Short title: Biosynthesis of sulfated salicinoids in poplar

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The occurrence of sulfated salicinoids in poplar and their formation by sulfotransferase 1

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One sentence summary

Sulfated salicinoids in poplar are produced by sulfotransferase 1 and may function in sulfur homeostasis and plant defense.

Author’s contributions
TGK, NDL, CPC, RLL, SBU, and JG designed the research. NDL, AM, TDUK, MR, AS, CPC, and KL carried out the experimental work. NDL, MR, AS, YN, CP, and TGK analyzed data. NDL and TGK wrote the manuscript. All authors read and approved the final manuscript.

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Abstract

Salicinoids form a specific class of phenolic glycosides characteristic of the Salicaceae. Although salicinoids accumulate in large amounts and have been shown to be involved in plant defense, their biosynthesis is unclear. We identified two sulfated salicinoids, salicin-7-sulfate and salirepin-7-sulfate, in black cottonwood (Populus trichocarpa). Both compounds accumulated in high amounts in above-ground tissues including leaves, petioles, and stems, but were also found at lower concentrations in roots. A survey of salicin-7-sulfate and salirepin-7-sulfate in a subset of poplar and willow (Salix sp.) species revealed a broader distribution within the Salicaceae. To elucidate the formation of these compounds, we studied the sulfotransferase (PtSOT) gene family in P. trichocarpa. One of the identified genes, PtSOT1, was shown to encode an enzyme able to convert salicin and salirepin into salicin-7-sulfate and salirepin-7-sulfate, respectively. The expression of PtSOT1 in different organs of P. trichocarpa matched the accumulation of sulfated salicinoids in planta. Moreover, RNA interference-mediated knockdown of SOT1 in gray poplar (P. x canescens) resulted in decreased levels of sulfated salicinoids in comparison to wild-type plants, indicating that SOT1 is responsible for their formation in planta. The presence of a non-functional SOT1 allele in black poplar (P. nigra) was shown to correlate with the absence of salicin-7-sulfate and salirepin-7-sulfate in this species. Food choice experiments with leaves from wild-type and SOT1 knockdown trees suggest that sulfated salicinoids do not affect the feeding preference of the generalist caterpillar Lymantria dispar. A potential role of the sulfated salicinoids in sulfur storage and homeostasis is discussed.

Key words: sulfotransferase, phenolic glycosides, salicinoids, sulfation, Populus trichocarpa, salicin-7-sulfate, salirepin-7-sulfate, Lymantria dispar, Salicaceae
Introduction

In response to biotic and abiotic stresses, plants produce a multitude of specialized metabolites, with more than 200,000 structures known to date (Pichersky and Lewinsohn, 2011). The high number and the structural diversity of specialized metabolites are mainly achieved by modifications of a limited number of core structures. Such modifications include glycosylation, methylation, acetylation, or sulfation (e.g. Varin et al., 1992; Saito et al., 2013). Many specialized metabolites such as terpenes, flavonoids, or cyanogenic glycosides are widely distributed through the plant kingdom; others, however, are restricted to single plant orders or families. Benzoxazinoids, for example, are mainly restricted to the Poaceae, while glucosinolates are exclusively formed in the Brassicales (Halkier and Gershenzon, 2006; Niemeyer, 2009). Salicinoids represent another example of family-specific plant specialized metabolites, being restricted to the Salicaceae (Böckler et al., 2011; Philippe and Bohlmann, 2007; Chen et al., 2009).

Like other specialized metabolites, salicinoids are defined by their chemical core structure (Böckler et al., 2011). The basic structural element is salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside), and derivatives of this core structure are referred to as complex salicinoids (Böckler et al., 2011). Common modifications of the core structure are mostly esterifications with organic acids at the primary alcohol function of the 2-hydroxybenzyl alcohol, or at the 2’ or 6’ position of the glucopyranose (Böckler et al., 2011). Thus far, more than 20 different salicinoids have been described in various poplars (Populus sp.), willows (Salix sp.), and a few other related species (Böckler et al., 2011). Some salicinoids, for instance salicin and salicortin, are widely distributed in the Salicaceae, whereas others show a more species-specific accumulation, for example idescarpin in the Japanese orange cherry (Idesia polycarpa) (Picard et al., 1994; Chou et al., 1997; Feistel et al., 2017).

Salicinoids have mainly been investigated because of their function as defense compounds against various herbivores (Lindroth and St. Clair, 2013). Several mammalian herbivores, e.g. mountain hares (Lepus timidus), field voles (Microtus
agrestis), and elk (Cervus elaphus) show a selective feeding behavior and prefer to feed on low salicinoid-containing plants over salicinoid-rich plants (Tahvanainen et al., 1985a; Bailey et al., 2006; Heiska et al., 2007). Salicinoids also function as efficient feeding deterrents and fitness reducers for generalist insects, including lepidopteran herbivores (e.g. Lindroth and Peterson, 1988; Lindroth et al., 1988; Feistel et al., 2017) and coleopteran herbivores (e.g. Tahvanainen et al., 1985b; Kelly and Curry, 1991). In contrast to induced defenses that are exclusively expressed rapidly upon herbivory, salicinoids are mainly considered as constitutive defense (Böckler et al., 2013; Rubert-Nason et al., 2015), although some studies reported moderately increased accumulation levels after herbivory (e.g. Clausen et al., 1989; Stevens and Lindroth, 2005; Young et al., 2010; Rubert-Nason et al., 2015; Fabisch et al., 2019). Whether such increased accumulation is caused by de novo biosynthesis or by translocation of preformed salicinoids is still unclear (Böckler et al., 2011; Massad et al., 2014).

Although salicinoids have been known and investigated for a long time, their biosynthesis remains elusive. Inhibition of the phenylalanine ammonia lyase in bay willow (Salix pentandra) shoot tips led to reduced accumulation of salicinoids (Ruuhola and Julkunen-Tiitto, 2003). Feeding experiments with isotopically-labelled precursors in purple willow (S. purpurea) (Zenk, 1967) and black poplar (Populus nigra) (Babst et al., 2010) revealed that salicinoids, as other C₆-C₁ benzenoids, are derived from cinnamic acid. Chain-shortening, ortho-hydroxylation, reduction, glucosylation, and esterifications have been proposed as reaction steps in the salicinoid pathway, however, their exact sequence and the involved enzymes are still not clear (Babst et al., 2010; Tsai et al., 2006). Recently, a poplar acyltransferase has been reported that catalyzes the formation of benzyl benzoate (Chedgy et al., 2015), a compound hypothesized as a key intermediate in the formation of complex salicinoids such as salicortin or tremulacin (Babst et al., 2010).

The recent identification of two sulfated salicinoids, salicin-7-sulfate in willows (Noleto-Dias et al., 2018) and idesin hydrogen sulfate in I. polycarpa (Chou et al., 1997), indicates that salicinoids can act as substrates for sulfotransferases (SOT; EC 2.8.2). In general, sulfotransferases catalyze the transfer of a sulfuryl group from the cofactor 3′-
phosphoadenosine 5’-phosphosulfate (PAPS) to hydroxyl groups of a variety of different substrates (Klein and Papenbrock, 2004; Hirschmann et al., 2014). In plants, sulfotransferases form mid-sized gene families with e.g. 35 members in rice (Oryza sativa) (Chen et al., 2012) and 21 members in Arabidopsis (Arabidopsis thaliana) (Klein and Papenbrock, 2004; Hirschmann et al., 2014). Sequence comparisons of sulfotransferases revealed common sequence features at the primary sequence level, determining four distinct regions, I to IV (Marsolais and Varin, 1995; Klein and Papenbrock, 2004; Hirschmann et al., 2014). The regions I, II, and IV are proposed to be involved in the binding of the cofactor PAPS, while the function of region III has not been completely elucidated (Kakuta et al., 1998; Hirschmann et al., 2014). However, the prediction of sulfotransferase substrate specificity based on the primary sequence is still limited (Klein and Papenbrock, 2004; Luczak et al., 2013; Hirschmann et al., 2014). So far, only a few plant sulfotransferases have been characterized in detail. Arabidopsis AtSOT10, for example, catalyzes the sulfation of brassinosteroids, AtSOT15 accepts 11- and 12-hydroxyjasmonate as substrate, and AtSOT12 and AtSOT16/17/18 produce sulfated salicylic acid and glucosinolates, respectively (Gidda et al., 2003; Piotrowski et al., 2004; Hirai et al., 2005; Marsolais et al., 2007; Baek et al., 2010). Sulfated metabolites are known to have various functions, being involved in plant defense and developmental processes, or acting as sulfur storage compounds (e.g. Faulkner and Rubery, 1992; Falk et al., 2007; Wang et al., 2014b). However, whether and how sulfation alters the impact of salicinoids as defense compounds is unclear. Moreover, SOTs that produce sulfated salicinoids in the Salicaceae have not been described. The aim of our study was (I) to investigate the occurrence of sulfated salicinoids in the Salicaceae family, and (II) to elucidate the biochemical and genetic mechanisms underlying the formation of sulfated salicinoids in black cottonwood (P. trichocarpa). Untargeted and targeted liquid chromatography/mass spectrometry enabled us to identify and quantify sulfated salicinoids in different Salicaceae species and plant tissues. Using phylogenetic analysis and RNA sequencing (RNAseq) data, we identified sulfotransferase genes in P. trichocarpa. Heterologous expression, biochemical characterization, and RNA interference (RNAi)-mediated knockdown of PtSOT1
revealed that the encoded enzyme catalyzes the sulfation of salicinoids in vitro and in vivo. To elucidate a potential function of sulfated salicinoids in plant defense, we performed feeding preference assays with gypsy moth (Lymantria dispar) caterpillars, a generalist insect herbivore known to feed on poplar trees. Moreover, we measured sulfur and sulfate in P. trichocarpa leaves and calculated the amounts of total sulfur and sulfate tied in to assess the role of salicinoids as sulfur-storage compounds.

