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Root isoprene formation alters lateral root development

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Abstract

Isoprene is a C5 volatile organic compound, which can protect aboveground plant tissue from abiotic stress such as short-term high temperatures and accumulation of reactive oxygen species (ROS). Here, we uncover new roles for isoprene in the plant belowground tissues. By analysing *Populus* x *canescens* isoprene synthase (*PcISPS*) promoter reporter plants, we discovered *PcISPS* promoter activity in certain regions of the roots including the vascular tissue, the differentiation zone and the root cap. Treatment of roots with auxin or salt increased *PcISPS* promoter activity at these sites, especially in the developing lateral roots (LR). Transgenic, isoprene non-emitting poplar roots revealed an accumulation of O_2^- in the same root regions where *PcISPS* promoter activity was localized. Absence of isoprene emission, moreover, increased the formation of LRs. Inhibition of NAD(P)H oxidase activity suppressed LR development, suggesting the involvement of ROS in this process. The analysis of the fine root proteome revealed a constitutive shift in the amount of several redox balance, signalling and development related proteins, such as superoxide dismutase, various peroxidases and linoleate 9S-lipoxygenase, in isoprene non-emitting poplar roots. Together our results indicate for isoprene a ROS-related function, eventually co-regulating the plant-internal signalling network and development processes in root tissue.

KEYWORDS

isoprene, isoprene promoter, lateral roots, poplar, *Populus* x *canescens*, reactive oxygen species, roots, ROS, VOC, volatile organic compounds

1 | INTRODUCTION

Plants interact with their environment by emitting a great variety of volatile organic compounds (VOCs), which are mainly comprised of terpenes, fatty acid derivates, phenylpropanoids and benzenoids (Maffei, Gertsch, & Appendino, 2011). Mono- and sesquiterpenes have multiple roles in plant signalling and communication (Heil, 2009; Riedlmeier et al., 2017; Simpraga, Takabayashi, & Holopainen, 2016; Wenig et al., 2019; Werner, Polle, & Birkmann, 2016). The function of the smallest and globally far most abundant terpene, isoprene, is, however, still under debate. Isoprene is a hemiterpene synthesized and emitted in large quantities by many tree species including poplars. Though the biological function of isoprene is still not fully understood, scientists agree on two physiological features: isoprene can enhance thermotolerance and reduce oxidative stress in leaf tissues (Behnke et al., 2007; Loreto et al., 2001; Sharkey & Singsaas, 1995; Vickers et al., 2009). The mechanism of actions might involve the modulation of reactive oxygen species (ROS) (for review see Sharkey &

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Monson, 2017) and reactive nitrogen oxide levels (Vanzo et al., 2016). Earlier studies on isoprene emitting (IE) and, transgenic, isoprene non-emitting (NE) grey poplar (*Populus* x *canescens*) revealed also altered ROS accumulation in NE leaves supporting isoprene's function in adjusting redox balance (Behnke et al., 2010).

Currently, there is rising interest to understand the biosynthesis, emission and biological/ecological functions of VOCs in the soil system (Peñuelas et al., 2014; Werner et al., 2016). Despite this interest, root and microbial VOC emissions, the involved biosynthetic genes and their spatial and temporal regulation are yet widely unexplored. In the *Arabidopsis thaliana* genome, 32 terpene synthase (*TPS*) genes were identified exhibiting diverse expression patterns (Chen et al., 2003; Chen et al., 2004; Herde et al., 2008; Ro et al., 2006). From the previously predicted 14 *AtTPS* genes with primary or exclusive expression in roots, four are functionally characterized to encode monoterpene or sesquiterpene synthases (Chen et al., 2004; Ro et al., 2006). Among these enzymes, *AtTPS-Cin* (synonym *AtTPS27/23*), synthesizing the monoterpene 1,8-cineole, is expressed in the vascular tissue, in epidermal cells and root hairs of primary and lateral roots (LR) (Chen et al., 2004). Cinege and colleagues (Cinege, Louis, Hänsch, & Schnitzler, 2009) observed that also the isoprene synthase (*PcISPS*) promoter is active in specific root regions of poplar—roughly in the tip of the fine roots and all over the vascular cylinder. The root system of poplars grown in hydroponic cultures also emits low amounts of isoprene (Ghirardo, Gutknecht, Zimmer, Brüggemann, & Schnitzler, 2011). These findings are interesting because isoprene is so far considered as a typical volatile in tree canopies and green parts of the plant but its role in root biology is unknown.

ROS are simple molecules having important roles in various plant developmental processes, such as cell proliferation and differentiation, programmed cell death and senescence but also in root development and LR growth (Dunand, Crèvecoeur, & Penel, 2007; Li et al., 2015; Manzano et al., 2014; Nanda, Andrio, Marino, Pauly, & Dunand, 2010; Singh et al., 2016; Tsukagoshi, 2016). In roots, ROS are shown to be active especially in developing plant organs (Carol & Dolan, 2006; Tsukagoshi, 2016). ROS are involved in remodelling root architecture to adjust to changing environment (Deak & Malamy, 2005; van der Weele, Spollen, Sharp, & Baskin, 2000; Zolla, Heimer, & Barak, 2010). ROS also have important roles in plant-microbe interactions. In some mutualistic relations ROS, such as O_2^- and H_2O_2, are crucial for the proper establishment of symbiosis (Babtista, Martins, Pais, Tavares, & Lino-Neto,- 2007; Jamet, Mandon, & Hérouart, 2007; Nanda et al., 2010). Many microbes produce ROS scavenging enzymes to overcome the plant defense system, but also plants themselves regulate the ROS concentration with enzymatic and non-enzymatic antioxidative mechanisms (Nanda et al., 2010). The activity of antioxidative systems might be especially important when plants are in contact with mutualistic microbes. For example, VOCs emitted by the ectomycorrhizal (ECM) fungi *Laccaria bicolor* alter plant internal signalling through ROS and trigger LR formation (Ditengou et al., 2015).

Because isoprene can adjust the plant redox balance in leaves (Sharkey & Monson, 2017), in the present study we elaborated the spatial and temporal activation of isoprene synthase (*PcISPS*) promoter in roots and aimed to define a biological function of isoprene in regulation of root ROS accumulation and LR development. We hypothesized that the redox balance in certain root parts is affected by isoprene. By modulating ROS, isoprene might eventually adjust plant developmental processes, such as LR growth. To explore these hypotheses, we used poplar and *Arabidopsis PcISPS::GFP-GUS* promoter reporter gene fusion lines (Cinege et al., 2009) as well as IE and NE transgenic poplar plants (e.g. Behnke et al., 2007, 2009, 2010; Vanzo et al., 2016). Previously Cinege et al. (2009) showed, that *PcISPS*-promoter can be detected in both, *Arabidopsis* and poplar reporter plant roots. This, and the easier detection of the promoter in the thin *Arabidopsis* root tissue encouraged us to employ both reporter plants in parallel, thought *Arabidopsis* is not an isoprene-emitting species. We investigated (a) whether isoprene emissions from roots differ between IE and NE poplar, (b) in which root cell types the *PcISPS* promoter is activated during LR development and abiotic stresses, (c) whether promoter activation is related to O_2^- production in specific regions of roots and (d) if isoprene in roots affects the fine root proteome and LR development. Based on our results we suggest a possible function for isoprene in adjusting ROS-mediated internal signalling in poplar roots.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Reporter plants carrying *Populus* x *canescens* isoprene synthase promoter gene (*PcISPS*) in front of a green fluorescence protein (GFP)::β-glucuronidase (GUS) -construct (Cinege et al., 2009) were used in the studies. Two transgenic *Arabidopsis thaliana* and two grey poplar (*Populus* x *canescens*) lines carrying *PcISPS::GFP-GUS* construct were chosen for the experiments: L2 and L4 (*Arabidopsis*) and L26 and L2 (poplar) (Figure S1). Additionally, two isoprene-non emitting lines (NE; *35S::PcISPS*-RNAi lines NE1 and NE2) and two naturally isoprene-emitting (IE) lines [empty vector control for *PcISPS*-RNAi constructs (EV) and wild type (WT)] were used (Behnke et al., 2007).

In all the experiments *A. thaliana* seeds were surface sterilized and placed on solid *Arabidopsis* medium (MS medium supplemented with Gamborg's vitamins) (similar as in Loivamäki et al., 2007). After stratification for 3 days at 4°C in the dark, the seeds were let to germinate [light: dark 16:8 h; 21°C, photosynthetically active photon flux density (*PPFD*) 100 μmol m^{-2} s^{-1}]. After 7 days the seedlings were transferred to square Petri dishes which were placed in vertical position in the same growth conditions.

Grey poplar plants were amplified by micro-propagation under sterile conditions as described by Leplé and colleagues (Leplé, Brasileiro, Michel, Delmotte, & Jouanin, 1992). Ten days after cutting, the plantlets were transferred into Petri dish systems with ½ MS medium solidified with gelrite as described in Ditengou et al. (2015). One third of the medium was cut out to have the green parts above the medium in vertical position. The growth conditions were as described for *Arabidopsis*.

2.2 | Plant treatments and experimental designs

To simulate salt stress, seven-day-old seedlings of *A. thaliana* (L2, L4) and poplar (L26, L2) were exposed to 125 mM NaCl in MS or 50 mM NaCl in ½ MS, respectively, for 10 days. For growth hormone treatment the *Arabidopsis* and poplar seedlings were exposed to 1 μM IAA (indole-3-acetic acid) in MS or 1/2MS, respectively.

