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Association genetics identifies a specifically regulated Norway spruce laccase gene, *PaLAC5*, linked to *Heterobasidion parviporum* resistance

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Abstract

It is important to improve the understanding of the interactions between the trees and pathogens and integrate this knowledge about disease resistance into tree breeding programs. The conifer Norway spruce (*Picea abies*) is an important species for the forest industry in Europe. Its major pathogen is *Heterobasidion parviporum*, causing stem and root rot.

In this study, we identified 11 Norway spruce QTLs (Quantitative trait loci) that correlate with variation in resistance to *H. parviporum* in a population of 466 trees by association genetics. Individual QTLs explained between 2.1 and 5.2% of the phenotypic variance. The expression of candidate genes associated with the QTLs was analysed in silico and in response to *H. parviporum* hypothesizing that (a) candidate genes linked to control of fungal sapwood growth are more commonly expressed in sapwood, and; (b) candidate genes associated with induced defences are respond to *H. parviporum* inoculation. The Norway spruce *laccase PaLAC5* associated with control of lesion length development is likely to be involved in the induced defences. Expression analyses showed that *PaLAC5* responds specifically and strongly in close proximity to the *H. parviporum* inoculation. Thus, *Pa*LAC5 may be associated with the lignosuberized boundary zone formation in bark adjacent to the inoculation site.

KEYWORDS

genome-wide association study (GWAS), lignosuberized boundary zone, mitochondrion, sapwood, secretory and endosomal trafficking pathways, suberin, TOM40

1 | INTRODUCTION

The importance of trees and forests for sustaining terrestrial life and biodiversity can probably not be exaggerated (Petit & Hampe, 2006).

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Pathogen and pest attacks on trees negatively impact the health and biodiversity of native forest ecosystems as well as forest plantations, which can have large economic, ecological and societal consequences (Cubbage, Pye, Holmes, & Wagner, 2000; Garbelotto & Gonthier, 2013; Pautasso, Schlegel, & Holdenrieder, 2015; Woodward, Stenlid, Karjalainen, & Hüttermann, 1998). Therefore, it is important to increase the understanding of interactions between the tree and a pathogen in order to incorporate traits that confer to increased resistance into forest tree breeding programs.

Norway spruce [*Picea abies* (L.) Karst.] is economically important for the forest industry in Europe. Its major pathogens are fungi in the species complex *Heterobasidion annosum* sensu lato (s.l.), which causes stem and root rot in Norway spruce and several other conifer tree species (Garbelotto & Gonthier, 2013; Woodward et al., 1998). Under natural conditions, airborne spores of *H. annosum* s.l. can infect stumps created after harvesting and thinning operations. Once the stump is infected, surrounding trees or stumps can be infected by secondary spread when *H. annosum* s.l. mycelium enters neighbouring trees through root grafts and contacts (Oliva, Bendz-Hellgren, & Stenlid, 2011; Redfern & Stenlid, 1998). In Norway spruce, resistance to the spruce-infecting congener *Heterobasidion parviporum* is quantitative in its nature (Arnerup, Swedjemark, Elfstrand, Karlsson, & Stenlid, 2010; Chen et al., 2018; Karlsson & Swedjemark, 2006; Steffenrem, Solheim, & Skrøppa, 2016), and classical interval mapping-based quantitative trait locus (QTL) analysis for resistance to *H. parviporum* identified 13 QTL linked to host resistance (Lind et al., 2014). *PaLAR3*, on the QTLs associated with control of fungal spread in the sapwood, has been validated and the function of the variation at the locus described (Nemesio-Gorriz et al., 2016).

A feature that Norway spruce has in common with all tree species is that a large fraction of the biomass is invested in the sapwood in the trunk (Petit & Hampe, 2006). The primary function of the sapwood is to transport water and nutrients to the crown and it is dominated by dead cells that have a limited capacity to respond to biotic or abiotic stress (Johansson & Theander, 1974; Oliva et al., 2015; Shain, 1971). To protect the sapwood, the trunk of a tree is clad in an impermeable barrier, bark. The term "bark" commonly refers to all tissues external to the vascular cambium of trees. The outer bark is highly suberized and lignified, making it extremely resistant to mechanical and chemical degradation. Only a few pathogenic microorganisms are capable of directly penetrating the outer bark (Lindberg & Johansson, 1991). Therefore, a common mode of entry for fungi that cause stem cankers and decays is via mechanical wounds, exposing the cortex, secondary phloem tissues or the xylem (Woodward & Pocock, 1996). The speed at which the tree is able to seal off the tissues exposed by wounding with wound periderm is critical in avoiding damaging infections and subsequent loss of water transport capacity. The process to heal the bark begins with rapid necrosis of cells closest to the wound or progressing infection. It then continues with programmed death of cells adjacent to the necrosis, forming the lignosuberized boundary zone (LSZ), and de-differentiation of cells next to the LSZ followed by differentiation of the wound periderm (Bodles, Beckett, & Woodward, 2007; Mullick, 1977; Woodward, Bianchi, Bodles, Beckett, & Michelozzi, 2007).

The trait control of lesion length extension (LL, with reported heritability values of 0.14–0.33) is measured as the size of the discernible necrosis cells closest to the wound or progressing infection (Arnerup, Lind, Olson, Stenlid, & Elfstrand, 2011; Chen et al., 2018; Steffenrem et al., 2016). It could be argued that LL provides a measure of how the induced defences and wound healing responses interact to control the spread of the necrotrophic pathogen (Arnerup et al., 2011; Chen et al., 2018; Danielsson et al., 2011; Lind et al., 2014; Steffenrem et al., 2016). The trait control of fungal spread in the sapwood (fungal sapwood growth, SWG) can be considered to provide a measure of how well the combination of constitutive defences and the induced defence responses in the parenchymatic cells can control the spread of *H. parviporum* in the exposed sapwood (Johansson & Stenlid, 1985; Oliva et al., 2015). The narrow-sense heritability of SWG has been estimated to vary between 0.11 and 0.42 depending on the material studied (e.g., experimental cross, natural population) (Arnerup et al., 2010; Chen et al., 2018).

