

RESEARCH PAPER

Volatile defense signaling: molecular basis of herbivory-induced terpene biosynthesis and emission in purple willow leaves

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Abstract

Volatiles play an important role in biotic plant–environment interactions. While major research has been conducted on the emission of herbivore-induced plant volatiles in annual herbaceous species, little comparable information is available about long-lived plant species. This study shows that herbivory by gypsy moth (*Lymantria dispar*) caterpillars or poplar leaf beetles (*Chrysomela populi*) on purple willow (*Salix purpurea*) leaves led to the induced emission of complex volatile bouquets, including a wide range of mono- and sesquiterpenes. Further comprehensive sequence and phylogenetic analyses enabled the identification of a mid-sized terpene synthase (TPS) family within the *S. purpurea* genome. The heterologous overexpression of identified *S. purpurea* TPS candidate genes in *E. coli* revealed their respective activities in the formation of the monoterpene alcohol linalool, as well as the sesquiterpenes (*E,E*)- α -farnesene and germacrene D, among others. Moreover, the majority of the herbivore-induced terpenoid volatile bouquet of *S. purpurea* leaves could be reconstituted in the volatile blend of the heterologous host *Nicotiana benthamiana* by overexpression of the respective TPS candidate genes.

Keywords: *Chrysomela populi*, herbivory, *Lymantria dispar*, Salicaceae, *Salix purpurea*, terpene synthases, terpenes, volatile organic compounds.

Introduction

Plants produce a diverse repertoire of specialized metabolites to withstand a broad spectrum of biotic stressors (Marone *et al.*, 2022; Wu *et al.*, 2023). These specialized metabolites play, for instance, vital roles in defense against herbivorous insects, aphids, and microbial pathogens (Wu *et al.*, 2023; Kliebenstein, 2024). Some of these metabolites are released as volatile organic compounds (VOCs) (Kessler and Baldwin, 2002; Dudareva *et al.*, 2006; Turlings and Erb, 2018). VOCs

emitted from flowers and fruits are well known for mediating interactions with pollinators and seed dispersers; however, vegetative tissues such as leaves and roots also emit VOCs (Rasmann *et al.*, 2005; Danner *et al.*, 2011; Lackus *et al.*, 2018; Lo *et al.*, 2024). Herbivory, for example, can substantially alter the VOC profiles of these tissues, leading to the release of herbivore-induced plant volatiles (HIPVs)—a class of VOCs emitted in response to insect feeding (Danner *et al.*, 2011;

Clavijo McCormick *et al.*, 2014; Lackus *et al.*, 2018; Turlings and Erb, 2018). HIPVs fulfill diverse defensive roles, ranging from directly deterring herbivores to indirectly attracting predators, inhibiting pathogens, enabling plant–plant signaling, or priming nearby plants (Turlings and Erb, 2018; Anjali *et al.*, 2023; Kutty and Mishra, 2023; Thompson *et al.*, 2024). They typically are made up of complex bouquets of compounds from various chemical classes, including green leaf volatiles, aromatics, nitrogen-containing compounds, and terpenes (Dudareva *et al.*, 2006, 2013; Mumm and Dicke, 2010). In addition to these volatile defenses, many specialized metabolites accumulate within plant tissues, contributing to the modulation of plant–environment interactions (Marone *et al.*, 2022; Kessler *et al.*, 2023; Wu *et al.*, 2023).

While HIPV-mediated defenses have been studied extensively in herbaceous species, the genetic and biochemical mechanisms underlying these responses in long-lived woody plants remain less well characterized. Throughout their lifetimes, trees are confronted with a wide range of herbivores (Turcotte *et al.*, 2014; Celedon and Bohlmann, 2019). This selective pressure requires them to develop robust, sustained defense strategies, making these plants ideal systems for studying the ecological and evolutionary aspects of plant defense (Oates *et al.*, 2015; Hsieh *et al.*, 2021). Despite considerable progress in herbaceous model species, comprehensive knowledge of defense strategies among different tree species—especially within the same plant family—remains limited. The *Salicaceae* family, which includes poplar (*Populus*) and willow (*Salix*) species, represents an excellent system for investigating tree defense mechanisms due to its ecological significance and importance in the renewable energy sector (Njakou Djomo *et al.*, 2015; Stolarski *et al.*, 2024). Chewing herbivory by generalist gypsy moth (*Lymantria dispar*) caterpillars or specialist poplar leaf beetles (*Chrysomela populi*) has been shown to induce major alterations in the volatile bouquet of poplar trees such as black cottonwood (*Populus trichocarpa*) and black poplar (*Populus nigra*) (Danner *et al.*, 2011; Irmisch *et al.*, 2013; Clavijo McCormick *et al.*, 2014; Lackus *et al.*, 2021b). The herbivore-induced volatile bouquets of poplar trees typically comprise nitrogen-containing compounds, aromatics, and a wide diversity of mono- and sesquiterpenoids (Danner *et al.*, 2011; Irmisch *et al.*, 2013; Clavijo McCormick *et al.*, 2014; Lackus *et al.*, 2021b). In addition to VOCs, members of the *Salicaceae* also produce non-volatile specialized metabolites, notably salicinoids, highly abundant, constitutively accumulating phenolic glycosides based on a salicin core (Boeckler *et al.*, 2011; Lackus *et al.*, 2020; Gordon *et al.*, 2022; Kaufman *et al.*, 2025). More complex salicinoid derivatives such as salicortin and tremulacin are widespread within the family and have proven effective activity against herbivorous insects like the forest tent caterpillar (*Malacosoma disstria*), as well as against mammalian herbivores such as field voles (*Microtus agrestis*) and mountain hares (*Lepus timidus*) (Tahvanainen *et al.*, 1985; Hwang and Lindroth, 1997; Heiska *et al.*, 2007; Kaufman *et al.*, 2025).

Together, volatile terpenes and non-volatile salicinoids form complementary defense systems that enhance the resistance of poplars and willows to herbivory. Despite their importance, species-specific defense responses within the *Salicaceae* family, such as those in purple willow (*Salix purpurea*), remain poorly understood.

Among the numerous classes of specialized metabolites, terpenes play a particularly central role in the plant–environment interaction. Terpenes constitute the largest and structurally most diverse group of plant specialized metabolites, with more than 30 000 known compounds (McCaskill and Croteau, 1998; Fürstenberg-Hägg *et al.*, 2013; Bergman and Dudareva, 2024). Terpenes are ubiquitous throughout the plant kingdom, occurring both within plant tissues and as components of volatile blends. Mono- and sesquiterpenes, in particular, are characteristic constituents of these volatile emissions (Karunanithi and Zerbe, 2019; Kessler *et al.*, 2023). Terpene structures are defined by their synthesis from the C₅ building blocks isopentenyl diphosphate and its allylic isomer dimethylallyl diphosphate (McGarvey and Croteau, 1995; Chen *et al.*, 2011; Bergman and Dudareva, 2024). These precursors are produced via two distinct pathways: the cytosolic mevalonate pathway and the plastidic 2-C-methylerythritol-4-phosphate pathway (Lichtenthaler, 1999; Sapir-Mir *et al.*, 2008; Bergman and Dudareva, 2024). Sequential head-to-tail condensations of the respective C₅ units, catalysed by isopentenyl diphosphate synthases, generate longer-chain intermediates such as geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅), and geranylgeranyl diphosphate (GGPP, C₂₀), which serve as precursors for mono-, sesqui-, and diterpenes, respectively (Chen *et al.*, 2011; Bergman and Dudareva, 2024). The resulting compounds are then converted into structurally diverse terpenes by terpene synthases (TPSs) (Chen *et al.*, 2011; Dudareva *et al.*, 2013; Bergman and Dudareva, 2024). TPS gene families are typically mid-sized in plants, with *Populus trichocarpa* and *Arabidopsis* possessing, for instance, 38 and 32 TPS genes, respectively (Aubourg *et al.*, 2002; Irmisch *et al.*, 2014), but genome-wide surveys have revealed considerable variation across lineages, ranging from only a few members in the bryophyte *Physcomitrella patens* to 113 annotated TPS genes in *Eucalyptus grandis* (Hayashi *et al.*, 2006; Külheim *et al.*, 2015). Plant TPSs are grouped into seven gene subfamilies: the angiosperm-specific TPS-a, TPS-b, and TPS-g, which predominantly form sesquiterpenes (TPS-a) and monoterpenes (TPS-b and TPS-g); the TPS-c subfamily, containing copalyl diphosphate synthases; the TPS-e/f subfamily, containing kaurene synthases; the gymnosperm-specific TPS-d; and the lycopod-specific TPS-h (Chen *et al.*, 2011; Bergman and Dudareva, 2024). Based on their reaction mechanisms, TPS enzymes are classified as class I or class II (Chen *et al.*, 2011; Bergman and Dudareva, 2024). The formation of volatile mono- and sesquiterpenes is typically catalysed by class I TPSs, which use divalent metal ions to

ionize GPP and FPP, generating reactive carbocation intermediates that subsequently undergo further rearrangements (Chen *et al.*, 2011; Bergman and Dudareva, 2024). In contrast, class II TPSs initiate cyclization through protonation, producing bicyclic prenyl diphosphates that subsequently serve as substrates for class I enzymes (Chen *et al.*, 2011; Bergman and Dudareva, 2024). Class I TPS enzyme activity depends on several conserved motifs, including a DDxxD motif for metal ion binding, an NSE/DTE motif for additional metal-ion coordination, and a RxR motif for stabilizing the released pyrophosphate (Chen *et al.*, 2011; Bergman and Dudareva, 2024). An additional RR_xW motif near the N-terminus is often involved in cyclization reactions, although it is frequently absent in TPS-g members (Bohlmann *et al.*, 1998; Chen *et al.*, 2024). Other notable aspects of TPS enzymes causing a huge structural diversity include the ability to produce multiple products from single substrates and the fact that minor sequence variations can cause substantial shifts in product profiles (Degenhardt *et al.*, 2009; Bergman and Dudareva, 2024; Li and Tao, 2024).

Despite characterization of TPS gene families in herbaceous model species, knowledge about their diversity and functional roles in willows remains scarce. To address this gap, we investigated the defense response of a long-lived woody plant to herbivory, focusing on purple willow (*Salix purpurea*). Thus, we analysed the volatilome and the accumulation of polar aromatic defense compounds (salicinoids) in *S. purpurea* leaves upon herbivory by chewing insects, namely *L. dispar* caterpillars and *C. populi* beetles. In addition, we performed phytohormone and gene expression analyses to gain insights into the regulation of herbivore-induced defense responses. Furthermore, in-depth *in silico* analyses were conducted to identify the molecular mechanisms underlying the formation of herbivore-induced volatile bouquets in *S. purpurea* leaves. These analyses led to the identification of a mid-sized TPS gene family in the *S. purpurea* genome. Finally, we functionally characterized eight TPS candidates *in vitro* and *in planta* through heterologous expression in *Escherichia coli* and *Nicotiana benthamiana*, respectively.

Materials and methods

Plant and insect material

Purple willow (*Salix purpurea*, kindly provided by the Max Planck Institute for Chemical Ecology) trees 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 (Klasmann-Deilmann, Geeste, Germany), until they reached about 100 cm in height. Gypsy moth (*Lymantria dispar*) caterpillars were hatched from eggs obtained from Hanna Nadel (US Department of Agriculture, Buzzards Bay, MA, USA) and were reared on an artificial diet (Gypsy moth diet, MP Biomedicals LLC, Illkirch, France). Poplar leaf beetles (*Chrysomela populi*) originated from egg clutches from old-growth black poplar (*Populus nigra*) trees collected in the field (Fabisch *et al.*, 2019)

and were further propagated and cultivated on *P. nigra* trees in the laboratory.

Herbivore treatment

Before the start of the experiment, *L. dispar* caterpillars were fed on poplar leaves for 1 week, and shortly before the start of the experiment, *L. dispar* caterpillars and *C. populi* beetles were starved for 13 h. For the herbivore treatment, four *L. dispar* caterpillars (fourth instar) or five *C. populi* beetles were released on a defined part of the *S. purpurea* trees (region between 5 cm and 30 cm below the apex), enclosed in PET bags, and allowed to feed for 24 h (08.00 h to 08.00 h), with each plant receiving a single herbivore species. On the second day, caterpillars, beetles, and PET bags were removed from the plants to avoid volatile contamination and leaves were enclosed in new PET bags (Bratschlauch, Toppits, Minden, Germany). Volatile collection was then performed from 10.00 h to 16.00 h as described in the section ‘Volatile collection and analysis’. After volatile collection, leaves were cut from the plant, arranged on a flat surface with a size reference for scale, and photographed. The photographs were used to determine herbivore-feeding intensity. All leaves were weighed to determine fresh weight, and chewed and intact leaves were harvested separately. Leaf material was flash-frozen in liquid nitrogen, ground using a chilled mortar and pestle, and divided for further processing: one portion was stored at –80 °C, while the other was lyophilized and stored at room temperature. For the herbivore treatment, only the chewed leaf material was used for phytochemical (salicinoid), phytohormone, and Rt-qPCR analyses.

Volatile collection and analysis

Volatiles were collected using a dynamic push-pull system (Tholl *et al.*, 2006). Air flow was maintained in the system through Teflon tubes. Charcoal-filtered air was pumped into the bags at a flow of 0.8 liters min^{–1}. A portion of the aspirated air (flow: 0.4 liters min^{–1}) was withdrawn with a second pump and passed through a filter packed with 30 mg Poropak Q (ARS, Inc., Gainesville, FL, USA) to adsorb the volatile compounds. Herbivory was allowed for 24 h, after which volatiles were collected for 6 h during the middle of the light period (see section ‘Herbivore treatment’). Seven biological replicates were measured per treatment. After volatile collection, the volatile compounds were desorbed by eluting the filter with 200 µl dichloromethane containing nonyl acetate as an internal standard (10 ng µl^{–1}). Samples were stored at –20 °C until gas chromatography analysis.

Qualitative and quantitative analyses of leaf volatiles were conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (Agilent Technologies, Santa Clara, CA, USA; interface temperature, 250 °C; quadrupole temperature, 150 °C; source temperature, 230 °C; electron energy, 70 eV, electron impact ionization) or a flame ionization detector operated at 300 °C, respectively. The constituents of the volatile bouquet were separated using a ZB5 column (Phenomenex, Aschaffenburg, Germany; 30 m×0.25 mm×0.25 µm) and He (MS) or H₂ (flame ionization detector) as carrier gas. The sample was injected without split at an initial oven temperature of 45 °C. The temperature was held for 2 min and then increased to 180 °C with a gradient of 6 °C min^{–1}, and then further increased to 300 °C with a gradient of 60 °C min^{–1} and a hold of 2 min. Compounds were identified by comparison of retention times and mass spectra to those of authentic standards, by determination of Kovats retention indices or by comparison with reference spectra in the Wiley and National Institute of Standards and Technology (NIST) libraries.

