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Research

Lack of antagonism between salicylic acid and jasmonate signalling pathways in poplar

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Summary

- Salicylic acid (SA) and jasmonic acid (JA) often play distinct roles in plant defence against pathogens. Research from *Arabidopsis thaliana* has established that SA- and JA-mediated defences are more effective against biotrophs and necrotrophs, respectively. These two hormones often interact antagonistically in response to particular attackers, with the induction of one leading to suppression of the other. Here, we report a contrasting pattern in the woody perennial *Populus*: positive SA–JA interplay.
- Using genetically engineered high SA lines of black poplar and wild-type lines after exogenous hormone application, we quantified SA and JA metabolites, signalling gene transcripts, antifungal flavonoids and resistance to rust (*Melampsora larici-populina*).
- Salicylic acid and JA metabolites were induced concurrently upon rust infection in poplar genotypes with varying resistance levels. Analysis of SA-hyperaccumulating transgenic poplar lines showed increased jasmonate levels, elevated flavonoid content and enhanced rust resistance, but no discernible reduction in growth. Exogenous application of either SA or JA triggered the accumulation of the other hormone. Expression of pathogenesis-related (*PR*) genes, frequently used as markers for SA signalling, was not correlated with SA content, but rather activated in proportion to pathogen infection.
- We conclude that SA and JA pathways interact positively in poplar resulting in the accumulation of flavonoid phytoalexins.

Introduction

Salicylic acid (SA), 2-hydroxybenzoic acid, is a phenolic phytohormone known to play essential roles in plant defence against pathogens and phloem-feeding insects (Vlot *et al.*, 2009). In response to pathogen attack, especially by biotrophs, plants increase SA concentrations at the infection sites to activate local defence, often associated with the hypersensitive response (Malamy *et al.*, 1990), as well as in healthy tissues distal to the infection, which induces systemic acquired resistance (Cao *et al.*, 1997; Wang *et al.*, 2006). Plants synthesise SA via two independent pathways: the chloroplast-localised isochorismate synthase (ICS) pathway and the cytosolic phenylalanine ammonia-lyase (PAL) pathway. The shikimic acid pathway intermediate chorismic acid is a common precursor for both pathways (Lefevere *et al.*, 2020). While all enzymes necessary for SA synthesis via the ICS pathway have been discovered in the model plant *Arabidopsis thaliana* (Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019; Lefevere *et al.*, 2020), several enzymatic steps required for the PAL pathway remain undiscovered (Ding & Ding, 2020). Although *Arabidopsis* synthesises a significant portion (*c.* 90%) of basal and pathogen-induced SA via the ICS pathway (Garcion *et al.*, 2008), the woody perennial poplar (*Populus* spp.) is primarily dependent on the PAL pathway for synthesising SA (Yuan *et al.*, 2009). This difference has been attributed to the fact that most plants, including poplar, harbour a single evolutionarily conserved *ICS* gene associated with phylloquinone biosynthesis, whereas *Arabidopsis* and other Brassicaceae possess an additional stress-inducible isoform derived from lineage-specific duplication (Yuan *et al.*, 2009).

Plants metabolise a substantial portion of constitutive and pathogen-induced SA into an inactive 2-*O*-β-D-glucoside (SAG) by a specific UDP-glucose : SA glucosyltransferase (Lee & Raskin, 1999; Song, 2006). However, SAG stored in the vacuole can readily be converted back to SA by an, as yet undescribed, β-glucosidase (Ding & Ding, 2020). Furthermore, specific hydroxylases convert SA into 2,3- and 2,5-dihydroxybenzoic acids, which can eventually be glycosylated and stored in the vacuole as inactive forms (Zeilmaker *et al.*, 2015; Chen & Li, 2017; Zhang *et al.*, 2017). The SA biosynthetic pathways are interconnected to other primary and secondary metabolic pathways, including the biosynthesis of the aromatic amino acids, phenylpropanoids and phylloquinone (Xue *et al.*, 2013; Widhalm & Rhodes, 2016). Therefore, changes in the rate of SA biosynthesis might modulate the formation of other metabolites.

In *Arabidopsis*, signal transduction downstream of immunity-induced SA is mostly regulated by the receptor Nonexpressor of

Pathogenesis-Related Gene 1 (NPR1), which upon SA perception binds to TGACG motif-binding (TGA) transcription factors resulting in the induction of a large number of defence genes, especially pathogenesis-related (*PR*) genes (Cao *et al.*, 1997; Wang *et al.*, 2006; Moore *et al.*, 2011). The NPR1 paralogues NPR3 and NPR4 also act as SA receptors, but negatively regulate TGA transcription factors (Castelló *et al.*, 2018; Ding *et al.*, 2018). When cellular SA contents are high, SA binds to NPR3 and NPR4, releasing their suppression of TGA transcription factors to activate defence-related genes (Ding *et al.*, 2018; Ding & Ding, 2020). NPR1 is also known to regulate the antagonism between the SA and jasmonic acid (JA) signalling pathways in *Arabidopsis* (Spoel *et al.*, 2003; Pieterse *et al.*, 2012). However, the two pathways are not always antagonists. Rice (*Oryza sativa*), for example, can bypass NPR1-dependent SA perception using the transcription factor WRKY45 (De Vleesschauwer *et al.*, 2013). In this case, both SA and JA directly activate WRKY45, resulting in the joint upregulation of *PR* genes and a broad-spectrum defence against biotrophs as well as necrotrophs (De Vleesschauwer *et al.*, 2013). In *Arabidopsis*, pathogen-induced SA and SA receptors NPR3 and NPR4 can activate the JA signalling pathway required for the effector-triggered immunity-mediated hypersensitive response (Liu *et al.*, 2016). Several other studies have also suggested synergistic or neutral interactions between the SA and JA signalling pathways (Rostás *et al.*, 2013; Lemarié *et al.*, 2015; Betsuyaku *et al.*, 2018; J. Chen *et al.*, 2021; Sun *et al.*, 2021). In poplar, NPR1 has reduced importance in SA signalling compared with in *Arabidopsis* (Xue *et al.*, 2013), but the interaction between SA and JA signalling pathways in trees and other woody plant defences is not yet well understood. Recent studies have shown that both SA and JA pathways are induced in poplar leaves infected by the biotrophic rust fungus *Melampsora larici-populina* (Ullah *et al.*, 2019a; Q. Chen *et al.*, 2021), suggesting that both hormone pathways might be involved in defence. However, this hypothesis has not been rigorously tested.

In addition to regulating defence-related genes such as PRs, vegetative storage proteins (VSPs), and *Plant Defensin 1.2* (*PDF1.2*), both SA and JA signalling pathways have been shown to induce the accumulation of several groups of low-molecular-weight plant defence metabolites, including flavonoids and camalexins (Ahuja *et al.*, 2012; Pieterse *et al.*, 2012; Jeandet *et al.*, 2014). Flavonoids are structurally diverse phenolic compounds that are ubiquitously present in terrestrial plants and have a long list of ecological functions. For example, many classes of flavonoids, such as flavanones (Duan *et al.*, 2014; Förster *et al.*, 2022), flavonols (Jia *et al.*, 2010; Chen *et al.*, 2019), flavan-3-ols (Ullah *et al.*, 2017, 2019b; Wang *et al.*, 2017) and isoflavonoids (Graham *et al.*, 2007; Jahan *et al.*, 2020) serve as effective antipathogen defences as shown by *in vitro* and *in planta* bioassays. A potential association between SA signalling and flavonoid accumulation was supported when downregulation of lignin biosynthesis in *Arabidopsis* and *Medicago truncatula* resulted in the enhanced accumulation of both SA and flavonoids (Gallego-Giraldo *et al.*, 2011a,b). In rice, the mutation of one of the *PAL* genes resulted in reductions in both SA and sakuranetin, a flavonoid phytoalexin (Duan *et al.*, 2014). More recently, poplar trees treated with the SA analogue benzothiadiazole (BTH) or containing endogenously elevated SA were shown to have increased levels of antifungal flavan-3-ols, including catechin and proanthocyanidins (Ullah *et al.*, 2019a). Similarly, jasmonate signalling also regulates flavonoid biosynthesis. For example, sakuranetin biosynthesis in rice is induced by methyl-JA (Ogawa *et al.*, 2017), and mutants defective in JA biosynthesis exhibit decreased concentrations of naringenin and sakuranetin (Miyamoto *et al.*, 2016; Xu *et al.*, 2021). It appears that both the SA and JA signalling pathways can promote flavonoid accumulation, but this proposition has not been rigorously demonstrated in any plant species.

Here, we investigated the interplay between SA and JA signalling pathways in *Populus*. We first profiled SA and JA metabolites and transcripts of some signalling genes from multiple poplar genotypes with differential rust susceptibility to determine their hormonal responses to rust infection. We then generated transgenic black poplar lines hyperaccumulating SA and observed an enhanced resistance to rust. Targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis showed significantly increased levels of jasmonates in high SA lines with a concomitant accumulation of antimicrobial flavonoids. Finally, we showed that treating wild-type trees with exogenous SA or JA resulted in the reciprocal induction of the other hormone. Therefore, poplar defence against the rust fungus is a consequence of a positive interaction between SA and JA. Our findings highlight the striking differences in SA signalling in this woody perennial from that in the annual herb *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

Black poplar (*Populus nigra*) genotypes, including NP1, Dornberg 4 (Dorn4) and Fritzlar 2, as well as hybrid poplar (*P.* × *canadensis*) genotypes, including Leipzig (Leip), Regenerata Kew (Kew) and Blanc du Poitou (Bla) were used in this study, unless otherwise stated. All genotypes were grown in the glasshouse facility of the Max Planck Institute for Chemical Ecology, Jena, Germany (50.9108°N, 11.5683°E). Trees were propagated from stem cuttings and grown at 22°C : 19°C, day : night temperature, 60% relative humidity and a 16 h : 8 h, light : dark cycle. Rooted stem cuttings were potted individually in 2 l pots containing standard substrate mix (Klasmann-Deilmann, Geeste, Germany), and fertilised as described previously (Ullah *et al.*, 2019b). Unless stated elsewhere, young trees with a height of *c.* 1 m were transferred to a controlled environment chamber 3 d before starting any experiment.