To date, the main focus of practical breeding in Norway spruce has been on climatic adaptation, growth and wood quality traits (Skrøppa, Solheim, & Steffenrem, 2015). In contrast, breeding for replantation material with improved resistance to *H.annosum* s.s. and *H. parviporum* is an overlooked objective because of limited information about genetic variation in resistance to these pathogens and the lack of reliable selection techniques (Skrøppa et al., 2015). There are, however, clearly sufficient phenotypic and genetic variation for resistance to *H. parviporum* in Norway spruce to allow for breeding (Arnerup et al., 2010; Chen et al., 2018; Karlsson & Swedjemark, 2006; Steffenrem et al., 2016), and no adverse correlations between resistance to *H. parviporum* and growth or wood properties traits (Chen et al., 2018; Steffenrem et al., 2016). Hence, the selection for *H. parviporum* resistance in breeding programmes could lead to considerable gain without compromising other breeding achievements (Chen et al., 2018).

To gain a deeper understanding of the heritability and genetic architecture of, for example, disease resistance traits, including the number, location, effect and nature of the loci involved, quantitative and molecular genetic approaches can be used to analyse the relationships between DNA polymorphism and phenotypic variation (Bartholomé et al., 2016; Neale & Savolainen, 2004). The two main approaches to detect QTLs: Interval mapping (IM) in experimental crosses or linkage disequilibrium (LD) mapping, commonly known as genome-wide association studies (GWAS) (Neale & Savolainen, 2004). GWAS, relying on historical recombination in the mapping population, overcomes the limited resolution of IM in experimental crosses (Baison et al., 2019; Neale & Savolainen, 2004). If enough markers can be analysed, this should be especially advantageous in conifers that have particularly short average distances of maintained LD, often even confined within genes (Namroud, Guillet-Claude, Mackay, Isabel, & Bousquet, 2010). The effects of LD are also influenced by the extreme physical distances separating genes in conifers (Nystedt et al., 2013).

It is likely that the Norway spruce genome harbours additional, yet undetected loci, to the 13 QTLs already identified by (Lind et al., 2014) controlling resistance to *H. parviporum* (Chen et al., 2018; Hall, Hallingbäck, & Wu, 2016). Identification of further loci would support the initiation of a breeding programme for the resistance to the pathogen in Norway spruce and, just as importantly, improve the understanding of the interactions between trees and necrotrophic pathogens. The short maintained LD and the polygenic nature of the traits controlling resistance suggest that GWAS could be a powerful method to identify further QTL regions associated with *H. parviporum* resistance in Norway spruce. Consequently, in this study, we aimed to identify Norway spruce loci that correlate with variation in resistance to *H. parviporum* in a population of 466 Norway spruce trees by GWAS. We identified candidate genes associated with the QTLs and analysed the expression patterns of the candidate genes in response to *H. parviporum* hypothesizing that (a) candidate genes linked to the SWG trait would be expressed in sapwood while candidate genes linked to LL are expressed in more peripheral tissues, and; (b) candidate genes that are part of the induced defence are induced in response to *H. parviporum* inoculation.

2 | MATERIALS AND METHODS

2.1 | Phenotyping of resistance traits in the progeny of 466 Norway spruce mother trees

We used the currently available largest Norway spruce resistance phenotyping dataset to perform the GWAS. The material, inoculation method and genetic analyses are described in detail in (Chen et al., 2018). On average ten 2-year-old, open-pollinated progenies derived from 466 tested plus trees in the Swedish breeding population were inoculated with *H. parviporum* Niemelä & Korhonen strain Rb175. A wooden dowel colonized by *H. parviporum* was fixated at a wound on the stem of the plant with Parafilm. The inoculated plants were kept under ambient light and temperature in the forest tree nursery and harvested 21 days post-inoculation. The induced defence responses (LL) in the phloem and inner bark were estimated by measuring the discernible lesion spread upwards and downwards from the edge of the inoculation point on the inside of the bark. SWG was estimated using established protocols (Arnerup et al., 2010; Stenlid & Swedjemark, 1988) (Table 1). The seedlings were cut up into five mm discs and placed on moist filter papers in Petri dishes. Plates were incubated in darkness under moist conditions at 21°C for 1 week to induce conidia formation. Thereafter, the presence or absence of *H. parviporum* conidia on each individual disc was determined under a stereomicroscope. For each seedling, the sum of the discs where conidia were observed multiplied by 5 (mm) was noted as SWG. Plates where no conidia could be observed on the discs, the inoculation point and on the inoculation plug, and that showed total lesion length of 2 mm or shorter, were treated as inoculation failures and were discarded (Lind et al., 2014). Chen et al. (2018) reported narrow-sense heritability values of 0.33 and 0.42, respectively, for LL and SWG and moderate phenotypic (0.48) and genetic (0.47) correlations between LL and SWG in this material.

TABLE 1 Summary statistics of the phenotype data used in the trait-marker association study (Details can be found in Chen et al. (2018))

Inoculation study	Acron.	Unit	N[a]	Mean
Diameter[b]	D	mm	4,628	4.0
Lesion length[c]	LL	mm	4,547	7.6
Fungal growth[d]	FG/SWG	mm	4,554	32.5
Vitality[e]	Vitality	Classes	4,376	1.9

[a]*N*: total number of progenies with valid recording of the trait.
[b]Diameter of the progenies at the inoculation site.
[c]Length of the necrotic lesion in the phloem and inner bark.
[d]Fungal growth in the sapwood of the progenies.
[e]Vitality of the progenies where score 1 was given to fully vital and worst score 3 was given to plants showing a pronounced loss of vitality.

2.2 | Norway spruce genotyping and SNP annotation

Dormant buds were collected from each of the mother trees. Total genomic DNA was extracted from the buds, using the Qiagen Plant DNA extraction kit (Qiagen, Hilden, Germany), and the DNA was quantified using the Qubit® ds DNA Broad Range (BR) Assay Kit (Oregon, USA). The generation and evaluation of exome capture for Norway spruce are described elsewhere (Vidalis et al., 2018). Sequence capture on the mother tree DNA was performed using 40,018 previously evaluated diploid probes (Baison et al., 2019; Vidalis et al., 2018). Probe design and sequence capture were done by RAPiD Genomics (Gainesville, FL, USA). In brief, Illumina sequencing compatible libraries were amplified with 14 cycles of PCR and the probes were then hybridized to a pool comprising 500 ng of eight equimolarly combined libraries following Agilent's SureSelect Target Enrichment System (Agilent Technologies). These enriched libraries were then sequenced to an average depth of 15x using an Illumina HiSeq 2,500 (San Diego, USA) on the 2 × 100 bp sequencing mode.