Results

Identification of salicin-7-sulfate and salirepin-7-sulfate in poplar

In our attempt to study the formation of salicinoids in the Salicaceae, we searched for possible intermediates in methanol (MeOH) extracts made from leaves of P. trichocarpa, gray poplar (P. x canescens), and the basket willow (S. viminalis) using liquid chromatography/mass spectrometry (LC-MS, Supplemental Fig. S1). Both poplar species produce a wide array of salicinoids when compared with S. viminalis, which was reported to be poor in salicinoids (Palo, 1984). One major peak in the chromatograms of P. trichocarpa and P. x canescens that was absent in S. viminalis, represented a compound with a molecular weight of 366 (Supplemental Fig. S1). The accurate mass of the unknown compound (measured as 365.0543, [M-H]– fragment) suggested two possible molecular formulae: C_{13}H_{20}O_{8}P_{2} (calculated [M-H]–, 365.0561, Δ = 4.7 ppm) and C_{13}H_{18}O_{10}S (calculated [M-H]–, 365.0548, Δ = 1.2 ppm). However, the abundant [M-H+2]– isotopic mass peak of [M-H]– (6.7%, Supplemental Fig. S2) indicated a sulfur-containing structure. The compound was purified from P. x canescens leaves and identified by nuclear magnetic resonance (NMR) spectroscopy as salicin-7-sulfate (Supplemental Fig. S3), which was recently reported in willows (Noleto-Dias et al., 2018).

In order to screen for further sulfated salicinoids, we performed a precursor ion scan experiment with the sulfate (m/z 97) as the target fragment using a MeOH extract made from P. trichocarpa leaves. Besides salicin-7-sulfate, two intense peaks appeared in the chromatogram (Fig. 1). One of the compounds had a molecular weight of 306 and was...
identified as 12-hydroxyjasmonic acid sulfate by comparison with an authentic standard kindly provided by C. Wasternack (Leibnitz Institute of Plant Biochemistry, Halle/S, Germany). The other compound had a molecular weight of 382 and was thus hypothesized to be a hydroxylated derivative of salicin-7-sulfate. The accurate mass of this compound was \( m/z \) 381.0497 \([\text{M-H}]^-\) with a proposed sum formula of \( \text{C}_{13}\text{H}_{18}\text{O}_{11}\text{S} \) (calculated \([\text{M-H}]^-\), \( m/z \) 381.0497, \( \Delta = 0.01 \) ppm). After purification of the compound from \( P. x \) canescens leaves, the structure was determined by NMR spectroscopy to be salirepin-7-sulfate, a previously undescribed compound (Supplemental Fig. S3).

Salicin-7-sulfate and salirepin-7-sulfate are broadly distributed within the Salicaceae and mainly accumulate in above-ground organs

To study the distribution of sulfated salicinoids within the Salicaceae, we measured and quantified salicin-7-sulfate and salirepin-7-sulfate in leaves and roots of five poplar and three willow species. Both compounds were detected in MeOH extracts made from leaves of \( P. \) trichocarpa, \( P. x \) canescens, silver poplar (\( P. \) alba), Simon's poplar (\( P. \) simonii), and \( S. \) purpurea (Fig. 2, Supplemental Table S1). Leaves of the goat willow (\( S. \) caprea) contained only salicin-7-sulfate and no salirepin-7-sulfate (Fig. 2, Supplemental Table S1). Common to all species able to produce sulfated salicinoids was a significantly higher concentration of these compounds in leaves in comparison to roots (Fig. 2, Supplemental Table S1). In \( S. \) purpurea, the accumulation of salirepin-7-sulfate was detectable in leaves but not in roots (Fig. 2, Supplemental Table S1). \( P. \) nigra and \( S. \) viminalis; however, showed no accumulation of sulfated salicinoids, either in leaves or in roots (Fig. 2). Further analysis of four additional \( P. \) trichocarpa genotypes confirmed the consistent occurrence of sulfated salicinoids in this species (Supplemental Table S2). Moreover, salicin-7-sulfate could be detected in leaves of fifty trembling aspen (\( P. \) tremuloides) genotypes (Supplemental Fig. S4) and in the bark of \( I. \) polycarpa (Supplemental Table S2), a species from the Flacourtiaceae (or Salicaceae sensu lato) that, together with the genus Bennettiodendron, forms the sister group to Populus-Salix (Liu et al., 2016). The presence of salicin-7-sulfate and salirepin-7-sulfate in \( I. \) polycarpa suggests that the formation of these compounds evolved early in the
evolution of the Salicaceae or even in a common ancestor of the Salicaceae and Flacourtiaceae.

A more detailed survey including leaf blades, petioles, stems, and roots was performed to determine the organ-specific distribution of salicin-7-sulfate and salirepin-7-sulfate in the two poplar species *P. trichocarpa* and *P. nigra*. In *P. trichocarpa*, highest accumulation of the two sulfated salicinoids was found in leaves, followed by petioles and stems, while roots accumulated only low amounts of these compounds (Fig. 3, Supplemental Table S3). The potential biosynthetic precursors of salicin-7-sulfate and salirepin-7-sulfate, salicin and salirepin, respectively, showed a similar pattern with highest accumulation in petioles, less abundant accumulation in leaves and stems, and lowest accumulation in roots of *P. trichocarpa* (Fig. 3, Supplemental Table S3). Although salicin and salirepin were found in all organs of *P. nigra* investigated, sulfated salicinoids could not be detected in any of them (Fig. 3, Supplemental Table S3).

**The accumulation of salicin-7-sulfate and salirepin-7-sulfate is not influenced by leaf herbivory of the generalist feeding caterpillar *Lymantria dispar***

Similar to non-sulfated salicinoids, salicin-7-sulfate and salirepin-7-sulfate might act as defense compounds against insect herbivores. To test whether their formation is influenced by insect herbivory, we analyzed the amounts of sulfated and non-sulfated salicinoids in undamaged *P. trichocarpa* leaves and leaves that had been fed upon by *L. dispar* caterpillars for nearly 24 h. LC-MS/MS analysis revealed that the herbivore treatment did not affect the accumulation of salicin-7-sulfate and salirepin-7-sulfate (Fig. 4). Furthermore, salirepin and the complex salicinoids salicortin, tremulacin, 6'-O-benzoylsalicortin, and homaloside D were not influenced by the herbivore treatment (Fig. 4, Supplemental Table S4). However, the accumulation of salicin was significantly increased in response to herbivory (Fig. 4).

**P. trichocarpa** sulfotransferase 1 (PtSOT1) catalyzes the formation of salicin-7-sulfate and salirepin-7-sulfate in vitro
In order to identify sulfotransferase genes responsible for the formation of salicin-7-sulfate and salirepin-7-sulfate, a BLAST search was performed with the previously characterized salicylic acid sulfotransferase AtSOT12 from Arabidopsis (Baek et al., 2010) as query and the *P. trichocarpa* genome as template. This analysis revealed 28 putative poplar sulfotransferase gene candidates that grouped together with sulfotransferase genes from other plants in a phylogenetic tree (Fig. 5). Since the sulfated salicinoids accumulated mostly in above-ground tissues of *P. trichocarpa* (Fig. 2; Fig. 3), we hypothesized that the responsible biosynthetic genes are highly expressed in leaves. The analysis of the identified sulfotransferase genes in a transcriptome made from *P. trichocarpa* leaves (Günther et al., 2019) revealed two candidates (*Potri.012G032700* and *Potri.003G189100*) with high expression in leaves (Supplemental Fig. S5). An amino acid alignment of *Potri.012G032700*, *Potri.003G189100*, and characterized sulfotransferases from rice and Arabidopsis confirmed that the poplar candidate enzymes contained the conserved regions important for the catalytic activity of sulfotransferases (Marsolais and Varin, 1995; Klein and Papenbrock, 2004; Hirschmann et al., 2014; Supplemental Fig. S6). For biochemical characterization of *Potri.012G032700* and *Potri.003G189100*, we heterologously expressed the full-length genes in *Escherichia coli* and performed enzyme assays with affinity-purified recombinant N-terminal His-tag fusion proteins, the potential substrates salicin or salirepin, and the cosubstrate PAPS. LC-MS/MS analysis of enzyme assays revealed that *Potri.012G032700* converted salicin and salirepin into salicin-7-sulfate and salirepin-7-sulfate, respectively, in vitro (Fig. 6). In contrast, recombinant *Potri.003G189100* showed no enzymatic activity with the tested substrates (Fig. 6). Interestingly, salicyl alcohol, the aglycone of salicin, was not accepted as substrate, neither by *Potri.012G032700* nor by *Potri.003G189100* (Supplemental Fig. S7). Based on its in vitro activity, *Potri.012G032700* was designated as *P. trichocarpa* sulfotransferase 1 (*PtSOT1*). Since *Potri.003G189100* showed no activity with the tested substrates (Fig. 6; Supplemental Fig. S7), the gene was not further considered in this study.
The expression of *PtSOT1* regulates the formation of sulfated salicinoids in *P. trichocarpa*
Reverse transcription quantitative PCR (RT-qPCR) was used to measure the transcript accumulation of *PtSOT1* in different tissues of *P. trichocarpa*. *PtSOT1* was highly expressed in leaf blades and petioles, but showed less expression in stems, and only trace expression in roots (Fig. 7). This expression pattern matched the accumulation of salicin-7-sulfate and salirepin-7-sulfate in the respective tissues of *P. trichocarpa* (Fig. 3), suggesting that the formation of the two compounds is mainly regulated by *PtSOT1* transcript levels.