Rust inoculation

A virulent strain of the rust fungus *Melampsora larici-populina* was multiplied on the highly susceptible clone Fritzlar 2 and urediniospores were harvested as described previously (Ullah *et al.*, 2017). The first inoculation experiment using five genotypes with varying rust resistance was carried out on potted trees grown just

outside the glasshouse during May to August 2015. For each genotype, 10 trees of similar size were chosen and placed in a separate receptacle for watering independently. Five plants per genotype were sprayed thoroughly using a rust spore suspension (*c.* 10^5 spores ml^{-1} water) onto the abaxial leaf surfaces. The other five trees were similarly sprayed with water only as a control. Inoculation experiments were performed at 18:00 h and trees were then kept in the dark for 12 h. All other infection experiments were carried out in the controlled environment chamber. Five prelabelled leaves from leaf plastochron index (LPI) 6–10 on each tree were harvested, midribs were removed and pooled leaf laminae were immediately frozen in liquid nitrogen and stored in −80°C until further use.

Generation of transgenic black poplar

To engineer black poplar trees with high levels of SA, the previously described *FD-Irp9* construct was used (Xue *et al.*, 2013). The binary vector harbours the *Yersinia enterocolitica Irp9* gene fused to the chloroplast-targeting sequence of the *Ferredoxin 2* (*FD2*) gene from *A. thaliana* and is driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter in pCambia 1302. Transformation of the *P. nigra* NP1 clone using pCambia1302-*FD-Irp9* was performed following a protocol optimised for *Populus tremula* × *alba* INRA 717-1B4 (Meilan & Ma, 2006). Transformation with only pCambia1302 served as the vector control. To verify transgene expression, reverse transcription (RT)-qPCR analysis was carried out on wild-type and *FD-Irp9* expressing transformants. Selected transgenic lines were micropropagated as described by Behnke *et al.* (2007). After being acclimatised in a controlled environment chamber, transgenic lines were moved to the glasshouse and the contents of SA and SAG were verified in a preliminary experiment. For subsequent experiments, each line was propagated by rooted stem cuttings from the transgenic parental lines.

Plant growth assessment

The height of poplar trees was measured at 10 wk old under glasshouse conditions. All plants were grown using nutrient-rich potting soil supplemented with standard fertilisers (Ullah *et al.*, 2019b). A separate experiment was carried out to measure multiple growth parameters, including tree height, leaf number per plant, shoot weight and root weight. Rooted stem cuttings were potted individually in 2 l pots containing sand mixed with perlite. Young poplar trees were fertilised periodically with half-strength Hoagland's solution. Growth data were taken at 12 wk after the rooted stem cuttings were transplanted into pots. After measuring fresh weight, shoots and roots of each tree were dried in a dryer at 50°C for 7 d. Final dry weight was recorded once the weight of shoots and roots were found to be similar on two consecutive days.

Extraction of phytohormones and phenolic metabolites

Flash-frozen plant materials were ground in liquid nitrogen to a fine powder using a mortar and pestle, and then lyophilised at 0.22 mbar pressure and −35°C temperature for 3 d. Here, *c.* 15 mg of dry tissue powder was extracted with 1 ml methanol containing 3 µl phytohormone standard mix (30 ng of D_4-SA (Sigma-Aldrich), 30 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), 6 ng D_6-jasmonoyl-L-isoleucine (JA-Ile) (HPC Standards GmbH)), 5 µg apigenin-7-glucoside (Sigma-Aldrich) and 0.3 mg phenyl β-D-glucopyranoside (Sigma-Aldrich) as internal standards. The contents were vortexed vigorously for a few seconds, incubated for 30 min at 20°C with continuous agitation, then centrifuged at 13 000 ***g*** at 4°C for 5 min. Here, *c.* 950 µl of the supernatant were transferred to a new microcentrifuge tube. Aliquots of each sample were transferred to high performance liquid chromatography (HPLC) vials and different metabolites were analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Phytohormone quantification using LC-MS/MS

Hormones were analysed using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a QTRAP 6500 tandem mass spectrometer (Sciex, Darmstadt, Germany) equipped with a turbo spray ion source operated in the negative ionisation mode. Hormones were separated on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm; Agilent) at 25°C, with two mobile phases consisting of 0.05% formic acid in water (A) and acetonitrile (B), at a flow rate of 1.1 ml min^{-1}, using the elution profile originally described by Vadassery *et al.* (2012) (Supporting Information Table S1). The parent ion and corresponding fragments of SA and jasmonates were analysed by multiple reaction monitoring (MRM) (Table S2). Concentrations of SA, JA and JA-Ile were determined relative to the internal standards of D_4-SA, D_6-JA and D_6-JA-Ile, respectively. Content of *cis*-OPDA was determined using D_6-JA, applying an experimental response factor (RF) of 1.0. Levels of OH-JA-Ile and COOH-JA-Ile were quantified relative to D_6-JA-Ile, applying an experimental RF of 1.0. JA-glucoside was determined relative to D_6-JA, applying a theoretical RF of 1.0. The content of SAG was determined relative to D_4-SA, applying a theoretical RF of 1.0.

RNA extraction and cDNA synthesis

Total RNA from leaf tissues was extracted using the Invitrap Spin Plant RNA Mini Kit (Stratec Biomedical, Birkenfeld, Germany) following the manufacturer's protocol, except that an additional DNase treatment was included (RNase-Free DNase Set; Qiagen) (Ullah *et al.*, 2017). Reverse transcription of 1 µg RNA into cDNA was achieved using the reverse transcriptase SuperScript III (Invitrogen) and 50 pmol oligo(dT)12-18 Primer (Invitrogen) in a reaction volume of 20 µl and following the manufacturer's protocol.

Analysis of gene expression and rust growth by RT-qPCR

To quantify the expression of target genes, a segment of *c.* 150 base pairs was amplified using gene-specific primers (Table S3). The RT-qPCR reactions were performed in a 20-µl volume as described by Ullah *et al.* (2019a). Transcript abundance was normalised to the abundance of the *PtUBQ* and was calculated from

four or five biological replicates, with each biological sample being analysed from two technical replicates. Relative colonisation of the rust fungus *M. larici-populina* in poplar leaves was determined using RT-qPCR (Ullah *et al.*, 2017), in which transcripts of the fungal housekeeping gene *MlpActin* were normalised to transcripts of the poplar housekeeping gene *PtUBQ*.

Quantification of flavonoids, salicinoids and other metabolites using LC-MS/MS

Flavan-3-ols, including the monomer (+)-catechin and (+)-gallocatechin, and the most abundant dimer procyanidin B1 (catechin-epicatechin), as well as the flavanone naringenin and the quercetin-glycoside were analysed using an Agilent 1200 HPLC system coupled to an API 3200 tandem mass spectrometer. The HPLC was equipped with a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm), and chromatographic separation was performed using 0.05% formic acid (v/v) and acetonitrile as mobile phases A and B, respectively (Table S4). The mass spectrometer equipped with a turbo spray ion source was operated in the negative ionisation mode to monitor the analyte parent ion to product ion formation (MRM, Table S5). Data acquisition and quantification were performed using the software Analyst 1.5.1.

Other flavonoids, including rutin, kaempferol, taxifolin, luteolin, luteolin-di-glycoside and kaempferol-rhamnoside, as well as salicinoids and phenolic acids, were analysed using an Agilent 1260 LC system coupled with a QTRAP 6500 tandem mass spectrometer. The HPLC was equipped with a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm), and chromatographic separation was performed using 0.05% formic acid (v/v) and acetonitrile as mobile phases A and B, respectively (Table S6) (Lackus *et al.*, 2021). The mass spectrometer equipped with a turbo spray ion source was operated in the negative ionisation mode to monitor analyte parent ion to product ion formation (MRM; Table S7). Data acquisition and quantification were performed using the software MultiQuant 3.0.3 (Sciex, Framingham, MA, USA). The relative content of each flavonoid metabolite was calculated equivalent to the internal standard apigenin-7-glucoside. Relative content of each salicinoid was calculated equivalent to the internal standard phenyl β-D-glucopyranoside.

Extraction and quantification of soluble sugars and free amino acids

To extract soluble sugars and free amino acids, 1 ml methanol was added to each microcentrifuge tube containing *c.* 10 mg of freeze-dried leaf tissue powder, vortexed vigorously, incubated for 25 min at 20°C with continuous agitation, and then centrifuged at 13 000 ***g*** at 4°C for 5 min. Here, *c.* 950 µl of the supernatant was transferred to a new microcentrifuge tube. The raw extract was diluted to 1 : 10 in water containing 5 µg ml^{-1} of ^{13}C-glucose (Sigma-Aldrich), 5 µg ml^{-1} of ^{13}C-fructose (Toronto Research Chemicals, North York, ON, Canada), and 10 µg ml^{-1} of ^{15}N or ^{13}C labelled amino acid standard mix (Isotec, Miamisburg, OH, USA) as internal standards. Sugars were analysed using an Agilent 1200 HPLC system equipped with an API 3200 tandem mass spectrometer (for details, please refer to Notes S1; Tables S8, S9). Free amino acids were analysed using an Agilent 1260 LC system coupled with a QTRAP 6500 tandem mass spectrometer (Notes S2; Tables S10, S11).

Exogenous chemical applications

To manipulate the hormone signalling pathways in poplar trees (*P. nigra* Fritzlar 2), the SA analogue BTH (Sigma-Aldrich), methyl jasmonate (MeJA; Sigma-Aldrich) and methyl salicylate (MeSA; Sigma-Aldrich) were used. BTH (0.5 mM) was dissolved in 0.2% methanol in water and solutions of MeSA and MeJA were prepared in water at final concentrations of 250 µM. All these solutions were sprayed thoroughly onto both surfaces of the leaves until saturation. Mock-treated trees were sprayed only with solvents. After spraying, the plants were covered with plastic bags for 12 h, opened from the top, and leaf laminae (LPI 5–7) were collected at different time points.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism v.9.1.3. The normality and variance of each data set were first determined using the Shapiro–Wilk test and the Levene test, respectively, to analyse data by parametric tests such as analysis of variance (ANOVA). For ANOVA, Tukey's multiple comparisons post-hoc test was performed if the test statistics were significant. Asterisks denote statistical significance (*, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Statistical results of a two-way ANOVA for hormone metabolites (Fig. 1) and gene expression (Fig. 2) are provided in Tables S12 and S13, respectively. All other *P*-values are mentioned in the respective figures or legends. Figures were prepared and organised using GraphPad Prism and Adobe Illustrator CS5.