Read mapping and initial variant calling as well as the recalibration of the quality of SNP calling were then applied to filter the raw variants, described in detail in Baison et al. (2019). In brief, the variant calling was made using GATK HaplotypeCaller v.3.6 as per the best practices protocol (Auwera et al., 2013) in gVCF output format. To increase accuracy, hard filters in the form of minor allele frequency (MAF) and "missingness" of <0.05 and >20%, respectively, were then performed on the final dataset.

3 | GWAS

The LASSO model as described by Li et al. (2014) was applied to the *H. parviporum* resistance trait data for the detection of QTLs.

The LASSO model:

$$\min_{(\alpha_0,\alpha_j)} \frac{1}{2n}\sum_{i=1}^{n}\left(y_i-\alpha_o-\sum_{j=1}^{P}x_y-\alpha_j\right)^2+\lambda\sum_{j=1}^{P}\alpha_j, \quad (1)$$

where y_i is the estimated breeding values (EBV) of an individual i (i = 1,...,n; n is the total number of individuals) for each trait, α_0 is the population mean parameter, x_{ij} is the genotypic value of individual i and marker j coded as 0, 1 and 2 for three marker genotypes AA, AB and BB, respectively, α_j is the effect of marker j (i = 1,...,n; n is the total number of markers) and λ (>0) is a shrinkage tuning parameter. A fundamental idea of LASSO is to utilize the penalty function to shrink the SNP effects towards zero, and only keep a small number of important SNPs that are highly associated with the trait in the model. The stability selection probability (SSP) of each SNP being selected to the model was applied as a way to control the false discovery rate and determine significant SNPs (H. Gao et al., 2014; Li & Sillanpää, 2015). For a marker to be declared significant, an SSP inclusion ratio (Frequency) was used with an inclusion frequency of all traits. This frequency inferred that the expected number of falsely selected markers was less than one, according to the formula of Bühlmann, Kalisch, and Meier (2014). Population structure was accounted for in all analyses by including principal components based on the genotype data as covariates into the model (Baison et al., 2019). An adaptive LASSO approach (Baison et al., 2019; Zou, 2006) was used to determine the percentage of phenotypic variance (PVE) (H^2_{QT}) of all the QTLs. The analyses were all performed in RStudio (Team, 2015).

3.1 | Identification of candidate genes associated with the QTLs

To assess putative functionality of SNPs with significant associations, a gene enrichment analysis of putative genes and their associated orthologs was performed against the *P. abies* v1.0 genome (http://congenie.org), collecting PFAM and GO term annotations and *Populus* and *Arabidopsis* orthologues. The position of the detected QTLs in Norway spruce genome was estimated by searching an ultra-dense genetic map (Bernhardsson et al., 2019) for markers derived from the same probes as the SNP markers holding the QTLs, identified based on tblastn sequence homology for the SNP array sequences in the Lind et al. (2014) study, as described by (Bernhardsson et al., 2019).

Information on the expression pattern of the putative candidate genes associated with the QTL, in the Norway spruce clone Z4006 (the clone sequenced in Nystedt et al. (2013)) and in wood, were collected from three sources. Firstly, expression data were downloaded from the publicly available *P.abies* exAtlas (https://www.congenie.org) and NorWood v1.0 (http://norwood.congenie.org) databases, respectively. Both these databases are comprised of expression profiles from approximately 50-year-old ramets of the genotype "Z4006." Then, we examined an RNAseq study of bark and phloem samples harvested at seven dpi proximal (0–5 mm from the wound) and distal to the inoculation site (10–15 mm away from the wound) from two Norway spruce genotypes (S21K0220126 and S21K0220184) inoculated with *H. parviporum* (Chaudhary et al., submitted manuscript). In brief, two-year-old branches on clones of S21K0220126 and S21K0220184 were inoculated and sampled as described above using wounding as a control. A total RNA from three biological replicates of each clone per treatment were sequenced on the Illumina HiSeq 2500 at the SNP&SEQ Technology Platform (SciLifeLab, Uppsala). Quality filtering was done using Nesoni 0.97 (http://www.vicbioinformatics.com/nesoni-cookbook/index.html#). Differential gene expression was identified using the Tophat-cufflinks pipeline (Trapnell et al., 2012, 2014; Trapnell et al., 2013) and the "*P. abies* v1.0-all-cds.fna" gene catalogue as a reference (Chaudhary et al., submitted manuscript).

3.2 | Branch inoculation with *H. parviporum*

We performed an inoculation experiment on six-year-old grafted cuttings of the Norway spruce genotype S21K7820222. Branches on healthy-looking potted plants were inoculated with wooden dowels colonized by *H. parviporum* Rb175 fixated to a wound on a two-year-old branch with Parafilm. Control treatment branches were wounded and covered with Parafilm. The inoculated plants kept at ambient light and temperature conditions in a greenhouse. At 7 days post-inoculation (dpi), bark surrounding the wounds and inoculation sites were cut into two sections and samples were collected at the inoculation site 0–5 mm around the wound and distal to the inoculation site 10–15 mm from the wound. The bark samples were frozen separately in liquid nitrogen and stored at −80°C until further use.

3.3 | Quantitative PCR analysis of expression patterns in response to H. parviporum inoculation

The total RNA was isolated according to the protocol by Chang, Puryear, and Cairney (1993). To eliminate genomic DNA contamination, samples were treated with DNase I (Sigma-Aldrich) according to the manufacturer's instructions. RNA integrity and quantity were analysed by using the Agilent RNA 6000 Nano kit (Agilent Technologies Inc.). The 1 μg of total RNA was reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad) in a total reaction volume of 20 μl according to the manufacturer's instructions, followed by a two-fold dilution of the cDNA and storage at − 20°C.

Quantitative PCR (qPCR) reactions were performed with the SsoFast™ EvaGreen® Supermix (Bio-Rad) according to the instructions in the manual, using 0.3 μM of each primer (Table S1 in Data S1) and Norway spruce cDNA equivalent to 25 ng of total RNA. The qPCRs were carried out in an iQ5™ Multicolor Real-Time PCR Detection System thermocycler (Bio-Rad) using a program with a 30 s initial denaturation step at 95°C, followed by 40 cycles of 5 s denaturation at 95°C and 10 s at 60°C. Melt curve analyses were used to validate the amplicon. Four biological replicates were used per treatment and two

technical repetitions per standard, sample and negative control were run.