The absence of sulfated salicinoids in *P. nigra* is caused by a loss-of-function allele of *SOT1*
In contrast to *P. trichocarpa*, *P. nigra* was found to contain no sulfated salicinoids, although the respective precursors salicin and salirepin could be readily detected in this species (Fig. 2; Fig. 3). To confirm this observation, we tested nineteen additional *P. nigra* genotypes and all showed no detectable accumulation of salicin-7-sulfate or salirepin-7-sulfate (Supplemental Fig. S8, Supplemental Table S5). Amplification of *PnSOT1* from genomic DNA isolated from two different *P. nigra* genotypes revealed a frame shift mutation in both alleles, leading to a non-functional PnSOT1 in *P. nigra* (Supplemental Fig. S9). Moreover, we could not detect any *PnSOT1* transcript accumulation in leaves, petioles, stems, and roots of *P. nigra* plants (Supplemental Table S6), suggesting that the mutated gene is not expressed in this species.

Downregulation of *SOT1* by RNAi in *P. x canescens* led to reduced formation of sulfated salicinoids
To study the role of *SOT1* in the formation of sulfated salicinoids in more detail, we used RNAi to downregulate its expression in *P. x canescens*, a species usually used for *Agrobacterium*-mediated poplar transformation (Leple et al., 1992). Two independent transgenic *SOT1* knockdown lines (RNAi-1 and RNAi-2) were generated and compared to wild-type (WT) trees and trees carrying an empty vector (EV). Transcript expression
analysis of SOT1 by RT-qPCR showed that the gene was significantly less expressed in leaves of the knockdown lines in comparison to WT and EV trees (Fig. 8). LC-MS/MS analysis of leaf methanol extracts made from 6-week-old trees revealed a significantly reduced concentration of salicin-7-sulfate and salirepin-7-sulfate in both transgenic SOT1 lines in comparison to WT and EV plants (Fig. 8). Along with the sulfated salicinoids, we measured the accumulation of their potential biosynthetic precursors salicin and salirepin. Whereas the salicin concentration was significantly increased in the SOT1 knockdown lines compared to WT and EV leaves, the concentration of salirepin was not influenced. Moreover, the accumulation of the complex salicinoids salicortin and tremulacin was not affected by the downregulation of SOT1 (Fig. 8). The analysis of salicin-7-sulfate and salirepin-7-sulfate in leaves of 9- and 12-week-old trees showed a pattern comparable to 6-week-old trees (Supplemental Fig. S10), indicating that the observed phenotype was stable for at least six weeks.

The reduced accumulation of salicin-7-sulfate and salirepin-7-sulfate in SOT1 RNAi plants did not influence the feeding preference of the generalist herbivore L. dispar

Salicinoids are effective feeding deterrents against various herbivores (e.g. Lindroth and Peterson, 1988; Lindroth et al., 1988). Since sulfation is known to alter the physical and biological properties of plant metabolites (e.g. Gidda et al., 2003; Wang et al., 2014b), the question arose whether sulfated salicinoids also act as feeding deterrents. To address this question, we performed feeding preference assays, offering L. dispar caterpillars leaf discs from P. x canescens WT and SOT1 knockdown lines. By analyzing the consumed leaf area, we could show that the caterpillars had no feeding preference for either the WT or the SOT1 knockdown line (Fig. 9).

As shown above, a side effect of the RNAi-mediated knockdown of SOT1 was a significantly increased accumulation of the salicin-7-sulfate precursor salicin (Fig. 8). To exclude the possibility that the increased salicin concentration compensated for a possible decrease in deterrence caused by reduced amounts of salicin-7-sulfate, we performed choice assays with artificially applied salicin. By applying increasing amounts
of salicin (from 0.1 mg/g fresh weight to 1.5 mg/g fresh weight) to *P. x canescens* WT leaf discs, we could show that an application of 1.5 mg/g fresh weight is required to shift the feeding preference of the caterpillars to control leaf discs (Supplemental Fig. S11). This amount corresponds to an increase of approximately 5 mg/g dry weight of salicin. Since the difference in salicin accumulation between WT and *SOT1* knockdown lines was smaller (~2.5 mg/g dry weight, Fig. 8), we assume that the altered accumulation of salicin did not influence the behavior of the caterpillars in our feeding preference assays.

**Salicin-7-sulfate and salirepin-7-sulfate represent a substantially sulfur pool in *P. trichocarpa* leaves**

Sulfur is an essential macronutrient, which is required for the synthesis of amino acids such as cysteine and methionine, but also for the formation of essential prosthetic groups (e.g. Takahashi et al., 2011). To gain insights into a potential role of sulfated salicinoids in sulfur homeostasis in poplar, we analyzed the amounts of total sulfur and free and hydrolysable sulfate in *P. trichocarpa* leaves. An elemental analysis of freeze-dried poplar leaf material revealed a concentration of 0.68% total sulfur, and sulfate measurements showed that 73.51% of the total sulfur was in the form of sulfate (0.50% of leaf dry weight). Considering the concentrations of salicin-7-sulfate (2.59 mg/g dry weight) and salirepin-7-sulfate (1.74 mg/g dry weight) (Fig. 3) analyzed in the same tissue, we calculated that 5.47% of the total sulfur and 7.47% of free and hydrolysable sulfate are tied up in sulfated salicinoids.

**Discussion**

Salicinoids are a group of highly abundant specialized metabolites in the Salicaceae (Böckler et al., 2011), investigated mainly for their function as defense compounds. However, little is known about their biosynthesis. As part of a search for intermediates of salicinoid biosynthesis in poplar, we encountered two sulfated salicinoids, the previously reported salicin-7-sulfate (Noleto-Dias et al., 2018) and salirepin-7-sulfate,
described here. Sulfation is mediated by sulfotransferases, a group of enzymes catalyzing the transfer of a sulfuryl group from PAPS to an acceptor molecule (Klein and Papenbrock, 2004; Hirschmann et al., 2014; Hirschmann et al., 2017). Thus, we hypothesized that sulfotransferase enzymes might contribute to the biosynthesis of sulfated salicinoids and aimed to identify and biochemically characterize the responsible proteins in poplar.

Salicin-7-sulfate and salirepin-7-sulfate are two widespread salicinoids in poplar

The structural diversity of specialized metabolites is mainly driven by modifications of common core structures by processes such as glycosylation, methylation, acetylation, or sulfation (e.g. Varin et al., 1992; Saito et al., 2013). Sulfated specialized metabolites are known to be involved in plant defense and plant development (e.g. Faulkner and Rubery, 1992; Wang et al., 2014b). A sulfated metabolite, salicin-7-sulfate, was recently described to occur in different willow species (Noleto-Dias et al., 2018) and was here isolated from various poplars (Fig. 2). Moreover, by performing LC-MS precursor ion scan analyses, we identified two further sulfated compounds in poplar, salirepin-7-sulfate (Fig. 1; Supplemental Fig. S3) and the jasmonic acid derivative 12-hydroxy jasmonic acid sulfate, a compound known from many other plant species (e.g. Gidda et al., 2003; Miersch et al., 2008). The sulfated salicinoids were found to occur in a range of poplar organs and species, and to be present in substantial amounts, sometimes at concentrations similar to those of their non-sulfated counterparts (Fig. 2; Fig. 3). Since precursor ion scan analyses rely on a specific fragmentation pattern, we cannot rule out the presence of other sulfated compounds in poplar. Sulfated flavonoids, for instance, are commonly distributed within the plant kingdom (Varin and Ibrahim, 1989; Varin et al., 1992; Falcone Ferreyra et al., 2012) and thus may also occur in poplar and willow species. Very recently, a novel method that uses neutral loss scan mass spectrometry for the identification of sulfated flavonoids in plant extracts has been described by Kleinenkuhnen et al. (2019). Applying this method to poplar and willow extracts might be a worthwhile approach for further discovery of sulfated compounds in the Salicaceae.
A functional SOT1 allele mediates the formation of salicin-7-sulfate and salirepin-7-sulfate in poplar

Sulfotransferases have been described in a number of plant species including Arabidopsis, rice, and clasping yellowtops (Flaveria chloraeifolia), where they are involved in the sulfation of desulfoglucosinolates, salicylic acid, and flavonoids, respectively (Varin et al., 1992; Piotrowski et al., 2004; Wang et al., 2014b). The genome of P. trichocarpa possesses 28 putative sulfotransferase genes (Fig. 5) that form a typical mid-sized gene family as described for other species (Klein and Papenbrock, 2004; Chen et al., 2012; Hirschmann et al., 2014). Phylogenetic and transcriptome analysis led to the identification of two putative poplar SOT candidate genes mainly expressed in leaves, the organs with the highest amounts of sulfated salicinoids (Fig. 2; Fig. 5; Supplemental Fig. S5). Biochemical characterization revealed that one of the candidates, PtSOT1, catalyzes the sulfation of salicin and salirepin in vitro using PAPS as the cofactor (Fig. 6). Notably, PtSOT1 did not accept the aglycone salicyl alcohol as substrate, indicating that sulfation occurs after glucosylation as the last step in the biosynthesis of sulfated salicinoids (Supplemental Fig. S7). The RNAi-mediated knockdown of SOT1 in P. x canescens revealed that SOT1 is responsible for the formation of salicin-7-sulfate and salirepin-7-sulfate in planta (Fig. 8; Supplemental Fig. S10). Moreover, the presence of a non-functional SOT1 allele in P. nigra, a species not able to produce sulfated salicinoids (Fig. 2; Fig. 3; Supplemental Fig. S8; Supplemental Fig. S9; Supplemental Table S5), suggests that the SOT1 locus is likely responsible for the formation of salicin-7-sulfate and salirepin-7-sulfate in other poplar and willow species.