Results

Salicylic acid and jasmonate content of poplar leaves both increase in response to rust infection

Using a highly compatible poplar–rust system, we have previously shown that levels of SA and JA metabolites increased over the course of an infection with *M. larici-populina* (Ullah *et al.*, 2019a). To determine if the induction of SA and jasmonates after rust infection is a common defence response in poplar, we conducted a controlled-inoculation experiment using five genotypes with varying levels of rust susceptibility grown outdoors in pots. The genotype NP1 was highly susceptible, while Leip and Dorn4 were moderately susceptible, and Kew and Bla were resistant to rust, as previously determined by quantifying *in planta* rust colonisation using RT-qPCR (Ullah *et al.*, 2017). Concentrations of SA and SAG increased at least five-fold in all genotypes in response to rust infection (Fig. 1a,b). Jasmonates, including JA, JA-glucoside and JA-Ile, increased significantly in all genotypes after rust infection, whereas the content of *cis*-OPDA increased significantly in all genotypes except NP1 (Fig. 1c–f). Taken together, the simultaneous induction of both SA and JA

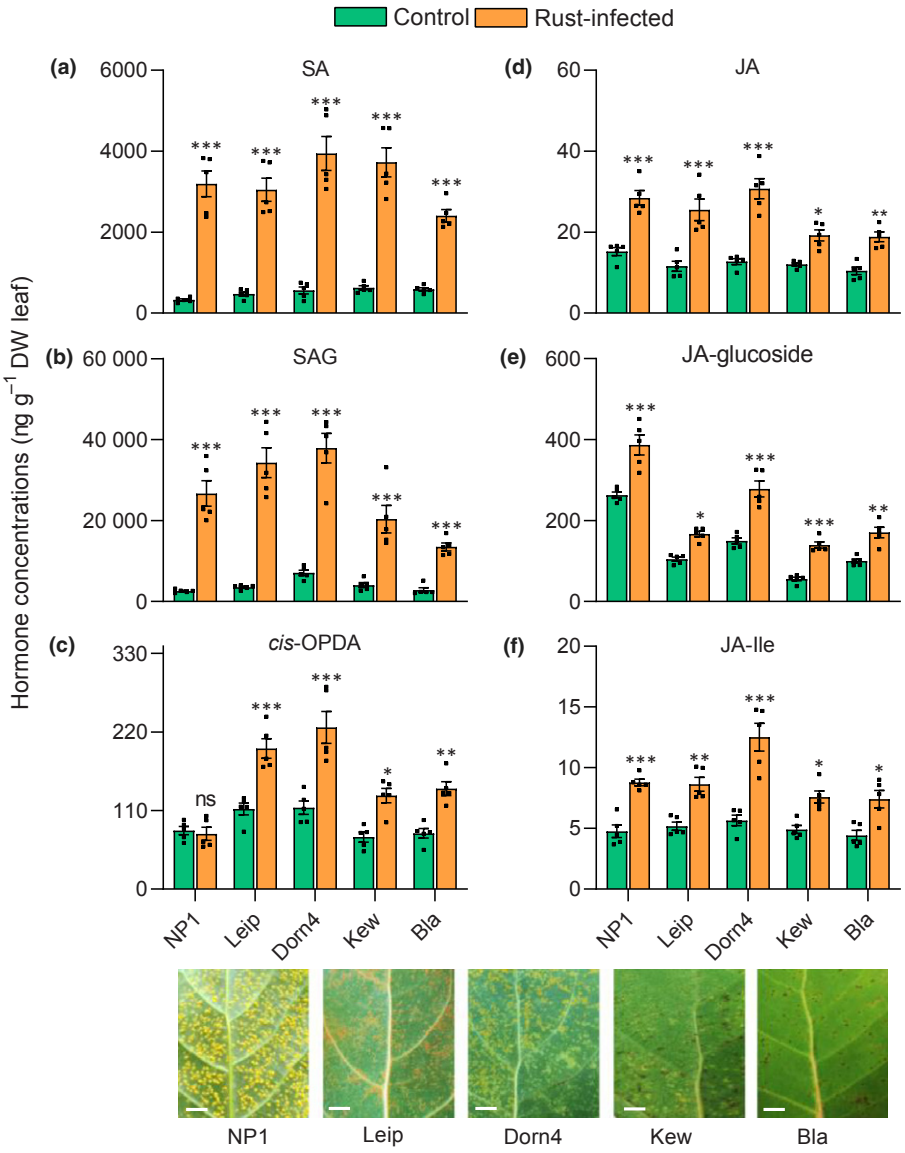

Fig. 1 Accumulation of salicylic acid (SA) and jasmonates in poplar after infection with a virulent rust fungus, *Melampsora larici-populina*. (a, b) Concentrations of SA and its glucoside (SAG) in poplar leaves with and without rust infection. (c–f) Jasmonate content in poplar leaves with and without rust infection. Young trees belonging to five different poplar genotypes with varying resistance levels were grown outdoors in pots. NP1 is highly susceptible, Leip and Dorn4 are moderately susceptible, and Kew and Bla are resistant to rust. Representative photographs of rust-infected poplar leaves below the graphs show the degree of rust resistance (for quantification of resistance, please refer to Ullah *et al*., 2017). Leaf samples were collected from both rust-infected and the corresponding control trees at 7 d after inoculation, and hormones were quantified using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system. Data were analysed using a two-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Statistical details of a two-way ANOVA for the levels of hormone metabolites are provided in the supplemental information (Supporting Information Table S12). Asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate pairwise significance between control vs rust-infected means. Bars represent the mean with standard error ($n = 5$). All data points are plotted on the graph as black dots. Bar, 3 mm. *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; ns, nonsignificant; SAG, salicylic acid-glucoside.

metabolites in rust-infected poplar leaves suggested that these two hormone signalling pathways are not necessarily antagonistic in this woody plant.

Rust induces *WRKY* transcription factors and *PR* genes differentially in poplar lines of differential susceptibility

An increase in SA and jasmonate concentrations often results in the induction of genes encoding *WRKY* transcription factors and *PR* proteins. We previously showed that one highly susceptible black poplar genotype also responded to rust infection by upregulating *WRKY* and *PR* genes (Ullah *et al*., 2019a). Here transcripts of *WRKY89*, a putative marker for SA, increased *c*. 10-fold in all genotypes after rust infection (Fig. 2a). *WRKY18*, which is known to be induced by both SA and JA (Jiang *et al*., 2014), was also induced in all poplar genotypes after rust infection (Fig. 2b). We found *PR1* and *PR2* gene transcripts increased by 50- to 60-fold in NP1, the most susceptible genotype to rust infection (Fig. 2c,d). A modest induction of *PR1* and *PR2* was found in the moderately susceptible genotypes Leip and Dorn4. The levels of *PR* transcripts in the resistant genotypes Kew and Bla were also significantly induced after infection, but the magnitude of their induction was much lower than in the susceptible genotypes (Fig. 2c,d). Taken together, these results indicated that poplar trees respond to rust attacks by activating SA and JA signalling pathways. *PR* genes were also induced, but the extent of induction was positively correlated with the amount of pathogen growth.

Engineering black poplar trees with high levels of SA and SA-glucoside

To more directly test SA effects on poplar defence against rust, we generated a transgenic black poplar with constitutively elevated SA in the rust-sensitive *P. nigra* NP1 background. We adopted the approach of Xue *et al*. (2013) by constitutively expressing a bacterial SA synthase (*Irp9*) gene from *Yersinia*

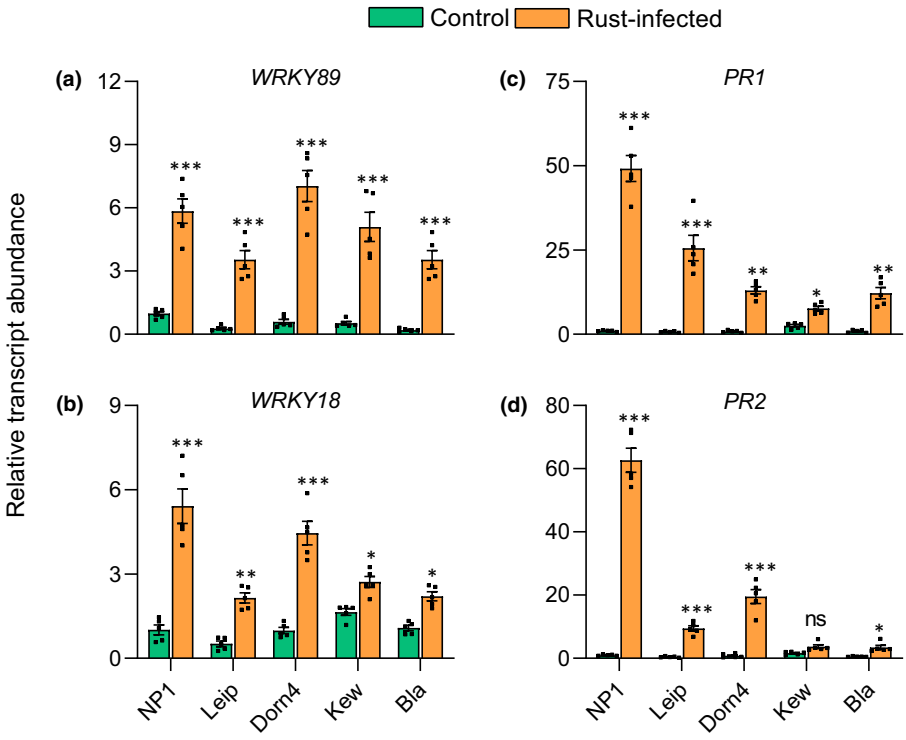

Fig. 2 Induction of *WRKY* transcription factor and pathogenesis-related (*PR*) protein-encoding genes in different poplar genotypes infected with rust. (a, b) Relative expression of *WRKY* genes. (c, d) Relative expression of *PR* genes in poplar leaves with and without rust infection. Transcripts of each gene were determined using RT-qPCR and normalised to the transcript levels of the housekeeping genes *Actin* and *Ubiquitin*. Data were analysed using a two-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Statistical details of a two-way ANOVA for gene expression are provided in the supplemental information (Supporting Information Table S13). Asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate pairwise significance between control vs rust-infected means. Bars represent mean ± standard error ($n = 5$, each containing two technical replicates). All data points are plotted on the graph as black dots. ns, nonsignificant.

enterocolitica with a chloroplast-targeting sequence from ferredoxin (*FD*). The FD-Irp9 enzyme converts chorismic acid in the chloroplast directly into SA, which is then transported to the cytosol, thereby minimising interference with the native SA biosynthetic pathways (Xue *et al.*, 2013; Fig. 3a). Six independent transgenic lines were confirmed by RT-qPCR and SA analysis and four were propagated for further analyses. All four lines had high levels of *Irp9* transcripts, even higher than those of the housekeeping gene *Actin* (Fig. 3b). The level of SA in *FD-Irp9* overexpressing leaves was 4–6 times higher than in wild-type (Fig. 3c). These high SA lines also accumulated significantly higher amounts of SAG than the wild-type, *c.* 16-fold higher, indicating that a major proportion of SA was conjugated (Fig. 3d).