The relative expression was calculated from threshold cycle (Ct) values using the 2ΔΔCT-method (Livak & Schmittgen, 2001) by using the geometric mean of *Phosphoglucomutase* (Vestman et al., 2011) and *elongation factor 1-α* (ELF1α) (Arnerup et al., 2011) to normalize transcript abundance. The gene expression experiments were performed with four biological and two technical replicates. One-way ANOVA with Dunns Post-test (GraphPad Prism 5.0) was used to detect differences in expression levels between treatments.

4 | RESULTS

4.1 | Trait association mapping identifies novel QTLs for resistance to *H. parviporum*

From an average of 1.5 million paired end sequence reads per individual, 197,399 high confidence SNPs from 23,837 probes were identified. The majority of the SNPs were missense (61%) and silent (36%), the highest percentage being either upstream or downstream variants (68% total).

Employing a Stability Selection Probability (SSP) on the estimated breeding values (EBVs) for SWG and LL of the offspring on the 466 trees, we identified six SNPs with significant associations for SWG and five SNPs associating with LL (Table 2). The QTLs for control of sapwood growth of *H. parviporum* (SWG) explained similar fractions of the observed phenotypic variation (H^2_{QTL}) 2.4 to 5.2% (Table 2). The five QTLs for control of the LL development in bark explained between 2.1 and 4.4% of the observed phenotypic variation (Table 2).

To investigate if the identified QTLs are independent from previously identified QTLs for resistance to the same isolate of *H. parviporum* using IM (Lind et al., 2014), we searched an ultra-dense genetic map (Bernhardsson et al., 2019) for the probes the SNP markers originated from. This allowed us to estimate the position of the detected QTLs and the original IM-based QTLs in the Norway spruce genome. We could estimate the position in the Norway spruce genome for six of the SNPs/probes (Table S2.I and Figure S2.II in Data S1). All of the identified SNPs/probes were positioned >30 cM away from the original IM-based QTLs in the genetic map. Given that the maintained LD is estimated to only 109 bp across all the tagged genomic sequences in this study (Table S2 in Data S1), it is likely that they are independent. The SNP MA_53835_9763, associating with the trait SWG, presented a potential exception as the probe MA_14663 is positioned 4 cM away from MA_53835 in the map (Bernhardsson et al., 2019). The probe MA_14663 corresponds to the SNP array sequence for an IM-based QTL for infection prevention (Lind et al., 2014; Chaudhary et al., submitted manuscript).

On the scaffolds holding the SNPs associated with the resistance traits, a total of 14 gene models were identified, including 11 high- or medium-quality Norway spruce gene models (Table 3). On the scaffolds holding more than one gene model, the SNPs were positioned in MA_5978g0020, MA_25569g0020 and MA_97119g0010. Seven of the candidate genes associated with SWG QTLs and seven with LL (Tables 2 and 3). PFAM and GO term annotations and *Populus* and *Arabidopsis* orthologues were collected from *P. abies* v1.0 genome portal (Table 3). These metrics suggested that the gene models MA_97119g0010 and MA_97119g0020, found on the scaffold harbouring the SNP MA_97119_12277, indeed represented one gene. BlastN searches against the NCBI database essentially confirmed this suggestion as both gene models match JX500691.1 (*Picea abies*

TABLE 2 Significant association in the GWA study

Phenotype[a]	QTL	SNP[b]	Allele[c]	SNP feature[d]	Frequency[e]	PVE (%)[f]
SWG_tot	8675	MA_5978_21,011	T/C	Missense	0.71	4.83
	26756	MA_17884_58584	A/G	Upstream variant	0.72	3.41
	54184	MA_53072_3732	G/A	Synonymous	0.551	2.88
	54695	MA_53835_9763	G/A	Upstream variant	0.567	2.40
	56105	MA_56128_7752	C/A	Upstream variant	0.545	5.21
	71928	MA_84091_11329	C/A	Upstream variant	0.534	2.23
LL_tot	21105	MA_14352_27165	G/A	Missense variant	0.603	3.82
	27795	MA_18316_3165	G/T	Upstream variant	0.618	2.11
	31060	MA_19645_22184	C/T	Missense	0.682	2.73
	37057	MA_25569_28091	T/C	Upstream variant	0.667	2.77
	81488	MA_97119_12277	T/C	Upstream variant	0.742	4.39

[a]Phenotype specifies the trait upon which the marker associate.
[b]SNP: The SNP name was composed of the contig (MA_number) and SNP position on contig. For example, the first SNP MA_5978_21011 was located on contig MA_5978 at position 21011 bp.
[c]Allele indicates the biallelic SNP.
[d]SNP feature allelic variation associated with the SNP.
[e]Frequency, stability selection probability inclusion ratios for markers declared significant.
[f]PVE, phenotypic variance explained, only values larger than 1.0% are displayed.

TABLE 3 Candidate Norway spruce gene models associated with the QTL markers

SNP[a]	Candidate gene[b]	Description (Blast2Go)[c]	PFAM-Description/GO term[d]	Orthologs populus/ Arabidopsis[e]
MA_5978_21,011	MA_5978g0010	Phenylcoumaran benzylic ether reductase	PF00106-short chain dehydrogenase, PF01073-3-beta hydroxysteroid dehydrogenase/isomerase family PF01118-Semialdehyde dehydrogenase, NAD binding domain, PF01370-NAD-dependent epimerase/ dehydratase family, PF02719-Polysaccharide biosynthesis protein, PF03435-Saccharopine dehydrogenase, PF03807-NADP oxidoreductase coenzyme F420-dependent, PF05368-NmrA-like family, PF07993-Male sterility protein, PF08659-KR domain, PF13460-NADH(P)-binding	Potri.009G118100.1/ AT1G75280.1
	MA_5978g0020	Nuclear factor 1 A-type isoform 2	PF06219-Protein of unknown function (DUF1005)	Potri.013G071000.3/ AT5G17640.1
MA_17884_58584	MA_17884g0010	Mitochondrial import receptor subunit TOM40-1	PF01459-Eukaryotic porin	Potri.007G000200.1/ AT3G20000.1
MA_53072_3732	MA_53072g0010			
MA_53835_9763	MA_53835g0010	Probable tocopherol O- chloroplastic	PF01209-ubiE/COQ5 methyltransferase family, PF01728-FtsJ-like methyltransferase, PF02353-Mycolic acid cyclopropane synthetase, PF03059-Nicotianamine synthase protein, PF05175-Methyltransferase small domain, PF05891-AdoMet dependent proline di-methyltransferase, PF07021-Methionine biosynthesis protein MetW, PF08003-Protein of unknown function (DUF1698), PF08241-Methyltransferase domain, PF08242-Methyltransferase domain, PF12847-Methyltransferase domain, PF13489-Methyltransferase domain, PF13578-Methyltransferase domain, PF13649-Methyltransferase domain, PF13659-Methyltransferase domain, PF13679-Methyltransferase domain, PF13847-Methyltransferase domain	Potri.013G077000.1 AT1G64970.1
MA_56128_7752	MA_56128g0010			Potri.006G130600.1
MA_84091_11329	MA_84091g0010			
MA_14352_27165	MA_14352g0010	Transcription factor bHLH118	PF00010-Helix–loop–helix DNA-binding domain	Potri.015G134300.1/ AT4G25400.1