The plasmalemma NADPH oxidase was inhibited by diphenylene iodonium (DPI; Sigma-Aldrich, Deisenhofen, Germany) treatment similar as described in Ditengou et al. (2015). Possible suitable DPI concentrations (0.0, 0.1, 0.5 or 1.0 μM DPI) were at first tested for WT poplar and the final concentration of 0.1 μM DPI was chosen for the experiment. The poplar plantlets were transferred to the DPI containing media in Petri dishes as described above.

To analyse the LR development, LRs were counted under the microscope every second day, as described in Ditengou et al. (2015), and the plates were scanned for later root physiological analyses. For determination of ROS and proteomic analyses, subsets of the plants were harvested on days 0, 4, 7 and/or 10.

2.3 | In silico analyses

In silico analysis was performed for isoprene synthase (*PcISPS*) promoter of *Populus* x *canescens* and for 1,8-cineole terpene synthase (At3g25820/At3g25830; *AtTPS-Cin*) promoter of *Arabidopsis thaliana*. Regulatory elements located in the 1.6-kb or 2.5-kb region upstream of the start codon in *PcISPS* and *AtTPS-Cin* promoters, respectively, were predicted by searching against the PlantCare (Lescot et al., 2002) and PLACE (Higo, Ugawa, Iwamoto, & Korenaga, 1999) database.

The comparative analysis of homologous gene sequences from 32 predicted *Arabidopsis* terpene synthases (*TPS*) and poplar *PcISPS* was performed using the Molecular Evolutionary Genetics Analysis (MEGA 7) software package (Kumar, Stecher, & Tamura, 2016). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. Codon positions included were first, second, third and non-coding. All positions containing gaps and missing data were eliminated.

2.4 | Isoprene emission analysis of roots

For isoprene measurements roots of 7–9 poplar plants grown in sterile cultures were harvested and pooled together for isoprene emission measurements. After 30 minutes stabilization time the roots were incubated in 5 ml glass vials for 4 hr under 32°C and photosynthetically active radiation of 300 μmol m^{-2} s^{-1}, similar as described earlier in Loivamäki et al. (2007). Isoprene was measured as headspace by proton transfer reaction—time of flight—mass spectrometer (PTR-ToF-MS, Ionicon Analytic, Innsbruck, Austria). The headspace of the samples was injected into the PTR-ToF-MS by using N_2 as carrier gas at the flow rate of 100 ml min^{-1}. As standard 10.9 ppmv isoprene in N_2 was used (BASI Schöberl GmbH, Germany). Isoprene emission was calculated per gram of root dry weight.

2.5 | GUS and NBT staining of roots

The plant material was fixed 20 min on ice in 90% cold acetone and washed with a rinsing solution. Roots were then vacuum infiltrated in desiccator for 10 min with X-Glucuronidase (X-Gluc) as described in Cinege et al. (2009) followed by overnight incubation at 37°C in staining solution. On the following day the samples were washed with the ethanol series (30, 50, 70, 85, 95 and 100%), each for 1 hr at room temperature. The stained tissue was stored in the fridge at 4°C.

Superoxide anion radicals were detected with a nitroblue tetrazolium salt (NBT; Carl Roth, Karlsruhe, Germany) assay (Ditengou et al., 2015). NBT was freshly prepared in 20 mM phosphate buffer (pH 6.1) and diluted to a final concentration of 2 mM. Harvested roots were vacuum infiltrated for 30 s and incubated for 60 min on a shaker at 250 rpm at room temperature in the dark. After incubation, the NBT was removed by washing twice for 5 min with 20 mM phosphate buffer. The plants were then dehydrated in 100% ethanol for 15 min and re-hydrated in decreasing concentrations of ethanol.

For microscopy the samples were carefully cut in smaller pieces in a drop of sterile water. Single LR were isolated and placed on microscope slides with few drops of a mounting solution [glycerol: sterile water (50:50)] and covered with a lid. The different samples were then observed and photographed under a microscope Olympus BX61 (Olympus life and material science Europa GmbH, Hamburg, Germany) equipped with F-View II digital camera system (Soft Imaging System GmbH, Münster, Germany). For measurement of different parameters and image processing Cell P Life Science Imaging Software version 2.2 (Olympus life and material science Europa GmbH, Hamburg, Germany) was used. The exposure time was always adjusted in the range of 200–400 ms.

2.6 | Embedding and microtome sectioning of roots

For embedding in Technovit® 7,100 (Morphisto GmbH, Frankfurt am Main, Germany) the root tissue was first fixed in a FAA solution (5% acetic acid, 5% formaldehyde and 50% ethanol) under vacuum for 1 hr. The root tissue was dehydrated by washing in series of ethanol (2 × 70% and 2 × 100% for 30 min) after which the tissue was infiltrated with Technovit:ethanol [50:50 (4 hr), 70:30 (4 hr), 100:0 (12 hr), respectively]. The tissue was embedded in silicon mold or microcentrifuge tube with Technovit solution including the hardener. The samples were incubated for polymerization overnight at 37°C.

Solidified resin blocks were at first trimmed and cut out with a fine coping saw, in order to orient the tissue properly. Cross sections

were cut with glass knives on the rotary microtome (Carl Stuart UK Limited, Frimley, UK). The thickness of the cross sections was in the range of 5–10 μm. The cutting was done over the water surface and then placed in a drop of water on a microscope slide, heated on a warming plate until the water evaporates and the sections adhere firmly to the slide. Microscopy was done as described above for whole roots.

2.7 | Quantification of the staining intensity

For the intensity quantification of the dye signals the colour deconvolution plugin as part of ImageJ software (Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2016) was used in order to implement stain separation using Ruifrok and Johnston's method (Ruifrok & Johnston, 2001, modified by Landini, Positano, & Santarelli, 2005). Background subtraction with colour correction was applied to all images before processing. The corrected total cell fluorescence (CTCF) was calculated using following formula: CTCF = Integrated Density − (Area of selected cell × Mean fluorescence of background).

2.8 | Label-free analysis of fine root proteomes using Progenesis LC–MS

For proteomics analysis 50 mg of frozen, homogenized, grinded root tissue was dissolved in 500 μl of 1x Laemmli buffer (240 mM Tris–HCl, pH 6.8; 8% wt/vol SDS, 40% vol/vol glycerol, 0.08% wt/vol bromphenolblue, 20% vol/vol β-mercaptoethanol) and incubated at 95°C for 5 min. After centrifugation (14,000 × g for 10 min) supernatant was separated and protein content was determined using the Bradford assay (Bradford, 1976). Aliquots that contained at least 10 μg of protein in 50 μl of solution were prepared for further analysis for liquid chromatography—tandem mass spectrometry (LC–MS/MS). Eight ± two independent, biological replicates were analysed for IE and NE plants.

Each 10 μg of root extract were digested with a modified Filter-Aided Sample Prep (FASP) procedure (Grosche et al., 2015; Wiśniewski, Zougman, Nagaraj, & Mann, 2009). Briefly, the proteins were diluted, reduced and alkylated (using dithiothreitol and indole-3-acetic acid). Then the proteins were centrifuged through a 30-kD cutoff filter device (PALL), and washed three times [once with UA buffer (8 M urea in 0.1 M Tris–HCl, pH 8.5) and twice with 50 mM ammoniumbicarbonate]. The proteins were digested for 2 hr at room temperature using 1 mg of Lys-C (WakoChemicals) and for 16 h at 37°C using 2 mg of trypsin (Promega). The further details have been described in Vanzo et al. (2016).

For mass spectrometric analysis the samples were thawed and centrifuged (14,000 × g, 5 min, 4°C). LC–MS/MS analysis was performed as described previously on Ultimate 3,000 nano RSLC coupled to a LTQ OrbitrapXL (Thermo Fischer Scientific, Hauck et al., 2010; Merl, Ueffing, Hauck, & von Toerne, 2012). Every sample was automatically injected and loaded onto the C18 trap column. After 5 min, the peptides were eluted and separated on the analytical column (75 μm i.d. × 25 cm, Acclaim PepMap100 C_{18}, 3 μm, 100 Å, Dionex) by a linear 135 min acetonitrile gradient from 4 to 30% at 300 nl min^{-1} flow rate. From the MS pre-scan, the 10 most abundant peptide ions were fragmented by collision induced dissociation in the linear ion trap if they showed an intensity of at least 200 counts and if they were at least doubly charged. During fragmentation a high-resolution (60,000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 300 to 1,500 Da and a dynamic exclusion of 60 s.

The acquired spectra were loaded to the Progenesis QI for proteomics software (version 2.0, Nonlinear Dynamics, part of Waters) for label free quantification and analysed as described previously (Hauck et al., 2010; Merl et al., 2012). Features with only one charge or more than eight charges were excluded. The raw abundances of the remaining features were normalized to allow correction for factors resulting from experimental variation. All MS/MS spectra were exported as Mascot generic file (mgf) and used for peptide identification with Mascot (version 2.5.1) in the *Populus trichocarpa* protein database (30,197,159 residues, 73,016 sequences). Search parameters were: 10 ppm peptide mass and 0.6 Da MS/MS tolerance, one missed cleavage allowed, carbamidomethylation of cysteins was set as fixed modification, methionine oxidation and asparagine or glutamine deamidation were allowed as variable modifications. A Mascot-integrated decoy database search calculated a false discovery rate of 1.36% when searches were performed with a mascot percolator score cut-off of 13 and a significance threshold $p = .05$. Peptide assignments were re-imported into the Progenesis QI software. After summing up the abundances of all unique peptides allocated to each protein, the identification and quantification results were exported.