RNA extraction and reverse transcription

Total RNA was isolated from ground fresh plant material using the InviTrap Spin Plant RNA Mini kit (Invitek, Berlin, Germany) according

to manufacturer's instructions. RNA concentration and purity were determined using a spectrophotometer (NanoDrop One, Thermo Fisher Scientific, Waltham, MA, USA). Single stranded cDNA was prepared from 2 µg of RNA using the HiScript III 1st Strand cDNA synthesis kit with implemented DNase-treatment (NeoBiotech, Nanterre France) according to the manufacturer's instructions.

Identification of putative willow terpene synthase genes

To identify putative purple willow *TPS* genes, a BLAST search against the *Salix purpurea* genome database (https://phytozome-next.jgi.doe.gov/info/Spurpurea_v5_1; Zhou *et al.*, 2020) was performed using PtTPS1, 2, 3, 4 (Danner *et al.*, 2011) and PtTPS5–15 (Irmisch *et al.*, 2014; Lackus *et al.*, 2021a) as inquiry sequences. A total of 42 *SpTPS* genes were identified, including full length genes and tentative pseudogenes.

Phylogenetic analysis and amino acid alignment

An alignment of all putative *S. purpurea* *TPS* candidate genes and characterized *TPS* genes from *Populus trichocarpa* (Danner *et al.*, 2011; Irmisch *et al.*, 2014, 2015; Lackus *et al.*, 2018, 2021a) was constructed using the MUSCLE (codon) algorithm (gap open, −2.9; gap extend, 0; hydrophobicity multiplier, 1.2; clustering method, UPGMB algorithm) implemented in the program MEGA11 (Tamura *et al.*, 2021). Tree reconstruction was done with MEGA11 using a Maximum Likelihood algorithm (model/method, Tamura–Nei model; substitutions type, amino acid; rates among sites, uniform rates; gaps/missing data treatment, use all sites; Tamura and Nei, 1993). A bootstrap resampling analysis with 750 replicates was performed to evaluate the tree topology. Amino acid alignments of Sapur.016G273600, Sapur.011G018900, and Sapur.011G019500 (<https://phytozome-next.jgi.doe.gov/>) with the corresponding amplified and cloned candidates SpTPS1-var1/SpTPS1-var2, SpTPS2-var1/SpTPS2-var2, and SpTPS3-var1/SpTPS3-var2, respectively, were performed with MEGA11 (Tamura *et al.*, 2021) and visualized with BioEdit (Hall, 1999).

Cloning of identified candidate genes

Open reading frames of the *SpTPS* candidate genes, Sapur.016G273600 (*SpTPS1*), Sapur.011G018900 (*SpTPS2*), Sapur.011G019500 (*SpTPS3*), Sapur.007G105100 (*SpTPS6*), Sapur.019G015400 (*SpTPS13*), Sapur.004G018300 (*SpTPS15*), Sapur.019G014500 (*SpTPS22*), and Sapur.019G047500 (*SpTPS23*) were amplified from herbivore induced purple willow leaf cDNA, inserted into the *E. coli* expression vector pOPINF, and into the pCAMBIA 2300U vector for heterologous overexpression in *N. benthamiana*. In brief, pOPINF and pCAMBIA2300U vectors were digested with HindIII-HF/KpnI-HF or PacI/Nt.BbvCI (New England Biolabs, Ipswich, MA, USA), respectively. PCR products were amplified using Phusion™ High-Fidelity DNA Polymerase (for pOPINF) and Phusion U Hot Start DNA Polymerase (for pCAMBIA 2300U) (Thermo Fisher Scientific), and inserted into the digested vectors using In-Fusion HD Cloning (Takara Bio Inc., Kusatsu, Shiga, Japan, for pOPINF) or USER cloning (USER enzyme, New England Biolabs, for pCAMBIA 2300U). Based on *in silico* subcellular localization analysis, truncated versions of candidate *SpTPS3* (Δ30), *SpTPS6* (Δ57), and *SpTPS13* (Δ51) were cloned into pOPINF vector for heterologous expression in *E. coli* (Supplementary Table S1). For candidate gene *SpTPS22*, a full-length version (1722 bp) and a shorter version (1677 bp) were both cloned in the pOPINF and pCAMBIA 2300U vector designated as *SpTPS22-fl* and *SpTPS22-tr*, respectively. Oligonucleotide sequence information is given in Supplementary Table S2. The cloned genes were fully sequenced. For the candidate genes *SpTPS1*, *SpTPS2*, and *SpTPS3*, two different sequence variants, presumably alleles, were obtained by PCR amplification and cloning, which are further designated as variants 1 and 2 (var1/var2), respectively (Supplementary Figs S1–S3).

Heterologous expression in *E. coli* and *SpTPS* enzyme assays

The *E. coli* strain BL21 Star™ (DE3) (New England Biolabs) was used for expression of *SpTPS* candidates cloned in the expression vector pOPINF. Cultures were grown at 37 °C, induced at OD₆₀₀=0.6 with 1 mM isopropyl β-D-1-thiogalactopyranoside, subsequently placed at 18 °C and grown for another 16 h. Cells were collected by centrifugation (10 min, 5000 g, 4 °C) and disrupted by sonication (4×30 s; Labsonic®, Sartorius Stedim Biotech SA, France) in chilled extraction buffer [10 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 10% (v/v) glycerol]. Cell fragments were removed by centrifugation (30 min, 15 286 g, 4 °C) and supernatant was desalted by passage through Illustra NAP-5 columns (Cytiva, Marlborough, MA, USA).

Enzymatic activity was determined by enzyme assays containing 50 µl of the bacterial raw extract, 44 µl assay buffer, 10 mM MgCl₂, and 1 µg substrate (GPP, (*E,E*)-FPP or (*E,E,E*)-GGPP, Merck KGaA, Darmstadt, Germany) in a 1.5 ml Teflon-sealed, screw capped glass vial. For enzyme assays with GPP or (*E,E*)-FPP, the reaction mixture was incubated at 30 °C, and a 100 µm polydimethylsiloxane solid-phase microextraction (SPME) fiber (Supelco, Bellefonte, PA, USA) was placed into the vial headspace for 1 h to adsorb volatiles. The fiber was then directly inserted into the gas chromatography (GC)–mass spectrometry (MS) injector for analysis (see ‘Analysis of *in vitro* enzyme products’). Assays with (*E,E,E*)-GGPP were overlaid with 100 µl *n*-hexane and incubated for 1 h at 30 °C, and the *n*-hexane phase was taken and analysed using a GC–MS system (see ‘Analysis of *in vitro* enzyme products’). As negative controls, raw protein extracts from *E. coli* expressing the empty vector pOPINF were tested with the substrates as described above. Additional negative controls were performed as described above, but without protein.

Analysis of *in vitro* enzyme products

In vitro *TPS* enzyme activity was analysed using an Agilent 6890N series gas chromatograph coupled to an Agilent 5975 quadrupole mass selective detector (interface temperature, 250 °C; quadrupole temperature, 150 °C; source temperature, 230 °C; electron energy, 70 eV, electron impact ionization). Compounds were separated using a DB5 column (OPTIMA 5 MS, Macherey–Nagel, Düren, Germany; 30 m×0.25 mm×0.25 µm) and He as carrier gas. For monoterpene analysis, the chromatographic separation was achieved by the following gradient: the temperature was held at 45 °C for 2 min, increased with a gradient of 7 °C min^{−1} to 130 °C, and then further increased to 300 °C with a gradient of 100 °C min^{−1} and a hold of 1 min. For the analysis of sesquiterpene formation, chromatographic separation was achieved using a gradient starting at 80 °C with a hold time of 2 min, followed by an increase to 180 °C at a rate of 7 °C min^{−1}, continued by an increase of 100 °C min^{−1} to 300 °C, and a final hold time of 1 min. To determine diterpene formation, the temperature gradient started at an initial oven temperature of 80 °C for 2 min, followed by an increase to 280 °C at a rate of 5 °C min^{−1}, and then further increased to 320 °C at a rate of 100 °C min^{−1} and a hold time of 2 min.

Transient overexpression of *SpTPS* genes in *N. benthamiana*

For gene expression in *N. benthamiana*, obtained *SpTPS* pCAMBIA 2300U constructs (see ‘Cloning of identified candidate genes’), *eGFP* and *p19::pBIN* constructs (both kindly provided by Dr Tobias G. Köllner; Max–Planck–Institute for Chemical Ecology) were separately transformed into *Agrobacterium tumefaciens* strain GV3101. Liquid cultures of transformed *A. tumefaciens* were grown at 28 °C for 2 d at 200 rpm, after incubation pelleted by centrifugation (4000 g, 14 °C, 20 min), respective pellets resuspended in infection medium (10 mM MES, pH 5.7, 10 mM MgCl₂, 100 µM acetosyringone) to an OD₆₀₀ of 0.4–0.6, and further incubated at room temperature for additional 1–3 h at 200 rpm. For infiltration, equal volumes of the constructs-of-interest

and the *p19::pBIN* vector. *A. tumefaciens* cultures were mixed and infiltrated with a 1 ml syringe into the lower side of all leaves of 3- to 4-week-old *N. benthamiana* plants. Transiently transformed plants were kept for one night in the dark and were then transferred to the greenhouse. Volatile sampling and analysis of the *N. benthamiana* plants is described in the sections 'Analysis of *in vitro* enzyme products' and 'Analysis of *in planta* enzyme products'.

Volatile collection of transiently transformed *N. benthamiana* plants

Volatiles from transiently transformed *N. benthamiana* plants were sampled using a dynamic push-pull system with the settings described above in the section 'Volatile collection and analysis', with the following exceptions: volatiles were collected for 24 h from the fourth to the fifth day after transformation from whole *N. benthamiana* plants packed in PET bags (Bratschlauch, Toppits, Minden, Germany). The soil pots of the *N. benthamiana* plants were previously wrapped in aluminum foil to avoid volatile contamination. After sampling, volatiles were desorbed by eluting the filter with 200 µl of dichloromethane containing nonyl acetate as internal standard (10 ng µl⁻¹). Samples were stored at -20 °C until gas chromatographic analysis. The respective transformed leaf material was harvested and weighted for quantification of emitted volatiles. For each construct combination, three biological replicates were investigated, and each transformation repeated twice.

Analysis of *in planta* enzyme products

The volatile sample was injected without splitting using the GC-MS system, setup and column as described above in the section 'Analysis of *in vitro* enzyme products' and chromatographic parameters used for separation as described in the section 'Volatile collection and analysis'. In addition, to quantify the emitted volatiles from the *N. benthamiana* plants, the samples were measured and analysed using a GC-flame ionization detection (FID) (Agilent 6850 Series II System, Agilent Technologies) system operating at 250 °C with H₂ as carrier gas. Chromatographic separation was performed using a DB5 column (OPTIMA 5 MS, Macherey-Nagel; 30 m×0.25 mm×0.25 µm) and oven parameters as described in 'Volatile collection and analysis'.

Quantitative real-time PCR analysis of *SpTPS* expression

For quantitative real-time PCR (RT-qPCR) analysis, cDNA was prepared as described above and diluted 1:2 with water. Oligonucleotides for amplification of respective *SpTPS* candidates were designed to have a *T_m* of approximately 60 °C, a GC content between 40% and 60%, a length of 19–25 nt, and a fragment length between 110 and 250 bp (Supplementary Table S2). Specificity of the oligonucleotides was confirmed by agarose gel electrophoresis, melting curve analysis, standard curve analysis, and sequence verification of cloned PCR amplicons. Due to the high sequence similarity, only one oligonucleotide pair was used for the amplification of *SpTPS22* and *SpTPS23*. *Ubiquitin (UBQ)*, *actin*, *elongation factor1 α (EF1α)*, *histone superfamily protein H3 (HIS)*, and *tubulin* were tested as reference genes (Ramírez-Carvajal *et al.*, 2008; Xu *et al.*, 2011; Wang *et al.*, 2014; Zhang *et al.*, 2017). Comparison of Δ*C_q* values and the corresponding SD revealed *EF1α* as the most suitable reference gene for expression analysis (Supplementary Table S3). The following PCR conditions were used: initial incubation at 95 °C for 15 min followed by 45 cycles of amplification (95 °C for 15 s, 55 °C for 20 s, 72 °C for 20 s). 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 (0.2 °C increment). All samples were run on the Bio-Rad CFX Duet Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) in an optical 96-well plate. Three biological replicates were run in duplicates using the Absolute

QPCR SYBR Capillary Mix (Thermo Fisher Scientific) for each of the three treatments.

Quantification of jasmonic acid, jasmonic acid isoleucine conjugates, 12-oxophytodienoic acid, abscisic acid, and salicylic acid

For the quantification of jasmonic acid (JA), JA isoleucine conjugates (JA-Ile), 12-oxophytodienoic acid (OPDA), abscisic acid (ABA), and salicylic acid (SA), 100 mg of lyophilized *S. purpurea* leaf material was used and extracted with 950 µl of ethyl acetate: formic acid (99:1; v/v) containing additional 50 µl of internal standard [dihydro-JA, [¹⁸O₂]-OPDA, JA-norvaline conjugate, [D₄]-SA, and [D₆]-ABA; 0.1 ng/µl each in acetonitrile (Supelco)]. Samples were homogenized in a ball mill (3 min, 20 Hz), centrifuged (20 min, 20 817 g), and the supernatant taken. The supernatant was dried (45 °C; 252 g) and the remaining pellet dissolved in 40 µl 1:1 acetonitrile:water (v/v) (Supelco). Upon further centrifugation (10 min, 20 817 g) the supernatant was taken and analysed by ultra performance liquid chromatography (UPLC)–electrospray ionization–tandem mass spectrometry (MS/MS) using a Waters (Milford, MA, USA) Acquity I-Class ultra-high-performance liquid chromatography system coupled to an AB Sciex 6500+ QTRAP® tandem mass spectrometer (AB Sciex, Framingham, MA, USA). Separation was achieved on an Acquity UPLC BEH RP C18 column (2.1×50 mm, 1.7 µm particle size, Waters). Formic acid (0.1%) (Biosolve B.V., Valkenswaard, The Netherlands) in water (v/v) and acetonitrile (Supelco) were employed as mobile phases A and B, respectively. The elution profile was: 0–7 min, 3–100% B; 7–9 min 100% B; 9–12 min 3% B. The mobile phase flow rate was 0.25 ml min⁻¹, and the column temperature maintained at 40 °C. The ion spray voltage was maintained at 4.0 kV in a negative ionization mode, curtain gas was kept at 30 psi and nebulizer gas (GS1) and drying gas (GS2) were adjusted to 50 and 80 psi, respectively, at a temperature of 600 °C. Selected reaction monitoring was used to monitor analyte parent ion→product ion formation as follows: *m/z* 137→93 [collision energy (CE) -22 V, declustering potential (DP) -25 V] for salicylic acid; *m/z* 141→97 (CE -22 V, DP -10 V) for D₄-salicylic acid; *m/z* 263→153 (CE -14 V, DP -40 V) for ABA; *m/z* 269→159 (CE -14 V, DP -25 V) for D₆-abscisic acid; *m/z* 209→59 (CE -14 V, DP -35 V) for JA; *m/z* 322→130 (CE -38 V, DP -55 V) for JA-Ile; *m/z* 211→59 (CE -16 V, DP -35 V) for dihydro-JA; *m/z* 308→116 (CE -26 V, DP -60 V) for JA-norvaline conjugate; *m/z* 291→165 (CE -26 V, DP -85 V) for 12-OPDA; *m/z* 295→165 (CE -26 V, DP -55 V) for [1,1-¹⁸O₂] 12-oxo-phytyldienoic acid.