TABLE 3 (Continued)

SNP[a]	Candidate gene[b]	Description (Blast2Go)[c]	PFAM-Description/GO term[d]	Orthologs populus/ Arabidopsis[e]
MA_18316_3165	MA_18316g0010	IST1 homologue	PF03398-Regulator of Vps4 activity in the MVB pathway	Potri.019G087400.1/ AT1G34220.2
MA_19645_22184	MA_19645g0010			
MA_25569_28091	MA_25569g0010		*GO:0005618-cell wall*, *GO:0016020-membrane*, *GO:0044444-cytoplasmic part*	Potri.002G054900.1/ AT1G03230.1
	MA_25569g0020			Potri.001G266500.1
MA_97119_12277	MA_97119g0010	Laccase	PF07732-Multicopper oxidase	Potri.019G124300.1 / AT2G30210.1
	MA_97119g0020	Laccase 12	PF00394-Multicopper oxidase, PF07731-Multicopper oxidase	Potri.010G183500.1 / AT5G05390.1

[a]SNP: The SNP name was composed of the contig (MA_number) and SNP position on contig.
[b]Candidate gene.
[c]Description (Blast2Go).
[d]PFAM-Description or GO terms when PFAM descriptions were missing.
[e]*Populus/Arabidopsis* orthologs identified in the *P. abies* v1.0 genome portal.

laccase LAC5a) with E = $4^{*}10^{-135}$ and E = 0 and 99.62 and 99.71% identity, respectively. This laccase, *PaLAC5*, was originally isolated from lignin-forming Norway spruce suspension cultures. Apart from MA_97119, two other QTL holding scaffolds (MA_5978 and MA_25569) harboured more than one gene model (Table 3). Both of these scaffolds appear to hold two different gene models as judged by the PFAM annotations and *Populus* or *Arabidopsis* orthologs (Table 3). MA_5978g0010 appears to encode a phenylcoumaran benzylic ether reductase (PCBER) with similarity to PicgIPPR21 (Porth, Hamberger, White, & Ritland, 2011). The gene model MA_14352g0010 may belong to the *basic helix–loop–helix (bHLH) DNA-binding* superfamily since the PFAM-ID PF00010 (Helix–loop–helix DNA-binding domain) is associated with the gene model. The candidate gene MA_18316g0010 is associated with PF03398 (regulator of Vps4 activity in the MVB pathway), indicating that this gene too may be involved in regulatory activities. The gene model MA_53835g0010 appears to encode a protein with methyltransferase capacities based on its PFAM annotation and its *Arabidopsis* orthologue (Table 3), and based on its PFAM annotation (PF01459) and the annotation of the *Arabidopsis* orthologue, AT3G20000.1 (Table 3) which encodes β-barrel protein, TOM40, forming channels in the outer mitochondrial membranes, it is likely that the candidate gene MA_17884g0010 encodes a Norway spruce TOM40-like protein.

4.2 | A majority of the candidate genes associated with SWG are expressed in stem and wood forming tissues

To gain a better understanding of the functionality of the candidate genes, we assessed the expression in silico using available resources

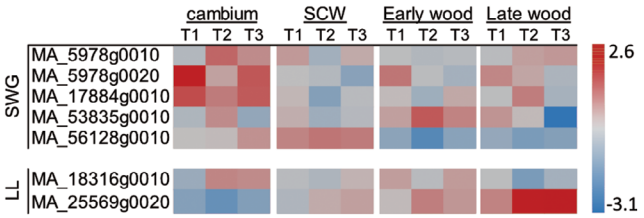

FIGURE 1 Relative expression levels of candidate genes associated to *H. parviporum* resistance QTLs through different stages of xylem development including cambium and expanding early wood (cambium), secondary cell wall-forming xylem (SCW), first dead early wood cells (Early wood) and the previous year's latewood (late wood). Data collected from NorWood v1.0 (http://norwood.congenie.org) database, T1-T3 represent the expression level in each of the three analysed trees (Jokipii-Lukkari et al., 2017). The bar to the left indicates the relative expression level of the candidate gene in the heat map

such as NorWood and *P. abies* exATLAS databases. It predicted that the candidate genes linked to SWG would more commonly be expressed in sapwood than genes linked to LL. Only seven candidate genes (MA_5978g0010, MA_5978g0020, MA_17884g0010, MA_53835g0010, MA_56128g0010, MA_18316g0010 and MA_25569g0020) were expressed in any of the libraries in NorWood (Figure 1). Of the expressed candidate genes, five were linked to SWG. This indicated a trend (Chi-square = 3.233, p = .07) where candidate genes linked to the SWG QTLs were expressed more often in wood compared to candidate genes linked to LL.

NorWood is a database of transcript abundances in high spatial resolution section series throughout the cambial and woody tissues of Norway spruce (Jokipii-Lukkari et al., 2017). Three of the five candidate genes associated with control of SWG (MA_5978g0010, MA_5978g0020 and MA_17884g0010) showed the highest transcript

levels in the cambial region. MA_56128g0010, also associated with SWG, appeared to be more active in the expanding early wood and secondary cell wall-forming tissues (Figure 1). One of the two candidate genes associated with the LL extension in the phloem and inner bark that were detected in the NorWood libraries, MA_25569g0020 showed very high activity in the samples collected at the visual appearance of dead early wood cells and in latewood (Figure 1). The inspection of the expression patterns in the *P. abies* exATLAS indicated that all candidate genes but MA_84091g0010 and MA_19645g0010 were expressed in at least one tissue of the clone Z4006 (Figure S3 in Data S1). Apart from the candidate genes that were also detected in the NorWood database, several candidate genes (MA_14352g0010, MA_25569g0010, MA_97119g0010 and MA_97119g0020) associated with LL were found to be expressed in samples derived from stem tissues (Figure S3 in Data S1).