The annotation and functional classification were achieved by using several databases. Firstly, the list of primary IDs identified by Mascot was exported into Phytozome v12.1.6 (Goodstein et al., 2012) using the Biomart tool. The *Arabidopsis* reference genome was chosen as a template and poplar genes were blast searched against potential matching *Arabidopsis* genes, resulting in a list of *Arabidopsis* orthologs and GO terms. Subsequently, the list of gene ID was exported in PopGenIE (The Populus Genome Integrative Explorer) to confirm previously predicted functions (Sundell et al., 2015). The PPDB (The Plant Proteome Database) was used to allocate in MapMan BIN categories (Thimm et al., 2004; Usadel et al., 2009).

Data deposition: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD019789.

2.9 | Statistics and visualization of the data

To test the differences in LR density, stain intensities and isoprene emission Student's *t* test or two-way-analysis of variance (ANOVA) with post hoc tests was performed using IBM SPSS Statistics version 19.0 for Windows (Armonk, NY).

The differences in the proteome of IE and NE genotypes were analysed using Orthogonal Partial Least Square regression (OPLS) statistical methods as previously described in details (Vanzo et al., 2016; Velikova et al., 2014). The results were validated by "full cross-validation" (Eriksson et al., 2006) using a 95% confidence level.

Raw abundances from the label-free analysis of proteome were extracted from the Progenesis QIsoftware (v2.0, Nonlinear Dynamics, part of Waters). Protein intensities were normalized to the corresponding (averaged) protein abundance in whole-cell extracts of the IE and NE roots. The size of the analysed matrix was 2,765-by-32. Skew data (limit ±2) were removed from analysis. Variable of Importance for the Projection (VIP) greater than 1 and the uncertainty bars of the jack-knifing method smaller than the respective VIP value were defined as discriminant proteins that can separate IE from NE samples. Additionally, discriminant proteins were tested for significance difference ($p < .05$) between IE and NE samples using Student's t test and two-way-ANOVA (SPSS, v22.0, SPSS Inc., Chicago, IL). Proteins with VIP scores <1 were accepted when they showed a significant difference (t-test $p \leq .05$) between the lines. For mapping the discriminant proteins we applied Mapman 3.6.0 RC1 (Usadel et al., 2009). The mapped BINs are based on gene functional assignments as taken from the corresponding *A. thaliana* orthologs (http://www.arabidopsis.org/tools/bulk/go/index.jsp), which were obtained via the POPGENIE (http://www.popgenie.org) database. Expression change was encoded by using a blue via red colour gradient with blue for decreased and red for increased expression.

3 | RESULTS

3.1 | Isoprene synthase promoter activity and ROS accumulation show tissue- and cell type-specificity in the roots

Histological studies in *PcISPS*::*GFP-GUS* reporter plants of *Arabidopsis* and poplar revealed development-, tissue- and cell type-specific expression of the promoter in roots (Figures 1 and 2). Particularly high β-glucuronidase (GUS) staining intensity was detected in the root vascular tissue, root cap, root hairs and, in general in the differentiation- and partly in the elongation zone of *Arabidopsis PcISPS*-promoter reporter plants (Figure 1). Transverse and cross sections of the poplar roots verified the high promoter activity in the vascular tissue and in root cap in poplar reporter plants (Figure 2a–c). Nitroblue tetrazolium (NBT)-staining of superoxide anion radicals (O_2^{-}; Kumar, Yusuf, Singh,

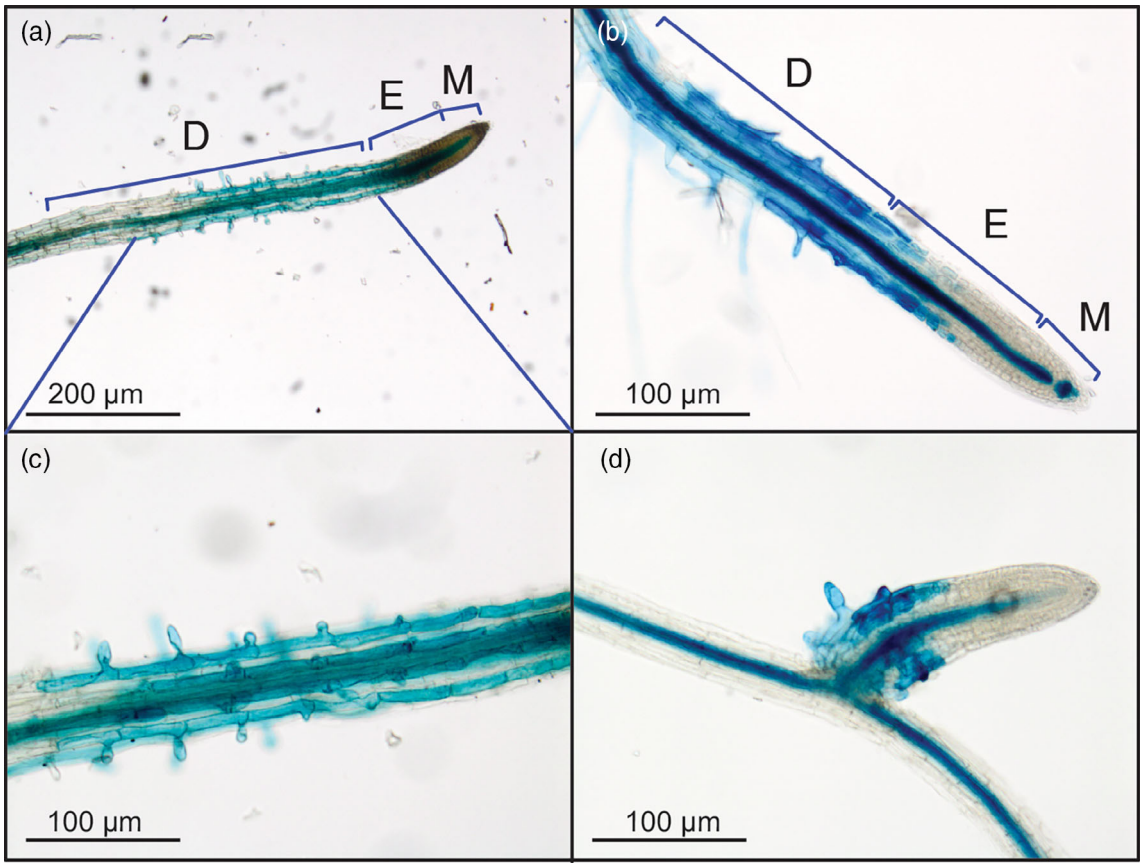

FIGURE 1 *PcISPS* promoter activity as β-glucuronidase (GUS) stain in lateral roots of *Arabidopsis* reporter plant (line L4). Representative images of promoter activity in differentation zone, elongation zone and meristem (a, b), root hairs of the differentation zone (c) and lateral root primordium (developmental stage VIII) (d) are shown. M: meristem; D: differentiation zone; E: elongation zone [Colour figure can be viewed at wileyonlinelibrary.com]

