Identification of Genes Underlying the Resistance to *Melampsora larici-populina* in an *R* Gene Supercluster of the *Populus deltoides* Genome

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Abstract

Identification of the particular genes in an *R* genes supercluster underlying resistance to the rust fungus *Melampsora larici-populina* in poplar genome remains challenging. Based on the de novo assembly of the *Populus deltoides* genome, all of the detected major genetic loci conferring resistance to *M. larici-populina* were confined to a 3.5-Mb region on chromosome 19. The transcriptomes of the resistant and susceptible genotypes were sequenced for a timespan from 0 to 168 hours postinoculation. By mapping the differentially expressed genes to the target genomic region, we identified two constitutive expression *R* genes and one inducible expression *R* gene that might confer resistance to *M. larici-populina*. Nucleotide variations were predicted based on the reconstructed haplotypes for each allele of the candidate genes. We also confirmed that salicylic acid was the phytohormone mediating signal transduction pathways, and *PR-1* was identified as a key gene inhibiting

rust reproduction. Finally, quantitative reverse transcription PCR assay revealed consistent expressions with the RNA-sequencing data for the detected key genes. This study presents an efficient approach for the identification of particular genes underlying phenotype of interest by the combination of genetic mapping, transcriptome profiling, and candidate gene sequences dissection. The identified key genes would be useful for host resistance diagnosis and for molecular breeding of elite poplar cultivars exhibiting resistance to *M. larici-populina* infection. The detected *R* genes are also valuable for testing whether the combination of individual *R* genes can induce durable quantitative resistance.

Keywords: disease resistant gene, forest, fungi, genetic mapping signal transduction, salicylic acid, trees, techniques, transcriptome sequencing

Melampsora species (the biotrophic basidiomycetous rust fungi) are the most devastating foliar pathogens, causing considerable yield and economic losses in commercial poplar plantations (Pinon and Frey 2005); >10 *Melampsora* species have been discriminated (Van Kraayenoord et al. 1974). The *Melampsora* species lifecycle is a complex heteroecious macrocycle involving two hosts and five spore types. In the sexual phase, the opposite mating pycniospores fuse to produce dikaryotic aeciospores. The windborne aeciospores infect poplar leaves and differentiate into a uredinium that forms orange pustules on poplar leaves (Hacquard et al. 2011a). On the infected poplar leaves, the polycyclic vegetative infection by asexual urediniospores leads to severe epidemics on compatible poplar trees under favorable conditions (Duplessis et al. 2009).

Quantitative and molecular genetic approaches have been intensively used to identify genetic loci conferring resistance to *Melampsora* rusts. A major quantitative genetic locus, *Mmd1*, conferring resistance to the North American poplar leaf rust *Melampsora medusae* f. sp. *deltoidae* was mapped in an interspecific *Populus trichocarpa* × *Populus deltoides* pedigree (Newcombe et al. 1996). Additionally, *MXC3*, a genetic locus

underlying the qualitative resistance to the hybrid rust *Melampsora* × *Columbiana* pathotype 3, was finely mapped to a genomic region associated with a substantial decrease in recombination frequency (Stirling et al. 2001). Moreover, *MXC3* was detected on chromosome 4 of the poplar genome with sequence-tagged site markers (Yin et al. 2004). Molecular markers for the resistance to the European poplar leaf rust *Melampsora larici-populina* have been identified (Cervera et al. 1996; Lefèvre et al. 1998; Villar et al. 1996). On the basis of multiple lines of evidence, two qualitative genetic loci (*Mer* and *R1*; both inherited from *P. deltoides*) and two large-effect quantitative genetic loci (one from *P. deltoides* and the other from *P. trichocarpa*) conferring resistance to *M. larici-populina* were mapped to the peritelomeric end of chromosome 19 (Bresson et al. 2011; Jorge et al. 2005; Yin et al. 2004; Zhang et al. 2001). In the *P. trichocarpa* genome (Tuskan et al. 2006), approximately 400 nucleotide-binding site (NBS)-encoding *R* genes have been identified (Kohler et al. 2008), with >70 of them detected in a supercluster in the peritelomeric region of chromosome 19 (Bresson et al. 2011). The specific genes mediating resistance to *M. larici-populina* appear to be embedded in such a supercluster (Bresson et al. 2011; Lescot et al. 2004; Zhang et al. 2001). A previous study revealed that the *R* gene supercluster on chromosome 19 colocalized with the poplar sex-determination locus, and the recombination of the target genomic region spanning a physical distance of several million base pairs was repressed (Yin et al. 2008). The *R* gene supercluster and repressed recombination make it difficult to identify the specific gene conferring disease resistance from among the many closely linked candidates.