Beside salicin-7-sulfate and salirepin-7-sulfate, P. trichocarpa accumulates significant amounts of 12-hydroxy jasmonic acid sulfate in leaves (Fig. 1). Potri.003G189100, the enzyme inactive with salicin and salirepin (Fig. 6), was found to be highly expressed in leaves (Supplemental Fig. S5); however, it is only distantly related to the Arabidopsis 12-hydroxy jasmonic acid sulfotransferase At2G14920.1 (Fig. 5) and thus most likely not involved in the formation of 12-hydroxy jasmonic acid sulfate. Phylogenetic analysis
revealed a small subgroup of four putative poplar sulfotransferase genes that cluster together with At2G14920.1 (Fig. 5). Thus, these genes might encode 12-hydroxy jasmonic acid sulfotransferases in poplar. Future research is needed to elucidate the involvement of specific sulfotransferases in the formation of 12-hydroxy jasmonic acid sulfate.

Sulfated salicinoids do not act as strong feeding deterrents against the generalist herbivore *Lymantria dispar*

Salicinoids are known as effective defense compounds acting against various folivorous insects (e.g. Lindroth et al., 1988; Hemming and Lindroth, 1995; Feistel et al., 2017). To test if sulfated salicinoids act as feeding deterrents, we performed preference assays with leaf discs of *P. x canescens* WT and *SOT1* knockdown lines offered to *L. dispar* caterpillars. However, we could not observe a feeding preference for reduced sulfated salicinoid accumulation (Fig. 9). Since downregulation of *SOT1* led to an increased salicin concentration (Fig. 8), the deterrence of this compound might obscure a feeding preference for low levels of sulfated salicinoids. However, preference assays with artificially applied salicin revealed that about 5 mg/g dry weight of salicin are required to shift the feeding preference of the caterpillars towards control leaf discs. This amount is above the observed salicin increase in the *SOT1* knockdown lines of around 2.5 mg/g dry weight and even above the overall salicin concentration in the plant tissue itself (Fig. 3; Fig. 8). Thus, it seems unlikely that the increased amount of salicin masked the effect of decreased sulfated salicinoids in the *SOT1* knockdown plants. Altogether, our results imply that sulfated salicinoids and salicin itself are not strong feeding deterrents against *L. dispar* caterpillars.

In general, the available data on the effectiveness of salicin, the structurally simplest salicinoid, as a defense compound are contradictory. For example, for the tiger swallowtail (*Papilio glaucus glaucus*) and the southern armyworm (*Spodoptera eridania*) it was shown that salicin had only marginal effects on growth rate and developmental time, whereas the two complex salicinoids salicortin and tremulacin had a significant negative impact (Lindroth and Peterson, 1988; Lindroth et al., 1988). However, artificial
application of salicin let to a significantly decreased weight gain in performance assays with *L. dispar* caterpillars (Böckler et al., 2016). Similar to salicin, it might be possible that sulfated salicinoids have an impact on caterpillar development, although *L. dispar* caterpillars had no pronounced feeding preference towards decreased levels of sulfated salicinoids (Fig. 9). Besides generalist feeders, salicin-7-sulfate and salirepin-7-sulfate may also influence the behavior of specialized feeding herbivorous insects. Larvae of the red poplar leaf beetle (*Chrysomela populi*), for instance, are able to sequestrate and metabolize salicin to salicylaldehyde and use this compound as a protectant against predators and microbial infection (Pasteels et al., 1983; Gross et al., 2002; Michalski et al., 2008). Sulfation may inhibit larval sequestration by competitive inhibition of larval transporters and metabolic enzymes, and thus impact the feeding preference of the beetle larvae.

**Salicin-7-sulfate and salirepin-7-sulfate may act as sulfur storage pools in poplar**

Sulfur is an essential macronutrient in primary metabolism for the synthesis of cysteine, methionine, coenzymes, and prosthetic groups among other compounds (e.g. Takahashi et al., 2011). Because of its importance in the primary metabolism, plants tightly regulate their sulfur status and uptake (Kopriva, 2006; Davidian and Kopriva, 2010; Takahashi et al., 2011). Sulfur deprivation, for instance, usually leads to an increased sulfur uptake, while an excess amount of reduced sulfur (e.g. hydrogen sulfide (H$_2$S)) leads to a decreased sulfur uptake (Herschbach et al., 1995; Lappartient and Touraine, 1996; Westerman et al., 2001). The main sulfur source for plants is sulfate, which is taken up from the soil by the plant roots (Davidian and Kopriva, 2010). However, the release of sulfate from sulfated specialized metabolites might also be important for sulfur homeostasis. We could show that substantially amounts of total sulfur (5.47%) and sulfate (7.47%) are tied up in sulfated salicinoids in poplar leaves. Notably, a similar number (6.4% total sulfur) has been reported for glucosinolates, a group of highly abundant sulfur-containing specialized metabolites in the Brassicaceae (Blake-Kalff et al., 1998; Halkier and Gershenzon, 2006). Principally known as defense compounds, it has been long-term speculated that glucosinolates may function as a
sulfur-storage pool (Hirai et al., 2004; Hirai et al., 2005; Falk et al., 2007). Glucosinolate accumulation is highly dependent on the sulfur availability and sulfur status of the plant (Hirai et al., 2004; Hirai et al., 2005; Falk et al., 2007). Moreover, the expression of glucosinolate biosynthetic genes and genes involved in the primary sulfur metabolism are regulated by the same R2R3-MYB transcription factors (Yatusevich et al., 2010). Since the amounts of total sulfur tied up in glucosinolates and salicinoids in Arabidopsis and poplar, respectively, are comparable, it is tempting to speculate that salicin-7-sulfate and salirepin-7-sulfate act as sulfur storage compounds in poplar. Like glucosinolates, the formation of sulfated salicinoids might also be dependent on the sulfur status of the plant and is likely influenced by changing environmental conditions. Flooding, for example, can alter the microbiome community by increasing the abundance of sulfur-reducing bacteria, which use sulfate as electron acceptors and produce H$_2$S (Muyzer and Stams, 2008; Randle-Boggis et al., 2018). Poplar and willow trees often grow in wetland or even riparian soils, where flooding can occur. Thus, storing high amounts of sulfur as sulfated salicinoids could be a strategy to overcome episodes of flooding with low sulfate availability. This might be a common pattern, because sulfation of specialized metabolites is known to occur more frequently near watery landscapes (Barron et al., 1988), and we showed that sulfated salicinoids broadly occur within the Salicaceae family (Fig. 2). Investigating the exact roles of sulfated salicinoids in sulfur metabolism and the sulfur status of poplar trees are worthwhile aims for future research.

**Materials and Methods**

**Plant and insect material**

Trees of black cottonwood (*Populus trichocarpa*, clone Muhle-Larsen, P&P Baumschule, Eitelborn, Germany), black poplar (*P. nigra*, clones 1 to 19 and f169 (Böckler et al., 2013)), gray poplar (*P. x canescens*, clone INRA 7171-B4, Jörg-Peter Schnitzler, German Research Centre for Environmental Health, Munich, Germany), silver poplar (*P. alba*, cultivar Nivea, Eggert Baumschulen, Vaale, Germany), Simon’s poplar (*P. simonii*, Eggert Baumschulen), basket willow (*Salix viminalis,*...
www.pflanzmich.de), purple willow (*S. purpurea*, Max Planck Institute for Chemical Ecology, Jena, Germany), and goat willow (*S. caprea*, Max Planck Institute for Chemical Ecology) were propagated from monoclonal stem cuttings and grown under summer conditions in the greenhouse (day, 23-25°C; night, 19-23°C; 50-60% rel. humidity; 16 h/8 h light/dark cycle) in a 1:1 mixture of sand and clay granules (Klassmann-Deilmann, Geeste, Germany), until they reached about 60-90 cm in height. Bark from the *P. trichocarpa* clones ‘Muhle Larsen’, ‘625’, ‘262/63’, ‘Brühl4’, ‘606’, and from the Japanese orange cherry (*Idesia polycarpa*) (Max Planck Institute for Chemical Ecology, Jena, Germany) were harvested from young twigs of approximately 1.5 m tall trees. Trembling aspen (*P. tremuloides*) leaf material was harvested in June 2017 from fifty randomly selected genotypes of the WisAsp common garden project in Wisconsin (Barker et al. 2019). Leaf material of *P. tremuloides* × *P. grandidentata* was obtained from the same resource.

Leaves were numbered according to the leaf plastochron index (LPI) (Frost et al., 2007). For analysis of different Salicaceae species, leaf blades of LPI3 to LPI10, and roots were harvested, immediately flash-frozen in liquid nitrogen, lyophilized, pulverized, and stored at 4°C. For analysis of the tissue-specific distribution of the sulfated salicinoids in *P. trichocarpa* and *P. nigra*, leaves of LPI3 to LPI10, their petioles, the stem in between these leaves, as well as the roots were harvested, immediately flash-frozen in liquid nitrogen and ground with one part stored at -80°C until further processing, while the other part was lyophilized, and stored at 4°C. For analysis of the sulfated salicinoids in different *P. nigra* genotypes and in *P. x canescens* SOT1 knockdown lines, leaves (LPI5 and LPI6, *P. nigra*; LPI3 to LPI5, *P. x canescens*) were harvested and further processed as described above for *P. trichocarpa* and *P. nigra* tissues. Leaves of *P. x canescens* SOT1 knockdown plants, wild-type plants, and empty vector plants were harvested at a plant age of 6-, 9-, and 12-weeks (corresponding to a tree height of approximately 30 cm, 70 cm, and 120 cm, respectively).