We next asked whether SA hyperaccumulation affected JA accumulation as both SA and jasmonate levels increased concurrently in poplar trees in response to rust infection (Fig. 1). Both JA and JA-Ile levels were significantly higher in *FD-Irp9* lines than in wild-type plants (Fig. 3e,f), suggesting that elevated SA resulted in an enhanced accumulation of JA and its metabolites. By contrast, no large magnitude changes were observed in soluble sugars (Fig. S1), free amino acids (Fig. S2), shikimate, quinate and benzoate derivatives (Figs S3, S4), and salicinoids (Fig. S5). The only exceptions were increases in 3-hydroxy and 2,5-dihydroxy benzoic acid, potential SA catabolites (Fig. S6), as well as flavonoid aglycones such as naringenin, kaempferol, taxifolin, luteolin and flavan-3-ols (Figs S7, S8). These data corroborated our previous report that flavonoid biosynthetic pathway genes were upregulated in SA-hyperaccumulating hybrid poplar and BTH-treated black poplar (Ullah *et al.*, 2019a). Taken together, SA hyperaccumulation in black poplar resulted in elevated pools of jasmonate and flavonoid metabolites.

High SA levels do not affect the expression of rust-induced *PR* genes in poplar leaves

To test whether the constitutive accumulation of SA and SAG in poplar resulted in the activation of signalling genes known to be induced after rust infection, we analysed the expression of *WRKY89* and two *PR* genes in *FD-Ipr9* transgenic lines. Transcripts of *WRKY89* were significantly higher in the high SA transgenic lines (Fig. 4a). However, transcript levels of *PR1* and *PR2* were unchanged, suggesting that elevated SA levels did not activate *PR* genes in the absence of other stresses (Fig. 4b,c). To further validate these unexpected observations, we sprayed poplar trees with the SA analogue BTH and leaf samples were collected at three different time points. BTH treatment induced *WRKY89* by more than 12-fold in 1 d and the transcript levels remained steady over 7 d (Fig. 4d), indicating that the SA signalling pathway positively regulated the *WRKY89* transcription factor gene in poplar. However, the transcripts of *PR1* and *PR2* genes did not change in response to BTH treatment (Fig. 4e,f), mirroring the response to SA hyperaccumulation (Fig. 4b,c). Taken together, our results suggested that rust-inducible *PR* genes in poplar were not directly regulated by elevated SA levels in the absence of pathogens.

SA hyperaccumulation does not show any adverse effects on black poplar growth

In *Arabidopsis*, SA overproduction results in severe defects in plant growth (Huot *et al.*, 2014). However, black poplar trees hyperaccumulating SA did not show any growth defects in terms of plant height when trees were grown in nutrient-rich potting soil under optimal growth conditions (Fig. S9). We then grew

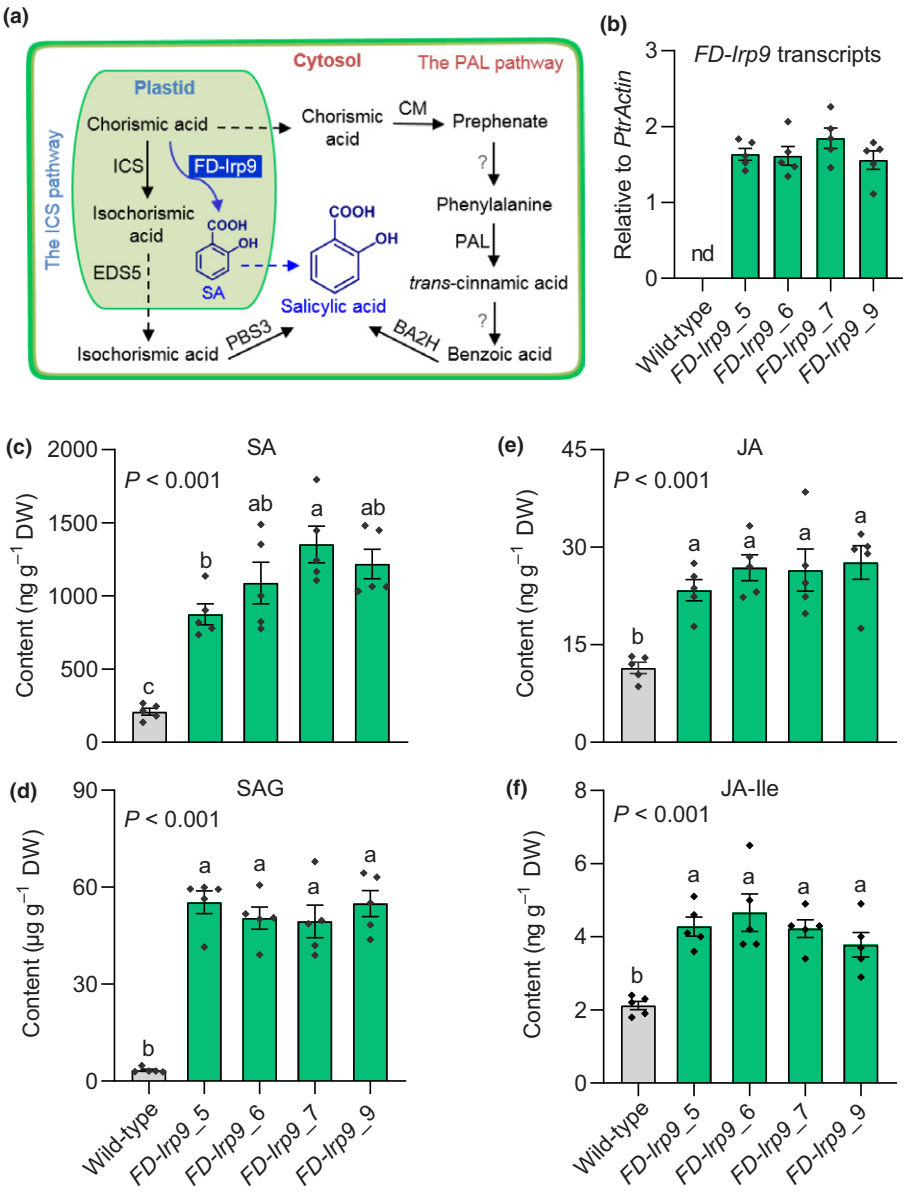

Fig. 3 Engineering black poplar trees to synthesise high levels of salicylic acid (SA) and its glucoside (SAG). (a) Biosynthesis of SA in plants occurs via two distinct routes: the isochorismate synthase (ICS) pathway and the phenylalanine ammonia-lyase (PAL) pathway, but black poplar trees were transformed to use a third route, catalysed by a bacterial SA synthase (*Irp9*) with the plastid-targeting sequence of a ferredoxin (*FD*) gene. Irp9 utilises chorismic acid present in the plastid and converts it directly to SA. (b) Relative transcripts of the *Irp9* gene in transgenic black poplar leaves. (c, d) Accumulation of high amounts of SA and SAG in *FD-Irp9*-expressing transgenic black poplars. (e, f) Accumulation of jasmonic acid (JA) and jasmonyl-L-isoleucine (JA-Ile) in transgenic black poplar. Leaf samples (LPI 5-6) were collected from 3-month-old trees, and hormones were quantified using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Data were analysed using a one-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Different letters above bar plots indicate groups were significantly different ($P < 0.05$). Data are represented as the mean ± standard error ($n = 5$). Individual data points are plotted on the graph as black dots. BA2H, benzoic acid 2-hydroxylase; CM, chorismate mutase; EDS5, ENHANCED DISEASE SUSCEPTIBILITY 5; PBS3, avrPphB SUSCEPTIBLE3; nd, not detected.

young black poplar trees in sand medium amended with perlite, supplying nutrients periodically through half-strength Hoagland's solution. Under these nutrient-limiting conditions, poplar trees grew more slowly than in potting soil, without showing any nutrient deficiency symptoms (Fig. 5a). Plant height and the number of fully expanded leaves were similar between SA-hyperaccumulating and wild-type lines (Fig. 5b,c), and fresh and dry above-ground biomass were identical (Fig. 5d,e). Fresh root weights were also statistically similar in high SA lines as in wild-type plants (Fig. 5f). Whereas dry root weights varied slightly among the transgenic lines, the means were not significantly different between high SA and wild-type lines (Fig. 5g). Collectively, these results supported the idea that constitutive activation of the SA signalling pathway in poplar did not result in any negative growth consequences, even under resource-limited conditions. A previous investigation conducted on lines of a hybrid poplar (*P. tremula* × *alba*) that hyperaccumulated SA also reported no negative growth consequences when plants were grown under different temperature regimes (Xue *et al.*, 2013).