Methanol-extraction and salicinoid analysis

Metabolites were extracted from 10 mg of freeze-dried plant material using 1 ml of 100% methanol (Supelco) containing 0.8 mg ml⁻¹ phenyl-β-D-glucopyranoside (Sigma-Aldrich, St Louis, MO, USA) as an internal standard. Samples were shaken for 30 min at 1200 rpm on a ThermoMixer® C (Eppendorf, Hamburg, Germany) and centrifuged. The supernatant was diluted 1:1 (v/v) with water and analysed by high-performance liquid chromatography coupled with UV detection (HPLC-UV; 200 nm; Agilent Technologies). Chromatographic separation was performed on a NUCLEODUR Sphinx RP column (5 µm, 4.6×250 mm; Macherey-Nagel) at 25 °C. Water (eluent A) and acetonitrile (eluent B, Supelco) served as mobile phases at a flow rate of 1 ml min⁻¹. The gradient started at 14% B, increased linearly to 58% B over 22 min, then to 100% B for 3 min, followed by re-equilibration to starting conditions for 5 min. Compound quantification was performed by determining response factors for salicin (Alfa Aesar GmbH & Co. KG, Karlsruhe, Germany), salicortin (PhytoLab GmbH & Co. KG, Vestenbergreuth, Germany), and tremulacin (PhytoLab) relative to the internal standard phenyl-β-D-glucopyranoside.

Chemicals

Chemicals used in this study were purchased from Carl Roth GmbH +Co. KG (Karlsruhe, Germany) or Merck KGaA (Darmstadt, Germany), unless otherwise indicated. Details of suppliers for analytical standards are provided in [Supplementary Table S4](#).

Statistical analysis

Throughout the article, data are presented as means $\pm$ standard errors (SE). Statistical analysis was done with RStudio (version 2023.12.1-402, R Development Core Team, <http://R-project.org>). One-way ANOVA was conducted to determine statistically significant differences between control, *L. dispar*-, and *C. populi*-treated *S. purpurea* leaves. Whenever necessary, the data were log-transformed to meet statistical assumptions such as normality and homogeneity of variances. In cases where the statistical assumptions could not be fulfilled, nonparametric one-way ANOVA on ranks (Kruskal–Wallis test) analyses were conducted. Post-hoc multiple comparisons were performed using Tukey's HSD test for parametric analyses or Dunn's test with Bonferroni correction for nonparametric analyses. The statistical analyses performed are reported for each dataset in the corresponding figure and table legends or within the tables themselves, including global test statistics (F or χ^2), degrees of freedom, and P -values.

Accession numbers

Sequence data for *SpTPS1-var1* (PX656970), *SpTPS1-var2* (PX656971), *SpTPS2-var1* (PX656972), *SpTPS2-var2* (PX656973), *SpTPS3-var1-tr* (PX656974), *SpTPS3-var2-tr* (PX656975), *SpTPS3-var2-fl* (PX656976), *SpTPS6-fl* (PX656977), *SpTPS6-tr* (PX656978), *SpTPS13-fl* (PX656979), *SpTPS13-tr* (PX656980), *SpTPS15* (PX656981), *SpTPS22-fl* (PX656982), *SpTPS22-tr* (PX656983), and *SpTPS23* (PX656984) can be found in the NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under the corresponding identifiers.

Results

Herbivory by *L. dispar* caterpillars or *C. populi* beetles on *S. purpurea* leaves induces the emission of a complex volatile bouquet, but has only a minimal effect on salicinoid accumulation

To investigate how herbivory shapes induced defenses within the *Salicaceae*, we analysed the chemical profile of purple willow (*Salix purpurea*) leaves following feeding by the generalist *L. dispar* caterpillars and the specialist *C. populi* beetles. Volatile compounds were collected and analysed using GC–MS and FID to characterize the herbivore-induced volatile profiles. The analysis identified a total of 28 compounds in the herbivore-induced volatile blends, including terpenes (both mono- and sesquiterpenes), N-containing compounds, and aromatic compounds ([Fig. 1](#); [Supplementary Table S5](#)). Among the most abundant herbivore-induced volatiles were the monoterpene (*E*)- β -ocimene and the sesquiterpenes germacrene D and (*E,E*)- α -farnesene for both investigated herbivore-treatments ([Fig. 1](#); [Supplementary Table S5](#)). In contrast, most compounds induced by herbivory were absent from the volatile blend of the corresponding control leaves ([Fig. 1](#); [Supplementary Table S5](#)). Structurally the group of the N-containing compounds encompasses a diverse range of

compounds including aldoximes, nitriles, and amines such as 2-methylbutyraldoxime, benzyl cyanide, and indole, respectively ([Fig. 1](#); [Supplementary Table S5](#)). The group of aromatic compounds consisted mainly of phenylpropanoid-related compounds such as 2-phenylethanol ([Fig. 1](#); [Supplementary Table S5](#)). Interestingly, the composition of the herbivore-induced volatile blends differed mainly in quantity rather than quality between the two herbivore treatments ([Fig. 1](#); [Supplementary Table S5](#)). This quantitative variation may be attributed to the lower feeding intensity of *C. populi* beetles compared with *L. dispar* caterpillars on *S. purpurea* leaves ([Supplementary Fig. S4](#)).

In addition to analysing herbivore-induced volatile defense responses, the accumulation of the non-volatile salicinoids was assessed using HPLC–UV. The analysis revealed a significantly increased accumulation of the structurally simplest salicinoid, salicin, in response to herbivory by both *L. dispar* caterpillars and *C. populi* beetles ([Supplementary Fig. S5](#)). In contrast, the accumulation levels of the complex salicinoids salicortin and tremulacin remained unaffected by herbivory, regardless of the feeding herbivore ([Supplementary Fig. S5](#)).

Accumulation of jasmonates and salicylic acid is induced upon herbivory in *S. purpurea* leaves

Accumulation of jasmonates, together with ABA and SA, was quantified in *S. purpurea* leaves by LC–MS/MS to assess herbivory-induced changes. Herbivory of *L. dispar* caterpillars resulted in a significantly increased accumulation of all measured analytes, including jasmonates, ABA, and SA, in comparison with the respective control leaves ([Fig. 2](#)). The strongest induction was observed for the jasmonates upon *L. dispar* herbivory, with OPDA, JA, and JA-Ile exhibiting 40-, 56-, and 130-fold increases, respectively, relative to control leaves ([Fig. 2](#)). In contrast, herbivory by *C. populi* beetles induced a less pronounced response. Among the measured metabolites, only OPDA and SA showed statistically significant increases, whereas the levels of JA, JA-Ile, and ABA were elevated upon herbivory but did not reach statistical significance ([Fig. 2](#)).

In silico analyses revealed the presence of a mid-sized terpene synthase gene family in *S. purpurea*

To elucidate the molecular mechanisms underlying volatile terpene formation in *Salix purpurea* leaves, we aimed to identify and characterize members of the TPS gene family. Thus, BLAST analyses were performed against the *S. purpurea* genome (https://phytozome-next.jgi.doe.gov/info/Spurpurea_v5_1) using previously characterized TPS sequences from black cottonwood (*Populus trichocarpa*) as queries ([Danner et al., 2011](#); [Irmisch et al., 2014](#)). This analysis resulted in the identification of 42 putative TPS genes within the *S. purpurea* genome. Among these, 34 genes were classified as full-length

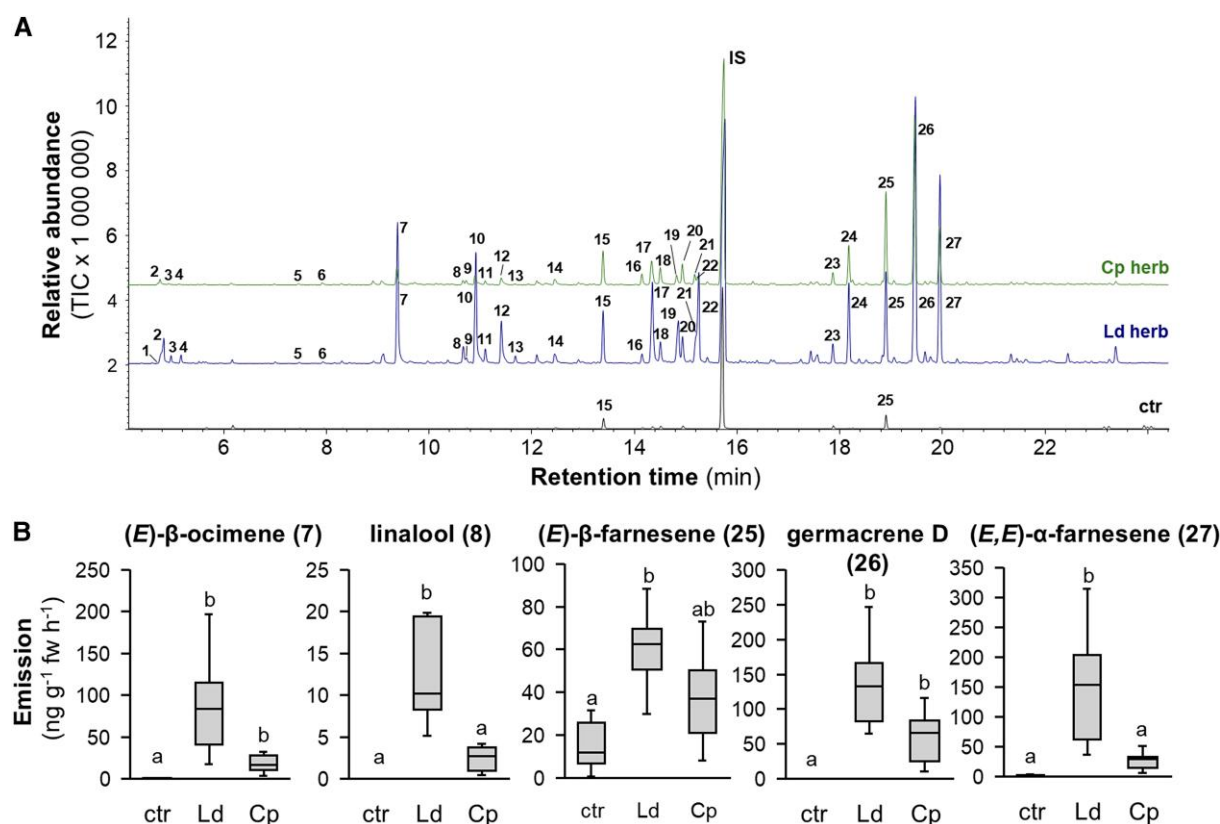


Fig. 1. Volatiles emitted by undamaged (ctr), gypsy moth caterpillar (*Lymantria dispar*)-damaged (Ld) or poplar leaf beetle (*Chrysomela populi*)-damaged (Cp) purple willow (*Salix purpurea*) leaves. (A) Volatile profiles of control and herbivore-damaged *S. purpurea* leaves. (B) quantification of selected terpenes. Volatile compounds were collected using a push–pull system and subsequently analysed and quantified by gas chromatography–mass spectrometry and gas chromatography–flame ionization detection, respectively. Box plots show median, interquartile range, and outliers ($n=7$ biological replicates). 1, (*E*)-2-hexanal; 2, 2- and 3-methylbutyraldoxime isomers 1; 3, 2-methylbutyraldoxime isomer 2; 4, 3-methylbutyraldoxime isomer 2; 5, sabinene; 6, β-pinene; 7, (*E*)-β-ocimene*; 8, linalool*; 9, nonanal; 10, 2-phenylethanol; 11, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 12, benzyl cyanide; 13, epoxy-ocimene; 14, nonanol; 15, octyl acetate; 16, unidentified compound 1; 17, phenylacetaldoxime isomer 1; 18, ethylacetophenone isomer 1; 19, phenylacetaldoxime isomer 2; 20, ethylacetophenone isomer 2; 21, indole; 22, 2-phenylnitroethane; 23, unidentified compound 2; 24, (*E*)-β-caryophyllene; 25, (*E*)-β-farnesene; 26, germacrene D; 27, (*E,E*)-α-farnesene. Compounds marked with '*' were identified by comparison of retention time and mass spectrum to those of authentic standards. Other compounds were identified by database comparisons. Differences between treatments were analysed by a one-way ANOVA and a post-hoc test (Tukey test) or Kruskal–Wallis and a post-hoc test (Dunn's test). Different letters indicate significant differences ($P<0.05$). Details on statistical analysis are given in [Supplementary Table S5](#). fw, fresh weight; IS, internal standard; TIC, total ion count.

(f) *TPS* genes, each encoding a putative functional enzyme with a minimum sequence length of 520 amino acids. To gain further insights into the evolutionary relationships of the *S. purpurea* *TPS* family, a phylogenetic analysis was conducted including all identified *SpTPS* candidates and the described *TPS* family members from *P. trichocarpa* (Fig. 3; Danner *et al.*, 2011; Irmisch *et al.*, 2014, 2015; Lackus *et al.*, 2018). The majority of *SpTPS* sequences clustered within the *TPS*-a subfamily, comprising putative sesquiterpene synthases (STS) with 18 members, and the *TPS*-b subfamily, comprising putative monoterpene synthases (MTS) with 14 members. Only a few candidates were assigned to the *TPS*-c, -e, and -f subfamilies, which typically include copalyl diphosphate synthases, kaurene synthases, and other diterpene synthases (DTS) (Fig. 3). Additionally, five *SpTPS* candidates clustered within the *TPS*-g

subfamily (Fig. 3). Regarding their genomic localization, a notable enrichment of *SpTPS* genes was observed on chromosome 19, which contains 15 candidates, and chromosome 16, which contains six candidates (Supplementary Fig. S6). The remaining *SpTPS* genes were more evenly distributed across several chromosomes, including chromosomes 2, 4, 5, 7, 8, 13, 15, and 17, whereas one tentative *SpTPS* gene could not yet be assigned to a specific chromosomal location.

