on transport of a fluid phase that seems to be governed by ER- or dictyosomes-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; Jurgens and Geldner, 2002). To study the mechanisms that underlie the secretion of extrafloral nectar we in the present study 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 in 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*, 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 (Stpiczynska et al., 2005; Kaczorowski et al., 2008). Detailed studies have shown that decrease in herbivory rates associated with extrafloral nectar secretion results from mechanical leaf damage, jasmonic acid (JA) production, and/or volatiles 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, or alkaloids (Jones, 1983). Given the enormous variability in nectar features regarding volume, concentration, and composition, the latter hypothesis appears a kind of oversimplified (Pacini et al., 2003; Heil, 2011).

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

**RESULTS**

**Two types of nectary structures harbor unique secretion systems**

In the two poplar species *Populus trichocarpa* (*Ptr*) and *Populus tremula x Populus tremuloides* (*Ptt*) pairs of nectaries are located on each side of the leaf blade near the petiole (Figure 1). *Ptt* nectaries were bigger, while those of *Ptr* released more nectar (Figure 1C and F).

Light microscopy with *P. trichocarpa* nectaries documented the structure described for many floral nectaries. They contain an epidermis in addition to a nectar- and a subnectary parenchyma (Stpiczynska et al., 2005; Wist and Davis, 2006; Kaczorowski et al., 2008; Wenzler et al., 2008) (Figure 2A). *Ptt* nectaries develop a nectar- and subnectary parenchyma as well. In contrast to *P. trichocarpa*, however, the outer layer consisted of modified epidermal cells (Figure 2G). In TEM analyses we found the surface of nectary epidermal cells of *Populus trichocarpa* covered by a cuticle entirely. Therein micro-channels appeared as narrow tubular interruptions in the continuity with the cell wall. These fibrillar outgrowths of the outer epidermal cell walls might represent a path for passive nectar flow (Figure 2B and Supplemental Figure 1). 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 (Figure 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 *Populus tremula x P. tremuloides* these cells possessed unusual thick walls with numerous pits and associated plasmodesmata connecting the protoplasts of adjacent cells (Figure 2D and E). Around the symplastic connections we could visualize numerous chloroplasts, plastids containing plastoglobuli, mitochondria along with rough ER, and dictyosomes (Golgi apparatus). The numerous vacuoles of the nectary parenchyma appeared small and surrounded by dark-stained matter (Figure 2).

With *P. tremula x P. tremuloides* nectaries, surprisingly, in TEM and light microscopy - following wax staining by Sudan III - no cuticle was found covering the secretory epidermal cells (Supplemental Figure S2). Ultrastructural analyses of *Ptt* nectaries showed that the secretory cells (Figure 2H), representing specialized epidermal cells, were interconnected at their lateral side by a large number of plasmodesmata (Figure 2I). Such connections were absent between the nectary parenchyma at the basolateral side (Figure 2L and Supplemental Figure 1). In *Ptt* extrafloral nectaries vesicles are located in the outer apoplastic space as well as in the tip of the secretory cells (Figure 2J, K, and Supplemental Figure 1). As with floral nectaries these structures were associated with epidermal cell endocytosis in the process of reabsorption of non-consumed nectar (Nepi and Stpiczynska, 2007, 2008).

*Ptt* nectaries were treated with membrane staining but membrane impermeable fluorescent dye FM4-64 (Fischer-Parton et al., 2000). In longitudinal-sections staining of the secretory cells traces pronounced endocytosis episodes of FM dye contained membrane vesicles (Supplemental Figure S3). FM4-64, however, failed to cross the apoplastic cleft between secretory cells and parenchymal neighbors.
Stress, nectary development and activity