SA hyperaccumulation enhances poplar resistance against rust

To determine the effects of high SA accumulation in transgenic black poplar lines against pathogens, we inoculated trees using the biotrophic rust fungus *M. larici-populina* and compared pathogen growth between genotypes. Rust colonisation on high SA lines was much lower compared with that on wild-type plants, as visually observed by lower numbers of uredinia and reduced sizes of the orange uredinial pustules at 7 d post inoculation

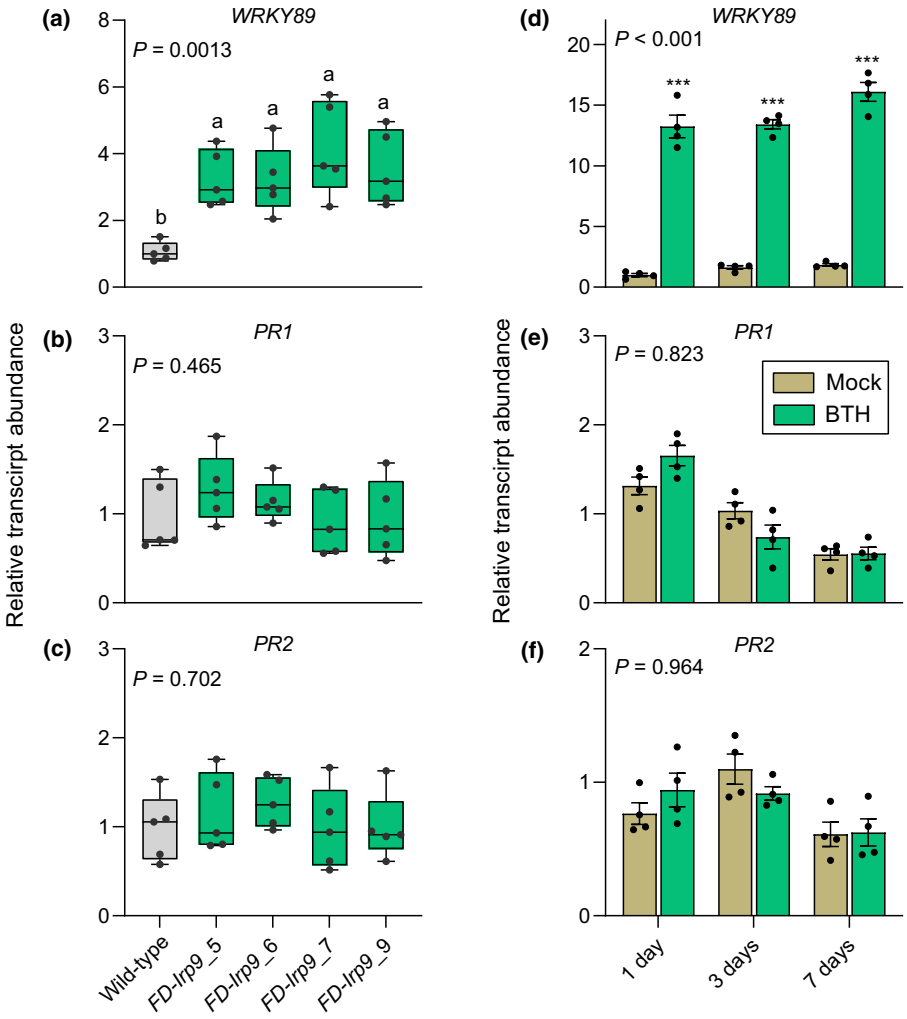

Fig. 4 Expression of *WRKY* and *PR* genes in high salicylic acid (SA) poplar lines and in wild-type trees after treatment with the SA analogue benzothiadiazole (BTH). (a–c) Relative transcripts in the leaves of transgenic black poplar hyperaccumulating SA. Leaf samples (the first and second fully expanded leaf from the apex) were collected from 3-month-old trees, and transcript levels were determined using RT-qPCR. Data were analysed using a one-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Different letters above box plots indicate groups were statistically significant ($P < 0.05$). Each box extends from the 25th to 75th percentiles, and the horizontal line inside the box represents the median. Whiskers were plotted down to the minimum and up to the maximum value, and all data points are shown on the graph as black dots ($n = 5$, two technical replicates). (d–f) Relative transcripts in the leaves of black poplar at different time points after being treated with the SA analogue BTH at a concentration of 0.5 mM. Data were analysed using a two-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. *P*-value (Factor: BTH treatment) on each graph denotes statistical significance obtained from a two-way ANOVA. Asterisks (***, $P < 0.001$) indicate pairwise significance between mock vs BTH-treated means. Data are presented as the mean ± standard error ($n = 4$, two technical replicates). Individual data points are plotted as black dots.

(Fig. 6a, left panel). Moreover, we quantified *in planta* rust colonisation by RT-qPCR. The relative colonisation of *M. larici-populina* was less than half as much on the high SA lines compared with wild-type (Fig. 6a, right panel), indicating that the SA signalling pathway is involved in poplar defence against biotrophs.

Although transcripts of *PR* genes known to be induced by rust were found to be similar in high SA lines compared with wild-type plants (Fig. 4), we hypothesised that *PR* genes might be induced strongly in high SA lines when infected by rust. Surprisingly, both *PR1* and *PR2* gene expression levels were found to be significantly lower in SA-hyperaccumulating poplar lines compared with wild-type trees after rust infection (Fig. 6b). *PR* gene transcripts were well correlated with rust colonisation (Figs S10, S11). As shown for uninfected control trees, the expression of the *WRKY* genes measured was significantly higher in rust-infected high SA lines than in rust-infected wild-type trees (Fig. 6c). These results were consistent with the induction pattern of *PR* genes in different poplar genotypes with varying levels of rust resistance, as shown previously (Figs 1, 2). We concluded that pathogen-inducible *PR* gene expression did not necessarily correspond with SA levels in poplar, but closely matches the extent of pathogen infection.

High SA lines accumulate elevated concentrations of jasmonates and flavonoids after rust infection

The lower *PR* gene transcript levels in *FD-Irp9*-overexpressing vs wild-type black poplar trees raised the question of whether elevated SA and SAG levels persisted after rust infection. Indeed, we found higher levels of both SA and SAG in *FD-Irp9*-overexpressing lines compared with in the wild-type following rust infection (Fig. 7a). Higher SA accumulation was also associated with enhanced jasmonate accumulation in these lines, as concentrations of JA, JA-Ile, OH-JA-Ile and JA-glucoside increased significantly after rust infection relative to the wild-type (Fig. 7a). These data further supported the positive effects of SA signalling on jasmonate accumulation.

As mentioned above and reported previously (Ullah *et al.*, 2019a), flavonoid biosynthesis is stimulated in high SA poplars.

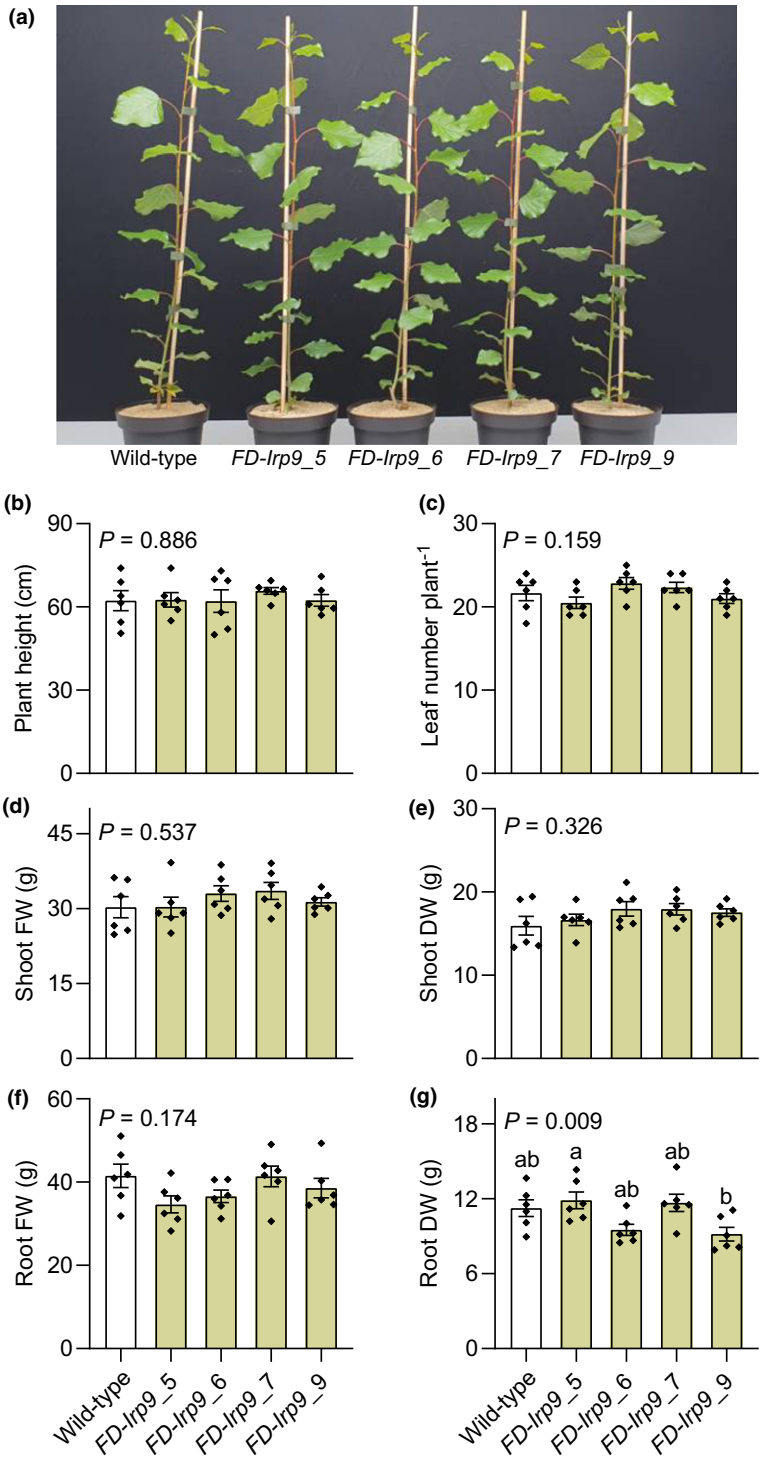

Fig. 5 Effect of salicylic acid (SA) hyperaccumulation on poplar growth. Plants were grown for 12 wk in 2 l pots containing sand and fertilised periodically with half-strength Hoagland's nutrient solution. (a) Representative photograph of *FD-Ipr9* transgenic lines and wild-type black poplar. (b–g) Different growth parameters of poplar trees. Data were analysed using a one-way ANOVA and Tukey's multiple comparison test with a 95% confidence interval. Different letters above bar plots (g) indicate groups that are significantly different ($P < 0.05$). Data are presented as the mean ± standard error ($n = 6$). Individual data points are plotted as black dots.

Following rust infection, the amounts of naringenin, kaempferol, rutin, quercetin-glycoside and flavan-3-ols (the monomer catechin and the dimer procyanidin B1), remained significantly higher in high SA lines compared with in wild-type plants (Fig. 7b). Levels of rust colonisation were negatively correlated with the content of SA and antimicrobial flavan-3-ols (Fig. S10). Therefore, both elevated jasmonates and antimicrobial flavonoids might have contributed to enhanced rust resistance of black poplar trees with high SA levels.