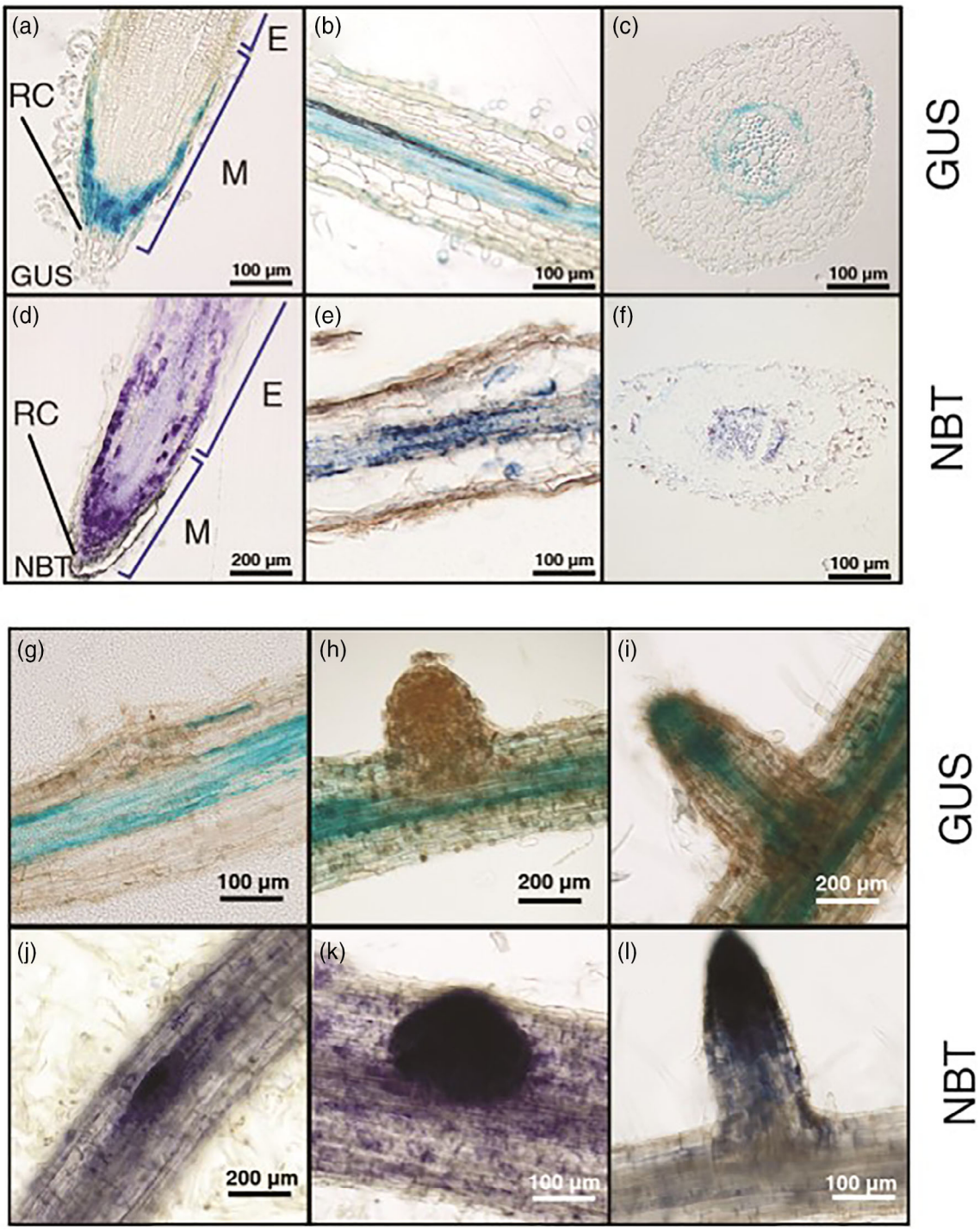

FIGURE 2 *PcISPS* promoter activity as β-glucuronidase (GUS) stain and O_2^- accumulation as nitroblue tetrazolium (NBT) stain in poplar lateral roots (LR). Promoter activity is detected in isoprene emitting poplar reporter line L2 and O_2^- accumulation in transgenic, isoprene non-emitting poplar. (a–f) The upper six pictures show promoter activity (a–c) and O_2^- accumulation (d–f) within vascular tissue and LR tip of poplar. Representative pictures of longitudinal section of LR tip (a, d), longitudinal section of vascular tissue (b, e) and cross sections 1 cm from LR tip (c, f) are shown. M: meristem; E: elongation zone; RC: root cap. (g–l), The lower six pictures show promoter activity (g–i) and O_2^- accumulation (j–l) during poplar lateral root primordia (from left to right: stages II, VI and VIII). Representative images are shown [Colour figure can be viewed at wileyonlinelibrary.com]

Sardar, & Sarin, 2014) revealed the accumulation of O_2^- in transgenic NE-poplars largely in similar root cell types as in which GUS activity of *PcISPS* promoter reporter plants was observed (Figure 2a–f). Though not entirely overlapping, we detected high stain intensities of both, NBT and GUS in vascular tissue and root cap in poplar (Figure 2a–f) and in *Arabidopsis* (Figure 1; Figure S2).

During the development of lateral root primordia (LRP) we observed GUS staining only starting in later developmental stages (*Arabidopsis* LRP stage V and poplar LRP stage VIII), suggesting that the *PcISPS* promoter was only active starting in these stages (Figure 2g–i; Figure S2). During the lateral root (LR) development promoter activity was observed as GUS stain especially in the root vascular tissue, that is, in the core cells of the LRP that already have a distinct elongated shape characteristic for vascular elements (Figure 2i; Figure S2). In *Arabidopsis* LRs, GUS stain was observed also in areas of developing root hairs (Figure 1d, Figure S2), whereas in LRs of poplar no root hairs were detected in the early developing stages (Figure 2). NBT stain largely coincided with the pattern of GUS stain in LRP starting approximately from the developmental stage VIII in NE-poplars (Figure 2g–l). Compared to poplar, in *Arabidopsis* GUS and NBT stains were detected a few developmental stages earlier during the LRP (Figure S2, left side), which might be simply due to the thinner *Arabidopsis* root tissue. When the *Arabidopsis* plants were stressed by NaCl or stimulated by the growth hormone indole-3-acetic acid (IAA) the promoter activity was detected even earlier in developing LR, starting from LRP stage III and IV (Figure S2, right side).

All *Arabidopsis* and poplar reporter lines challenged with NaCl (125 or 50 mM, respectively) showed increased GUS staining due to enhanced *PcISPS* reporter activity in the roots (Figure 3, Figure S5a,b). GUS stain intensity was significantly stronger in salt treated roots than in control in all the tested lines (Figure 3, Student's *t* test, $p < .001$; $n = 10$; mean ± se).

Similar to NaCl, also 1 μM IAA application to roots stimulated *PcISPS* promoter activity leading into stronger GUS stain in all reporter lines. The staining intensity was at least doubled in the IAA-treated roots compared to controls (Figure 3a,b, Student's *t* test, $p \leq .001$; $n = 10$; mean ± se, Figure S5c,d). After the IAA treatment, the highest GUS activity was observed especially in developing LRs (Figure S2, right side). Since auxin and ROS signals are involved in LR growth promotion (Huang, She, Cao, & Ren, 2011; Lavenus et al., 2013; Müller, Linkies, Leubner-Metzger, & Kermode, 2012; Swanson & Gilroy, 2010) we analysed also the ROS accumulation in IAA-treated LRs. The application of IAA significantly increased NBT stain in poplar roots (Figure S5e–g, Student's *t* test, $p < .018$; $n = 12$; mean ± se).

3.2 | Abiotic stresses induce isoprene synthase promoter activity in the roots

We tested the *PcISPS* promoter response to salt stress and growth hormone indole-3-acetic acid (IAA; auxin) using the *PcISPS::GFP-GUS Arabidopsis* and poplar reporter gene plants. These treatments were chosen as previous in silico analysis of the promoter revealed several defense and hormone, such as auxin and abscisic acid responsive elements in *PcISPS* promoter (Figure S3). The elements were largely similar as in another terpene synthase gene promoter: the phylogenetically closely related *1,8-cineole synthase* (*AtTPS-Cin*) promoter of *Arabidopsis* which is as well active in roots (Figures S3 and S4, Chen et al., 2004).

3.3 | Isoprene impairs ROS and LR growth in poplar roots

To establish a causal relationship between isoprene and LR formation, we tested whether the lack of isoprene in transgenic NE poplars stimulated LR development compared to naturally isoprene emitting [IE; wild type (WT) and empty vector control (EV)] plants. Indeed, the roots of the NE poplars developed higher LR densities compared to roots of IE poplar plants (Figure 4a,b,e, One way ANOVA and Tukey test, $^{**}p \leq .01$; $^{*}p < .05$; $n = 29 \pm 1$; mean ± se). Accordingly, ROS accumulation in NE roots was significantly higher than in IE roots

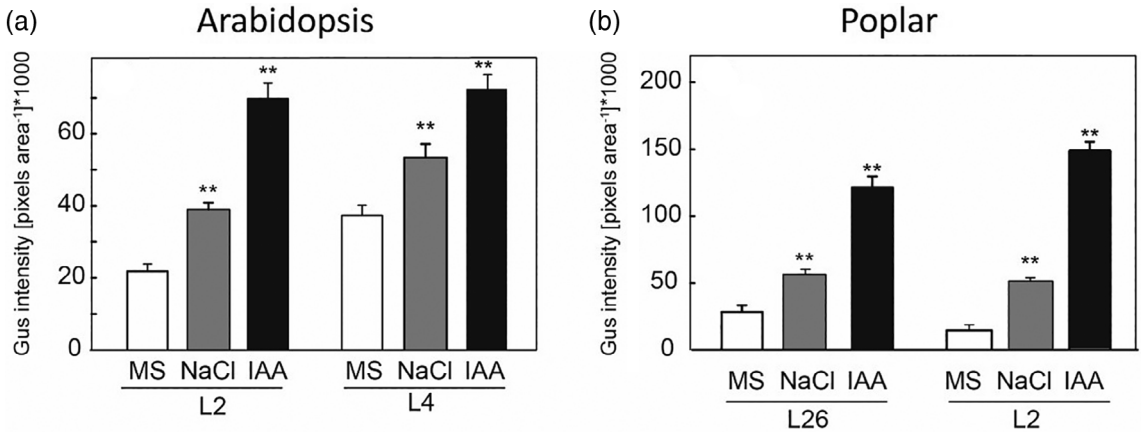

FIGURE 3 *PcISPS* promoter activity under NaCl- or indole-3-acetic acid (IAA) treatment in the lateral root (LR) tips. The β-glucuronidase (GUS) stain intensity (pixels area^{-1}*1,000) in control (Murashige and Skoog medium (MS); white bars) and in NaCl (grey bars) or IAA (black bars) treated plants is shown for two *Arabidopsis* lines (L2, L4) (a) and two poplar lines (isoprene emitting reporter lines L26, L2) (b). The intensity was measured in the root tip (i.e. root cap, meristematic- and elongation zone) of the LR. Different letters indicate the significant differences between control and treatment by Student's *t* test ($p < .001$; $n = 10$; mean ± se)