In spring many emerging *Populus tremula* × *P. tremuloides* and *Populus trichocarpa* leaves harbor nectaries (Figure 3B). To test whether this phenomenon results from heritable genetics extrafloral nectaries densities of field-grown *Ptt* trees were determined. The populations of leaves with (38%) and without nectaries (62%) were highly conserved among individual trees (Figure 3A). Similar data were obtained with *Ptr* (Supplemental Figure S4). These results are consistent with general effectiveness of nectaries against herbivore attack (Heil et al., 2001; Heil et al., 2005; Kost and Heil, 2008). 60% (*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) had been severely affected (Figure 3C and Supplemental Figure S3B). Nectary-free leaves, however, exhibited a higher percentage of damages with *Ptt* and *Ptr* (Figure 3 and Supplemental Figure 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 following nectar release. When, however, 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 (Figure 4).

So, how the secondary nectar production of *P. trichocarpa* was induced? To test whether in *P. trichocarpa* extrafloral nectar production results of mechanical stress, leaves of *Ptr* were wounded by puncturing the leaf blade with a needle (Figure 5A). Following 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 (Figure 5B and C). The computer-controlled mechanical caterpillar “MecWorm” mimics the damage caused by herbivores in terms of spatio-temporal 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 by simply wounding of leaves but requires another stimulus instead.
The same result was obtained when the MecWorm-damaged tissue was additionally treated with the oral secretion of the mediterranean climbing cutworm (*Spodoptera littoralis*). Finally caterpillars of *Spodoptera littoralis*, *Spodoptera exigua*, and *Lymantria dispar*, which are polyphagous insects and thus feed on poplar leaves, were placed on *Populus trichocarpa* (Figure 5D). Again, neither nectary nor nectar production was observed within 48 hours 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 (Figure 5E 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 (not shown). In most cases the secondary nectar production occurred upon attack by sucking insects (Figure 5E - H). Thus *Ptr* nectar formation seemed to be confined to specific types of herbivores.

**Volatile are not involved in P*tr* 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 leaves after herbivore damage are able to induce EFN flow in undamaged neighbored plants (Heil et al., 2008). Herbivory or even mechanical leaf damage elevates endogenous levels of jasmonic acid (JA) stimulating volatile biosynthesis. Consequently also externally added JA 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 *P. trichocarpa* by jasmonates or herbivory-related stressors, poplar leaves were
continuously damaged by the MecWorm or by caterpillars of *Spodoptera littoralis*, *Spodoptera exigua*, and *Lymantria dispar*. After mechanical damage, herbivory, and application of JA or coronalon, which acts as a mimic of jasmonoyl-isoleucine (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 \((E)-\beta\text{-ocimene}, 4,8\text{-dimethyl-1,3,7-nonatriene (DMNT)}, farnesene, nerolidol, and 4,8,12\text{-trimethyltridec-1,3,7,11-tetraene (TMTT)}\) (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)-\beta\text{-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 visitor’s attraction**

Nectar is a complex mixture of metabolites. The sweet nectar blend is dominated by sucrose, glucose, and fructose. Nectar with high sucrose content attracts generalists, while nectars with higher hexose contents are preferred by specialists (Heil et al., 2005). The amounts of sucrose, fructose, and glucose in poplar EFN appeared relatively constant in the different samples from the same species, but differed remarkably between species (Supplemental Figure S5). In *Ptt* the ratios between glucose, fructose, and sucrose were equally, while in contrast the percentage of sucrose in *Ptr* nectar was rather low. Thus EFN of *Ptt* likely attract generalists such as honey bees, wasps, and parasitic wasps, while those of *Ptr* attract more specialized visitors, like e.g. ants (Supplemental Figure 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 which enable them to sense amino acids (Shiraishi and Kuwabara, 1970). Interestingly, amino acids responsible for sweet taste like phenylalanine,
tryptophan, and valine were well presented in both *Ptt* and *Ptr* nectar samples, but low or absent in leaves (Figure 7, and Supplemental Figure S7). Proline which has a “salty taste” was only found in nectar samples but not in leaf extracts (Figure 7, and Supplemental Figure S7). In *Ptt* phenylalanine and glutamine dominated, while in *Ptr*, asparagine, histidine, and tyrosine 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 were sampled (inset Figure 8). 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%) down-regulated. Array data were validated by qPCR with a set of randomly selected and a set of further analyzed transcripts (see below and Supplemental Table S2). For an unbiased view on the impact of differential expression on nectar biology, gene functions were analyzed by using MapMan (Usadel et al., 2009) (Figure 8). Among the 27 different gene clusters found, nine (signaling, stress, transport, development, CHO-, 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- and TE-microscopy (Figure 2). Genes related to hormone action and metabolism, lipids (vesicle transport), as well as sugar metabolism and cell wall appeared up-regulated, in many cases to very high levels.