SA and JA signalling pathways interact positively in poplar after external hormone application

To further investigate the interplay between SA and JA signalling pathways in poplar, we manipulated these pathways by external spraying with MeSA and MeJA. Poplar trees treated with MeSA increased their contents of SA and SAG in leaves, and the effect was greater when MeJA was co-applied (Fig. 8a,b). External spraying with MeSA also increased the contents of jasmonates,

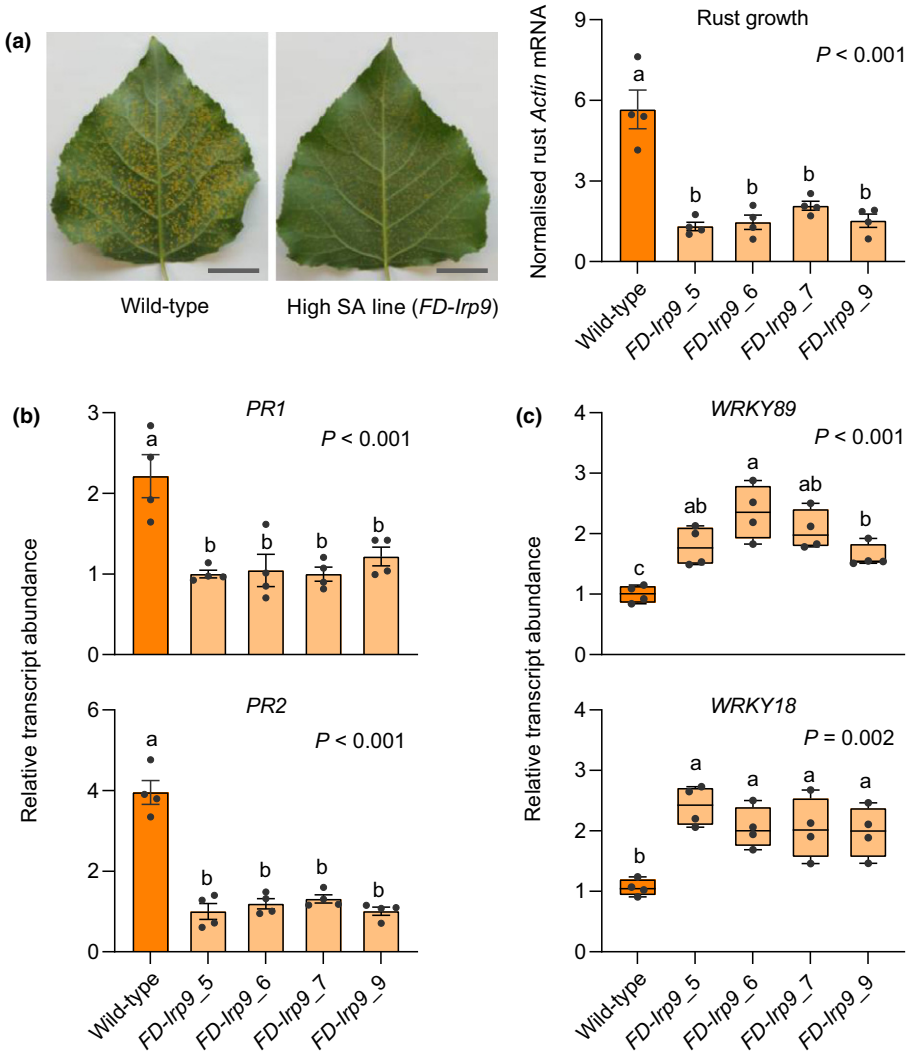

Fig. 6 Salicylic acid (SA) overproducing black poplar trees are more resistant to rust. Comparisons of leaves of transgenic SA-hyperaccumulating vs wild-type lines infected by the rust fungus *Melampsora larici-populina*. (a) Representative photographs (left panel), and relative colonisation of rust fungus determined by RT-qPCR (right panel). Transcripts of the rust house-keeping gene *MlpActin* were normalised to transcripts of the poplar house-keeping gene *Ubiquitin*. (b) Relative transcript levels of *PR* genes. Data were analysed using a one-way ANOVA and Tukey's multiple comparison test with a 95% confidence interval. Different letters above bar plots indicate groups were significantly different ($P < 0.05$). Data are presented as the mean ± standard error ($n = 4$, two technical replicates). Individual data points are plotted as black dots. Bar, 15 mm. (c) Relative expression of *WRKY* transcription factor genes. Transcripts of each gene were determined using RT-qPCR and normalised to the transcript levels of the housekeeping gene *Ubiquitin*. Data were analyzed using a one-way ANOVA and Tukey's multiple comparison test with a 95% confidence interval. Different letters above box plots indicate groups were significantly different ($P < 0.05$). Each box extends from the 25th to 75th percentiles, and the horizontal line inside the box represents the median. Whiskers were plotted down to the minimum and up to the maximum value, and all data points are shown on the graph by dots ($n = 4$).

including *cis*-OPDA, JA and JA-Ile (Fig. 8c–e), consistent with the results for SA-hyperaccumulating lines (Fig. 3). MeJA alone increased the concentrations of jasmonates, as would be expected, while co-application with MeSA increased the accumulation of all hormone metabolites (Fig. 8). Levels of flavonoids, including naringenin, quercetin-glycoside and the three flavan-3-ols measured were all increased by spraying with either MeSA or MeJA separately (Fig. 8f–j) and increased in greater amounts when MeSA and MeJA were co-applied (Fig. 8), suggesting a positive interplay between SA and JA signalling pathways in poplar.

Discussion

Salicylic acid and jasmonates are extensively studied plant hormones that play crucial roles in plant defence against pathogens (Pieterse *et al.*, 2012). These two hormones regulate the induction of a plethora of defence molecules, including the *PR* proteins and low-molecular-weight secondary metabolites (Pieterse *et al.*, 2012; Jeandet *et al.*, 2014). SA and JA are generally thought to mediate the defence against biotrophic and necrotrophic pathogens, respectively, with the two signalling pathways antagonistic to one another (Pieterse *et al.*, 2009). However, this dichotomy is not conserved in plants (De Vleesschauwer *et al.*, 2013). Here we provide evidence that the two pathways are not antagonistic in the woody perennial *Populus*. In response to infection by the rust fungus *M. larici-populina*, poplar trees concomitantly induced the accumulation of SA and JA metabolites. This positive interplay regulates the flavonoid defence against *M. larici-populina*, an obligate biotroph (Fig. 9).

SA and JA biosynthetic pathways interact positively in poplar

Multiple lines of evidence support the absence of SA–JA antagonism in poplar. First, both SA and JA metabolites accumulated to greater amounts in leaves after rust infection, and this was observed in different poplar genotypes with varying levels of rust susceptibility. Previous studies on poplar have also reported the simultaneous induction of SA and jasmonates after herbivore and pathogen attacks (Clavijo McCormick *et al.*, 2014; Ullah *et al.*, 2019a,b). Second, transgenic black poplar with endogenously elevated SA levels showed an increased jasmonate content, regardless

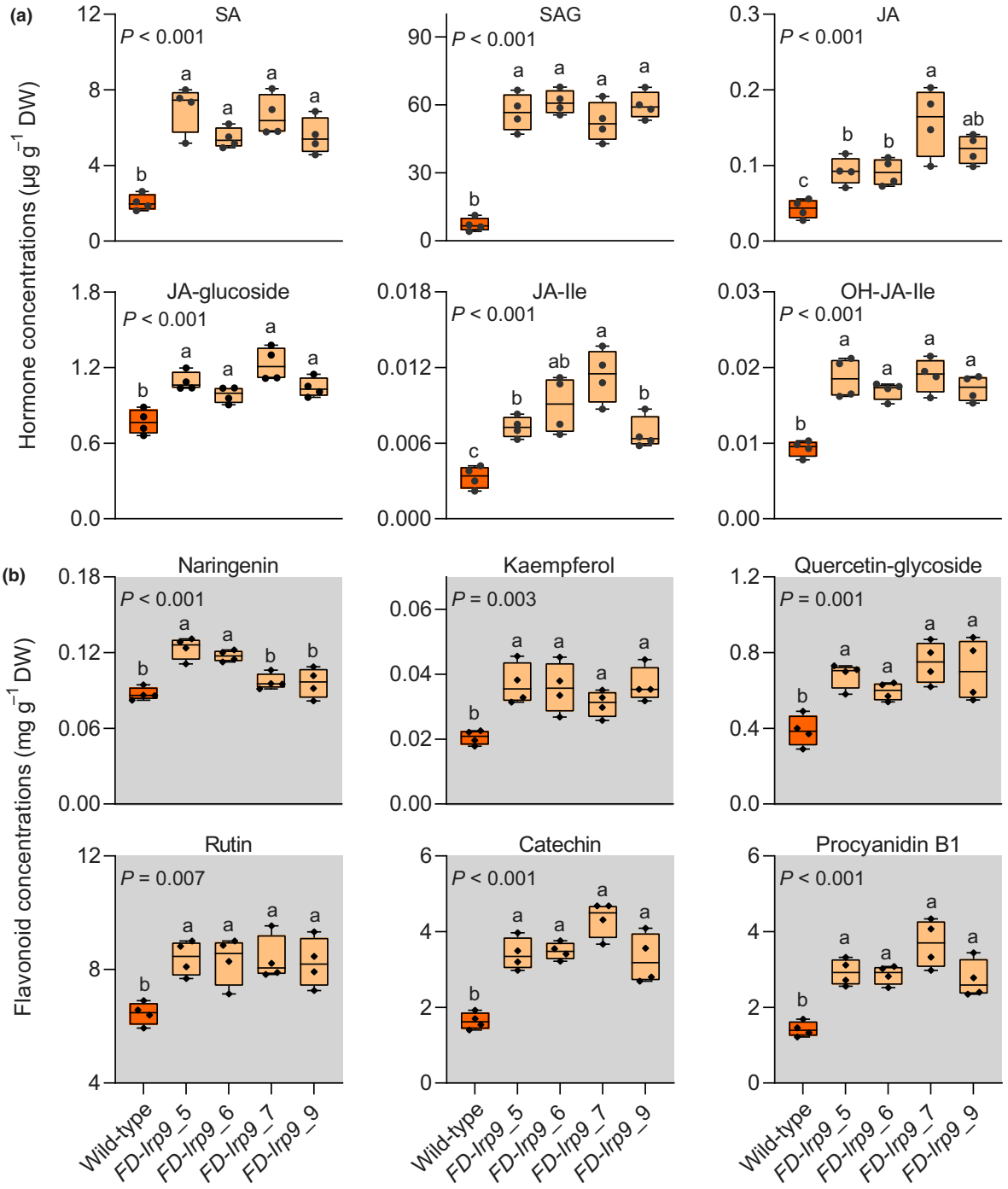

Fig. 7 Salicylic acid (SA)-hyperaccumulating poplar lines accumulate higher levels of jasmonates and antimicrobial flavonoids after rust infection than wild-type line. (a) Concentrations of SA, jasmonic acid (JA) and derivatives. (b) Relative concentrations of flavonoids. Hormones and flavonoids were quantified using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Data were analysed using a one-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Each box extends from the 25th to 75th percentiles, and the horizontal line inside the box represents the median. Whiskers were plotted down to the minimum and up to the maximum value, and all data points are shown on the graph as black dots ($n = 4$). Different letters above box plots indicate groups that were significantly different ($P < 0.05$). JA-Ile, jasmonoyl-L-isoleucine; OH-JA-Ile, hydroxy-JA-Ile; SAG, salicylic acid-glucoside.

of rust inoculation. Third, exogenous applications of MeSA or MeJA resulted in increases in both SA and JA metabolites. A mild increase in jasmonates had been previously observed after treatment with the SA analogue BTH (Ullah *et al.*, 2019a). Exogenously applied MeJA in an *in vitro* culture of the poplar species *P. davidiana* also elevated SA content (Park *et al.*, 2017).