Isolation and structural *in silico* characterization of *S. purpurea* terpene synthase

To explore the molecular basis of herbivore-induced terpene formation in *S. purpurea* leaves, we isolated candidate genes likely involved in terpene biosynthesis. Analysis was restricted to

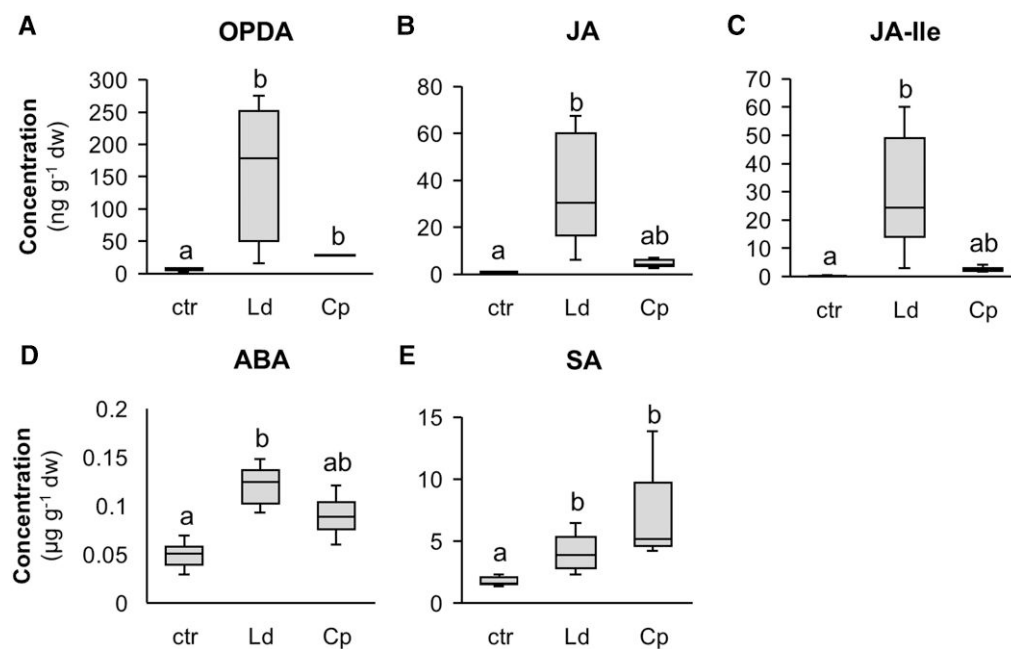


Fig. 2. Phytohormone content in undamaged (ctr), gypsy moth caterpillar (*Lymantria dispar*)-damaged (Ld), and poplar leaf beetle (*Chrysomela populi*)-damaged (Cp) purple willow (*Salix purpurea*) leaves. Compounds were extracted with ethyl acetate: formic acid (v:v; 99:1) from lyophilized plant material and analysed using liquid chromatography–tandem mass spectrometry. Box plots show median, interquartile range, and outliers ($n=7$ biological replicates). Differences between treatments were analysed by a Kruskal–Wallis and a post-hoc test (Dunn’s test). Different letters indicate significant differences ($P<0.05$). JA ($\chi^2=16.794$, $df=2$, $P=0.00023$), OPDA ($\chi^2=15.384$, $df=2$, $P=0.00046$), JA-Ile ($\chi^2=17.121$, $df=2$, $P=0.00019$), ABA ($\chi^2=10.338$, $df=2$, $P=0.00569$), SA ($\chi^2=14.924$, $df=2$, $P=0.00057$). ABA, abscisic acid; df, degrees of freedom; dw, dry weight; JA, jasmonic acid; JA-Ile, jasmonic acid isoleucine conjugates; OPDA, 12-oxo-phytodienoic acid; SA, salicylic acid.

full-length MTS and STS from the TPS-a, TPS-b, and TPS-g subfamilies, while DTS was excluded. For gene isolation, cDNA was synthesized from *S. purpurea* leaves treated with *L. dispar* and *C. populi*, and the open reading frames of eight candidate TPS genes were successfully amplified and cloned. Based on their phylogenetic relationship with previously characterized *P. trichocarpa* TPS enzymes, the poplar TPS nomenclature was adopted, and the *S. purpurea* candidates were designated as their respective orthologues. For *S. purpurea* TPS candidates lacking identifiable orthologues, nomenclature was extended by continuing the *P. trichocarpa* numbering scheme beyond *PrTPS21* (Lackus *et al.*, 2018). Accordingly, three genes of the TPS-b subfamily were identified as *SpTPS2* (*Sapur.011G018900*), *SpTPS6* (*Sapur.007G105100*), and *SpTPS13* (*Sapur.019G015400*); two genes of the TPS-g subfamily as *SpTPS3* (*Sapur.011G019500*) and *SpTPS15* (*Sapur.004G018300*); and three genes of the TPS-a subfamily as *SpTPS1* (*Sapur.016G273600*), *SpTPS22* (*Sapur.019G014500*), and *SpTPS23* (*Sapur.019G047500*). Amplification and cloning of *SpTPS1*, *SpTPS2*, and *SpTPS3* each yielded two sequence variants, which were considered allelic versions of the respective genes. These variants were designated as *SpTPS1-var1/-var2*, *SpTPS2-var1/-var2*, and *SpTPS3-var1/-var2*, and both alleles were included in further *in vitro* characterizations. Amino acid sequence alignments revealed sequence identities of 98.6%,

99.7%, and 99.3% between the corresponding variants of *SpTPS1*, *SpTPS2*, and *SpTPS3*, respectively (Supplementary Figs S1–S3).

To gain further insight into the putative functionality of the identified TPS candidates, additional *in silico* analyses were conducted to assess the presence of conserved catalytic motifs and to predict subcellular localization. Signal peptide predictions were performed using the TargetP 2.0 server (<https://services.healthtech.dtu.dk/services/TargetP-2.0/>; Almagro Armenteros *et al.*, 2019). Typically, MTS and DTS enzymes harbor N-terminal signal peptides directing them to the plastids, whereas STS enzymes, generally localized in the cytosol, lack such sequences (Bohlmann *et al.*, 1998; Chen *et al.*, 2011; Dudareva *et al.*, 2013). Consistent with this, plastidial targeting peptides were predicted for *SpTPS3*, *SpTPS6*, and *SpTPS13*, supporting their proposed function as MTSs (Supplementary Table S1). In contrast, no signal peptides were predicted for *SpTPS1*, *SpTPS2*, *SpTPS15*, and *SpTPS23*, suggesting cytosolic localization and a likely role as STSs (Supplementary Table S1). For *SpTPS22*, signal peptide prediction was slightly ambiguous, with a cytosolic localization likelihood of only 0.85 (Supplementary Table S1). Moreover, further *in silico* analysis confirmed the presence of several conserved motifs essential for TPS enzymatic activity in the respective candidates. All candidates contained the aspartate-rich DDxxD

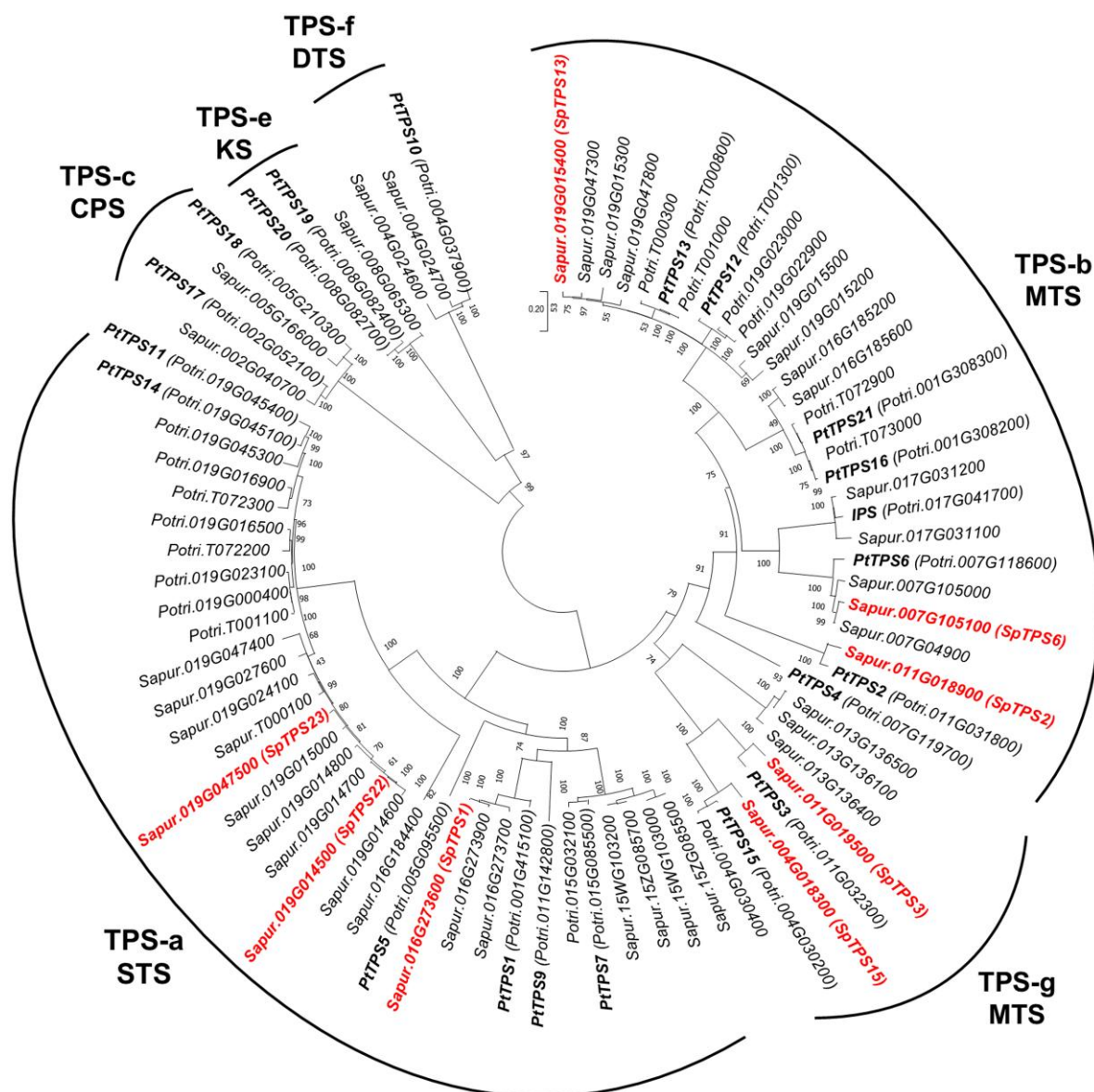


Fig. 3. Phylogenetic analysis of putative purple willow (*Salix purpurea*, Sp) terpene synthases (TPS), and TPS from black cottonwood (*Populus trichocarpa*, Pt). The tree was inferred by using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993) implemented in MEGA11 (Tamura et al., 2021). Bootstrap values ($n=750$) 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 first+second+third+noncoding. SpTPS candidate genes further considered in this study are highlighted in red. PtTPS in bold have previously been described in (Danner et al., 2011; Irmisch et al., 2014, 2015; Lackus et al., 2018, 2021a, b). CDS, copalyl diphosphate synthase; DTS, diterpene synthase; IPS, isoprene synthase; KS, kaurene synthase; MTS, monoterpene synthase; STS, sesquiterpene synthase. TPS-a to h represent TPS subfamilies (Chen et al., 2011).

motif and the NSE/DTE motif, both critical for metal cofactor binding (Chen et al., 2011; Bergman and Dudareva, 2024). For SpTPS22 and SpTPS23, however, the conserved serine/threonine residue of the NSE/DTE motif was replaced by glycine (Supplementary Table S1). Moreover, all SpTPS candidates possess the RxR motif, which facilitates pyrophosphate complexation, though in SpTPS3 and SpTPS15 the second arginine was substituted by glutamine (Supplementary

Table S1). In addition, all SpTPS candidates possess the conserved RR_xW motif, except for SpTPS3 and SpTPS15 (Supplementary Table S1).

Herbivory induces expression of SpTPS genes in *S. purpurea* leaves

To further elucidate the contribution of the isolated SpTPS candidates to the herbivore-induced volatile bouquet, gene

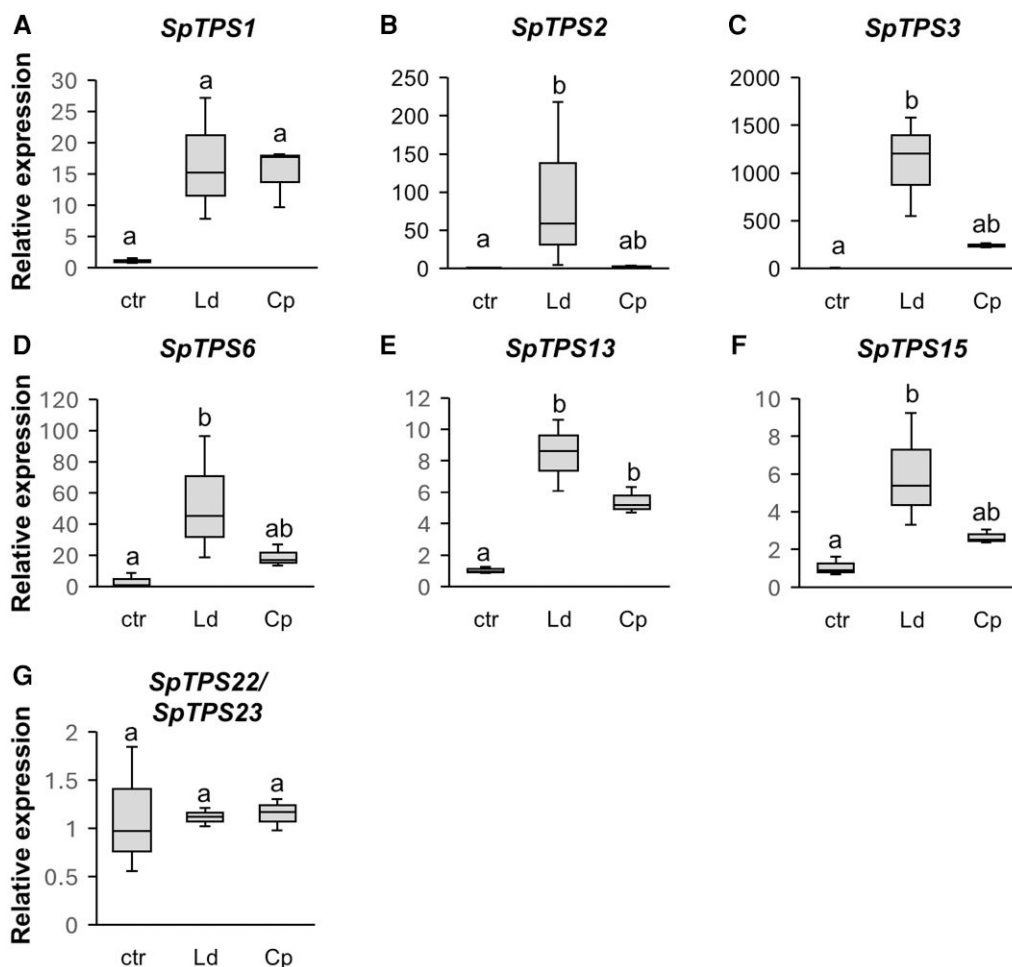


Fig. 4. Gene expression of purple willow (*Salix purpurea*, Sp) candidate terpene synthases (*SpTPS*) in undamaged (ctr), gypsy moth caterpillar (*Lymantria dispar*)-damaged (Ld), and poplar leaf beetle (*Chrysomela populi*)-damaged (Cp) purple willow (*Salix purpurea*) leaves. Gene expression was determined by RT-qPCR with *elongation factor 1- α* (Xu et al., 2011) as housekeeping gene. Box plots show median, interquartile range, and outliers ($n=3$ biological replicates). Differences between treatments were analysed by a one-way ANOVA and a post-hoc test (Tukey test) or a Kruskal–Wallis and a post-hoc test (Dunn's test). Different letters indicate significant differences ($P<0.05$). *SpTPS1* ($F=5.664$, $df=2$, $P=0.04150$), *SpTPS2* ($\chi^2=7.200$, $df=2$, $P=0.02732$), *SpTPS3* ($\chi^2=7.200$, $df=2$, $P=0.02732$), *SpTPS6* ($\chi^2=6.489$, $df=2$, $P=0.03899$), *SpTPS13* ($F=21.250$, $df=2$, $P=0.00189$), *SpTPS15* ($F=6.030$, $df=2$, $P=0.03670$), *SpTPS22/23* ($F=0.006$, $df=2$, $P=0.99400$). df, degrees of freedom.