**Exocytosis associated genes (Table 1):** 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 prerequisite for
ongoing nectar flow. Secretory vesicles appeared thus to be very prominent in secretory cells from *Populus tremula x P. tremuloides* nectaries (Figure 2). Accordingly, in the full array data set we could identify at least 21 genes related to exocytosis (Table 1). Among them five were remarkably higher expressed in the nectaries (2 to 7.9-fold) and eight slightly induced (1.5 to 1.9-fold). In this population, seven nectary genes were linked to multivesicular body (MVP) 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 (4), RabE, and VHA-a1 class. We identified a number of genes which according to Zarsky et al. (2009) seemed to be involved in vesicle or TPC (TGN to plasma membrane carriers) formation. Among them three genes appeared remarkably up-regulated. The latter species of *Ptt* nectary expressed genes belong to the SEC14, Ala3, and Dynamin classes. SEC14 encodes a phosphatidylinositol transfer protein and Ala3 a flippase. Both are essential for vesicle budding from the Golgi complex (Sha et al., 1998; Litvak et al., 2005; Poulsen et al., 2008), while 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 *P. trichocarpa*. With this poplar species none of the genes appeared to be induced in nectaries (Table 2) supporting the hypothesis of different secretion mechanisms used by *Ptt* and *Ptr* extrafloral nectaries.

**Cell Wall (Table 3):** Among the cell wall cluster transcripts for esterases and lyases - enzymes involved in pectin metabolism - were found increased. Within the same category leucine-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-sugars metabolism point to increased carbohydrate synthesis for cell wall formation (Gibeaut and Carpita, 1994).

**Hormones/defense (Table 4):** Within the hormone cluster 6 genes appeared to be associated with auxin signaling. This group contained PIN genes that have been associated with auxin distribution, cell division, cell expansion, and polar growth
(Blilou et al., 2005; Petrasek et al., 2006). Linked to vascular differentiation three brassinosteroid metabolism genes were found up-regulated in nectaries. Within the hormone cluster two genes associated to ethylene-, two jasmonic acid-, and five to 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 Figure S8).

**Sugar metabolism and transport (Table 5):** Nectar production demands high amounts of sugars very likely provided by the phloem. Since *Ptt* nectar secreting cells are apoplastically separated from the parenchyma and phloem cells (Figure 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 analysed 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 induced (2 to 4 fold), but only two sucrose transporters of the suc3-type were present and not induced. In addition, a sucrose synthase (SUS3) was induced by a factor of five. Furthermore, the analysis of the released nectar blend revealed almost equal amounts of glucose, fructose, and sucrose (Supplemental Figure S5). From these data one might speculate that (i) sucrose released from the phloem is mainly cleaved by invertases within the extracellular space, and (ii) the secretory cells predominantly import hexose via the monosaccharide transporters, of which a part is re-converted to sucrose by SUS3. Interestingly, further sugar metabolism related enzymes like raffinose synthase, myo-inositol oxygenase, and α-glucosidase that 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 provided evidence that two tree species of the same genera might have evolved different mechanisms to control herbivory. *Populus tremula x P. tremuloides* and *Populus trichocarpa* 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 production of new nectaries on demand in *Ptr* is associated to short-term but massive holocrine nectar release. The nectars composition in both nectary types is rich in tastes that are known to attract different kinds of visitors including body guards.