4.3 | The transcriptional responses to *H. parviporum* inoculation identifies candidates responding specifically to the pathogen

If the candidate gene models associated with QTLs contribute to the control of the *H. parviporum* infection, they may be involved in either the constitutive or induced defence in the tissue (or both) (Arnerup et al., 2011; Danielsson et al., 2011; Oliva et al., 2015). Assuming that genes associated with the induced defences respond to inoculation with the pathogen, it is relevant to assess the candidate genes expression pattern in response to *H. parviporum* (Arnerup et al., 2011; Danielsson et al., 2011; Oliva et al., 2015). We used an RNASeq study of transcriptional responses in bark and phloem response to wounding and *H. parviporum* inoculation (Chaudhary et al., submitted manuscript). Five candidate genes showed constitutive expression at seven dpi irrespective of the treatment: MA_5978g0020, MA_17884g0010, MA_53835g0010, MA_56128g0010 and MA_25569g0020 (Figure 2). Most of these showed moderate expression levels, but MA_17884g0010 expression was relatively high in all samples. Four gene models associated with LL were differentially expressed at seven dpi: MA_14352g0010, MA_18316g0010, MA_97119g0010 and MA_97119g0020 (Figure 2). Interestingly, the two candidate gene models, (MA_97119g0010 and MA_97119g0020, i.e., *PaLAC5*) that showed the largest induction in response to the inoculation treatment compared to the wounding control proximal to the inoculation site, were not induced but rather downregulated distally at seven dpi (Figure 2). To validate the transcriptional responses estimated from the RNAseq data, we set up a separate inoculation experiment in a single Norway spruce genotype for qPCR validation of the expression patterns at seven dpi. The qPCR verified the transcriptional regulation patterns between *H. parviporum* inoculation and wounding treatment for most genes (Figures 2 and 3). This included the absence of a transcriptional activity of the candidate genes MA_53072g0010, MA_84091g0010, MA_19645g0010 and MA_25569g0010. The repression of the putative bHLH transcription factor MA_14352g0010 in response to *H. parviporum* was not detected in the qPCR experiment. The qPCR did confirm that *PaLAC5* (MA_97119g0010 and MA_97119g0020) is strongly and specifically upregulated in close proximity to the *H. parviporum* inoculation site (Figure 3d). Two of the candidate genes linked to the SWG QTLs with detected expression in the Norwood database, MA_17884g0010 and MA_53835g0010, were shown to be induced in response to *H. parviporum* compared to the control (Figure 3f,g). None of the tested candidate genes, including MA_17884g0010 and MA_53835g0010, were differentially expressed between H. *parviporum* inoculation and wounding in sapwood in early interactions (Table S4 and Method Section in Data S1).

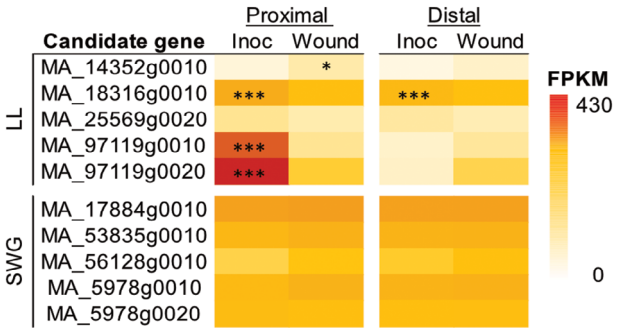

FIGURE 2 Expression profile of candidate genes for *H. parviporum* resistance in response to *H. parviporum* inoculation and wounding at seven dpi proximally (0–5 mm from the inoculation site) and distally (10–15 mm from the inoculation site) in the clones S21K0220126 and S21K0220184 (Chaudhary submitted MS). Asterisks indicate significant different expression levels between the inoculation treatment and the wounding control in *Cuffdiff*. The bar to the left indicates the FPKM values associated with the gene model

5 | DISCUSSION

5.1 | Twelve distinct QTLs for resistance to *H. parviporum* identified by GWAS

In this study, the GWAS identified 11 significant associations across the two traits for *H. parviporum* resistance. QTLs for LL and SWG traits detected in the GWAS explained similar fractions of the observed phenotypic variation, as in the IM-based QTL study by Lind et al. (2014). However, the narrow-sense heritability of the phenotypic traits was considerably higher among the 466 Norway spruce half-sib families than in the single family used in the IM-based QTL study, 0.42 compared to 0.11 for SWG (Arnerup et al., 2010; Chen et al., 2018; Lind et al., 2014). The fact that the Norway spruce genome v 1.0 assembly was highly fragmented comprising >10 million scaffolds over 500 bp (Bernhardsson et al., 2019; Nystedt et al., 2013) made it difficult to evaluate how the QTLs identified by GWAS relate to the previously identified QTLs (Lind et al., 2014), or to each other. However, the newly published ultra-dense genetic map (Bernhardsson et al., 2019) showed that five of the QTLs were independent from the other QTL regions as they were found in different linkage groups. Only one of the QTL regions that was identified in the linkage map