Gypsy moth (*Lymantria dispar*) egg batches were kindly provided by Hannah Nadel, APHIS, Buzzards Bay, MA, USA, and hatched caterpillars were reared on an artificial diet (Gypsy moth diet, MP Biomedicals LLC).
**Herbivore treatment**

*L. dispar* caterpillars were fed with *P. trichocarpa* leaves for one week and starved for 24 hours prior to the onset of the experiment. For the herbivore treatment, each caterpillar (fourth to fifth instar) was encaged on the LPI5, LPI7, LPI9, and LPI11 leaf of a single *P. trichocarpa* tree. Caterpillars were allowed to feed for 21 h (5 pm – 2 pm). For the control treatment, empty cages were placed on the LPI5, LPI7, LPI9, and LPI11 leaves. Plant material was harvested as described above for different Salicaceae species.

**Methanol extraction of plant material**

Metabolites were extracted from 10 mg freeze-dried plant material by adding 1 ml 100% methanol (MeOH) containing 0.8 mg/ml phenyl-β-D-glucopyranoside (Sigma Aldrich) and 40 ng/ml D$_6$-abscisic acid (D$_6$-ABA) (Santa Cruz Biotechnology) as internal standards. Samples were shaken for 30 sec in a paint shaker (Scandex) and afterwards for 30 min at 200 rpm on a horizontal shaker (IKA Labortechnik). After centrifugation, the supernatants were split for high performance liquid chromatography (HPLC)-UV and LC-MS/MS measurements and were subsequently analyzed.

**Liquid chromatography-mass spectrometry analysis of plant methanol extracts and enzyme products**

*Full scan analysis* – Chromatographic separation was achieved on an Agilent 1100 Series LC system, using an EC 250/4.6 Nucleodur Sphinx column (RP 5 μm, Macherey-Nagel), with aqueous formic acid (0.2% (v/v)) and acetonitrile as mobile phases A and B, respectively. The mobile phase flow rate was 1 ml/min. The elution profile was: 0-22 min, 14-58% B; 22.1-25 min, 100% B; 25.1-30 min, 14% B. The column temperature was maintained at 25°C. The LC system was coupled with an Esquire 6000 ESI-ion trap mass spectrometer (Bruker Daltonics). Compounds were analyzed in alternating ionization (positive/negative) mode with a skimmer voltage of 40 V/-40 V, a capillary exit
voltage of 113.5 V/-121 V, and a capillary voltage of -3000/3000 V. Nitrogen was used as drying gas (11 l/min, 330°C) and nebulizer gas (pressure 35 psi).

**Full scan analysis for purification** – Chromatography and mass spectrometry analysis was done as described above for ‘**Full scan analysis**’, except the mobile phases consisted of 0.05% (v/v) formic acid in water (A), and acetonitrile (B), and the elution profile was: 0-8 min, 14-30% B; 8.1-11 min, 100% B; 11.1-15 min, 14% B.

**Precursor ion scan analysis** – For the precursor ion scan analysis, an Agilent 1200 HPLC system (Agilent Technologies) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) was used. Chromatographic separation was achieved as described above for ‘**Full scan analysis**’, with the exception that the column temperature was maintained at 20°C. The mass spectrometer was equipped with a turbospray ion source, operated in negative ionization mode. The ion spray voltage was maintained at -4500 eV and the turbo gas temperature was set at 700°C. Nebulizing gas was set at 70 psi, curtain gas at 30 psi, heating gas at 60 psi and collision gas at 10 psi. A precursor ion scan (precursor of m/z 97) was performed to identify further sulfated compounds. A mass range from 80 to 800 Da was scanned, the declustering potential (DP) was set at -35 V, and the collision energy (CE) ranged between -55 V to -60 V.

**Accurate mass determination** – Analytes were separated using a Dionex Ultimate 3000 RS pump system (Thermo Fisher Scientific) equipped with an EC 100/2 Nucleodur C18 Isis column (1.8 µm, Macherey-Nagel). The mobile phases consisted of 0.1% (v/v) formic acid in water (A) and acetonitrile (B), with a flow rate of 0.3 ml/min. The column temperature was maintained at 25°C. The elution profile was: 0-0.5 min, 5% B; 0.5-11 min, 5-60% B; 11.1-12 min, 100% B; 12.1-15 min, 5% B. The liquid chromatography system was coupled to a timsTOF mass spectrometer (Bruker Daltonics) equipped with a turbospray ion source (capillary voltage, 3500 V). Nitrogen was used as drying gas (10 l/min, 230°C) and nebulizer gas (1.8 bar). Analysis was done in negative ionization mode, scanning a mass range from m/z 50 to 1500. Sodium formate adducts were used as internal calibrators.
Targeted analysis for quantification – Chromatographic separation was achieved using an Agilent 1260 infinity II LC system (Agilent) equipped with a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent), using aqueous formic acid (0.05% (v/v)) and acetonitrile as mobile phases A and B, respectively. The mobile phase flow rate was 1.1 ml/min. The elution profile was: 0-0.5 min, 5% B; 0.5-6.0 min, 5-37.4% B; 6.02-7.5 min, 80-100% B; 7.5-9.5 min, 100% B; 9.52-12 min, 5% B. The column temperature was maintained at 20°C. The LC system was coupled to a QTRAP 6500 tandem mass spectrometer (Sciex) equipped with a turbospray ion source, operated in negative ionization mode. The ion spray voltage was maintained at -4500 eV and the turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi and collision gas at medium level. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion formation for salicin-7-sulfate: m/z 365→97.0 (DP -30 V; CE -25 V); salirepin-7-sulfate: m/z 381→97.0 (DP -30 V; CE -55 V); salirepin: m/z 301→139 (DP -30 V; CE -18 V), and D₆-ABA: 269→159.2 (DP -30 V; CE -22 V). Sulfated salicinoids and salirepin were quantified relative to the signal of the internal standard D₆-ABA, by applying experimentally determined response factors (RF) (salicin-7-sulfate RF: 1.9; salirepin-7-sulfate RF: 3.0; salirepin RF: 111.47). Analyst 1.6.3 software (Applied Biosystems) was used for data acquisition and processing.

Analysis of enzyme products – Products of sulfotransferase enzymes were measured using an Agilent 1200 HPLC system coupled to an API 3200 tandem mass spectrometer (Applied Biosystems). For analysis of enzyme assays with salicin and salirepin as substrates, the chromatographic separation was achieved with 0.2% (v/v) formic acid in water (A) and acetonitrile (B) as mobile phases and an EC 250/4.6 Nucleodur Sphinx column (RP 5 μm, Macherey-Nagel). The column temperature was maintained at 20°C. The elution profile was: 0-0.5 min, 5% B; 0.5-20.5 min, 5-45% B; 20.52-22 min, 100% B, 22.1-26 min, 5% B, with a flow rate of 1 ml/min. The mass spectrometer was operated in negative ionization mode, with the same settings as described for the ‘Precursor ion scan analysis’. For analysis of enzyme assays with salicyl alcohol as substrate, the chromatographic separation was conducted using a
Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent), and aqueous formic acid (0.05% (v/v) (A)) and acetonitrile (B) as mobile phases. The elution profile was: 0-0.5 min, 5% B; 0.5-6 min, 5-37.4% B; 6.02-7.5 min, 100% B, 7.6-10 min, 5% B. The flow rate was 1.1 ml/min, and the column temperature was maintained at 25°C. The mass spectrometer was operated in negative ionization mode. The ion spray voltage was maintained at -4500 eV and the turbo gas temperature was set at 650°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. For both chromatographic separations, MRM mode was used to monitor precursor → product ion reactions for each analyte as follows: salicin-7-sulfate: m/z 365→97.0 (DP -35 V; CE -55 V); salirepin-7-sulfate: m/z 381→97.0 (DP -35 V; CE -55 V); salicyl alcohol-sulfate m/z 203→97.0 ( DP -35 V; CE -25 V). Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing.

**HPLC-UV analysis for salicinoid quantification and purification of sulfated salicinoids**

*Salicinoid quantification* – Salicinoids were analyzed and quantified by HPLC-UV (200 nm) as described previously in Böckler et al. (2013) for the compounds salicin, salicortin, tremulacin, and homaloside D. 6'-O-Benzoylsalicortin was analyzed and quantified by HPLC-UV (200 nm) as described in Lackner et al. (2019).

*Purification of sulfated salicinoids* – The purification of sulfated salicinoids via preparative HPLC was conducted on an Agilent 1200 HPLC system (Agilent Technologies), with UV/VIS-detector, connected to an Advantec SF-2120 Super fraction collector (Advantec MSF, Inc.). Chromatographic separation was achieved as described above in ‘Full scan analysis for purification’ using an EC 250/4.6 Nucleodur 100-5 C_{18} ec column (Macherey-Nagel). The UV absorption was detected at 200 nm and used for determination and collection of the respective peaks.