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 depends on vascular supply of basic nectar compositions, very likely specified by factors produced as defense in the secretory cell on demand (Diaz-Castelazo et al., 2005).

*P. trichocarpa* nectaries are characterized by cells covered by a remarkably thick wall which 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 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 *Helleborous foetidus* (Ranunculaceae) (Kronestedt et al., 1986; Stpiczynska et al., 2004; Koteeva, 2005). Nectaries from *P. trichocarpa* following the release of large volumes of nectar, eventually died. The fact that i) short term secretion with *Ptr* nectaries results in cell death and this organ type ii) does not express granulocrine secretion-related transcripts point to a
self-destructing holocrine secretion. This mode of secretion they thus have in common with e.g. floral nectaries of *Helleborus foetidus* or *Digitalis purpurea* (Gaffal et al., 2007; Vesprini et al., 2008).

**Nectaries from** *P. tremula x P. tremuloides*, in contrast, developed just one layer of brush border-like large secretory cells (Figure 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 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 *Ptt* nectaries lipid metabolism gene clusters up-regulated (Figure 8) point to a granulocrine nectary type. In this context one should mention that transcriptome analysis of *Arabidopsis* 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 Stpiczynska, 2008). A similar situation exists in *Ptt* extrafloral nectaries. The lack of a cuticle and the distribution of membrane FM-dye would support nectar re-absorption via endocytosis of cells in the outer layer of the secretory organ. Here bulk secretion seems to be mediated by multivesicular bodies (Figure 2), components of the plant exocytosis system (Foresti et al., 2008; Foresti and Denecke, 2008; Robinson et al., 2008; Zarsky et al., 2009). Accordingly microarray studies with *Ptt* nectaries identified 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 few days. Another nectar release cycle therefore depends on the production of a new set of holocrine secreting nectaries. Our findings with the tree model *Populus* seem thus support the notion that *Populus tremula x P. tremuloides* and *Populus trichocarpa* operate two different nectary types and secretion mechanisms.
Appearance of nectaries and nectar

In wild cotton (*Gossypium thurberi*) size and density of extrafloral nectaries are heritable (Rudgers and Strauss, 2004). One report focusing on *Populus 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 findings that four year old *Ptt*-trees expressed nectaries on about 40% of the leaves are well in agreement with data that were found with four to five year old trees (Wooley et al., 2007). We can, however, not 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 seem to protect *Ptt*, as well as *Ptr* leaves from herbivore attack (Figure 3 and Supplemental Figure 4 online). Secondary nectar release from *Ptr* was observed in response to invasion of phloem-feeding insects. Poplar species are known to have several direct defense mechanisms against herbivores like 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 *P. trichocarpa* might thus be a strategy to avoid interference of the different defense systems. Recent studies indicated that continuous mechanical tissue damage is sufficient to trigger jasmonic acid (JA) production and subsequent volatile emission (Mithöfer et al., 2005). In addition to wounding, chemical elicitors present in insect saliva may play an important role in the extrafloral nectary response too (Radhika et al., 2010). Upon wounding *Ptr*, a typical JA or herbivory-linked VOC spectrum was emitted, but new nectaries or the release of nectar from existing nectaries was 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 may, however, be used directly by carnivorous arthropods as 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 extrafloral nectar of myrmecophyte acacia contains just glucose and fructose. It has been shown that addition of sucrose significantly changed the attractiveness of this nectar (Heil et al., 2005). Cell wall invertases are important determinants of sink strength and thus phloem sugar unloading and 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 10fold up-regulated. In agreement with the afore mentioned *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 fructose, glucose, and sucrose strengthens this theory. Nectar of *P. tremula* x *P. tremuloides* might thus be considered as tasty for generalists which appeared to be reflected by the diversity of visitors described for *Populus tremula* (Wooley et al., 2007). *P. trichocarpa* 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 function of nectary type, nectar composition, and bodyguards represents a future goal. Thereby the question will be addressed whether or not 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 extrafloral nectars from *Ptt*, and *Ptr* were rich in “sweet taste” amino acids like phenylalanine which is the most abundant amino acid in nectars of bee pollinated plants, and also known to serve as bee phagostimulant (Inouye and
Waller, 1984; Petanidou, 2007). Proline 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 proline in extrafloral nectar of both poplar species but not in leaf extracts. It is by far the most abundant amino acid in honeybee hemolymph, important for egg laying (Hrassnigg et al., 2003), and regulates the conversion of nectar into honey (Davies, 1978). The composition of Ptt nectar seemed therefore suitable to support honeybees live style. It has been shown that honeybees, parasitic flies (Tachinidae), parasitic wasps (Ichneumonidae), and bees are common visitors of all poplar nectaries (Supplemental Figure 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 might therefore be an effective strategy to reduce poplar leaf damage by herbivore infestation.