expression analyses were performed using RT-qPCR, comparing relative transcript levels in *S. purpurea* leaves upon *L. dispar* or *C. populi* herbivory with those in untreated control leaves. For the isolated candidates *SpTPS2*, *SpTPS3*, *SpTPS6*, *SpTPS13*, and *SpTPS15*, feeding by *L. dispar* caterpillars resulted in a significant up-regulation of gene expression compared with the respective controls (Fig. 4B–F). Among these, *SpTPS3* exhibited the strongest induction, with transcript levels increasing approximately 650-fold, followed by *SpTPS6*, which showed a 17-fold increase upon *L. dispar* herbivory (Fig. 4C, D). In contrast, feeding by *C. populi* beetles led to a less pronounced up-regulation of the same *SpTPS* genes (Fig. 4C, D). This expression pattern mirrored the quantitative differences in herbivore-induced volatile emissions and the extent of feeding damage observed between the two herbivore species (Fig. 4; Supplementary Fig. S4). Further RT-qPCR

analysis of *SpTPS1* revealed a strong trend toward increased expression in response to both *L. dispar* and *C. populi* herbivory; however, the induction did not reach statistical significance (Fig. 4A). Due to the high sequence similarity between *SpTPS22* and *SpTPS23*, gene-specific primers could not be designed, and a shared primer pair targeting both genes was used. RT-qPCR analysis showed no herbivore-induced change in the combined expression pattern of these two *TPS* candidates (Fig. 4G).

In vitro functional characterization reveals terpene synthase activity of *SpTPS* candidates

For functional characterization of the *SpTPS* candidates, the isolated genes were heterologously expressed in *E. coli*. Based on the *in silico* predicted N-terminal signal peptides

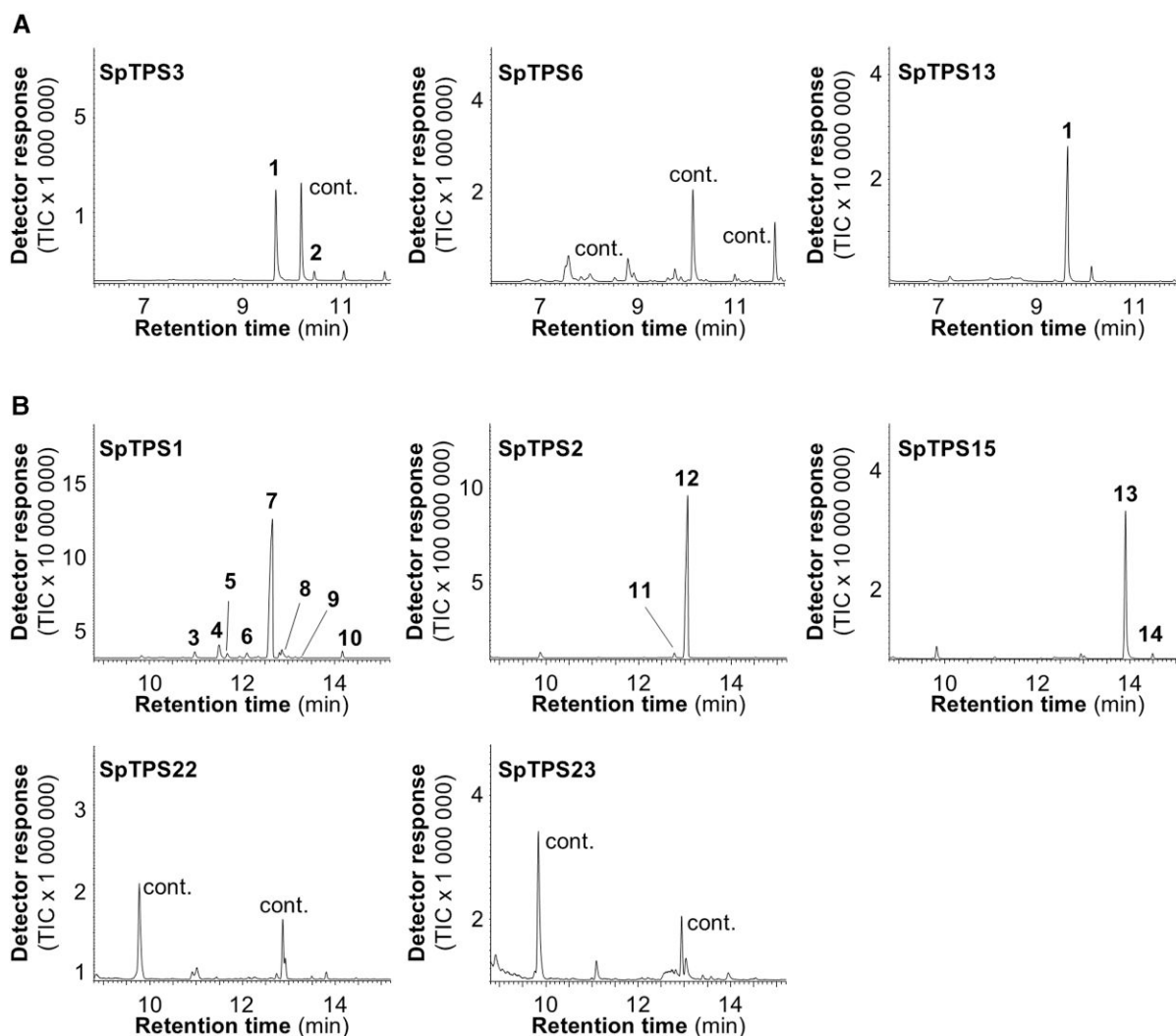


Fig. 5. *In vitro* enzymatic characterization of purple willow (*Salix purpurea*) candidate terpene synthases (SpTPS). The enzymes were heterologously expressed in *Escherichia coli*, extracted, partially purified, and incubated with the substrates geranyl diphosphate (GPP) and (*E,E*)-farnesyl diphosphate ((*E,E*)-FPP) and the cofactor Mg^{2+} . Products were collected with a solid-phase microextraction (SPME) fiber and analysed by gas chromatography–mass spectrometry. (A) Monoterpene products with substrate GPP. (B) Sesquiterpene products with substrate (*E,E*)-FPP. 1, linalool*; 2, unidentified monoterpene 1; 3, β -elemene; 4, calarene; 5, epi-bicyclosesquiphellandrene; 6, unidentified sesquiterpene 1; 7, germacrene D*; 8, bicyclogermacrene; 9, δ -cadinene; 10, unidentified sesquiterpene alcohol 1; 11, α -bergamotene; 12, (*E,E*)- α -farnesene*; 13, nerolidol; 14, unidentified sesquiterpene 2; cont., contamination. Compounds marked with "*" were identified by comparison of retention time and mass spectrum to those of authentic standards. Other compounds were identified by database comparisons. TIC, total ion count.

(Supplementary Table S1), truncated constructs were generated for *SpTPS3* ($\Delta 30$ nt), *SpTPS6* ($\Delta 57$ nt), and *SpTPS13* ($\Delta 51$ nt), whereas the remaining candidates were expressed as full-length constructs. For *SpTPS22*, both a full-length (*SpTPS22-fl*) and a truncated version (*SpTPS22-tr*, $\Delta 45$ nt) were expressed. The heterologously expressed proteins were partially purified, and their enzymatic activities were tested using GPP, (*E,E*)-FPP, and (*E,E,E*)-GGPP as substrates for MTS, STS, and DTS activities, respectively, with Mg^{2+} as cofactor.

SpTPS1-var1, predicted to function as an STS, catalysed the formation of multiple sesquiterpenes from (*E,E*)-FPP *in vitro*,

with germacrene D as the main product (Fig. 5B). Interestingly, it also converted GPP into six monoterpenes, with myrcene, limonene, and α -terpinolene being the most abundant (Supplementary Fig. S7A). The *SpTPS1-var2* variant displayed a similar product profile with both substrates, confirming that both alleles encode enzymes with comparable functional activity (Fig. 5B; Supplementary Fig. S8A). No activity was detected with (*E,E,E*)-GGPP as substrate for either *SpTPS1-var1* or *SpTPS1-var2* (Supplementary Figs S8A, S9). Similarly, *SpTPS2-var1* and *SpTPS2-var2* both acted as STS enzymes, producing (*E,E*)- α -farnesene as the major product and α -bergamotene as a minor product with (*E,E*)-FPP as

substrate (Fig. 5B). Both variants showed only low MTS activity with GPP, forming small amounts of (*E*)- β -ocimene, and possessed no detectable activity with (*E,E,E*)-GGPP (Supplementary Figs S7A, S8B, S9). SpTPS3-var1/var2, SpTPS13, and SpTPS15 exhibited comparable activity with GPP and (*E,E*)-FPP, producing the monoterpene alcohol linalool and the sesquiterpene alcohol nerolidol as the predominant products, respectively (Fig. 5A, B; Supplementary Figs S7A, B, S8C). A slight difference was observed for SpTPS13, which additionally produced minor amounts of (*E*)- β -farnesene from (*E,E*)-FPP (Supplementary Fig. S7B). None of these candidates showed detectable activity with (*E,E,E*)-GGPP (Supplementary Fig. S9). Moreover, no enzymatic activity was detected for SpTPS6, SpTPS22-fl, SpTPS22-tr, or SpTPS23 with any of the tested substrates (Fig. 5A, B; Supplementary Figs S7A, B, S9). Subsequent SDS-PAGE analysis revealed that these candidates were not expressed in *E. coli*, providing a likely explanation for the lack of enzymatic activity (Supplementary Fig. S10). Notably, substitution of the cofactor Mg²⁺ with Mn²⁺ did not alter the product profiles of the TPS enzymes SpTPS1-var1, SpTPS2-var1, SpTPS13, and SpTPS15, whereas no product formation was detected for SpTPS3-var1 in the presence of Mn²⁺ (Supplementary Fig. S11). Negative controls, including assays with protein extracts from empty-vector *E. coli* and enzyme-free reactions, showed no formation of mono-, sesqui-, or diterpenes (Supplementary Fig. S12A, B).

Transient expression of *SpTPS* genes in *N. benthamiana* results in terpene formation *in planta*

To assess the *in planta* functional activity of the isolated *SpTPS* candidates, full-length constructs were heterologously expressed in *N. benthamiana* leaves through *Agrobacterium tumefaciens*-mediated transient transformation. For *SpTPS1*, *SpTPS2*, and *SpTPS3*, only the respective *var-2* forms were expressed. Expression was enhanced by co-infiltration with the p19 silencing suppressor, and an *eGFP*-expressing construct served as a negative control. Volatile compounds emitted from the transformed leaves were subsequently collected and analysed by GC-MS and GC-FID to characterize the terpene profiles produced *in planta*.

To characterize the volatile profiles associated with the *SpTPS* candidate genes, *N. benthamiana* plants were transiently transformed with all eight full-length *SpTPS* constructs. Analysis of the resulting volatile bouquets revealed a total emission of three monoterpenes and six sesquiterpenes (Fig. 6A). To determine the specific product spectrum of each enzyme, individual *SpTPS* constructs were expressed separately (Fig. 6B). Overexpression of *SpTPS1-var2* resulted amongst others in the emission of the sesquiterpenes germacrene D and (*E*)- β -caryophyllene, whereas *SpTPS2-var2* expression led mainly to the formation of (*E,E*)- α -farnesene (Fig. 6B; Supplementary Fig. S13). Transient expression of *SpTPS3-var2*, *SpTPS13*, and *SpTPS15* resulted in linalool emission;

additionally, *SpTPS15*-expressing plants released comparable amounts of the sesquiterpene alcohol nerolidol (Fig. 6B). These *in planta* results for SpTPS1-var2, SpTPS2-var2, SpTPS3-var2, SpTPS13, and SpTPS15 were consistent with the enzyme activities determined *in vitro* (Figs 5A, B, 6B). For SpTPS6, SpTPS22-tr, and SpTPS23, which showed no detectable activity *in vitro*, volatile emissions were nevertheless observed *in planta*. *SpTPS6*-overexpressing plants produced the monoterpene (*E*)- β -ocimene and smaller amounts of its isomer (*Z*)- β -ocimene (Fig. 6B). Overexpression of *SpTPS22-tr* or *SpTPS22-fl* resulted in the emission of (*E*)- β -caryophyllene and β -elemene, the latter likely arising as a thermal rearrangement product of germacrene A during GC injection (Fig. 6B; Supplementary Fig. S13; de Kraker *et al.*, 1998). Moreover, *SpTPS23* overexpression led to the formation of a so far unidentified sesquiterpene alcohol within the volatile bouquet (Fig. 6B; Supplementary Fig. S14).

Discussion

This study aimed to elucidate the defense response of the long-lived woody species *Salix purpurea* (*Salicaceae*) to herbivory. Comparative analysis of volatile emissions from herbivore-damaged (*L. dispar* or *C. populi*) and untreated leaves revealed that herbivory triggers the release of diverse mono- and sesquiterpenes. To uncover the molecular basis of this response, we depicted the *TPS* gene family in *S. purpurea*. Several *SpTPS* candidates were identified and functionally validated *in vitro*, while heterologous expression in *N. benthamiana* reconstituted major components of the *S. purpurea* herbivore-induced terpenoid blend. These findings suggest that the characterized *SpTPS* enzymes might play a central role in the volatile defense response of *S. purpurea* to *L. dispar* or *C. populi* herbivory.

Volatile terpenoids are major compounds of the herbivore-induced volatile bouquet and might play a role as defense compounds

In response to herbivory, plants emit a complex blend of volatile organic compounds comprising nitrogen-containing compounds, green leaf volatiles, aromatic and phenylpropanoid-derived compounds, and, most prominently, terpenoids (Dudareva *et al.*, 2006, 2013; Mumm and Dicke, 2010). This pattern is also observed in the *Salicaceae* family, where complex volatile blends are emitted in response to herbivory, as reported for black poplar (*P. nigra*), black cottonwood (*P. trichocarpa*), and various willow species (Peacock *et al.*, 2001; Danner *et al.*, 2011; Fabisch *et al.*, 2019; Lackus *et al.*, 2021b).