**Purification of salicin-7-sulfate and salirepin-7-sulfate from *P. x canescens* leaves**

Ground and freeze-dried *P. x canescens* leaf material (12 g) was extracted twice with 100% MeOH (2x120 ml). The extract was evaporated using nitrogen gas to a volume of
10 ml and then diluted with water to a final concentration of 2% (v/v) MeOH. The extract was first purified by solid phase extraction (SPE) using a CHROMABOND HR-XA mixed-mode anion exchange column (85 µm, 6 ml/500 mg, Macherey-Nagel) and, after washing, eluted with 75% (v/v) formic acid in MeOH. The eluate was dried with nitrogen gas. After reconstitution with water, the mixture was further purified using a CHROMABOND C18 ec SPE column (45 ml/5000 mg, Macherey-Nagel). Sulfated salicinoids were sequentially eluted with 2% (v/v) and 10% (v/v) MeOH in water, respectively, and dried with nitrogen gas. Fractions containing the sulfated salicinoids were reconstituted with 100% MeOH and separated using HPLC on an EC 250/4.6 Nucleodur 100-5 C18 ec column (Macherey-Nagel). The purified sulfated salicinoids were collected, dried with nitrogen gas, and subjected to NMR analysis. For details on HPLC separation and LC-MS analysis of the fractions, please see the sections above ‘Purification of sulfated salicinoids’ and ‘Full scan analysis for purification’, respectively.

**NMR general experimental procedure**

NMR spectra (1H-NMR, 1H-1H COSY, 1H-13C HSQC and 1H-13C HMBC) were measured on a Bruker Avance III HD 500 NMR spectrometer, operating at 500.13 MHz for 1H and 125.75 MHz for 13C, and a Bruker Avance III HD 700 NMR spectrometer, operating at 700.13 MHz for 1H and 175.75 MHz for 13C (Bruker Biospin GmbH). Both spectrometers were equipped with a TCI cryoprobe (5 mm for Avance III HD 500 and 1.7 mm for Avance III HD 700). Spectrometer control and data processing was accomplished using Bruker Topspin ver. 3.6.1. All NMR measurements were recorded at 25°C in D2O, and chemical shifts were left uncorrected.

**Determination of total sulfur and sulfate in *P. trichocarpa* plant material**

Total sulfur was determined in 30 mg freeze-dried *P. trichocarpa* leaf material with a vario EL elemental analyzer (Elementar Analysensysteme GmbH). Sulfate was extracted from 40 mg freeze-dried *P. trichocarpa* leaf material by adding 4 ml of ddH2O. The extracts were incubated for 12 h at 40°C and 100 rpm. Afterwards, the extracts were centrifuged twice (21,000 g for 15 min at 4°C) and were incubated in between the
centrifugation steps for 10 min at 4°C. The collected supernatants were filtered over a 0.45 µm membrane (Filtropur S 0.45 µm, Sarstedt) and stored at 4°C until further analysis. The sulfate concentration was analyzed via ion chromatography using a Dionex DX-500 system (Thermo Fisher Scientific).

**RNA extraction and reverse transcription**

Total RNA was isolated from frozen and ground plant material using the InviTrap Spin Plant RNA Kit (Stratec) according to manufacturer’s instructions. RNA concentration was assessed using a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific). RNA was treated with DNaseI (Thermo Fisher Scientific) prior to cDNA synthesis. Single-stranded cDNA was prepared from 1 µg of DNase-treated RNA using SuperScript III reverse transcriptase and oligo (dT<sub>12-18</sub>) primers (Invitrogen).

**Identification and cloning of putative poplar sulfotransferase genes**

Putative poplar sulfotransferase genes were identified by BLAST analysis using AtSOT12 (NM_126423) from Arabidopsis (*Arabidopsis thaliana*) as query and the poplar genome as reference (Tuskan et al., 2006, http://www.phytozome.net/poplar). The complete open reading frames of the two candidate genes, Potri.012G032700 and Potri.003G189100, were amplified from leaf cDNA. Since only Potri.003G189100 could be inserted into the *Escherichia coli* expression vector pET100/D-TOPO (Thermo Fisher Scientific), Potri.012G032700 was synthesized as a codon-optimized sequence and subsequently cloned into the same expression vector (Thermo Fisher Scientific). The cloned Potri.003G189100 gene was fully sequenced.

**Phylogenetic analysis, and amino acid and DNA alignment**

An alignment of all putative *P. trichocarpa* sulfotransferase genes and characterized sulfotransferase genes from Arabidopsis, rice (*Oryza sativa*), and rapeseed (*Brassica napus*) was constructed using the MUSCLE (codon) algorithm (gap open, -2.9; gap extend, 0; hydrophobicity multiplier, 1.5; clustering method, UPGMB) implemented in MEGA6 (Tamura et al., 2013). Tree reconstruction was done with MEGA6 using a
Maximum Likelihood algorithm (model/method, Kimura 2-parameter model; substitutions type, nucleotide; rates among sites, uniform rates; gaps/missing data treatment, partial deletion; site coverage cutoff, 80%). A bootstrap resampling analysis with 1000 replicates was performed to evaluate the tree topology.

An amino acid alignment of Potri.012G032700 (PtSOT1) and Potri.003G189100, together with Arabidopsis SOT12 (NP_178471; Baek et al., 2010) and rice SOT1 (LOC_Os11g30910; Wang et al., 2014b) was constructed with MEGA6 and visualized with BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A DNA alignment of PtSOT1 and PnSOT1 (f41 and f169) was also constructed and visualized with MEGA6 and BioEdit, respectively.

**gDNA extraction and cloning of PnSOT1**

Genomic DNA (gDNA) was extracted from ground and freeze-dried *P. nigra* f41 and f169 leaf material using the Invisorb Spin Plant Mini Kit (Stratec) according to the manufacturer’s instructions. gDNA concentration was assessed using a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific). A PCR was conducted for amplification of PnSOT1 using gene-specific primers for PtSOT1 (Supplemental Table S7). The amplified PCR product was inserted into the expression vector pET100/D-TOPO (Thermo Fisher Scientific) and the cloned genes were fully sequenced.

**RNAseq and RT-qPCR analysis**

For gene expression analysis of the 28 putative poplar sulfotransferases, we reanalyzed the data set (SRA accession number PRJNA516861) of an RNAseq experiment as recently described in Günther et al. (2019).

For RT-qPCR analysis, cDNA was prepared as described above and diluted 1:10 with water. For the amplification of a SOT1 gene fragment with a length of 107 bp, primers were designed having a Tm ≥ 60°C, a GC content between 50 - 60%, and a primer length of 20 nucleotides (Supplemental Table S7). The specificity of the primers was confirmed by agarose gel electrophoresis, melting curve analysis, standard curve
analysis, and by sequence verification of cloned PCR amplicons. *Ubiquitin (UBQ)*, *actin*, *elongation factor 1 alpha (EF1α)*, *histone superfamily protein H3 (HIS)*, and *tubulin (TUB)* were tested as reference genes (Ramírez-Carvajal et al., 2008; Xu et al., 2011; Wang et al., 2014a). Comparison of ∆Cq values and the corresponding standard deviation revealed *HIS* as the most suitable reference gene for expression analysis in *P. trichocarpa* and *P. nigra* samples (Supplemental Table S8; Supplemental Table S9). Based on previous studies from Irmisch et al. (2013) and Günther et al. (2019), *UBQ* was used as the reference gene for expression analysis in (non-) transgenic *P. x canescens* leaves. The following PCR conditions were applied for analysis of *SOT1* expression: Initial incubation at 95°C for 3 min followed by 40 cycles of amplification (95°C for 10 sec, 57°C for 20 sec, 65°C for 10 sec). Reference gene expression analysis was performed with an initial incubation at 95°C for 3 min followed by 40 cycles of amplification (95°C for 10 sec, 60°C for 10 sec). For all measurements, plate reads were taken at the end of the extension step of each cycle and data for the melting curves were recorded at the end of cycling from 60°C to 95°C. All samples were run on the same PCR machine (Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratory)) in an optical 96-well plate, using Brilliant III SYBR Green QPCR Master Mix (Stratagene). For analyzing gene expression in *P. trichocarpa* and *P. nigra*, six biological replicates were analyzed, each as technical triplicates. Expression analysis of the *P. x canescens* knockdown, WT, and EV trees was conducted for all biological replicates in technical triplicates.

**Heterologous expression of sulfotransferase genes**

The *Escherichia coli* strain BL21 Star (DE3) (Thermo Fisher Scientific) was used for heterologous expression of Potri.012G032700 (PtSOT1) and Potri.003G189100. Cultures were grown at 37°C, induced at an OD$_{600}$ = 0.6 with 1 mM IPTG, and subsequently placed at 18°C and grown for another 20 hours. The cells were collected by centrifugation and disrupted by a 4 × 20 s treatment with a sonicator (Bandelin UW2070) in chilled extraction buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 20 mM imidazole, 1% (v/v) Tween 20, 10% (v/v) glycerol, 0.3 mg/ml of lysozyme, 2.5 U/ml
Benzonase Nuclease (Merck), and protease-inhibitor Mix HP (Serva, according to the manufacturer’s instructions)). After centrifugation (15,000 g for 30 min at 4°C), the supernatant was used for further affinity-based purification with the Ni-NTA Spin Kit (Qiagen), following the manufacturer’s instructions. To determine the catalytic activity of recombinant enzymes, assays containing 50 μl of the purified protein, 42 µl assay buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol), 3 µl of the tested substrates (10 mM salicin, salirepin, or salicyl alcohol), and 5 µl PAPS (4 mM) were incubated for 2 h at 30°C under shaking at 300 rpm. The reaction was stopped by adding 200 µl MeOH. After placing on ice for 30 min, the denatured enzymes were removed by centrifugation (15,000 g for 10 min at 4°C) and the supernatant was transferred into a glass vial for LC-MS/MS analysis. Reaction products were analyzed using LC-MS/MS.