CONCLUSION

The morphology of Populus tremula x P. tremuloides nectaries revealed granulocrine secretion via e.g. multivesicular bodies, while Populus trichocarpa 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 case of special insect attack produces secondary nectar by extrafloral nectaries. The emerging P. trichocarpa nectar seems to attract rather specialist visitors, maybe depending on the respective herbivore. P. tremula x P. tremuloides
in contrast provides nectar for generalists according to the unspecific nectar production.
These different defense strategies require varying secretion systems which we found confirmed by transcript and metabolite compositions, as well as morphology and physiology of nectaries, and nectar of both poplar species.

MATERIALS AND METHODS

Plant material and growing conditions
Populus tremula × P. 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 light (22°C) : 8 h darkness (17°C); TLD 58 W/840 Super 80; Philips, and 58 W L58/77, Osram). Field grown trees were about 3 - 4 years old, 15 - 20 feet high, used for effectiveness tests, and the visitors determinations. All other experiments were performed using cultivated trees of about 4 - 5 feet height. These plants were watered twice a week and fertilized frequently.

Light microscopy
Nectaries from P. trichocarpa or P. tremula × P. tremuloides were harvested and fixed by passing through ascending grades of ethanol for 45 min each. Following dehydration, nectaries were embedded in 2-hydroxyethyl methacrylate (HEMA)/GMA (Agar Scientific) according to the manufacture’s advice. 20μm sections of extrafloral nectaries were cut with a c-profile 16cm knife (Leica) in a Leica RM2165 microtome (Leica), and heat-fixed to microscope slides. Specimens were stained with toluidine blue, then mounted in immersion oil under coverslips, and examined with a Keyence VHX-100k digital microscope (Keyence Corporation).
Nectaries appearance and effectiveness
Nectaries appearance and leaf damage were quantified using 11 randomly chosen branches (1257 leaves in total) of three different trees (Ptt) or 6 branches (327 leaves in total) of two different trees. Damage was classified as severe (>75% damage of the leaf surface), mild damage (<75%), and healthy (no damage).

Transmission electron microscopy (TEM)
Small sections of leaf tissue (1-2mm) were cut with a razor blade, and immediately immersed for 4 h in fixation medium containing 1% (w/v) formaldehyde, 1mM EGTA, 50mM cacodylat buffer, and 5% glutaraldehyde. Subsequently the tissue was post-fixed with 2% (w/v) osmium tetroxide overnight at room temperature, then stained with 3% (w/v) uranyl acetate in 20% ethanol for one hour, dehydrated in a graded series of ethanol, and embedded in Spurr’s epoxy resin (Spurr, 1969). Ultra-thin sections with a thickness of 70-80nm 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 80kV.