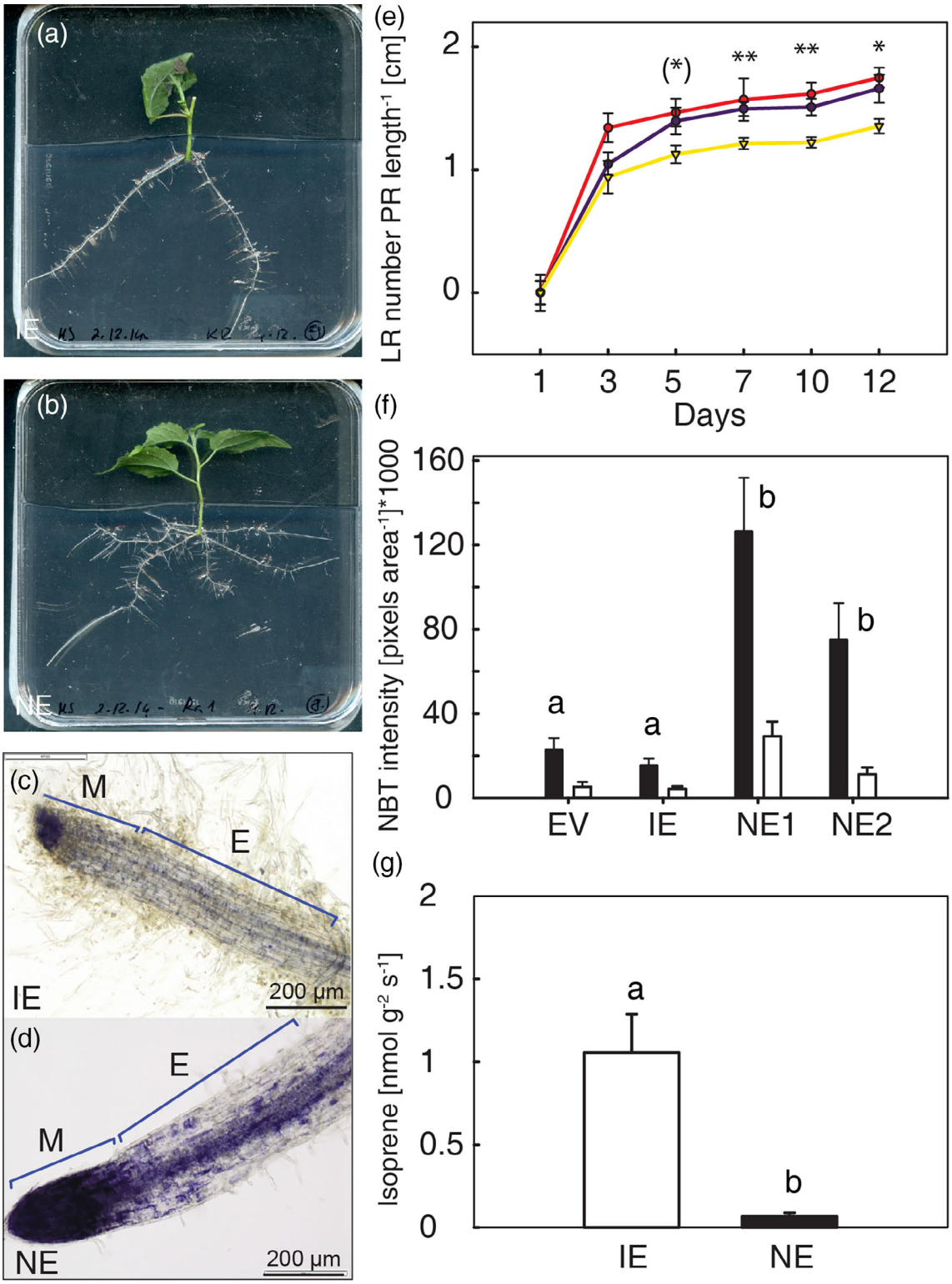

FIGURE 4 Lateral root (LR) growth, O_2^- accumulation and isoprene emission in isoprene emitting (IE) and non-emitting (NE) poplar roots. (a–d), Representative pictures of LR development (a, b) and O_2^- accumulation [as nitroblue tetrazolium (NBT) stain (c, d)] of IE (a, c) and NE (b, d) poplar. (e), LR number [normalized to primary root (PR) length] of IE (yellow) and two NE lines (red and blue). Asterisks indicate the statistical significance between lines; analysis of variance and Tukey test (**$p \leq .01$, *$p < .05$, [*]$p < .05$ for one of the lines; $n = 5 \pm 1$; mean ± se). (f), O_2^- stain intensity in two IE lines [empty vector (EV) and wild type (IE)] and 2 NE lines (NE1, NE2). The stain is shown under control conditions in LR tip (i.e. cap, meristematic—and elongation zone; black bars) and in the LR middle parts (i.e. differentiation zone; white bars). (g), Isoprene emission from roots of the IE and NE lines. Different letters indicate the statistical differences between the treatments by analysis of variance and Tukey test ($p < .05$; $n = 4$ (F); and $p = .013$; $n = 5$ (g); means ± se). M: meristem; E: elongation zone [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 4c,d,f, ANOVA and Tukey test; $p < .050$; $n = 4$; mean ± se). The NBT stain intensity was enhanced especially in the root tip but also in the vasculature of roots in NE lines compared to the IE lines (Figure 4c,d).

To demonstrate isoprene biosynthesis in roots, we analysed root isoprene emissions of NE and IE poplars by headspace collection and mass spectrometry. We found low isoprene emission, amounting 1.06 nmol g^{-2} s^{-1} from wild type roots in sterile culture, thus being approximately a factor of hundred lower than leaf emissions (Behnke et al., 2007). The roots of transgenic NE plants still emitted very low amounts of isoprene, the emission being approximately 5% of the emission of wild type roots (Figure 4g, Student's *t* test, $p = .013$; $n = 5$; mean ± se).

To further verify the involvement of ROS in LR formation we applied diphenylene iodonium (DPI), an inhibitor of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, leading to reduced ROS accumulation (Cross & Jones, 1986), to the culture medium. The increase in DPI concentration led into increasingly impaired primary root length, LR number and LR density of poplar roots (Supplemental Figure S6). From the tested concentrations (0.1, 0.5 and 1 μM DPI) we chose 0.1 μM DPI for further studies as the lowest possible, physiologically relevant concentration (same concentration as applied by Ditengou et al., 2015). Inhibition of NAD(P)H oxidase activity by DPI application hampered the LR growth in NE plants by 10%. Thus, DPI treatment resulted into similar LR growth in NE plants as was detected in the untreated IE plants (Figure 5, ANOVA and Fisher LSD test, $p \leq .05$; $n = 10$; mean ± se). The LR growth of IE plants was reduced only when higher DPI concentration was applied (reduction by 45 and 53% with 0.5 and 1 μM DPI, respectively; Figure S6c, One way ANOVA and Fisher LSD test on day 10 post treatment, $p \leq .001$; $n = 12 \pm 2$; mean ± se). Together, these results indicate a pivotal role for isoprene as a regulator of ROS accumulation in root cells of poplar.

To test whether the LR induction correlated with ROS accumulation in NE and IE poplars, we stained the roots with NBT at day 10 post DPI treatment. In accordance to the induced LR density, a higher NBT stain was observed in untreated NE lines compared to IE lines or DPI treated NE lines at day 10 (One way ANOVA and Tukey's test, $p \leq .05$; $n = 10$; mean ± se; Figure 5c). A linear relationship was found between LR density and NBT staining intensity in the roots (Figure 5d; $R^2 = 0.933$, $p = .0017$).

3.4 | Internal isoprene affects root proteome

In order to understand the biochemical and physiological changes in roots of NE and IE poplars, we performed a proteomic analysis. In the fine roots of NE and IE poplar 2,763 proteins could be annotated. Using orthogonal partial least square regression models (OPLS), we searched among the root proteomes for the most important proteins able to discriminate IE from NE lines ($p < .001$, cross-validated ANOVA, Figure 6a,b). Altogether we detected 121 significantly (Student's *t* test and two-way-ANOVA, $p \leq .05$, VIP score > 1, $\log_2$ fold change >0.5) downregulated and 27 significantly upregulated proteins in the fine roots of NE compared to IE poplars (Table S1). These proteins accounted for ~5% of the total annotated proteins. The discriminating proteins were functionally involved in different metabolic processes, the large part being linked to amino acid and protein metabolism and to redox and stress responses (Figure 6c).

For further visualization of proteomic differences between the lines, the discriminant proteins were mapped with ImageAnnotator (Usadel et al., 2009). The mapping highlights changes in biotic stress and signalling-related protein abundances in NE lines compared to IE (Figure 6d). Especially proteins from the antioxidative system were affected: superoxide dismutase proteins that are involved in superoxide radical degradation were upregulated and several other redox-related proteins were downregulated. To the downregulated proteins belong enzymes from peroxidase protein family (PRX) including L-ascorbate peroxidase 3 (APX; Potri.009G134100.3), thioredoxin superfamily protein (TRX; Potri.006G137500.1), several glutathione S-transferases (GST- tau 25, tau 7, lambda 2), the cytochrome P450 protein (Potri.005G220700.1) and other peroxidase superfamily proteins (Figure 6d; Table S1). These changes in protein abundance suggest changes in the redox signalling/regulation of NE fine roots. Also the abundances of several other, abiotic stress related proteins were adjusted, such as stress-related ozone-induced protein (Potri.006G051900.4), heat shock protein 101 (Potri.015G056900.2), MLP-like protein 423 (Potri.008G213100.1) and osmotin 34 (Potri.001G107600.1) (Figure 6d; Table S1) suggesting isoprene plays a role in stress related processes.