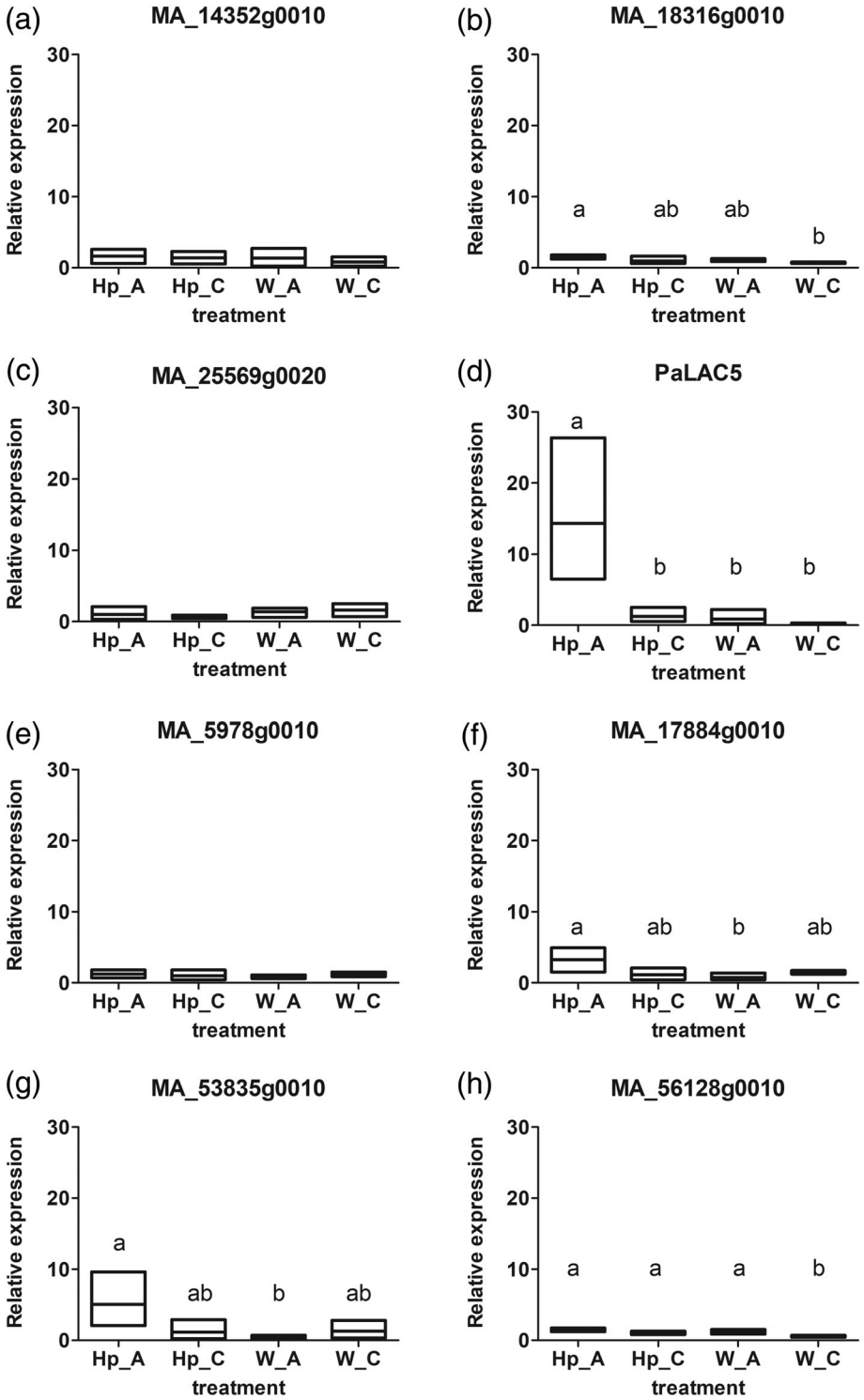

FIGURE 3 Expression profile of candidate genes for *H. parviporum* resistance in response to *H. parviporum* inoculation (Hp) and wounding (W) at seven dpi proximally (0–5 mm from the inoculation site, indicated by the letter "A" in, e.g., the treatment "Hp_A") and distally (10–15 mm from the inoculation site, indicated by the letter "C") in the Norway spruce clone S21K7820222 as detected by qPCR. Candidates a–d are associated with the trait LL and candidate genes e–g with trait the SWG. The floating bars in the graphs indicate min and max values, the line indicates mean, and different letters over the bars in the graph indicate significant differences in the statistical analyses ($N = 4$)

may possibly coincide with a previously identified resistance QTLs (Lind et al., 2014). The SNP MA_53835_9763 is positioned within 4 cM from a probe in the confidence region for the trait infection prevention (IP) on LG 11 (Lind et al., 2014; Chaudhary et al., submitted manuscript). Thus, the possibility that these markers target the same genomic region cannot be excluded, although it is not very likely given the short LD. Overall, the GWAS returned 11 new potential markers for resistance to *H. parviporum* in Norway spruce that could be used to aid selection in breeding programmes.

5.2 | Candidate genes have orthologues whose genetic variation is associated with the control of the responses to multiple stresses

Three of the candidate genes identified in the GWAS, MA_17884g0010, MA_5978g0020 and MA_18316g0010, have *Arabidopsis* orthologues AT3G20000.1, AT5G17640.1 and AT1G34220.2, respectively. These orthologues hold QTLs for responses to multiple stresses (Kawa et al., 2016; Thoen et al., 2017).

The candidate gene MA_18316g0010 was associated with control of lesion length in the inner bark and it was upregulated in response to *H. parviporum* inoculation compared to wounding alone, both proximally and distally. The *Arabidopsis* orthologue AT1G34220.2 encodes IST1-LIKE1 (ISTL1), a protein predicted to be the *Arabidopsis* homologue of yeast IST (Buono et al., 2016). ISTL1 is a regulator of the multivesicular bodies (MVB) pathway in which ubiquitinated and endocytically internalized membrane proteins are degraded (C. Gao, Zhuang, Shen, & Jiang, 2017). ISTL1, in interaction with LIP5 (LYST INTERACTING PROTEIN 5, AT4G26750), is essential for normal plant growth and repression of spontaneous cell death (Buono et al., 2016). The fungus *H. parviporum* is a necrotrophic pathogen and upon infection or inoculation in trees, it will create necrotic lesions in the phloem to gain access to the sapwood (Johansson & Stenlid, 1985; Lindberg & Johansson, 1991). It is, therefore, tempting to propose that the MA_18316g0010 protein fulfils the same role in the control of the cell death process as the ISTL1/LIP5 complex, MA_18316g0010 was upregulated in response to *H. parviporum* inoculation to repress cell death, a mechanism that must be integral to the LL trait. It would be interesting to test if the variation at MA_18316_3156 leads to differential accumulation of the transcript in response to *H. parviporum*.

The *Arabidopsis* orthologue to MA_17884g0010, AT3G20000.1, encodes translocase of the outer mitochondrial membrane 40, TOM40. *AtTOM40* is in LD with a QTL (Ch3:6968031) identified in a multi-trait QTL mixed models GWAS using the responses to a set of 30 biotic and abiotic stresses in 196 accessions of *Arabidopsis* (Thoen et al., 2017). TOM40 protein is the central channel forming units of the TOM complex (Hill et al., 1998). The TOM complex and the mitochondrial outer membrane play a central role in the interaction between the mitochondrion and the cytosol. It mediates the import of preproteins, the passage of small molecules and the transduction of signals between cellular compartments (Duncan, van der Merwe, Daley, & Whelan, 2013). Consequently, it is perhaps not unexpected that genetic variation associated with MA_17884g0010 and *TOM40* may influence plants responses to stress, or that MA_17884g0010 shows a ubiquitous expression in the surveyed Norway spruce tissues, with a slight upregulation in metabolically very active tissues (eg the cambium) and in response to *H. parviporum* inoculation.