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 1ml, boiled for 5 min, and centrifuged (10 min at 14.000g). The supernatant was treated with 10mg per 100μl sample Serdolit MB1 (Serva) and the sugar concentration was measured using a PED (pulse electrochemical detector) Dionex 4500i (Dionex). Amino acid quantity and quality was measured using an amino acid analyzer Biochrom 20 Plus (Biochrom).

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

**Collection and analysis of volatiles**

The collection of volatiles emitted from leaves of the test plants damaged by caterpillars, MecWorm, or treated with chemical elicitors (jasmonic acid or coronalon) was achieved using charcoal traps. Elicitor treated or insect-damaged areas of the plants were enclosed in PET foil bags that were tightly closed at both ends to guarantee 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 (1.5mg, CLSA Filter, Gränicher & Quarto, Daumazan sur Arize, France) by air circulation maintained by a circulation pump. The trapped volatiles (24 h; 48 h or 72 h) were desorbed from the filters using 2 x 20μl dichloromethane containing 1-bromodecane (50μg ml⁻¹) as an internal standard. An aliquot (1μl) of the stock solution was analyzed on a Finnigan Trace GC/MS equipped with an EC-5 column (15m×0.25mm ×0.25μm, Alltech, Unterhaching, Germany). Helium at a flow rate of 1.5ml min⁻¹ served as carrier gas. The GC 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 30°C min⁻¹ to 280°C. Split ratio of the stock solution was 1:10, the resulting split flow 15 ml min⁻¹ (10 times the column gas flow of 1.5 ml min⁻¹). Authentic standards were used for identification of compounds.

**Induction of volatile biosynthesis with jasmonic acid or coronalon**

Leaves of the test plants were sprayed with aqueous solutions of jasmonic acid (1mM, and 0.5mM) or coronalon (0.1mM) until run-off. After drying (1 h) the pre-treated 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 *Spodoptera 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 333mm² of damaged area using 4 punches min⁻¹. Volatiles were collected as described. Additional experiments were conducted by combining continuous mechanical damage with 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 (Omega bio-tek), nectaries total RNA with the RNeasy MicroKit (Qiagen,) according to the manufactures’ protocols with minor modifications. The binding buffers were supplemented with 2% β-mercaptoethanol, 1% PVP, 70mM K-ethylxantogenate, and added to 30mg 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. 1μg 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 pre-amplify full-length cDNAs prior to in vitro transcription, an additional 10 cycle PCR (95°C for 30 sec, 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 was monitored using the “Experion
Automated Electrophoresis System” (BIO-RAD) with the Experion RNA Highsense Analysis Kit according to the manufacture’s protocol.

**Quantitative real time PCR (qPCR)**

To remove remaining DNA total RNA was treated with RNase-Free DNase (Fermentas) according to the manufacturer’s protocol. First-strand cDNA was prepared using 2.5µg RNA with the M-MLV-RT kit (Promega, Mannheim, Germany). First-strand cDNA was 20-fold diluted for RT-PCR. qPCR was performed in a Mastercycler® ep Realplex²S (Eppendorf) with the ABsolute QPCR SYBR Green Capillary Mix (ABgene, Hamburg, Germany) in a 20 µl reaction volume. After ‘hot start’ (15 min at 95°C) a standard PCR program was applied: 40times (15 seconds at 95°C, 15 sec at primer specific annealing temperature, 20 sec at 72°C), followed by a dissociation curve (10 sec 95°C, 60 - 95°C with an increment of 0.3°C/sec). Primers used (TIB MOLBIOL, Germany) have been designed for *Populus tremula x P. tremuloides* or *Populus trichocarpa* and were tested regarding optimal annealing temperature, specificity by dissociation curves, and gel electrophoresis (not shown) prior to real time PCR. Primers are listed in the supplement (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* actins 2 and 8 (for details see An et al., 1996; Szyroki et al., 2001). Each transcript was quantified using individual standards. To enable 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, Germany. Samples of leaves and nectaries from *P. tremula tremuloides* field-culture were analyzed. All samples were amplified using the “One-Cycle Target Labeling Assay” (Affymetrix) according to the manufacture’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 hybridation artifacts and proper grind alignment. Files of quality control were generated using the program “Expression Console” (Affymetrix).