Interestingly, also LOX1 (Potri.005G032700.1), an enzyme that can be assigned to lipid metabolism, plant defense responses and LR development was downregulated (Table S1). Several other downregulated proteins suggest adjustment of development related processes. To such belong α-L-arabinofuranosidases (ARA; Potri.006G029900.1), cellulose synthase (Potri.001G266400.1), β-galactosidase 9 (Potri.017G057900.1), expansin-like B1 (Potri.003G083200.1) and pectin methylesterase 3 (Potri.001G162500.), all involved in cell-wall formation and lignin biosynthesis pathway (Table S1).

In accordance to Cinege et al. (2009) who found only low *ISPS* gene expression in poplar roots, the root isoprene synthase was under the detection limit in the proteomic analysis of IE and NE lines.

4 | DISCUSSION

4.1 | A distinct location of poplar isoprene synthase promoter suggests development related function in roots

The present study reveals new functions for isoprene in fine roots. So far isoprene's function has been related mainly to plant foliage and to protection against abiotic stresses, such as high temperature or oxidative stress (Behnke et al., 2007; Loreto et al., 2001; Sharkey & Singsaas, 1995; Vickers et al., 2009). Present data suggest that isoprene has a more general role in plant physiology than previously

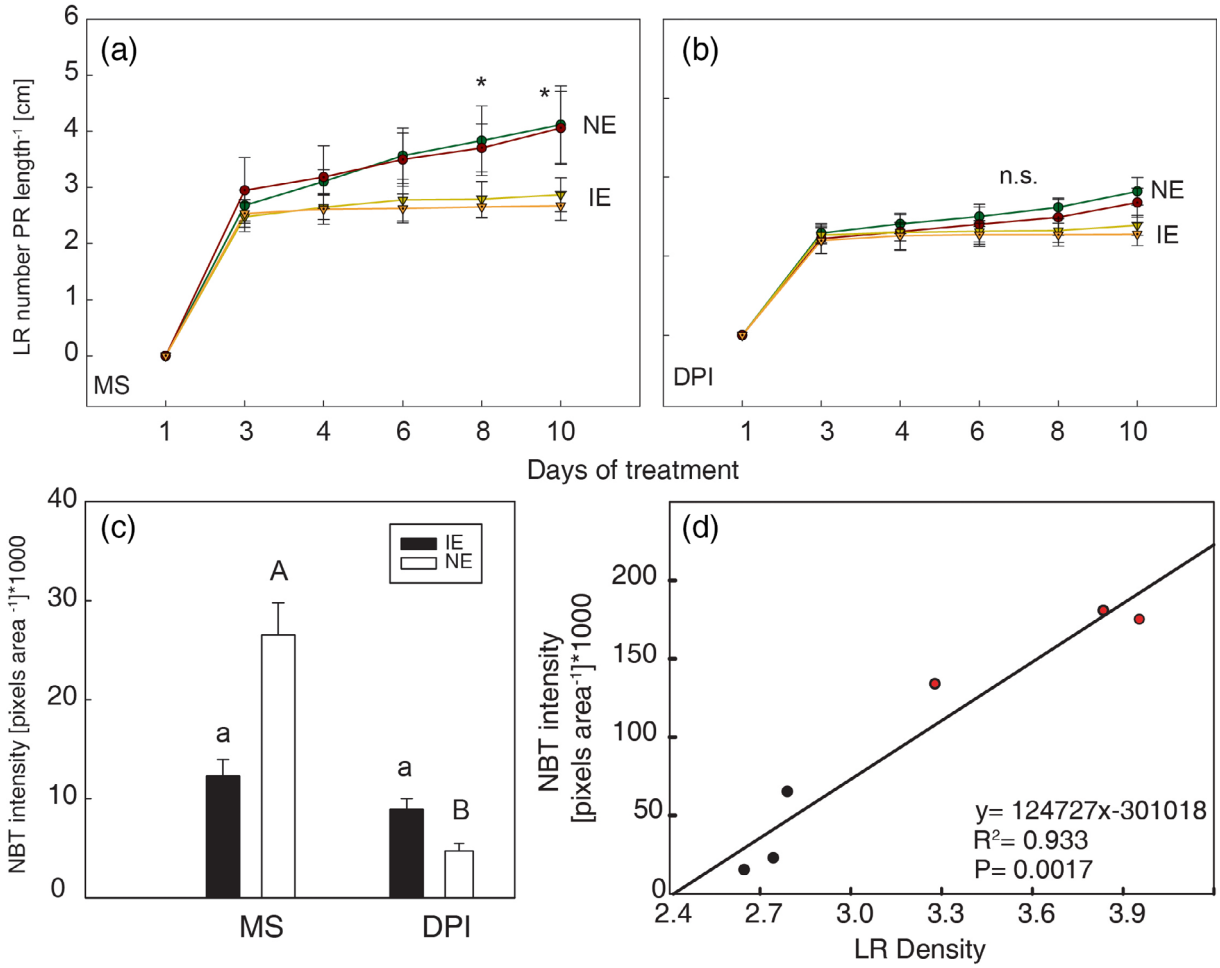

FIGURE 5 Lateral root (LR) growth and O_2^- accumulation as nitroblue tetrazolium (NBT) stain in isoprene emitting (IE) and non-emitting (NE) poplar roots in control conditions and influenced by diphenylene iodonium (DPI). (a, b), The LR number [normalized to primary root (PR) length] in isoprene (IE; wild type and empty vector control) and non-isoprene (NE; lines NE1 and NE2) emitting poplar lines under control conditions (a) and when treated by DPI (b). Asterisks indicate the statistical differences between the treatments by One way analysis of variance and Fisher LSD test (*p < .05; n = 10); n.s. = not significant. (c), The NBT intensity is given as bar charts for IE (white) and NE (black) under control conditions and when treated with 0.1 μM DPI; different letters indicate the statistical differences between the treatments by t-test (p < .05; n = 10). (d), The correlation between O_2^- accumulation (given as NBT-stain intensity, pixels area^{-1}*1,000) and LR density. Black dots: IE; red dots: NE [Colour figure can be viewed at wileyonlinelibrary.com]

thought. The histochemical analyses revealed a high *PcISPS* activity especially in vascular tissue, root hairs, root cap and developing LRs, proposing a function of isoprene in specific, eventually rapidly developing/expanding root regions. The location of *PcISPS* in roots is largely similar as that of *AtTPS-Cin* promoter, whose activity was shown especially in root vascular tissue, epidermal cells and root hairs of *Arabidopsis* (Chen et al., 2004). Also other terpene biosynthesis related promoters have been localized in partly similar, specific root regions: A semi-volatile diterpene rhizathalene activity was found in root stele, in emerging secondary roots and root tips of *Arabidopsis* (Vaughan et al., 2013) whereas cytochrome P450 monooxygenase [involved in biosynthesis of (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) in *Arabidopsis* roots] was localized to the root stele and meristematic zone (Sohrabi et al., 2015). In addition to the specific location, a development/growth related function of isoprene is in the present study supported by increased *PcISPS* promoter activity in IAA treated roots, and by the putative auxin and gibberellin responsive regulatory elements in the promoter. Development related function for isoprene was suggested also by some previous findings, showing enhanced growth of transgenic, IE *Arabidopsis* plants (Loivamäki et al., 2007; Sasaki et al., 2007; Zuo et al., 2019) and promotion of flowering time in several plant species when exposed to isoprene (Terry, Stokes, Hewitt, & Mansfield, 1995).

4.2 | Absence of isoprene alters ROS in distinct root cells

Though the mode of action is unknown, isoprene is suggested to reduce ROS in plant tissue (Affek & Yakir, 2002; Loreto &