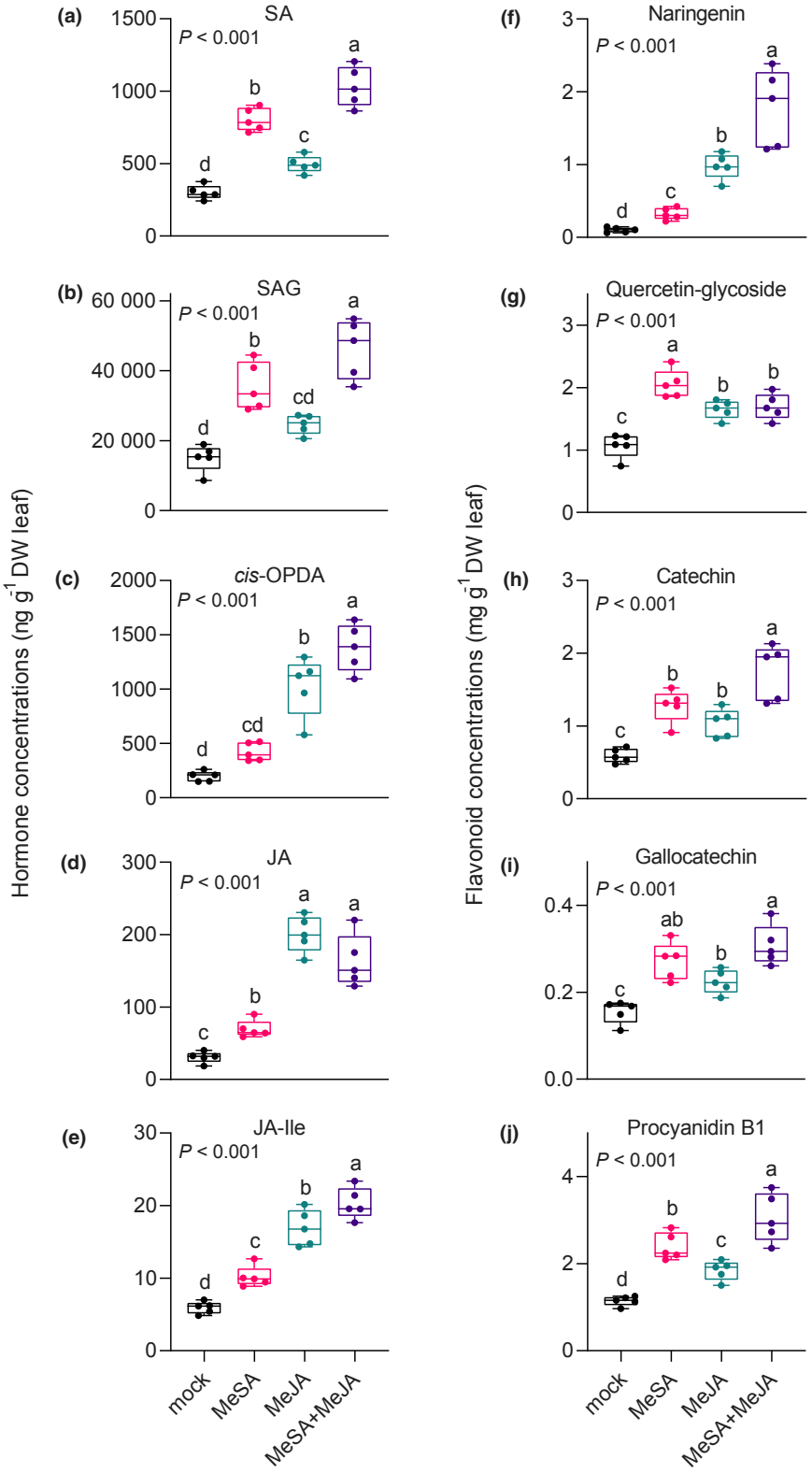

Fig. 8 Salicylic acid (SA) and jasmonate pathways interact positively in poplar. Black poplar saplings of 60–70 cm height were sprayed with 250 μM solutions of methyl salicylate (MeSA) and methyl jasmonate (MeJA), separately and together. Leaf samples were collected 2 d after spraying and analysed for specific hormone and flavonoid metabolites using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Accumulation of (a–e) SA and jasmonate metabolites and (f–j) representative flavonoids in leaves after exogenous hormone treatment. Data were analysed using a one-way ANOVA, followed by Tukey's multiple comparison test with a 95% confidence interval. Each box extends from the 25th to 75th percentiles, and the horizontal line inside the box represents the median. Whiskers were plotted down to the minimum and up to the maximum value, and all data points are shown on the graph by dots ($n = 5$). Different letters above box plots indicate groups were significantly different ($P < 0.05$). *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; SAG, salicylic acid-glucoside.

When we co-applied MeSA and MeJA at similar concentrations, the result was an additive effect on the accumulation of SA and JA metabolites, arguing against potential antagonistic regulation between SA and JA in poplar.

The antagonism between SA and JA signalling pathways is often demonstrated simply by the expression of a few marker genes, for example using *PR1* for the SA pathway and *PDF1.2* or *VSP1/2* for the JA pathway (Thaler *et al.*, 2012). However,

hormonal crosstalk is much more complex, involving many genes that reprogramme plant defences and other physiological processes. For instance, a microarray analysis of *Arabidopsis* identified more than 50 defence-related genes that were co-induced by SA and JA (Schenk *et al.*, 2000). A recent meta-analysis using 257 publicly available RNA-seq data sets collected after SA or JA treatment revealed that many genes involved in *Arabidopsis* growth and defence were coregulated by SA and JA (Zhang *et al.*, 2020). Unfortunately, such examples of positive SA–JA interplay have not received sufficient attention. In *Arabidopsis*, co-application of SA and JA at low concentrations resulted in transient synergism (Mur *et al.*, 2006). Aphid feeding on *Arabidopsis* also resulted in the concomitant induction of SA and JA biosynthetic genes and the upregulation of defence-related marker genes around feeding sites (Rubil *et al.*, 2022). Also, in *Arabidopsis*, SA was shown to activate the JA signalling pathway through the NPR3 and NPR4 receptors as a component of effector-triggered immunity (Liu *et al.*, 2016). Another study on effector-triggered immunity reported the activation of SA marker genes at the centre of hypersensitive lesions and JA marker genes in the peripheral regions, suggesting a coordinated contribution of both hormones in plant defence (Betsuyaku *et al.*, 2018). SA and JA may also complement each other in time. In wheat and oilseed rape, defence responses to pathogens involved sequential activation of SA signalling followed by JA signalling (Ding *et al.*, 2011; Wang *et al.*, 2012). Many more examples of the positive interplay between SA and JA signalling pathways will undoubtedly be reported in the future.

PR gene expression in poplar is not a consistent marker for SA signalling

Salicylic acid-mediated plant defence is frequently identified by its induction of *PR* genes, especially *PR1* (Vlot *et al.*, 2009). *Arabidopsis* markedly upregulates *PR1* transcripts in response to biotrophic pathogens or constitutively elevated SA levels. However, in rice, both SA and JA regulate *PR1* gene expression via *WRKY45*, resulting in enhanced defences against multiple pathogens of different lifestyles (De Vleesschauwer *et al.*, 2013). Our earlier study using the rust-sensitive poplar genotype NP1 revealed that *PR1* and *PR2* genes were significantly induced upon fungal infection, reaching their highest levels during the active proliferation of the fungus and uredinia formation (Ullah *et al.*, 2019a). In the current study, we demonstrated that elevated *PR1* and *PR2* expression is a common response to rust infection in five poplar genotypes with varying levels of rust resistance. Unexpectedly, the magnitude of *PR* gene induction was much greater in the susceptible compared with in the resistant genotypes (Fig. 2). By contrast, levels of SA and JA metabolites, as well as *WRKY89* and *WRKY18* transcripts, were similarly induced in infected leaves of all genotypes. These results raised the question of whether SA played a role in regulating *PR1* and *PR2* expression in poplar as it did in *Arabidopsis*. We addressed this question using transgenic black poplar lines with endogenously elevated SA and wild-type trees exogenously treated with the SA analogue BTH (Fig. 4). In both cases, *PR1* and *PR2* expression levels were not changed relative to the control, suggesting that they were insensitive to SA levels. In fact, upon rust infection, transgenic high SA poplar had significantly lower *PR* gene transcript levels than those found in the susceptible wild-type trees. We propose that pathogen abundance dictates the extent of *PR* gene induction in poplar, as shown by a strong positive correlation between rust growth and *PR* gene transcript levels (Fig. S10). The lack of correlation between SA signalling and *PR* gene expression that we found in poplar adds to the documented differences between poplar and *Arabidopsis* in SA biosynthesis and homeostasis (Yuan *et al.*, 2009; Xue *et al.*, 2013). NPR1, the master regulator of SA signalling for *PR* gene induction in *Arabidopsis*, is not responsive to high SA levels in poplar (Xue *et al.*, 2013) or to rust infection (Ullah *et al.*, 2019a). *Populus* NPR1 also lacks the SA-interacting Cys residues required for nuclear translocation (Tada *et al.*, 2008; Wu *et al.*, 2012; Xue *et al.*, 2013). We conclude that SA-mediated rust resistance is independent of NPR1 in poplar and that *PR* gene expression is not a reliable marker for SA signalling. Instead, rust-induced *PR* expression is well correlated with the extent of pathogen colonisation in this woody plant. The distinct SA biosynthesis and the lack of an NPR1 response to SA in poplar (Yuan *et al.*, 2009; Xue *et al.*, 2013) may be major features preventing the antagonism between SA and JA, as NPR1 is required for SA–JA antagonism in *Arabidopsis*.