**Biostatistic 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 has been performed using the variance stabilization method (vsnrma) (Huber et al., 2002) and probeset 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 has been 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 have been corrected for multiple testing by applying the false discovery rate from Benjamini and Hochberg (Benjamini and Hochberg, 2000).

The 500 most differentially expressed genes were analyzed by MapMan 3.1.1. The according Affymetrix probe set IDs were imported, 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 IDs was done by using BarleyBase/PLEXdb (Wise et al., 2007). Microarray data have been uploaded to GEO. Data are accessible using the following link:

SUPPLEMENTAL MATERIAL

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* nectaries density and effectiveness against herbivore attack.

**Supplemental Figure S5.** Sugar content in nectars of *Populus tremula x P. tremuloides* and *Populus trichocarpa*.

**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 *Ptt* nectaries versus leaves.

**Supplemental Table S2.** Validation of microarray data by qPCR.

**Supplemental Table S3.** Primers used in qPCR.

Supplemental Protocols

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Positioning of poplar extrafloral nectaries. In both species nectary pairs localize at the base of the leaf blade. (A) to (C) *Populus trichocarpa*, (D) to (F) *Populus tremula x P. tremuloides*. Leaf overview, arrowheads denote nectary position (A, D), leaf base enlargement, nectaries in detail (arrowheads) (B, E), *Ptr* release of large nectar amounts (C), large *Ptt* nectaries release small nectar amounts (F).

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 micro-channels marked by arrows. (C, D), overview of nectary parenchyma cells with some extent thick walls and symplastic connections (square) (E), plasmodesmata between cells (arrows), and numerous small vacuoles (F, left) and large nuclei (F, right). (G) to (L), *Ptt* nectary microscopy. (G), overview. (H), overview secretory cells. (I), plasmodesmata (arrows) between two secretory cells. (J, K), secreted vesicles and multivesicular bodies (arrows) occur within the epidermal cells and in the upper apoplastic space. Note partial loosening of the cell wall. (L), symplastic connections between the secretory cells and the basolateral neighbors are absent (black rectangles). cw= cell wall; cu= cuticle; NE= nectary epidermis; NP= nectary parenchyma; SNP= subnectary parenchyma; m= mitochondria; mvb= multivesicular bodies; ch= chloroplast; e= endoplasmic reticulum; v= vacuole.

Figure 3. Nectaries density and effectiveness against herbivore attack. (A), conserved percentage of *Ptt* leaves with and without nectaries. (B), both *Ptr* (bottom) and *Ptt* leaves (upper) emerging in spring presented nectaries (black arrows). (C), *Ptt* extrafloral nectaries effectiveness. Leaves with nectaries appeared less damaged by herbivores. Mean ± SE, n=11 branches (759 leaves).

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.
Figure 5. Induction of Ptr nectar release by sucking insects. (A), mechanical damage performed by a needle. (B), MecWorm set up for mimicking caterpillar caused mechanical leaf damage. (C), continuous leaf wounding by MecWorm. (D), Spodoptera exigua feeding on Ptr leaves. (E, F), mealy bugs on leaf blade with ongoing nectar secretion. (G), aphids on leaf bottom side. (H), topside of leaf shown in (G) with ongoing nectar secretion.

Figure 6. Volatiles emitted by Ptt after treatment with chemical elicitors and feeding larvae of Spodoptera littoralis. Control (crtl), n=11; jasmonic acid [1mM] (JA), n=13; coronalon [0,1mM] (Cor), n=9; Spodoptera littoralis (Slit), n=15; n-bromodecane as internal standard; C_{10}H_{14}, C_{10}H_{16}O are oxidation products of ocimene.