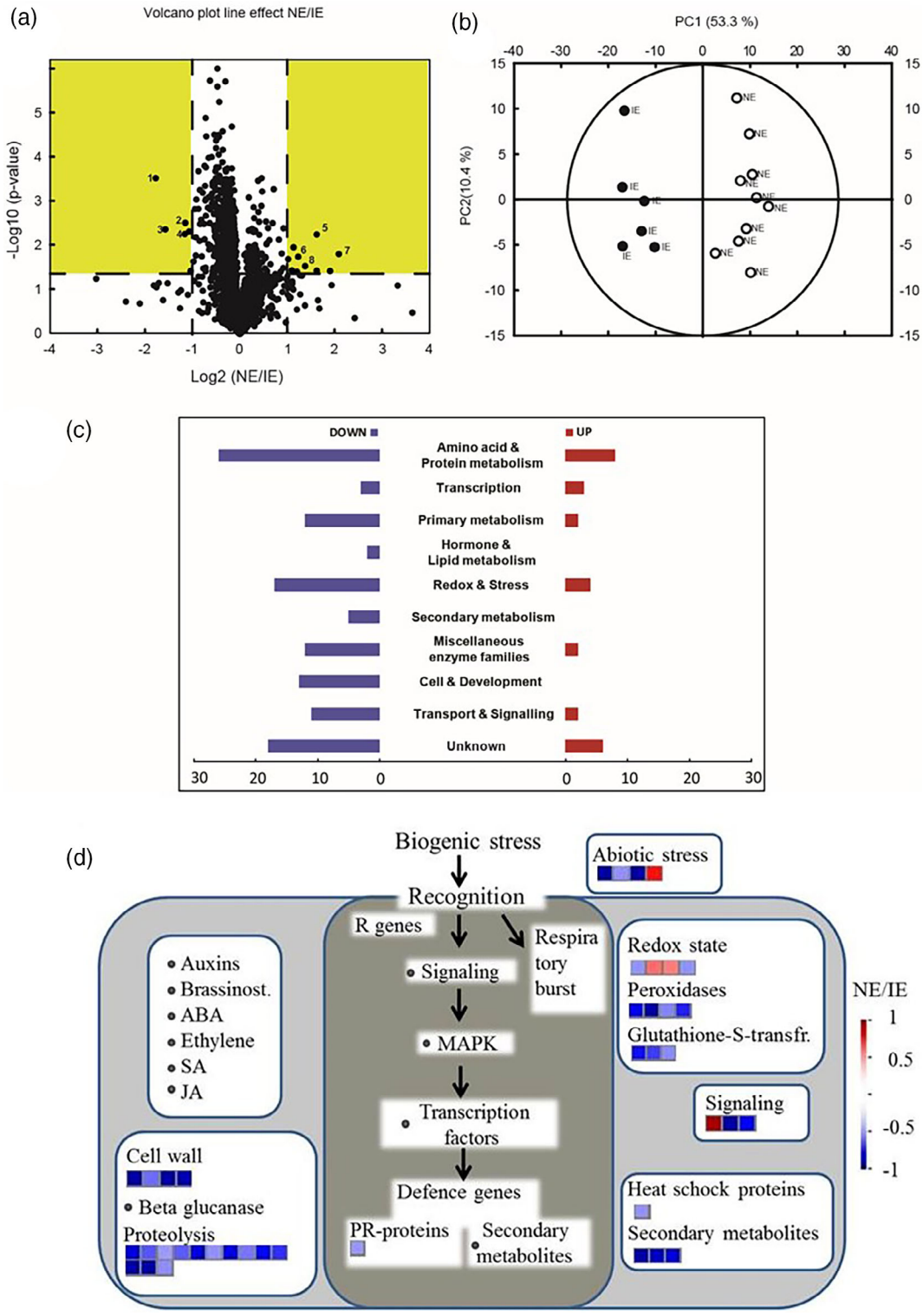

FIGURE 6 Legend on next page.

Velikova, 2001; Velikova, Pinelli, & Loreto, 2005). In roots, ROS are shown to be involved in several growth regulating processes being active especially in developing plant organs, such as root cap, root hairs and meristematic- and elongation zones (Carol & Dolan, 2006; Gapper & Dolan, 2006; Tsukagoshi, 2016). ROS are also involved in remodelling the plant root architecture to adjust to changing environment, such as osmotic or salt stress (Deak & Malamy, 2005; Møller & Tester, 2007; van der Weele et al., 2000; Zolla et al., 2010). The ROS

concentration in salt stressed tissues is strongly influenced by a coordinated action of different antioxidative enzymes (Munns & Tester, 2008). By altering ROS also isoprene might interfere several plant performance and development related processes. Previous analysis of post translational modifications (PTM) of proteins in poplar leaves (Vanzo et al., 2016) indicates that isoprene indirectly regulates ROS production via the control of the S-nitrosylation level of ROS-metabolizing enzymes, thus modulating the extent and velocity at which the ROS and NO signalling molecules are generated. In support, we located high *PcISPS* promotor activity largely in the same root regions as in which O_2^- accumulated in NE poplars. High *PcISPS* promoter activity was especially found in NaCl and the growth hormone IAA treated roots. These results suggest that isoprene might interfere or adjust signalling processes in which O_2^- are involved in the root tissue, similar to antioxidants that regulate ROS signalling and maintain the balance between toxic and beneficial ROS levels (Mittler, 2017). Though isoprene is rather a less effective antioxidant than the "classical" antioxidants such as ascorbate or glutathione (Hancock, 2016) it might have a function in fine tuning of gene expression (Zuo et al., 2019) or by altering enzyme activities by PTM (Vanzo et al., 2016) at specific sites. Isoprene is, moreover, rather small and highly volatile, and its biosynthesis and local cell-internal concentrations are therefore rapidly adjustable which might allow faster changes in effective concentrations compared to non-volatile antioxidants.

4.3 | Absence of isoprene alters root architecture in ROS dependent manner

ROS are also important components in LR development and accumulate especially in later stages of LRP starting from stage IV (Dunand et al., 2007; Li et al., 2015; Manzano et al., 2014). In the present study the *PcISPS* promoter was only active in later stages of LR development, namely when vascular tissue starts to develop, which fits well with previous knowledge on ROS signalling being active during LR emergence, but not in the primordium specification itself (Manzano et al., 2014). In our studies *PcISPS* promoter activity was especially high in developing LRs additionally facing salt stress indicating that isoprene may be simultaneously involved in both, development-related processes and in stress response.

The role of isoprene in these processes is further alleviated by the observed O_2^- accumulation and LR growth alteration in the isoprene-deficient NE poplar roots. Previously also a sesquiterpene, (−)-thujopsene was shown to alter root architecture: Ditengou et al. (2015) revealed induced LR growth and O_2^- accumulation in *Arabidopsis* and poplar after root external exposure to the fungal sesquiterpene (−)-thujopsene. This is an opposing outcome compared to internally produced isoprene in the present study. Our results together with those of Ditengou et al. (2015) suggest that both, internally produced isoprene and externally accessible (−)-thujopsene modify ROS signalling in the roots leading to altered LR growth. ROS proved to be important component in both kinds of signalling leading into altered LR development: DPI, an inhibitor of NAD(P)H oxidase, negatively affected O_2^- and LR no matter whether it was induced by internal isoprene (this study) or by external (−)-thujopsene (Figure 7; Ditengou et al., 2015).

4.4 | Absence of isoprene caused alterations in poplar root proteome

The proteomic survey of NE poplar roots revealed changes in the abundance of several proteins related to redox balance, signalling and development, suggesting a central role of isoprene in plant physiological processes. In previous studies large rearrangements of leaf gene expression (e.g. Behnke et al., 2010), leaf metabolism (Moritz, Kaling, Schnitzler, & Schmitt-Kopplin, 2017; Way et al., 2013) and chloroplast/leaf protein composition (Monson et al., 2020; Vanzo et al., 2016; Velikova et al., 2014) were detected in NE poplars. Together, these observations suggest an involvement of isoprene in many biochemical and physiological processes of foliage. Behnke

FIGURE 6 Whole-proteome comparison of isoprene emitting (IE) and transgenic, non-isoprene emitting (NE) poplar roots. (a), Volcano plot showing the magnitude of differential protein abundance in NE and IE genotypes ($\log_2$ [fold change]) compared with the measure of statistical significance ($-\log_{10}$ [*p* value, Student's *t* test]). Vertical, dashed lines indicate log fold change of ±1, and the horizontal line indicates a significance value of $p = .05$. The proteins with the highest and significant fold changes between NE and IE samples are highlighted and numbered as follows: 1 = Auxin-responsive protein, putative; 2 = Pathogenesis-related protein Bet v I family; 3 = Tetrahydroberberine oxidase; 4 = Prenylcysteine oxidase / Prenylcysteine lyase; 5 = HMG 1 protein; 6 = PHOSPHATASE SUBUNIT GENE G4-1; 7 = ABC transporter 1; 8 = Nodulin-like protein family (MTN26protein family). (b), Score plot of OPLS of the whole proteome of IE (black circles) and NE (white circles) poplar roots. PC, predictive component. The ellipse indicates the tolerance based on Hotelling's t^2 with a significance level of 0.05. OPLS model fitness is as follows: r^2 (*x*) = 53%, r^2 (*y*) = 100%, r^2 = 94%, and q^2 (cumulative) = 79% using one predictive component. RMSEE (root mean square error of estimation) = 0.134 and RMSEcv (root mean square error of cross validation) = 0.227. $p < .001$; cross-validated analysis of variance. (c), Discriminant proteins that explain the separation between IE and NE poplar (148 in total; Table S1) grouped according their functional categories. Blue bars indicate down-regulated and red bars indicate up-regulated proteins in NE sample. (d), Changes in the proteomics of NE compared to IE poplars visualized in a biotic stress response map. The map was created by uploading to the MapMan (Usadel et al., 2009) the gene functional assignments, taken from the corresponding *Arabidopsis* orthologs (http://www.arabidopsis.org/tools/bulk/go/index.jsp), which were obtained via the POPGENIE (http://www.popgenie.org) database. Protein expression changes in the biosynthetic pathways are displayed according to their functional categories. Changes are colour coded: red indicates increased and blue decreased in the NE genotype [Colour figure can be viewed at wileyonlinelibrary.com]

et al. (2009) describe, moreover, higher resistance of NE lines to oxidative stress by ozone due to adjusted ascorbate glutathione and α-tocopherol concentrations. The rearrangement of redox balance- and signalling related root proteins in the present study fit to these previous observations on leaves. Several peroxidases that can be linked to ROS detoxification were, for example, downregulated in NE plants. Peroxidases are proposed to play also an important role in controlling root growth (Dunand et al., 2007; Passardi, Tognolli, De Meyer, Penel, & Dunand, 2006). In addition, the strong upregulation of superoxide dismutases, well-known O_2^-—degrading proteins, (Monson et al., 2020; Vanzo et al., 2016) in NE poplars alleviate the ROS linkage of isoprene function in belowground. The upregulation of superoxide dismutases can be expected to decrease the ROS levels in NE plants. The present ROS-stain data, however, indicate that NE trees could not achieve comparable redox status to IE trees by adjustments of other redox balance related proteins. These complex changes in the NE poplar proteome, and the nevertheless upregulated ROS levels, stress the importance of isoprene function in poplar root physiology.