Vector construction and transformation of poplar
The construction of the binary vector was described by Levée et al. (2009). The transformation of the P. x canescens clone INRA 7171-B4 followed a protocol published by Meilan and Ma (2007). To target SOT1 mRNA, two fragments between position 114 and 389 (RNAi-1) or 327 and 471 (RNAi-2) of the coding sequence of PtSOT1 were selected. Transgenic RNAi plants were amplified by micropropagation as described by Behnke et al. (2007). Saplings of around 10 cm high were repotted to soil (Klasmann potting substrate) and propagated in a controlled environment chamber for six weeks (day, 22°C; night, 18°C; 65% rel. humidity; 16 h/8 h light/dark cycle) before they were transferred to the greenhouse. To test the level of gene silencing, RT-qPCR analysis was done on wild type plants, empty vector control (EV) plants, and RNAi-plants (line RNAi-1 and line RNAi-2).

Feeding preference assays with Lymantria dispar caterpillars
Feeding preference assays were performed as described previously in Lackner et al. (2019) with the following modifications: L. dispar caterpillars (2nd larval instar) were starved for 6.5 h prior to the assay and the caterpillars were then allowed to feed for 21
Two discs of *P. x canescens* leaves (LPI3-5) harvested from each WT and *SOT1* knockdown lines (RNAi-1) were offered. To test the feeding preference with artificial application of salicin (Alfa Aesar), different salicin solutions (corresponding to 0.1 mg/g fresh weight (FW) salicin, 0.5 mg/g FW, 1.0 mg/g FW, and 1.5 mg/g FW) in ethanol were prepared. Salicin solutions or pure ethanol were applied to *P. x canescens* WT leaf discs (LPI3-LPI5). The same set-up was used as described in Lackner et al. (2019), with the modifications described above.

**Statistical analysis**
Throughout the manuscript, data are presented as means ± SE. Statistical analysis was performed with SigmaPlot 11.0 for Windows (Systat Software Inc.) and is described in the figure and table legends for the respective experiments. Whenever necessary, the data were log transformed to meet statistical assumptions such as normality and homogeneity of variances.

**Accession Numbers**
Sequence data for PtSOT1 (MN729495), and Potri.003G189100 (MN729494) can be found in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the corresponding identifiers.

**Supplemental Data**
The following supplemental materials are available.

Supplemental Figure S1. Liquid chromatography/mass spectrometry (LC-MS) analysis of methanol extracts made from leaves of *Populus x canescens*, *P. trichocarpa*, and *Salix viminalis*.

Supplemental Figure S2. Isotopic cluster of [M-H]$^-$ peak for unknown compound ($m/z$ 365), later identified as salicin-7-sulfate.
Supplemental Figure S3. NMR chemical shift data (in D$_2$O) of salicin-7-sulfate (1) and salirepin-7-sulfate (2).

Supplemental Figure S4. Concentrations (µg/g dry weight) of salicin-7-sulfate in leaves of different *Populus tremuloides* and *P. tremuloides* x *P. grandidentata* genotypes.

Supplemental Figure S5. Expression of putative poplar sulfotransferase genes in *Populus trichocarpa* leaves.

Supplemental Figure S6. Amino acid sequence comparison of putative *Populus trichocarpa* sulfotransferases Potri.012G032700 and Potri.003G189100 with characterized sulfotransferases from other plants.

Supplemental Figure S7. Catalytic activity of putative *Populus trichocarpa* sulfotransferases Potri.012G032700 (PtSOT1) and Potri.003G189100.

Supplemental Figure S8. Concentrations of sulfated and non-sulfated salicinoids in leaves of different *Populus nigra* genotypes. are shown in panel A, and of

Supplemental Figure S9. DNA sequence comparison of *Populus trichocarpa* PtSOT1 with *P. nigra* SOT1 cloned from two different genotypes.

Supplemental Figure S10. RNAi-mediated knockdown of SOT1 in *Populus x canescens*.

Supplemental Figure S11. Feeding preference of *Lymantria dispar* caterpillars (L2) in food choice assays with *Populus x canescens* wild type (WT) leaf discs and WT leaf discs with artificially applied salicin (salicin).
Supplemental Table S1. Concentrations (mg/g dry weight) of salicin, salirepin, and complex salicinoids in leaves and roots of different poplar and willow species.

Supplemental Table S2. Concentrations of sulfated salicinoids (µg/g dry weight), salicin, and salirepin (mg/g dry weight) in the bark of different *Populus trichocarpa* genotypes and *Idesia polycarpa*.

Supplemental Table S3. Concentrations (mg/g dry weight) of complex salicinoids in different organs of *Populus trichocarpa* and *P. nigra* trees.

Supplemental Table S4. Concentrations (mg/g dry weight) of complex salicinoids in undamaged (control) and *Lymantria dispar* caterpillar-damaged (herbivory) *Populus trichocarpa* leaves.

Supplemental Table S5. Concentrations (mg/g dry weight) of sulfated salicinoids, salicin, and salirepin in leaves of different *Populus nigra* genotypes.

Supplemental Table S6. Expression levels of *SOT1* in different organs of *Populus trichocarpa* (*Pt*) and *P. nigra* (*Pn*) trees.

Supplemental Table S7. Oligonucleotides used for the amplification of full length *SOT1*, *Potri.003G189100*, and for gene expression analysis of *SOT1* by RT-qPCR.

Supplemental Table S8. Expression levels of potential housekeeping genes in different organs of *Populus trichocarpa* trees.

Supplemental Table S9. Expression levels of potential housekeeping genes in different organs of *Populus nigra* trees.
Abbreviations
RT-qPCR, reverse transcription quantitative PCR; LC-MS/MS, liquid chromatography-tandem mass spectrometry, SOT, sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

Declarations
Availability of data and materials
All supporting data are included as additional files. Constructs described in this work and datasets analyzed during the current study are available from the corresponding author upon request. Sequences were deposited in GenBank (see Methods section).

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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help with the analysis of sulfur and sulfate. The research was funded by the Max Planck Society.

Figure legends

**Figure 1.** Sulfated organic compounds accumulate in leaves of *Populus trichocarpa*. A precursor ion scan experiment with the target mass $m/z$ 97 [$M-H]^-$ (sulfate fragment) was performed to screen for sulfated organic compounds in *P. trichocarpa* leaves (A). Structural formulas of the three identified sulfated compounds (B). 12-hydroxyjasmonic acid sulfate was identified by comparisons with an authentic standard. Structures of salicin-7-sulfate and salirepin-7-sulfate were verified by NMR. cps, counts per second.

**Figure 2.** Salicin-7-sulfate and salirepin-7-sulfate concentrations in leaves (dark gray bars) and roots (gray bars) of different poplar and willow species. The compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS. Means and SE ($n = 5 - 7$) are shown. dw, dry weight; nd, not detected. Asterisks indicate statistical significance between the two parts of the same species as assessed by paired t-test or Wilcoxon Signed Rank Test. Salicin-7-sulfate (A): *Populus trichocarpa* ($p < 0.001$, $t = 34.372$); *P. x canescens* ($p < 0.001$, $t = 14.388$); *P. alba* ($p < 0.001$, $t = 18.303$); *P. simonii* ($p = 0.031$, $W = -21.00$); *Salix purpurea* ($p = 0.029$, $t = 2.843$); *S. caprea* ($p = 0.047$, $W = -24.00$). Salirepin-7-sulfate (B): *P. trichocarpa* ($p < 0.001$, $t = 17.009$); *P. x canescens* ($p < 0.001$, $t = 25.130$); *P. alba* ($p < 0.001$, $t = 13.306$); *P. simonii* ($p < 0.001$, $t = 7.045$); *S. purpurea* ($p = 0.001$, $t = 5.531$).

**Figure 3.** Salicin, salicin-7-sulfate, salirepin, and salirepin-7-sulfate concentrations in different organs of *Populus trichocarpa* (dark gray bars) and *P. nigra* (gray bars) trees. Leaf blades (leaves) and petioles originated from LPI 3 - 10 leaves. Stem material was harvested between leaf LPI 3 and LPI 10. Compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS.
and HPLC-UV. Means and SE (n = 6 - 8) are shown. dw, dry weight; nd, not detected.

Differences between organs and species were analyzed by a two-way repeated measures ANOVA and a post-hoc test (Tukey’s test). Different letters indicate significant differences ($p < 0.05$).

**Figure 4.** Salicin, salicin-7-sulfate, salirepin, and salirepin-7-sulfate concentrations in undamaged (control) and *Lymantria dispar* caterpillar-damaged (herbivory) leaves of *Populus trichocarpa*. Compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS or HPLC-UV. Means and SE (n = 7 - 8) are shown. dw, dry weight; ns, not significant. Asterisks indicate statistical significance as assessed by Student’s t-test. Salicin ($p = 0.009, t = -3.065$); salicin-7-sulfate ($p = 0.152, t = 1.523$); salirepin ($p = 0.846, t = 0.198$); salirepin-7-sulfate ($p = 0.588, t = 0.555$).