In *S. purpurea*, the herbivore-induced volatile bouquet was dominated by terpenes, accompanied by minor amounts of nitrogen-containing and phenylpropanoid-related compounds (Fig. 1A; Supplementary Table S5). Among the terpenoids, (*E*)- β -ocimene represented the most abundant monoterpene, while (*E,E*)- α -farnesene and germacrene D were the predominant

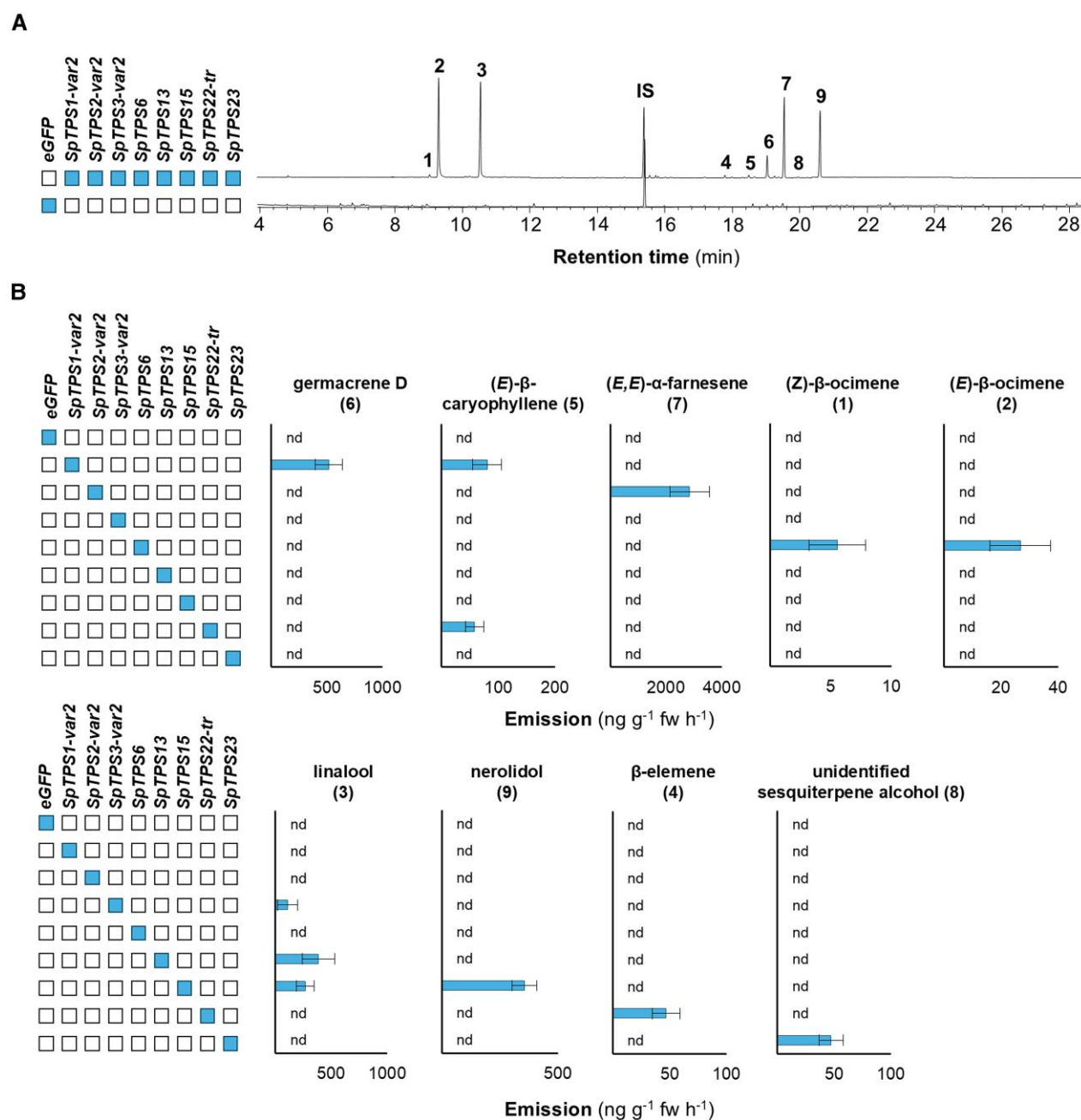


Fig. 6. Volatile emissions from *Nicotiana benthamiana* leaves transiently overexpressing *S. purpurea* candidate terpene synthases (*SpTPS*). (A) Volatile bouquet of transiently transformed tobacco plants. (B) Volatile emission of tobacco plants transiently overexpressing single *SpTPS* candidates. Plants were infiltrated with *Agrobacterium tumefaciens* containing 35S:eGFP, 35S:SpTPS1-var2, 35S:SpTPS2-var2, 35S:SpTPS3-var2, 35S:SpTPS6, 35S:SpTPS13, 35S:SpTPS15, 35S:SpTPS22-tr, and/or 35S:SpTPS23. Volatiles were collected on the fourth day after infiltration for 24 h using an active push-pull system. Volatile compounds were subsequently analysed and quantified by gas chromatography–mass spectrometry and gas chromatography–flame ionization detection, respectively. Data are the mean $\pm$ SE ($n=3$ biological replicates). IS, internal standard (nonyl acetate); 1, (Z)-β-ocimene; 2, (E)-β-ocimene*; 3, linalool*; 4, β-elemene; 5, (E)-β-caryophyllene*; 6, germacrene D*; 7, (E,E)-α-farnesene*; 8, unidentified sesquiterpene alcohol; 9, nerolidol*. nd, not detected. Compounds marked with “*” were identified by comparison of retention time and mass spectrum to those of authentic standards. Other compounds were identified by database comparisons.

sesquiterpenes (Fig. 1; Supplementary Table S5). These findings are consistent with previous reports from *P. trichocarpa*, where the same compounds were among the major herbivore-induced volatiles (Danner *et al.*, 2011; Irmisch *et al.*, 2014). Interestingly, the

composition of the volatile blend did not differ between *L. dispar* or *C. populi* feeding (Fig. 1; Supplementary Table S5), suggesting that the induced terpenoid emission represents a generalized defense response rather than a herbivore-specific reaction. Comparable

herbivore-independent volatile profiles have also been observed for *P. nigra* trees upon feeding by multiple lepidopteran or coleopteran species (Fabisch *et al.*, 2019). Notably, the herbivore-defense responses reported here and in *P. nigra* (Fabisch *et al.*, 2019) were investigated using chewing insects. However, other feeding guilds, such as leaf miners or sap-sucking insects, could elicit distinct defense patterns, reflecting the specificity of plant responses to different types of herbivory. Furthermore, exploring intraspecific variation in *S. purpurea* may provide additional insights into the specificity and magnitude of herbivore-induced defenses, as previous studies have demonstrated that genotypic differences can significantly influence plant chemical profiles and defensive traits (e.g. Moreira *et al.*, 2019). Such analyses could help clarify the ecological relevance of defense variability and contribute to understanding the evolutionary drivers of plant–herbivore interactions.

Several terpenes emitted by herbivore-induced *S. purpurea* leaves are known from other plant species to act as key mediators in diverse plant–environment interactions (De Moraes *et al.*, 2001; Schaub *et al.*, 2010; Zhuang *et al.*, 2012). The monoterpene (*E*)- β -ocimene, for example, is a well-characterized inducible defense volatile in *Lotus japonicus* following infestation by the two-spotted spider mite (*Tetranychus urticae*) (Arimura *et al.*, 2004b). While inducible emission alone does not demonstrate a defensive function, (*E*)- β -ocimene has been implicated in plant–herbivore interactions in other systems, where it can influence herbivore behavior and performance (Huang *et al.*, 2022). Consistent with this, deterrent effects on *Spodoptera litura* larvae and increased larval mortality have been reported in non-choice assays (Huang *et al.*, 2022). In addition to its role in direct defense, (*E*)- β -ocimene mediates plant–plant communication. Emission of this compound from transgenic tobacco plants, for example, enhanced aphid resistance in neighboring tomato plants (Cascone *et al.*, 2015). Likewise, the sesquiterpene (*E,E*)- α -farnesene has been reported as a herbivory-induced volatile in several plant species, including poplar, tea, and soybean (Danner *et al.*, 2011; Lin *et al.*, 2017; Wang *et al.*, 2019; Scott *et al.*, 2020). In soybean, for instance, enhanced (*E,E*)- α -farnesene emission has been associated with increased resistance to the soybean cyst nematode (Lin *et al.*, 2017; Constantino *et al.*, 2021). Moreover, another major constituent of the *S. purpurea* volatile blend, (*E*)- β -caryophyllene, has been shown to play multifaceted ecological roles. In maize roots, (*E*)- β -caryophyllene emission is induced by feeding of the western corn rootworm (*Diabrotica virgifera virgifera*), where it mediates an indirect defense response by attracting entomopathogenic nematodes (Rasmann *et al.*, 2005; Köllner *et al.*, 2008). Furthermore, in *Arabidopsis* flowers, (*E*)- β -caryophyllene emission contributes to pathogen resistance against *Pseudomonas syringae*, and loss of this volatile was linked to severely impacted seed size and misshaping following infection (Huang *et al.*, 2012). Given the described roles of volatile terpenes in plant defense (Turlings and Erb, 2018; Kutty and Mishra, 2023; Thompson *et al.*, 2024), it is likely that the terpenes emitted by herbivore-induced *S. purpurea* leaves contribute to both direct

and indirect defense against the feeding herbivores. Moreover, several terpenoids have been reported to exhibit antimicrobial activity *in vitro*, raising the possibility that wound-induced volatile emissions may have indirect effects on microbial interactions, although this remains speculative. Similar hypotheses regarding defensive or antimicrobial roles of plant-emitted terpenoids have been proposed previously (Lackus *et al.*, 2018).

Phytohormone signaling and transcriptional regulation as mediators in herbivore-induced volatile formation

In response to herbivore attack, plants typically activate complex hormonal signaling networks involving phytohormones such as JA and SA (Erb *et al.*, 2012; Singh *et al.*, 2016). JA is generally seen as the primary mediator of defense responses against chewing herbivores (Howe and Jander, 2008; Dicke *et al.*, 2009; Stroud *et al.*, 2022; Ali *et al.*, 2024), whereas SA is predominantly associated with resistance to biotrophic and hemibiotrophic pathogens (White, 1979; Yang *et al.*, 2015; Stroud *et al.*, 2022; Ali *et al.*, 2024). Although JA and SA signaling are generally considered antagonistic, increasing evidence suggests a more nuanced interaction involving synergistic cross-talk between the two pathways (Mur *et al.*, 2006; Smith *et al.*, 2009; Riemann *et al.*, 2013; Ullah *et al.*, 2022). For instance, studies in rice using jasmonate-deficient mutants demonstrated JA-dependent resistance to the fungal plant pathogen *Magnaporthe oryzae* (Riemann *et al.*, 2013; Ma *et al.*, 2022; Liu *et al.*, 2025), while in *Populus nigra*, simultaneous induction of SA and JA was observed during infection with the rust fungus *Melampsora larici-populina* (Ullah *et al.*, 2022). Similarly, in our study, herbivory by *L. dispar* significantly increased both JA-derived metabolites and SA levels in *S. purpurea* leaves, suggesting a potential synergistic activation of these signaling pathways (Fig. 2). In contrast, herbivory by *C. populi* resulted in a less pronounced induction of JA-related metabolites, which may be attributed to the comparatively lower feeding intensity of the beetles relative to *L. dispar* caterpillars (Supplementary Fig. S4). Interestingly, herbivore specificity in hormonal signaling has also been reported in *Nicotiana attenuata*, where feeding by the generalist beet armyworm (*Spodoptera exigua*) strongly induced SA accumulation, whereas the specialist tobacco hornworm (*Manduca sexta*) did not elicit a comparable response (Diezel *et al.*, 2009). These findings could imply that herbivore identity may influence distinct hormonal signaling cascades in *S. purpurea* as well. However, despite these differences in phytohormone profiles, the resulting volatile defense responses were largely similar between the two herbivores in our study, indicating that downstream integration of the signaling pathways may converge (Figs 1, 2). Further investigations into the specificity and cross-regulation of these hormonal networks will be required to fully elucidate the complexity of herbivore-induced defense responses in *S. purpurea*.

In connection with the broader hormonal signaling network, we also examined the gene expression profiles of the isolated TPS candidate genes (Fig. 5). The expression levels of most genes were substantially induced following herbivore treatment (Fig. 5). This observation is consistent with findings in other woody plant species, where herbivory similarly induces enhanced gene expression (Arimura *et al.*, 2004a; Köpke *et al.*, 2008; Vickers *et al.*, 2010; He *et al.*, 2022). These results mutually suggest that transcriptional regulation may represent a key and conserved mechanism underlying terpene biosynthesis in response to herbivore attack. Moreover, the herbivory-induced transcriptional regulation and the consequent production of volatiles illustrate a mechanism that may contribute to balancing growth and defense, although this trade-off is likely context-dependent (Lucas-Barbosa *et al.*, 2011; Huot *et al.*, 2014). In many cases, plants under stress can reduce growth and redirect resources toward defensive pathways, but the magnitude and occurrence of such responses can vary widely among species, genotypes, and environmental conditions (Lucas-Barbosa *et al.*, 2011; Huot *et al.*, 2014). In addition, other factors, including spatial variation within plant populations and differences in herbivore pressure, may influence the inducible formation of specialized metabolites, suggesting that both genetic and ecological context shape plant defense strategies (Rasmann *et al.*, 2015).

Terpene synthase gene family composition and functional diversification in *S. purpurea*

In this study, we identified a mid-sized TPS gene family in *Salix purpurea*, comprising 34 full length members. This family size is comparable to that reported for *Populus trichocarpa* and *Arabidopsis*, which harbor 38 and 32 TPS genes, respectively (Aubourg *et al.*, 2002; Chen *et al.*, 2011; Irmisch *et al.*, 2014). In contrast, other plant families display either expanded or reduced TPS families, reflecting lineage-specific diversification within this gene family (Jiang *et al.*, 2019; Wang *et al.*, 2025). Given the high sequence similarity among several *SpTPS* members, further genome annotation may refine the exact number of functional TPS genes in *S. purpurea* in the future. A notable finding was the clustering of a substantial proportion of *SpTPS* genes on chromosome 19 (Supplementary Fig. S6), suggesting recent tandem duplication events. Such duplications represent an important evolutionary mechanism driving chemical diversification in plant specialized metabolism (Chen *et al.*, 2011; Kroymann, 2011; Chae *et al.*, 2014; Ono and Murata, 2023). Subsequent neo- or sub-functionalization of duplicated genes often leads to enzymes with altered substrate specificity or product profiles, contributing to the vast structural diversity of plant specialized metabolism (Chen *et al.*, 2011; Kroymann, 2011; Ono and Murata, 2023). Conversely, partial redundancy within TPS gene families may also be a mechanism to maintain metabolic stability and ensure consistent production of key defense compounds.