Flavonoid defence against rust is a consequence of positive interplay between SA and JA signalling pathway

The constitutive activation of the SA signalling pathway in transgenic black poplar might be expected to lead to a dramatic change in plant defence responses against pathogens. Due to the compatible interactions between *M. larici-populina* and black poplar, we could perform a bioassay to look for increased resistance in hyperaccumulating SA lines. Fungal colonisation in SA-hyperaccumulating lines was significantly lower than in wild-type (Fig. 6). These observations were consistent with previous findings of lower rust colonisation in poplar trees pretreated with the SA analogue BTH (Ullah *et al.*, 2019a) and suggested a direct role of SA in poplar resistance against this obligate biotrophic pathogen. As black poplar *PR* gene transcripts were not well correlated with SA-mediated rust resistance, we focused on lower molecular weight metabolites when searching for the basis of resistance. Upon pathogen attack, various poplar lines have been reported to increase their accumulation of flavonoids, especially flavan-3-ols, including the monomeric catechin and the oligomeric proanthocyanidins (Ullah *et al.*, 2017, 2019b; Wang *et al.*, 2017). Here, the high SA lines displayed increased accumulation of flavan-3-ols and other flavonoids with or without rust inoculation. The concomitant JA increase in high SA lines could have further contributed to poplar defence against rust, as MeJA application alone also led to a flavonoid increase vs untreated controls (Figs 7, 8). This is consistent with the greater effects of MeSA and MeJA co-application on flavonoids, especially on flavan-3-ols, supporting a positive interplay between JA and SA signalling pathways in poplar antipathogen defence. Similarly, wild-type *Arabidopsis* seedlings inoculated with *Penicillium corylophilum*, increased both their SA and JA concentrations along with accumulation of anthocyanins, a

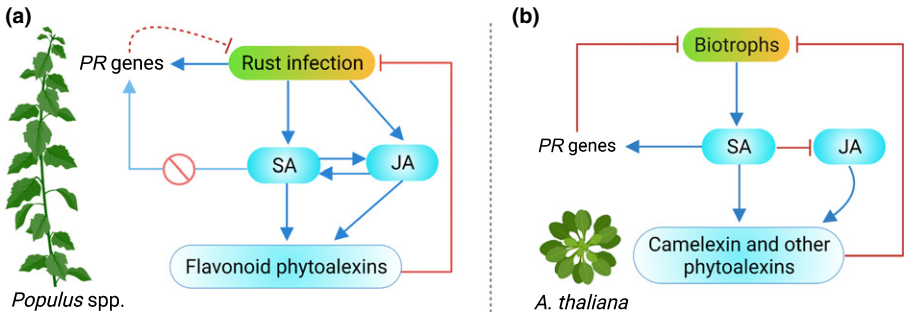

Fig. 9 Model contrasting the interactions between salicylic acid (SA) and jasmonic acid (JA) in *Populus* spp. compared with those in *Arabidopsis thaliana*. (a) Positive SA–JA interactions in poplar. (b) Antagonistic SA–JA interactions in *A. thaliana*. Unlike in *Arabidopsis*, both SA and jasmonate content in poplar leaves are induced in response to a biotrophic pathogen such as the rust fungus *Melampsora larici-populina*. In addition, transgenic black poplar lines engineered with high levels of SA also show increased content of jasmonates, unlike in high SA *Arabidopsis* lines, as well as an induction of flavonoid phytoalexins, including catechin and procyanidin B1. Exogenous applications of either SA or JA to poplar leaves both induce increases in the other hormone, but this does not occur in *Arabidopsis*. Finally, poplar *PR* genes are not activated in engineered high SA lines, or in wild-type trees after treatment with the SA analogue benzothiadiazole, in direct contrast with the situation in *Arabidopsis*. Lines with arrowheads depict positive relationships and lines with blunt ends represent inhibition or negative relationships. Dashed blunt-end line indicates that the relationship is not directly supported by the results of this study, but is predicted from studies on other plants.

class of flavonoids (Liu *et al.*, 2020). As flavonoid induction was abolished in both *npr1* and *coi1* mutants, a concerted function of both SA and JA signalling pathways was suggested.

Constitutive accumulation of SA does not lead to any discernible decline in black poplar growth

Under natural conditions, plants are thought to experience growth–defence trade-offs due to resource limitations. The prioritisation of growth or defence processes is regulated by phytohormones (Huot *et al.*, 2014; Guo *et al.*, 2018; van Butselaar & Van den Ackerveken, 2020). Therefore constitutive activation of defence signalling pathways often leads to increased resistance at the expense of growth. For instance, high SA-accumulating *Arabidopsis* plants showed a stunted growth phenotype even when grown without resource limitations (Gallego-Giraldo *et al.*, 2011a; Zeilmaker *et al.*, 2015). By contrast, SA and JA signalling mutants grew better than wild-type plants (Garcion *et al.*, 2008). However, in our study, high SA-accumulating poplar trees did not show compromised growth phenotypes (Fig. S9), consistent with a previous report on hybrid poplar (Xue *et al.*, 2013). Moreover, there were no differences in the growth parameters measured for high SA and wild-type lines when plants were raised under conditions of mild nutrient limitation (Fig. 5). In contrast with herbaceous plants, woody perennials such as poplar might be capable of defence responses without sacrificing growth. For example, whereas high SA *Arabidopsis* lines constitutively expressed *PR* genes, which may be metabolically costly, high SA poplar lines did not show constitutive upregulation of *PR* genes.

Conclusion

Salicylic acid and JA signalling were found to interact positively in poplar trees. Both SA and jasmonate content increased in multiple poplar genotypes under attack by the biotrophic rust fungus *M. larici-populina*. Poplar trees engineered with high levels of SA also increased their levels of jasmonates and antimicrobial flavonoids, and displayed enhanced resistance against rust. Furthermore, exogenous application of either SA or JA positively affected the other hormone. In contrast with the annual herb *Arabidopsis*, constitutive accumulation of SA in poplar did not negatively affect plant growth. SA–JA antagonism has been considered an evolutionarily conserved trait across the plant kingdom due to the presence of orthologous genes encoding proteins such as NPR1, WRKY70, ERF1 and MYC2, which are essential for the negative SA–JA crosstalk in *Arabidopsis* (Thaler *et al.*, 2012). However, this belief has been challenged by reports of simultaneous or sequential induction of SA and JA signalling under pathogen attack in plants, including poplar. Further studies are necessary to reveal under what conditions SA and JA signalling pathways interact positively or negatively. Factors such as tissue specificity, developmental stage, environmental stress, type of attacker and resource availability for defence might determine the outcome of SA–JA interplay. Woody perennials that can store large reserves of carbon might have evolved SA- and JA-mediated co-defence systems without antagonism, especially as such long-lived plants are subject to simultaneous attack by multiple herbivores and pathogens. By contrast, short-lived annuals may lack the resources to respond to multiple attackers, and so their defence apparatus is designed to respond to individual attacker. A further understanding of why the SA and JA signalling pathways do not antagonise each other may allow the exploitation of this phenomenon in agricultural settings to afford protection against different types of pathogens.

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Author contributions

CU and JG conceived this study. CU designed and carried out experiments. C-JT contributed the construct and AS assisted in poplar transformation. MR assisted in the analytical laboratory. CU and JG interpret the results. CU prepared figures, analysed data and wrote the manuscript. JG and C-JT edited the manuscript. All authors read, provided comments and approved the final version of the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Soluble sugars in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S2 Content of free amino acids in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S3 Relative concentrations of shikimic acid and quinic acid in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S4 Levels of phenolic acids in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S5 Relative levels of salicinoids in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S6 Accumulation of benzoic acid and its derivatives in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S7 Levels of flavonoids in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S8 Accumulation of flavan-3-ols in leaves of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S9 Growth of transgenic high salicylic acid vs wild-type black poplar lines.

Fig. S10 Correlation between rust colonisation with *PR* gene expression, salicylic acid and flavan-3-ol contents in leaves of black poplar lines.

Fig. S11 Induction of *PR* genes in transgenic high salicylic acid and wild-type black poplar lines after rust infection.

Notes S1 Quantification of soluble sugars using liquid chromatography–tandem mass spectrometry.

Notes S2 Quantification of free amino acids using liquid chromatography–tandem mass spectrometry.

Table S1 Chromatographic gradient for analysis of phytohormones by liquid chromatography–tandem mass spectrometry.

Table S2 Details of the analysis of phytohormones by liquid chromatography–tandem mass spectrometry.

Table S3 List of RT-qPCR primers used in this study.

Table S4 Chromatographic gradient for analysis of flavonoids by liquid chromatography–tandem mass spectrometry.

Table S5 Details of the analysis of flavonoids by liquid chromatography–tandem mass spectrometry.

Table S6 Chromatographic gradient for analysis of salicinoids and other metabolites.

Table S7 Details of the analysis of salicinoids and other phenolic metabolites by liquid chromatography–tandem mass spectrometry.

Table S8 Chromatographic gradient for analysis of soluble sugars by liquid chromatography–tandem mass spectrometry.

Table S9 Details of the analysis of soluble sugars.

Table S10 Chromatographic gradient for analysis of free amino acids by liquid chromatography–tandem mass spectrometry.

Table S11 Details of the analysis of amino acids by liquid chromatography–tandem mass spectrometry.

Table S12 Statistical results of a two-way ANOVA for the levels of hormone metabolites in poplar leaves infected with the rust fungus *Melampsora larici-populina*.

Table S13 Statistical results of a two-way ANOVA for the expression of *PR* and *WRKY* transcription factor genes in poplar leaves infected with *Melampsora larici-populina*.

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