Considering the enhanced LR growth rate in NE poplars several further proteins are interesting. LOX1, a protein related to root development and plant pathogen response (Melan et al., 1993; Vellosillo et al., 2007) was downregulated in NE. Vellosillo et al. (2007) showed increased LR numbers caused by cell wall modification and down-regulation of LOX1 function. This result is in line with our data showing increased LR growth under decreased LOX1. Several other proteins involved in cell-wall modification were also down-regulated in the NE genotype, such as α-L-arabinofuranosidases (ARA). ARA is a glycosyl hydrolase localized in the vascular tissue of roots and stems and connected to secondary cell wall formation and cell wall reorganization (Sumiyoshi et al., 2013). Cellulose synthases, expansins and pectin methylesterases (PMEs) were, moreover, altered in NE lines,

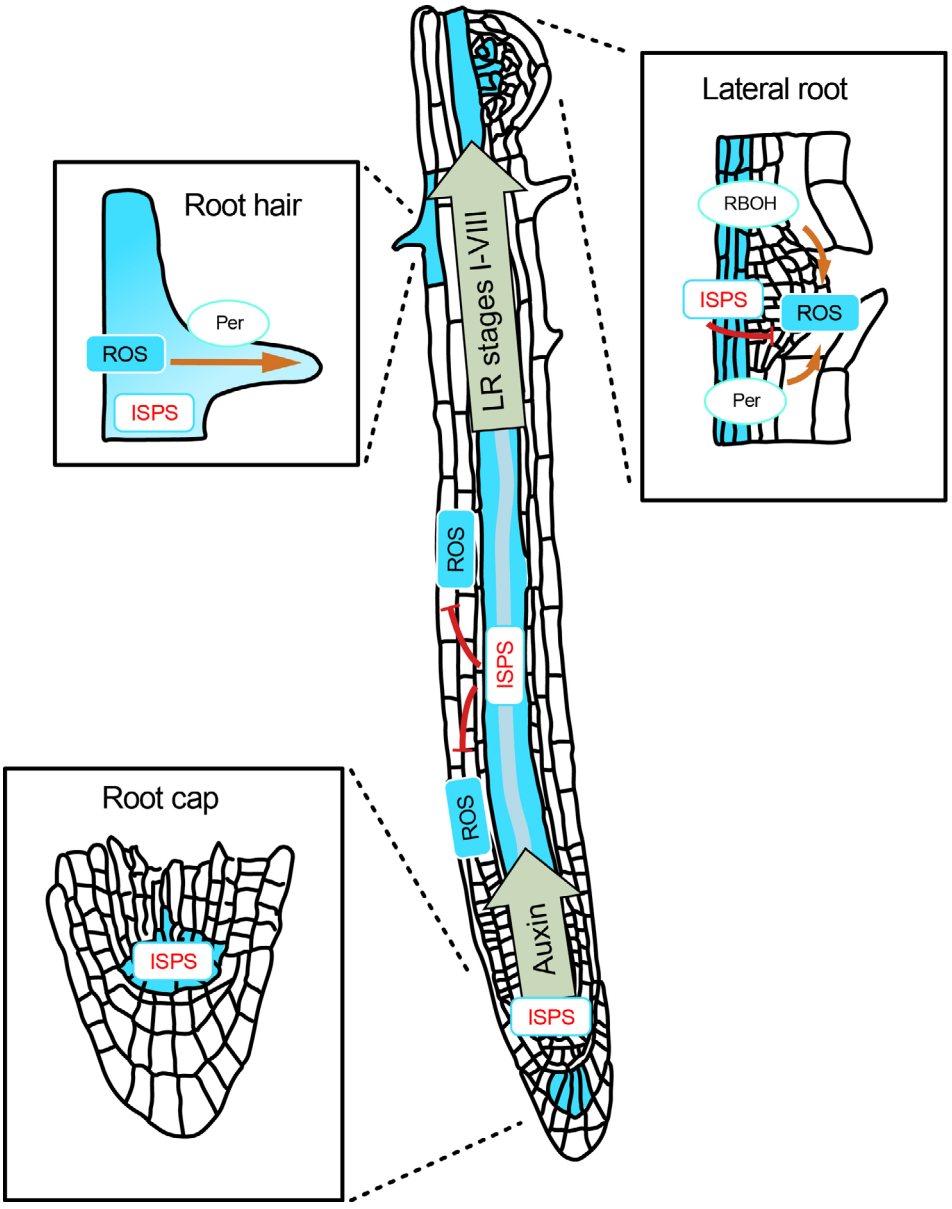

FIGURE 7 Scheme of possible signalling function of internal isoprene (ISPS) in roots. ROS: reactive oxygen species; Per: peroxidase; RBOH: respiratory burst oxidase homolog [Colour figure can be viewed at wileyonlinelibrary.com]

further supporting the role of isoprene in adjusting root growth and development. PMEs, for example, catalyse the demethylesterification of cell wall polygalacturonans (Ociepa, 2017) thereby releasing methanol, which emission is indicative for cell wall development (Vanzo et al., 2015). In support, Ociepa (2017) showed that the down-regulation of PMEs increased the number of LR in *Arabidopsis*. Cellulose synthases are required for the biosynthesis of cellulose, which follows peroxidase mediated cell wall loosening and secondary cell wall formation (Li et al., 2016) and, expansins are also known to be involved in cell-wall modification related developmental events (Sampedro & Cosgrove, 2005).

4.5 | Isoprene's potential to alter poplar internal signalling and plant performance

ROS signalling is integrated with hormonal signalling networks thereby regulating not only plant development but also responses to environmental cues (Mittler, 2017). ROS has been shown to alter at least SA-, gibberellin-, auxin- and ABA-mediated signalling processes (Mittler, 2017), the same phytohormones that have putative cis-responsive elements in *PcISPS* promoter. By adjusting O_2^-, isoprene might affect several plant hormonal signalling pathways in a yet unknown manner. The very recent gene expression data of Zuo et al. (2019) on transgenic, IE *Arabidopsis*, suggest that isoprene might execute its effects by altering specific, growth regulation and defense associated genes such as gibberellic acid and jasmonic acid. Previous studies have reported changed aboveground biotic interaction of NE poplars (Behnke et al., 2012; Müller et al., 2015). These changes may be explained by adjustments in plant internal signalling. Isoprene might also interfere root-microbe interactions: in early stages of plant root contact with mycorrhiza, both ROS and ROS scavenging enzymes (and the inhibition of them) play crucial roles (Babtista et al., 2007; Ditengou et al., 2015; Nanda et al., 2010). The intensity of the ROS burst being important in preparing for contact with mutualistic or pathogenic plant associated microbes (Babtista et al., 2007; Nanda et al., 2010), fine tuning of redox balance can be essential to differentiate between various microbes. Questions as how the lack of isoprene- or fungi-derived terpenes will affect poplar mycorrhization and the rhizosphere microbiome should be directed in the future. To regulate ROS in such interactions, isoprene might take over part of the job either indirectly by intervening the signalling routes for example, via modulation of gene expression (Zuo et al., 2019) or protein S-nitrosylation (Vanzo et al., 2016), or directly by supporting more effective O_2^- scavenging molecules.

4.6 | Conclusions

Taken together, the altered LR development, the ROS accumulation as well as the redox balance and signalling related adjustments in the proteome of NE poplar roots propose isoprene a ROS-related role. The localization of *PcISPS* promoter activity and O_2^- accumulation in distinct, expanding root regions further supports such function. Together our data suggest for isoprene a more important, ROS mediated functions in plant development and/or signalling than previously thought. Functions of isoprene are, thus, not restricted to plant foliage but can be relevant also belowground. The present results are in line with the suggestion of Sharkey and Monson (2017): isoprene does not only function for example, in protection against rising temperatures, but might rather have a more general function in adjusting plant physiological processes to changing environment.

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AUTHOR CONTRIBUTIONS

Maja Miloradovic van Doorn conducted promoter and root growth analyses and overall data integration, and together with Maaria Rosenkranz wrote the initial draft of the manuscript, Juliane Merl-Pham conducted the proteome analysis, Andrea Ghirardo performed multivariate data analysis, and Siegfried Fink performed part of microscopic analysis. Andrea Polle, Jörg-Peter Schnitzler, and Maaria Rosenkranz conceived and supervised the study, Maaria Rosenkranz supervised and finalized manuscript writing, all commented and agreed on the final version of manuscript. Maaria Rosenkranz agrees to serve as the author responsible for contact and ensures communication.

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SUPPORTING INFORMATION

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

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