TPS enzymes are known to be highly sensitive to single amino acid substitutions, which can dramatically alter product

specificity (Köllner *et al.*, 2009; Christianson, 2017; Whitehead *et al.*, 2023). For example, single amino acid variation in poplar PtTPS19 has been shown to shift the product formation from *ent*-kaurene to 16 α -hydroxy-*ent*-kaurane, while in poplar PtTPS16, conversion of four amino acids changed the product profile from γ -terpinene into a multi-product enzyme producing camphene, limonene, and α -/ β -pinene (Irmisch *et al.*, 2015; Lackus *et al.*, 2018). Similarly, in maize, closely related TPS4 and TPS5 produce overlapping product spectra in different proportions, determined largely by four amino acid substitutions, especially a highly important glycine residue, that affect enzyme stereoselectivity (Köllner *et al.*, 2004). In contrast, the allelic variants identified in this study (*SpTPS1-var1/var2*, *SpTPS2-var1/var2*, and *SpTPS3-var1/var2*) exhibited identical product profiles, indicating that the observed sequence polymorphisms do not influence catalytic function (Fig. 5; Supplementary Figs S7, S8). Interestingly, *SpTPS13* displayed a notable functional divergence from its poplar orthologue PtTPS13 (Fig. 5A; Irmisch *et al.*, 2014). While PtTPS13 produces 1,8-cineole (Irmisch *et al.*, 2014), *SpTPS13* formed linalool in the presence of GPP. This enzymatic difference likely reflects the relatively low sequence identity (92.2%) between the two orthologues. The observed enzyme activities, however, are consistent and might explain the absence of 1,8-cineole in the *S. purpurea* volatile bouquet (Fig. 1).

Moreover, of particular interest is the candidate *SpTPS23*, which produced an unidentified sesquiterpene alcohol in *N. benthamiana* upon heterologous expression (Fig. 6; Supplementary Fig. S14). This compound was not detected in the *S. purpurea* leaf volatile bouquet, indicating that the sesquiterpene alcohol might undergo further enzymatic modification *in planta*, potentially by cytochrome P450 monooxygenases or other tailoring enzymes. Similar metabolic diversification has been described for instance in maize, where β -macrocarypene-derived zealexins are formed through cytochrome P450 enzymes (Mao *et al.*, 2016; Ding *et al.*, 2020). Structural elucidation of this novel sesquiterpene alcohol and identification of tentative associated modifying enzymes represent worthwhile aims for future research.

Conclusion

In conclusion, herbivory on *Salix purpurea* leaves induces a complex volatile blend of mono- and sesquiterpenes, demonstrating that long-lived woody plants are capable of mounting sophisticated chemical defenses. Through integrated genomic and phylogenetic analyses, we identified a mid-sized TPS gene family tentatively underlying these emissions. Functional characterization in *E. coli* and *in planta* reconstitution in *N. benthamiana* provided functional evidence for the contribution of these TPS enzymes to the herbivore-induced terpenoid bouquet. Collectively, these findings establish a comprehensive framework for understanding volatile-mediated plant-insect interactions in

perennial species and provide a foundation for future investigations into the ecological and evolutionary roles of terpenoid emissions in woody plants.

Supplementary data

The following supplementary data are available at [JXB](#) online.

Fig. S1. Amino acid sequence alignment of cloned *Salix purpurea* terpene synthase 1 (*SpTPS1*) alleles and Sapur.016G273600 (Phytozome v13).

Fig. S2. Amino acid sequence alignment of cloned *Salix purpurea* terpene synthase 2 (*SpTPS2*) alleles and Sapur.011G018900 (Phytozome v13).

Fig. S3. Amino acid sequence alignment of cloned *Salix purpurea* terpene synthase 3 (*SpTPS3*) alleles and Sapur.011G019500 (Phytozome v13).

Fig. S4. Percentage of leaf area remaining in undamaged (ctr), *Lymantria dispar*-damaged (Ld), and *Chrysomela populi*-damaged (Cp) *Salix purpurea* leaves.

Fig. S5. Salicinoid content in undamaged, *Lymantria dispar*-, or *Chrysomela populi*-damaged *Salix purpurea* leaves was measured by HPLC-UV.

Fig. S6. Schematic representation of *Salix purpurea* terpene synthase (*SpTPS*) gene locations on chromosomes 16 and 19 (v5.1 genome assembly).

Fig. S7. *In vitro* enzymatic activity of *Salix purpurea* terpene synthase (*SpTPS*) candidates heterologously expressed in *E. coli*, incubated with GPP or (*E,E*)-FPP, with products collected by SPME and analysed by GC-MS.

Fig. S8. *In vitro* enzymatic activity of *Salix purpurea* terpene synthase (*SpTPS*) candidate variants heterologously expressed in *E. coli*, incubated with GPP, (*E,E*)-FPP, or (*E,E,E*)-GGPP, with products collected by SPME or *n*-hexane and analysed by GC-MS.

Fig. S9. *In vitro* enzymatic activity of *Salix purpurea* terpene synthase (*SpTPS*) candidates heterologously expressed in *E. coli*, incubated with (*E,E,E*)-GGPP, with products collected with *n*-hexane and analysed by GC-MS.

Fig. S10. SDS-PAGE analysis of protein raw extracts from *E. coli* expressing purple willow (*Salix purpurea*) terpene synthases.

Fig. S11. *In vitro* enzymatic activity of *Salix purpurea* terpene synthase (*SpTPS*) candidates heterologously expressed in *E. coli*, incubated with GPP or (*E,E*)-FPP, cofactor Mn^{2+} with products collected by SPME and analysed by GC-MS.

Fig. S12. *In vitro* control assays using empty vector (EV) extracts from *E. coli* or no-protein, incubated with GPP, (*E,E*)-FPP, or (*E,E,E*)-GGPP, with products collected by SPME or *n*-hexane and analysed by GC-MS.

Fig. S13. Volatile emission of transiently transformed *Nicotiana benthamiana* plants overexpressing *Salix purpurea* candidate terpene synthases.

Fig. S14. Mass spectrum of the unidentified sesquiterpene alcohol produced by *Salix purpurea* terpene synthase 23 (*SpTPS23*).

Table S1. *In silico* characterization of *Salix purpurea* terpene synthase (TPS) candidates showing predicted nucleotide and amino acid lengths, TPS types, conserved domains, and subcellular localizations.

Table S2. Oligonucleotides used for amplification and gene expression analyses of *Salix purpurea* terpene synthase genes.

Table S3. Expression levels (ΔC_q mean $\pm$ SD, $n=3$) of housekeeping genes in undamaged (ctr), *Lymantria dispar*-damaged (Ld-herb), and *Chrysomela populi*-damaged (Cp-herb) *Salix purpurea* leaves.

Table S4. Compounds used as standards for GC-MS analysis.

Table S5. Volatile composition ($ng\ g^{-1}\ FW\ h^{-1} \pm SE$, $n=7$) of undamaged (ctr), *Lymantria dispar*-damaged (Ld), and *Chrysomela populi*-damaged (Cp) *Salix purpurea* leaves.

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Author contributions

NDL: conceptualization; NDL: methodology; NDL and MP: formal analysis; NDL, MP, SS, and MK: investigation; NDL and MJM: resources; NDL: data curation; NDL and MP: writing—original draft; NDL: writing—review & editing; NDL and MP: visualization; NDL: supervision; NDL: funding acquisition. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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Data availability

All supporting data are included as [Supplementary data](#) files. Constructs described in this work and datasets analysed during the current study are available from the corresponding author upon request. Sequences were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

References

- Ali J, Tonga A, Islam T, Mir S, Mukarram M, Konopkova AS, Chen R. 2024. Defense strategies and associated phytohormonal regulation in *Brassica* plants in response to chewing and sap-sucking insects. *Frontiers in Plant Science* **15**, 1376917.
- Almagro Armenteros JJ, Salvatore M, Emanuelsson O, Winther O, von Heijne G, Elofsson A, Nielsen H. 2019. Detecting sequence signals in targeting peptides using deep learning. *Life Science Alliance* **2**, e201900429.
- Anjali, Kumar S, Korra T, Thakur R, Arutselvan R, Kashyap AS, Nehela Y, Chaplygin V, Minkina T, Keswani C. 2023. Role of plant secondary metabolites in defence and transcriptional regulation in response to biotic stress. *Plant Stress* **8**, 100154.
- Arimura G-I, Huber DP, Bohlmann J. 2004a. Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa* $\times$ *deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (–)-germacrene D synthase, *PtdTPS1*. *The Plant Journal* **37**, 603–616.
- Arimura G-I, Ozawa R, Kugimiya S, Takabayashi J, Bohlmann J. 2004b. Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (*E*)- β -ocimene and transcript

- accumulation of (*E*)- β -ocimene synthase in *Lotus japonicus*. Plant Physiology **135**, 1976–1983.
- Aubourg S, Lechamy A, Bohlmann J.** 2002. Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. Molecular Genetics and Genomics **267**, 730–745.
- Bergman ME, Dudareva N.** 2024. Plant specialized metabolism: diversity of terpene synthases and their products. Current Opinion in Plant Biology **81**, 102607.
- Boeckler GA, Gershenzon J, Unsicker SB.** 2011. Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. Phytochemistry **72**, 1497–1509.
- Bohlmann J, Meyer-Gauen G, Croteau R.** 1998. Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proceedings of the National Academy of Sciences, USA **95**, 4126–4133.
- Cascone P, Iodice L, Maffei ME, Bossi S, Arimura G-I, Guerrieri E.** 2015. Tobacco overexpressing β -ocimene induces direct and indirect responses against aphids in receiver tomato plants. Journal of Plant Physiology **173**, 28–32.
- Celedon JM, Bohlmann J.** 2019. Oleoresin defenses in conifers: chemical diversity, terpene synthases and limitations of oleoresin defense under climate change. New Phytologist **224**, 1444–1463.
- Chae L, Kim T, Nilo-Poyanco R, Rhee SY.** 2014. Genomic signatures of specialized metabolism in plants. Science **344**, 510–513.
- Chen F, Tholl D, Bohlmann J, Pichersky E.** 2011. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. The Plant Journal **66**, 212–229.
- Chen X, Xu M, Han J, Schmidt-Dannert M, Peters RJ, Chen F.** 2024. Discovery of bifunctional diterpene cyclases/synthases in bacteria supports a bacterial origin for the plant terpene synthase gene family. Horticulture Research **11**, uhae221.
- Christianson DW.** 2017. Structural and chemical biology of terpenoid cyclases. Chemical Reviews **117**, 11570–11648.
- Clavijo McCormick A, Irmisch S, Reinecke A, Boeckler GA, Veit D, Reichelt M, Hansson BS, Gershenzon J, Köllner TG, Unsicker SB.** 2014. Herbivore-induced volatile emission in black poplar: regulation and role in attracting herbivore enemies. Plant, Cell & Environment **37**, 1909–1923.
- Constantino N, Oh Y, Sennik E, Andersen B, Warden M, Oralkan O, Dean RA.** 2021. Soybean cyst nematodes influence aboveground plant volatile signals prior to symptom development. Frontiers in Plant Science **12**, 749014.
- Danner H, Boeckler GA, Irmisch S, Yuan JS, Chen F, Gershenzon J, Unsicker SB, Köllner TG.** 2011. Four terpene synthases produce major compounds of the gypsy moth feeding-induced volatile blend of *Populus trichocarpa*. Phytochemistry **72**, 897–908.
- de Kraker J-W, Franssen MCR, de Groot A, König WA, Bouwmeester HJ.** 1998. (+)-Germacrene A biosynthesis. The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. Plant Physiology **117**, 1381–1392.
- De Moraes CM, Mescher MC, Tumlinson JH.** 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature **410**, 577–580.
- Degenhardt J, Köllner TG, Gershenzon J.** 2009. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry **70**, 1621–1637.
- Dicke M, van Loon JJ, Soler R.** 2009. Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology **5**, 317–324.
- Diezel C, von Dahl CC, Gaquerel E, Baldwin IT.** 2009. Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiology **150**, 1576–1586.
- Ding Y, Weckwerth PR, Poretsky E, et al.** 2020. Genetic elucidation of interconnected antibiotic pathways mediating maize innate immunity. Nature Plants **6**, 1375–1388.
- Dudareva N, Negre F, Nagegowda DA, Orlova I.** 2006. Plant volatiles: recent advances and future perspectives. Critical Reviews in Plant Sciences **25**, 417–440.
- Dudareva N, Klempien A, Muhlemann JK, Kaplan I.** 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytologist **198**, 16–32.
- Erb M, Meldau S, Howe GA.** 2012. Role of phytohormones in insect-specific plant reactions. Trends in Plant Science **17**, 250–259.
- Fabisch T, Gershenzon J, Unsicker SB.** 2019. Specificity of herbivore defense responses in a woody plant, black poplar (*Populus nigra*). Journal of Chemical Ecology **45**, 162–177.
- Fürstenberg-Hägg J, Zagrobelny M, Bak S.** 2013. Plant defense against insect herbivores. International Journal of Molecular Sciences **14**, 10242–10297.
- Gordon H, Fellenberg C, Lackus ND, Archinuk F, Sproule A, Nakamura Y, Köllner TG, Gershenzon J, Overy DP, Constabel CP.** 2022. CRISPR/Cas9 disruption of *UGT71L1* in poplar connects salicinoid and salicylic acid metabolism and alters growth and morphology. The Plant Cell **34**, 2925–2947.
- Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series **41**, 95–98.
- Hayashi K-I, Kawaide H, Notomi M, Sakigi Y, Matsuo A, Nozaki H.** 2006. Identification and functional analysis of bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens*. FEBS Letters **580**, 6175–6181.
- He J, Verstappen F, Jiao A, Dicke M, Bouwmeester HJ, Kappers IF.** 2022. Terpene synthases in cucumber (*Cucumis sativus*) and their contribution to herbivore-induced volatile terpenoid emission. New Phytologist **233**, 862–877.
- Heiska S, Tikkanen O-P, Rousi M, Julkunen-Tiitto R.** 2007. Bark salicylates and condensed tannins reduce vole browsing amongst cultivated dark-leaved willows (*Salix myrsinifolia*). Chemoecology **17**, 245–253.
- Howe GA, Jander G.** 2008. Plant immunity to insect herbivores. Annual Review of Plant Biology **59**, 41–66.
- Hsieh J-F, Krause ST, Kainer D, Degenhardt J, Foley WJ, Külheim C.** 2021. Characterization of terpene biosynthesis in *Melaleuca quinquenervia* and ecological consequences of terpene accumulation during myrtle rust infection. Plant-Environment Interactions **2**, 177–193.
- Huang M, Sanchez-Moreiras AM, Abel C, Sohrabi R, Lee S, Gershenzon J, Tholl D.** 2012. The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (*E*)- β -caryophyllene, is a defense against a bacterial pathogen. New Phytologist **193**, 997–1008.
- Huang X, Zhang H, Li H, et al.** 2022. Functional characterization of a terpene synthase responsible for (*E*)- β -ocimene biosynthesis identified in *Pyrus betulaeifolia* transcriptome after herbivory. Frontiers in Plant Sciences **13**, 1077229.
- Huot B, Yao J, Montgomery BL, He SY.** 2014. Growth-defense tradeoffs in plants: a balancing act to optimize fitness. Molecular Plant **7**, 1267–1287.
- Hwang SY, Lindroth RL.** 1997. Clonal variation in foliar chemistry of aspen: effects on gypsy moths and forest tent caterpillars. Oecologia **111**, 99–108.
- Irmisch S, McCormick AC, Boeckler GA, et al.** 2013. Two herbivore-induced cytochrome P450 enzymes CYP79D6 and CYP79D7 catalyze the formation of volatile aldoximes involved in poplar defense. The Plant Cell **25**, 4737–4754.
- Irmisch S, Jiang Y, Chen F, Gershenzon J, Köllner TG.** 2014. Terpene synthases and their contribution to herbivore-induced volatile emission in western balsam poplar. BMC Plant Biology **14**, 270.
- Irmisch S, Müller AT, Schmidt L, Günther J, Gershenzon J, Köllner TG.** 2015. One amino acid makes the difference: the formation of *ent*-kaurene and 16- α -hydroxy-*ent*-kaurene by diterpene synthases in poplar. BMC Plant Biology **15**, 262.
- Jiang S-Y, Jin J, Sarojam R, Ramachandran S.** 2019. A comprehensive survey on the terpene synthase gene family provides new insight into its evolutionary patterns. Genome Biology and Evolution **11**, 2078–2098.