Figure 7. Most abundant amino acids in poplar extrafloral nectar. (A), Populus tremula x P. tremuloides and (B) Populus trichocarpa. Of the aromatic amino acids particularly the “sweet” phenylalanine and tryptophan were present in higher quantities. Proline was not detectable in leave extracts of both species. (Mean ± SD, n=4)

Figure 8. Clustering of Populus tremula x P. tremuloides differently expressed genes (extrafloral nectaries vs. leaves). The 500 highest differentially expressed genes were imported into MapMan 3.1.1 and classified accordingly. (inset) Ptt nectary, only the upper third (line) was used for RNA isolation. (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 the according category (blue squares = up-regulated, red squares = down-regulated genes). Note that genes in these clusters are almost all up-regulated.
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**Table 1.** Exocytosis associated genes in *Ptt* nectaries. a accession number of 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 IDs. b AGI code of the nearest Arabidopsis homolog
according to a. c Annotation according to b. d FC, fold change. e logFC, log2 of fold change. f adj. P-val., adjusted P-values. g type of protein, abbreviations according to Zarsky et al., (2009). h class of proteins; TGN, trans-Golgi network; MVB, multivesicular body; RE, recycling endosome; VF, vesicle formation.
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<td>SEC14</td>
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**Table 2.** qPCR-monitoring of exocytosis associated transcripts in *Ptr* nectaries. Comparison with Microarray data from Ptt (Table 1). ① type of protein, abbreviations according to Zarsky et al., (2009). ② class of proteins; TGN, trans-Golgi network; MVB, multivesicular body; RE, recycling endosome; VF, vesicle formation. ③ FC, fold change, microarray data *Ptt*. ④ FC, fold change, quantitative real time PCR *Ptr*.
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<th>adj.P-val.</th>
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<td>At1g69530</td>
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**Table 3.** Differentially expressed cell wall metabolism associated genes in *Ptt* nectaries. a accession number of 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 IDs. b AGI code of the nearest Arabidopsis homolog according to a. c Annotation according to b. d FC, fold change. e logFC, log2 of fold change. f adj. P-val., adjusted P-values.
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<th>Annotation</th>
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<th>logFC</th>
<th>adj.P-val.</th>
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<td>aux</td>
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**Table 4.** Differentially expressed hormone metabolism associated genes in *Ptt* nectaries. a accession number of corresponding sequence at (www.ncbi.nlm.nih.gov/guide/) received from
with Affymetrix Probe Set IDs. \(^b\) AGI code of the nearest Arabidopsis homolog according to \(^a\). \(^c\) Annotation according to \(^b\). \(^d\) FC, fold change. \(^e\) logFC, log2 of fold change. \(^f\) adj. P-val., adjusted P-values. \(^g\) related to the hormone auxin (aux), brassinosteroids (bra), ethylene (eth), jasmonic acid (JA), salicylic acid (SA).
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**Table 5.** Sugar metabolism and transport associated genes in *Ptt* nectaries. 

- **AGI code** of the nearest Arabidopsis homolog according to a. 
- **Annotation** according to b. 
- **FC**, fold change. 
- **logFC**, log2 of fold change. 
- **adj.P-val.**, adjusted P-values.
ORIGINAL FIGURE FILES

Figure 1: Figure 1 PP
Figure 2: Figure 2 PP
Figure 3: Figure 3 PP
Figure 4: Figure 4 PP
Figure 5: Figure 5 PP
Figure 6: Figure 6 PP
Figure 7: Figure 7 PP
Figure 8: Figure 8 PP

SUPPLEMENTAL DATA FILES

PP_Revision_196014 supplemental_material
A pie chart showing that 38% of leaves have nectaries and 62% do not. Images in B show different levels of damage to the leaves, with arrows indicating nectary presence or absence.

C shows a bar chart comparing the percentage of total leaves with nectaries to those without across different conditions: healthy leaves, mild damage, and severe damage. The chart indicates that the presence of nectaries decreases as the level of damage increases.