**Figure 5.** Cladogram analysis of putative poplar sulfotransferase genes and characterized sulfotransferase genes from other plants. The tree was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model implemented in MEGA6 (Tamura et al., 2013). Bootstrap values (n = 1000) are shown next to each node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 80% site coverage were eliminated. Arabidopsis *AtTPST* (NM_100677) was used as an outgroup. Poplar sulfotransferases investigated in this study are highlighted in red. Substrates of characterized sulfotransferases are shown next to the tree on the right site. *OsSOT1*, *Oryza sativa* LOC_Os11g30910; *BnST3*, *Brassica napus* AF000307; *BnST4*, *B. napus* AY442306; *AtSOT12*, *A. thaliana* NM_126423.

**Figure 6.** Catalytic activity of *Populus trichocarpa* sulfotransferases Potri.012G032700 (PtSOT1) and Potri.003G189100. PtSOT1 and Potri.003G189100 were heterologously expressed in *Escherichia coli* as His-tag fusion proteins and
recombinant proteins were purified using affinity chromatography. Purified proteins were incubated with the potential substrates salicin or salirepin, and the co-substrate PAPS as sulfate group donor. Reaction products were analyzed using liquid chromatography/tandem mass spectrometry (LC-MS/MS). cps, counts per second.

Figure 7. Gene expression analysis of PtSOT1. Transcript accumulation of PtSOT1 in different organs of Populus trichocarpa was measured using RT-qPCR. Means and SE are shown (n = 6). Leaf blades (leaves) and petioles originated from LPI 3 - 10 leaves. Stem material was harvested between leaf LPI 3 and LPI 10. HIS (histone superfamily protein H3) was used as a housekeeping gene (Wang et al., 2014a). Differences between organs were analyzed by a one way repeated measures ANOVA and a post-hoc test (Tukey’s test). Different letters indicate significant differences (p < 0.05).

Figure 8. RNAi-mediated knock-down of sulfotransferase 1 in Populus x canescens. Transcript accumulation of P. x canescens sulfotransferase 1 (A), and salicin-7-sulfate (B), salirepin-7-sulfate (C), salicin (E), salirepin (F), salicortin (H), and tremulacin (I) concentration in leaves of wild type and transgenic P. x canescens trees are shown. The chemical structures are displayed in (D) and (G). Data are displayed for 6-week-old trees. Gene expression was measured using RT-qPCR, with ubiquitin (Ramírez-Carvajal et al., 2007) as a housekeeping gene. Compounds were extracted with methanol from freeze-dried leaf material and analyzed using LC-MS/MS and HPLC-UV. Medians ± quartiles, and outliers are shown. dw, dry weight; EV, empty vector; WT, wild type. Differences between WT, EV, and RNAi knockdown lines were analyzed by a one-way ANOVA and a post-hoc test (Tukey’s test) or by Kruskal-Wallis One Way ANOVA on Ranks (n = 3 - 6). Different letters above each box indicate significant differences (p < 0.05).

Figure 9. Feeding preference of Lymantria dispar caterpillars (L2) for leaf discs of Populus x canescens wild type (WT) versus sulfotransferase 1 (RNAi) knockdown
lines. Consumed leaf area was measured after 21 h of feeding. Medians ± quartiles are shown. ns, not significant. Asterisks indicate statistical significance as assessed by paired t-test or Wilcoxon Signed Rank Test (n = 11 - 14). experiment 1 (p = 0.172, t = -1.470); experiment 2 (p = 0.380, W = 24.00).
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Figure 1. Sulfated organic compounds accumulate in leaves of *Populus trichocarpa*. A precursor ion scan experiment with the target mass \( m/z \) 97 [M-H]- (sulfate fragment) was performed to screen for sulfated organic compounds in *P. trichocarpa* leaves (A). Structural formulas of the three identified sulfated compounds (B). 12-hydroxyjasmonic acid sulfate was identified by comparisons with an authentic standard. Structures of salicin-7-sulfate and salirepin-7-sulfate were verified by NMR. cps, counts per second.
Figure 2. Salicin-7-sulfate and salirepin-7-sulfate concentrations in leaves (dark gray bars) and roots (gray bars) of different poplar and willow species. The compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS. Means and SE (n = 5 - 7) are shown. dw, dry weight; nd, not detected. Asterisks indicate statistical significance between the two parts of the same species as assessed by paired t-test or Wilcoxon Signed Rank Test. Salicin-7-sulfate (A): *P. trichocarpa* (p < 0.001, t = 34.372); *P. x canescens* (p < 0.001, t = 14.388); *P. alba* (p < 0.001, t = 18.303); *P. simonii* (p = 0.031, W = -21.00); *Salix purpurea* (p = 0.029, t = 2.843); *S. caprea* (p = 0.047, W = -24.00). Salirepin-7-sulfate (B): *P. trichocarpa* (p < 0.001, t = 17.009); *P. x canescens* (p < 0.001, t = 25.130); *P. alba* (p < 0.001, t = 13.306); *P. simonii* (p < 0.001, t = 7.045); *S. purpurea* (p = 0.001, t = 5.531).
Figure 3. Salicin, salicin-7-sulfate, salirepin, and salirepin-7-sulfate concentrations in different organs of *Populus trichocarpa* (dark gray bars) and *P. nigra* (gray bars) trees. Leaf blades (leaves) and petioles originated from LPI 3 - 10 leaves. Stem material was harvested between leaf LPI 3 and LPI 10. Compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS and HPLC-UV. Means and SE (n = 6 - 8) are shown. dw, dry weight; nd, not detected. Differences between organs and species were analyzed by a two-way repeated measures ANOVA and a post-hoc test (Tukey’s test). Different letters indicate significant differences (p < 0.05).
Figure 4. Salicin, salicin-7-sulfate, salirepin, and salirepin-7-sulfate concentrations in undamaged (control) and *Lymantria dispar* caterpillar-damaged (herbivory) leaves of *Populus trichocarpa*. Compounds were extracted with methanol from freeze-dried plant material and analyzed using LC-MS/MS or HPLC-UV. Means and SE (n = 7 - 8) are shown. dw, dry weight; ns, not significant. Asterisks indicate statistical significance as assessed by Student’s t-test. Salicin (p = 0.009, t = -3.065); salicin-7-sulfate (p = 0.152, t = 1.523); salirepin (p = 0.846, t = 0.198); salirepin-7-sulfate (p = 0.588, t = 0.555).
Figure 5. Cladogram analysis of putative poplar sulfotransferase genes and characterized sulfotransferase genes from other plants. The tree was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model implemented in MEGA6 (Tamura et al., 2013). Bootstrap values (n = 1000) are shown next to each node. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 80% site coverage were eliminated. Arabidopsis AtTPST (NM_100677) was used as an outgroup. Poplar sulfotransferases investigated in this study are highlighted in red. Substrates of characterized sulfotransferases are shown next to the tree on the right site. OsSOT1, Oryza sativa LOC_0s11g30910; BnST3, Brassica napus AF000307; BnST4, B. napus AY442306; AtSOT12, A. thaliana NM_126423.

Figure 5
Figure 6. Catalytic activity of *Populus trichocarpa* sulfotransferases Potri.012G032700 (PtSOT1) and Potri.003G189100. PtSOT1 and Potri.003G189100 were heterologously expressed in *Escherichia coli* as His-tag fusion proteins and recombinant proteins were purified using affinity chromatography. Purified proteins were incubated with the potential substrates salicin or salirepin, and the co-substrate PAPS as sulfate group donor. Reaction products were analyzed using liquid chromatography/tandem mass spectrometry (LC-MS/MS). cps, counts per second.
Figure 7. Gene expression analysis of *PtSOT1*. Transcript accumulation of *PtSOT1* in different organs of *Populus trichocarpa* was measured using RT-qPCR. Means and SE are shown (n = 6). Leaf blades (leaves) and petioles originated from LPI 3 - 10 leaves. Stem material was harvested between leaf LPI 3 and LPI 10. *HIS* (*histone superfamily protein H3*) was used as a housekeeping gene (Wang et al., 2014a). Differences between organs were analyzed by a one way repeated measures ANOVA and a post-hoc test (Tukey’s test). Different letters indicate significant differences (*p* < 0.05).
Figure 8. RNAi-mediated knock-down of sulfotransferase 1 in Populus x canescens. Transcript accumulation of P. x canescens sulfotransferase 1 (A), and salicin-7-sulfate (B), salirepin-7-sulfate (C), salicin (E), salirepin (F), salicortin (H), and tremulacin (I) concentration in leaves of wild type and transgenic P. x canescens trees are shown. The chemical structures are displayed in (D) and (G). Data are displayed for 6-week-old trees. Gene expression was measured using RT-qPCR, with ubiquitin (Ramírez-Carvajal et al., 2007) as a housekeeping gene. Compounds were extracted with methanol from freeze-dried leaf material and analyzed using LC-MS/MS and HPLC-UV. Medians ± quartiles, and outliers are shown. dw, dry weight; EV, empty vector; WT, wild type. Differences between WT, EV, and RNAi knockdown lines were analyzed by a one-way ANOVA and a post-hoc test (Tukey’s test) or by Kruskal-Wallis One Way ANOVA on Ranks (n = 3 - 6). Different letters above each box indicate significant differences (p < 0.05).
Figure 9. Feeding preference of *Lymantria dispar* caterpillars (L2) for leaf discs of *Populus x canescens* wild type (WT) versus sulfotransferase 1 (RNAi) knockdown lines. Consumed leaf area was measured after 21 h of feeding. Medians ± quartiles are shown. ns, not significant. Asterisks indicate statistical significance as assessed by paired t-test or Wilcoxon Signed Rank Test (n = 11 - 14). experiment 1 (p = 0.172, t = -1.470); experiment 2 (p = 0.380, W = 24.00).
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