- Karunanithi PS, Zerbe P.** 2019. Terpene synthases as metabolic gatekeepers in the evolution of plant terpenoid chemical diversity. *Frontiers in Plant Science* **10**, 1166.
- Kaufman NJ, You J, Fox BG, Mansfield SD.** 2025. *Populus* salicinoids: a thriving subfield in the -omics era. *Tree Physiology* **45**, 45–56.
- Kessler A, Baldwin IT.** 2002. Plant responses to insect herbivory: the emerging molecular analysis. *Annual Reviews of Plant Biology* **53**, 299–328.
- Kessler A, Mueller MB, Kalske A, Chauta A.** 2023. Volatile-mediated plant-plant communication and higher-level ecological dynamics. *Current Biology* **33**, R519–R529.
- Kliebenstein DJ.** 2024. Specificity and breadth of plant specialized metabolite-microbe interactions. *Current Opinion in Plant Biology* **77**, 102459.
- Köllner TG, Schnee C, Gershenzon J, Degenhardt J.** 2004. The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distributions. *Phytochemistry* **65**, 1895–1902.
- Köllner TG, Held M, Lenk C, Hiltbold I, Turlings TC, Gershenzon J, Degenhardt J.** 2008. A maize (*E*)- β -caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *The Plant Cell* **20**, 482–494.
- Köllner TG, Gershenzon J, Degenhardt J.** 2009. Molecular and biochemical evolution of maize terpene synthase 10, an enzyme of indirect defense. *Phytochemistry* **70**, 1139–1145.
- Köpke D, Schröder R, Fischer HM, Gershenzon J, Hilker M, Schmidt A.** 2008. Does egg deposition by herbivorous pine sawflies affect transcription of sesquiterpene synthases in pine? *Planta* **228**, 427–438.
- Kroymann J.** 2011. Natural diversity and adaptation in plant secondary metabolism. *Current Opinion in Plant Biology* **14**, 246–251.
- Külheim C, Padovan A, Hefer C, Krause ST, Köllner TG, Myburg AA, Degenhardt J, Foley WJ.** 2015. The *Eucalyptus* terpene synthase gene family. *BMC Genomics* **16**, 450.
- Kutty NN, Mishra M.** 2023. Dynamic distress calls: volatile info chemicals induce and regulate defense responses during herbivory. *Frontiers in Plant Science* **14**, 1135000.
- Lackus ND, Lackner S, Gershenzon J, Unsicker SB, Köllner TG.** 2018. The occurrence and formation of monoterpenes in herbivore-damaged poplar roots. *Scientific Reports* **8**, 17936.
- Lackus ND, Müller A, Kröber TDU, et al.** 2020. The occurrence of sulfated salicinoids in poplar and their formation by sulfotransferase1. *Plant Physiology* **183**, 137–151.
- Lackus ND, Morawetz J, Xu H, Gershenzon J, Dickschat JS, Köllner TG.** 2021a. The sesquiterpene synthase PtTPS5 produces (1S,5S,7R,10R)-guaia-4(15)-en-11-ol and (1S,7R,10R)-guaia-4-en-11-ol in oomycete-infected poplar roots. *Molecules* **26**, 555.
- Lackus ND, Schmidt A, Gershenzon J, Köllner TG.** 2021b. A peroxisomal β -oxidative pathway contributes to the formation of C₆-C₁ aromatic volatiles in poplar. *Plant Physiology* **186**, 891–909.
- Li M, Tao H.** 2024. Enhancing structural diversity of terpenoids by multisubstrate terpene synthases. *Beilstein Journal of Organic Chemistry* **20**, 959–972.
- Lichtenthaler HK.** 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology* **50**, 47–65.
- Lin J, Wang D, Chen X, et al.** 2017. An (*E,E*)- α -farnesene synthase gene of soybean has a role in defence against nematodes and is involved in synthesizing insect-induced volatiles. *Plant Biotechnology Journal* **15**, 510–519.
- Liu C, Han L-B, Wen Y, Lu C, Deng B, Liu Z, Deng X, Shen N, Tang D, Li Y-B.** 2025. The *Magnaporthe oryzae* effector MoBys1 suppresses rice immunity by targeting OsCAD2 to manipulate host jasmonate and lignin metabolism. *New Phytologist* **246**, 280–297.
- Lo M-M, Benfodda Z, Molinier R, Meffre P.** 2024. Volatile organic compounds emitted by flowers: ecological roles, production by plants, extraction, and identification. *Plants* **13**, 417.
- Lucas-Barbosa D, van Loon JJ, Dicke M.** 2011. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. *Phytochemistry* **72**, 1647–1654.
- Ma J, Morel J-B, Riemann M, Nick P.** 2022. Jasmonic acid contributes to rice resistance against *Magnaporthe oryzae*. *BMC Plant Biology* **22**, 601.
- Mao H, Liu J, Ren F, Peters RJ, Wang Q.** 2016. Characterization of CYP71Z18 indicates a role in maize zealexin biosynthesis. *Phytochemistry* **121**, 4–10.
- Marone D, Mastrangelo AM, Borrelli GM, Mores A, Laidò G, Russo MA, Ficco DBM.** 2022. Specialized metabolites: physiological and biochemical role in stress resistance, strategies to improve their accumulation, and new applications in crop breeding and management. *Plant Physiology and Biochemistry* **172**, 48–55.
- McCaskill D, Croteau R.** 1998. Some caveats for bioengineering terpenoid metabolism in plants. *Trends in Biotechnology* **16**, 349–355.
- McGarvey DJ, Croteau R.** 1995. Terpenoid metabolism. *The Plant Cell* **7**, 1015–1026.
- Moreira X, Abdala-Roberts L, Nell CS, Vazquez-Gonzalez C, Pratt JD, Keefover-Ring K, Mooney KA.** 2019. Sexual and genotypic variation in terpene quantitative and qualitative profiles in the dioecious shrub *Baccharis salicifolia*. *Scientific Reports* **9**, 14655.
- Mumm R, Dicke M.** 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. *Canadian Journal of Zoology* **88**, 628–667.
- Mur LA, Kenton P, Atzorn R, Miersch O, Wasternack C.** 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology* **140**, 249–262.
- Njakou Djomo S, Ac A, Zenone T, De Groot T, Bergante S, Facciotto G, Sixto H, Ciria Ciria P, Weger J, Ceulemans R.** 2015. Energy performance of intensive and extensive short rotation cropping systems for woody biomass production in the EU. *Renewable & Sustainable Energy Reviews* **41**, 845–854.
- Oates CN, Külheim C, Myburg AA, Slippers B, Naidoo S.** 2015. The transcriptome and terpene profile of *Eucalyptus grandis* reveals mechanisms of defense against the insect pest, *Leptocybe invasa*. *Plant & Cell Physiology* **56**, 1418–1428.
- Ono E, Murata J.** 2023. Exploring the evolvability of plant specialized metabolism: uniqueness out of uniformity and uniqueness behind uniformity. *Plant & Cell Physiology* **64**, 1449–1465.
- Peacock L, Lewis M, Powers S.** 2001. Volatile compounds from *Salix* spp. varieties differing in susceptibility to three willow beetle species. *Journal of Chemical Ecology* **27**, 1943–1951.
- Ramirez-Carvajal GA, Morse AM, Davis JM.** 2008. Transcript profiles of the cytokinin response regulator gene family in *Populus* imply diverse roles in plant development. *New Phytologist* **177**, 77–89.
- Rasmann S, Köllner TG, Degenhardt J, Hiltbold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TC.** 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* **434**, 732–737.
- Rasmann S, Chassin E, Bilat J, Glauser G, Reymond P.** 2015. Trade-off between constitutive and inducible resistance against herbivores is only partially explained by gene expression and glucosinolate production. *Journal of Experimental Botany* **66**, 2527–2534.
- Riemann M, Haga K, Shimizu T, et al.** 2013. Identification of rice *Allene Oxide Cyclase* mutants and the function of jasmonate for defence against *Magnaporthe oryzae*. *The Plant Journal* **74**, 226–238.
- Sapir-Mir M, Mett A, Belasov E, Tal-Meshulam S, Frydman A, Gidoni D, Eyal Y.** 2008. Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiology* **148**, 1219–1228.
- Schaub A, Blande JD, Graus M, Oksanen E, Holopainen JK, Hansel A.** 2010. Real-time monitoring of herbivore induced volatile emissions in the field. *Physiologia Plantarum* **138**, 123–133.

- Scott ER, Li X, Wei J-P, et al.** 2020. Changes in tea plant secondary metabolite profiles as a function of leafhopper density and damage. *Frontiers in Plant Science* **11**, 636.
- Singh A, Singh S, Singh IK.** 2016. Recent insights into the molecular mechanism of jasmonate signaling during insect-plant interaction. *Australasian Plant Pathology: APP* **45**, 123–133.
- Smith JL, De Moraes CM, Mescher MC.** 2009. Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants. *Pest Management Science* **65**, 497–503.
- Stolarski MJ, Gil Ł, Krzyżaniak M, Olba-Zięty E, Wu A-M.** 2024. Willow, poplar, and black locust debarked wood as feedstock for energy and other purposes. *Energies* **17**, 1535.
- Stroud EA, Jayaraman J, Templeton MD, Rikkerink EHA.** 2022. Comparison of the pathway structures influencing the temporal response of salicylate and jasmonate defence hormones in *Arabidopsis thaliana*. *Frontiers in Plant Sciences* **13**, 952301.
- Tahvanainen J, Helle E, Julkunen-Tiitto R, Lavola A.** 1985. Phenolic compounds of willow bark as deterrents against feeding by mountain hare. *Oecologia* **65**, 319–323.
- Tamura K, Nei M.** 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526.
- Tamura K, Stecher G, Kumar S.** 2021. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution* **38**, 3022–3027.
- Tholl D, Boland W, Hansel A, Loreto F, Röse US, Schnitzler J-P.** 2006. Practical approaches to plant volatile analysis. *The Plant Journal* **45**, 540–560.
- Thompson MN, Arriaga J, Bradford BJ, Kurian R, Strozier G, Helms AM.** 2024. Belowground insect herbivory induces systemic volatile emissions that strengthen neighbouring plant resistance aboveground. *Plant, Cell & Environment* **47**, 714–725.
- Turcotte MM, Davies TJ, Thomsen CJ, Johnson MT.** 2014. Macroecological and macroevolutionary patterns of leaf herbivory across vascular plants. *Proceedings of the Royal Society of London: Series B, Biological Sciences* **281**, 20140555.
- Turlings TCJ, Erb M.** 2018. Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. *Annual Review of Entomology* **63**, 433–452.
- Ullah C, Schmidt A, Reichelt M, Tsai C-J, Gershenzon J.** 2022. Lack of antagonism between salicylic acid and jasmonate signalling pathways in poplar. *New Phytologist* **235**, 701–717.
- Vickers CE, Possell M, Hewitt CN, Mullineaux PM.** 2010. Genetic structure and regulation of isoprene synthase in poplar (*Populus* spp.). *Plant Molecular Biology* **73**, 547–558.
- Wang H-L, Chen J, Tian Q, Wang S, Xia X, Yin W.** 2014. Identification and validation of reference genes for *Populus euphratica* gene expression analysis during abiotic stresses by quantitative real-time PCR. *Physiologia Plantarum* **152**, 529–545.
- Wang Q, Jiang J, Liang Y, Li S, Xia Y, Zhang L, Wang X.** 2025. Expansion and functional divergence of terpene synthase genes in angiosperms: a driving force of terpene diversity. *Horticulture Research* **12**, uhae272.
- Wang X, Zeng L, Liao Y, Li J, Tang J, Yang Z.** 2019. Formation of α -farnesene in tea (*Camellia sinensis*) leaves induced by herbivore-derived wounding and its effect on neighboring tea plants. *International Journal of Molecular Sciences* **20**, 4151.
- White RF.** 1979. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* **99**, 410–412.
- Whitehead JN, Leferink NGH, Johannissen LO, Hay S, Scrutton NS.** 2023. Decoding catalysis by terpene synthases. *ACS Catalysis* **13**, 12774–12802.
- Wu M, Northen TR, Ding Y.** 2023. Stressing the importance of plant specialized metabolites: omics-based approaches for discovering specialized metabolism in plant stress responses. *Frontiers in Plant Science* **14**, 1272363.
- Xu M, Zhang B, Su X, Zhang S, Huang M.** 2011. Reference gene selection for quantitative real-time polymerase chain reaction in *Populus*. *Analytical Biochemistry* **408**, 337–339.
- Yang L, Li B, Zheng X-Y, Li J, Yang M, Dong X, He G, An C, Deng XW.** 2015. Salicylic acid biosynthesis is enhanced and contributes to increased biotrophic pathogen resistance in *Arabidopsis* hybrids. *Nature Communications* **6**, 7309.
- Zhang Y, Han X, Chen S, Zheng L, He X, Liu M, Qiao G, Wang Y, Zhuo R.** 2017. Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in *Salix matsudana* under different abiotic stresses. *Scientific Reports* **7**, 40290.
- Zhou R, Macaya-Sanz D, Carlson CH, et al.** 2020. A willow sex chromosome reveals convergent evolution of complex palindromic repeats. *Genome Biology* **21**, 38.
- Zhuang X, Köllner TG, Zhao N, Li G, Jiang Y, Zhu L, Ma J, Degenhardt J, Chen F.** 2012. Dynamic evolution of herbivore-induced sesquiterpene biosynthesis in sorghum and related grass crops. *The Plant Journal* **69**, 70–80.