5.3 | Candidate genes linked to SWG QTLs are more commonly expressed in wood

Despite the economic and ecological importance of conifers, we know surprisingly little about the genetic basis of resistance to decay pathogens compared to canker-forming pathogens in conifers (Kinloch, Sniezko, & Dupper, 2003; Liu et al., 2017; Sniezko, Smith, Liu, & Hamelin, 2014). Examining the regions under selection in response to given pathogens or stressors, identifying and testing candidate genes, can lead to better understanding of the interaction between the host and the pathogen (Liu et al., 2017; Martin, Rönnberg-Wästljung, Stenlid, & Samils, 2016; Nemesio-Gorriz et al., 2016; Thoen et al., 2017). Under the expectation that candidate genes linked to the control of SWG are involved in processes shaping the cell wall or in production of, for example, specialized metabolites in wood (Oliva et al., 2015; Popoff, Theander, & Johansson, 1975; Stenlid & Johansson, 1987), we predicted that the expression of the candidate genes linked to SWG QTLs should be more commonly detected in the wood-forming tissues than the genes linked to the LL QTLs. A trend for this was observed in the NorWood database (Jokipii-Lukkari et al., 2017), although a larger number of QTLs and candidate genes for both traits studied would probably have been needed to gain conclusive evidence. It is, however, important to point out that none of the QTLs identified for SWG, or LL, coincide with the 52 QTLs for important wood quality traits in Norway spruce reported by Baison et al. (2019). An observation that is fully in agreement with the absence of significant correlations between wood quality, or growth, traits and resistance to *H. parviporum* in this material (Chen et al., 2018), suggesting that the detected SWG QTLs may be associated to distinct defence-related processes. Several of the expressed candidate genes showed their highest transcriptional activity in the cambium and expanding early wood libraries. The candidate gene MA_25569g0020, associated with LL, showed increased transcriptional activity during visual appearance of dead early wood cells in the sapwood. The transcript is also specifically expressed in the phloem in the autumn/winter (Jokipii-Lukkari et al., 2018), but it was not induced by *H. parviporum* inoculation. This points to that the role of MA_25569g0020 in resistance may be associated to the constitutive defence.

5.4 | The Norway spruce laccase *PaLAC5* responds specifically to *H. parviporum* inoculation

Two candidate genes associated with the LL trait in bark, MA_53835g0010 and *PaLAC5*, are likely to be members of the induced defence to *H. parviporum*. The Norway spruce *laccase* gene *PaLAC5* (MA_97119g0010 and MA_97119g0020) was originally isolated from lignin-producing Norway spruce suspension cultures (Koutaniemi, Malmberg, Simola, Teeri, & Kärkönen, 2015), and transcriptome analyses of these lignin-producing Norway spruce suspension cultures under different conditions suggest that *Pa*LAC5 is associated with the activation of stress associated lignin production (Laitinen et al., 2017). *PaLAC5* has a very specific spatial expression pattern in response to *H. parviporum* inoculation. It is strongly, and specifically, upregulated proximally to the *H. parviporum* inoculation site but not regulated 10 mm away from the developing necrotic lesion or in response to the wounding control. In contrast to the induction of *PaLAC5* in stress associated lignin production conditions in vitro, the transcriptional activity of *PaLAC5* is very low in sapwood (Blokhina et al., 2019; Jokipii-Lukkari et al., 2017; Laitinen et al., 2017). Therefore, *Pa*LAC5 is not likely to be associated with lignifying tracheids or ray parenchyma cells indicating that the induction of *PaLAC5* expression under lignin-forming conditions in the cell cultures is stress-associated and not directly connected to lignification processes in wood (Blokhina et al., 2019; Jokipii-Lukkari et al., 2017;

Laitinen et al., 2017). However, if *PaLAC5* would be responding to stress in general, it would likely have had an expression pattern similar to many other studied defense genes, which often show upregulation in proximal to both mechanical wounding sites and to inoculation points (Arnerup et al., 2011; Danielsson et al., 2011; Ralph et al., 2006). Instead, it showed a distinct expression pattern. Thus, it is probable that *PaLAC5* expression is associated with specific cell types or processes such as the formation of the LSZ in the bark adjacent to the inoculation site. The LSZ is characterized by deposition of phenolics and suberin, and an early development of a discernible LSZ is crucial in stopping fungal invasions (Bodles et al., 2007; Lindberg & Johansson, 1991; Solla, Tomlinson, & Woodward, 2002; Woodward et al., 2007). Recently, it was suggested that specific isoforms of peroxidase and laccases may be involved in cross-linking aromatics to form lignin-like polyphenolics in the suberin in bark (Rains, Molina, & Gardiyehewa de Silva, 2017). The expression pattern of *PaLAC5* responding to *H. parviporum* and lignin-forming conditions (Laitinen et al., 2017) clearly makes it an interesting candidate for such a role. It remains to be seen if *PaLAC5*, indeed, is involved in the LSZ formation and if genetic variation associated with *PaLAC5* influences the formation of the LSZ.

6 | CONCLUSIONS

Our large sample sizes and a relatively high number of markers allowed us to link traits to SNPs with GWAS and to identify candidate genes associated with the QTLs. These candidate genes present new insights into the interaction between Norway spruce and *H. parviporum*, such as a putative involvement of the secretory and endosomal trafficking pathways and the *laccase PaLAC5*, in the control of lesion extension in the inner bark or the potential role of mitochondrial protein import and biogenesis in controlling *H. parviporum* spread in the sapwood.

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The R scripts used for the GWAS are publicly available at https://github.com/RosarioGarciaLab. Genotypic data and SNP position files are available upon contacting Rosario Garcia-Gil (m.rosario.garcia@slu.se). The Norway spruce genome assemblies and resources are available from http://congenie.org/pabiesgenome.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived the study: H.W., J.S., B.K., M.R.G.G. and M.E.

Planned the study: B.K., M.R.G.G., M.E., I.V.

Performed experiments: K.L., I.V., M.E., H.C., M.S.Å., R.C., Å.O.

Analysed data: L.Z., J.B., Z.-Q.C., M.E., K.L., R.C.

Drafted the MS: M.E. and J.B.

Commented on MS: J.B., Z.-Q.C., K.L., B.K., M.E., H.C., M.S.Å., R.C., J.S., Å.O. and M.R.G.G.

Wrote the final MS: M.E., all authors read and approved the final version.

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