Transcriptome profiling of host–pathogen interactions may enable researchers to identify disease resistance genes. For example, RNA sequencing (RNA-seq) is a fairly widespread technique used to study transcriptomes. It has considerably enhanced investigations into poplar–pathogen interactions (Liang et al. 2014; Zhu et al. 2012), especially regarding the molecular processes controlling the pathogen recognition and defense responses in poplar leaves infected by rust-causing *Melampsora* species (Azaiez et al. 2009; Duplessis et al. 2011b; Joly et al. 2010; Miranda et al. 2007; Petre et al. 2012; Rinaldi et al. 2007). A comparison of the transcriptome profiles of

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early to late defense responses during the poplar–rust pathogen interaction indicated that the expression of host-specific resistance genes differs greatly during the early (24 to 48 h postinoculation [hpi]), intermediate (48 to 96 hpi), and late (96 to 168 hpi) infection stages. During a compatible interaction, the biotrophic growth of *M. larici-populina* in infected poplar leaves results in a substantial increase in the fungal biomass between 48 and 96 hpi. In contrast, during an incompatible interaction, fungal growth is arrested during the early colonization stage, which coincides with increased plant defense reactions (Hacquard et al. 2011a). The time course transcriptome profiles of contrasting genotypes are useful for analyzing the changes in *R* gene expression during the early to late infection stages. Mapping the differentially expressed genes (DEGs) in the genome region containing disease resistance loci may clarify the expression of candidate genes in the target intervals.

The diverse physiological races of *M. larici-populina* vary in their specificity to individual poplar genotypes (Pinon 1992; Prakash and Thielges 1987). The highly specific resistance of the host to certain pathotypes is commonly mediated by *R* genes. In the incompatible host–pathogen interaction, the proteins encoded by *R* genes may activate plant immune responses after specifically recognizing the pathogen avirulence (AVR) products, after which a hypersensitive response occurs around the site of the pathogen infection (Jones and Dangl 2006; Kushalappa et al. 2016; Mur et al. 2007). The subsequent production and release of systemic signaling molecules induce systemic acquired resistance in plants (Fu and Dong 2013). Salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) are phytohormones that mediate signal transduction pathways involved in defenses against biotic and abiotic stresses and plant development (Pieterse et al. 2012; Santner and Estelle 2009). The results of a study suggested that there is a strong association among SA, flavan-3-ol biosynthesis, and rust resistance in poplar. Additionally, ABA was observed to promote poplar defenses against rust infections, but this is likely through stomatal immunity, independent of flavan-3-ols. In contrast, JA apparently does not induce defense responses to rust infections (Ullah et al. 2019). Transcriptome profiling may generate useful information regarding the expression of key genes contributing to signaling pathways associating with *M. larici-populina* resistance.

P. deltoides was introduced to southern China in the 1970s, and it (along with its hybrids) currently dominates the forest plantations along the middle and lower reaches of the Yangtze River. Since the 1990s, epidemics of rust disease have occurred in most of these poplar plantations. The morphological characteristics of urediniospores and species-specific genetic sequences enabled the identification of *M. larici-populina* as the causative agent of the rust infections of the *P. deltoides* trees in southern China (Wan et al. 2013, 2015). In our *P. deltoides* germplasm collection, we identified genotypes resistant and susceptible to *M. larici-populina*. This study involved the de novo assembly of the *P. deltoides* target genomic region containing the *R* gene supercluster. Moreover, time course transcriptome profiles of the resistant and susceptible poplar genotypes infected with rust pathogens over time were used to compare the dynamic transcriptional expression between the contrasting poplar genotypes at different infection times. Our objectives were to (i) identify the specific genes conferring resistance to *M. larici-populina* from among the many closely linked *R* genes, (ii) detect the allelic variations of the identified *R* genes between the contrasting genotypes, and (iii) investigate the key hormones and genes mediating the signaling pathway associated with *M. larici-populina* resistance. The results provided herein can be applied for diagnosis of host resistance to rust infection, and also, they are useful for developing a marker-aided selection toolkit for breeding of the new poplar lines exhibiting rust resistance.

Materials and Methods

Plant materials and rust inoculation. The long-term empirical observations under field conditions at four different sites, including Nanjing and Sihong in Jiangsu province and Wuhan and Shishou in Hubei province, found that *P. deltoides* clone T-120 was highly resistant to *M. larici-populina*, whereas clone D-896 was the most

susceptible genotype. Resistance to *M. larici-populina* was further tested with a rust mixture collected from poplar clone NL895, a widely planted commercial *P. deltoides* cultivar in the south of China, by the leaf disc inoculation method (Barrès et al. 2008). A total of 88 genotypes from the *P. deltoides* germplasm were tested. According to the disease index described in Wan et al. (2013), rust disease indices were classified into six ranks (0 to 5) based on the appearance of uredinia on leaf surface (Supplementary Fig. S1). T-120 was ranked with a disease index of 0, and it was consistently found that no uredinium appeared on leaf discs after inoculation, whereas D-896 was consistently observed with numerous large uredinia on the surface of leaf discs. D-896 was verified to be the most susceptible genotype with disease index ranked 5. In this study, *P. deltoides* clones T-120 and D-896 were selected as the contrasting genotypes to the infection of *M. larici-populina* for transcriptomic sequencing. Cuttings of the two genotypes were soil cultured in greenhouse. After 12 weeks, young trees grew up to 1-m tall and had >10 fully expanded leaves. Eight ramets from each genotype were inoculated for a time course analysis.

For rust inoculation, rust pathogens were collected on leaves of NL895, and the collected rust mixture was confirmed to be *M. larici-populina* by morphological characteristics and PCR amplification. The abaxial surfaces of three fully expanded leaves (the fifth, seventh, and ninth leaves from the shoot apical bud) on T-120 and D-896 ramets were sprayed with *M. larici-populina* urediniospore suspension prepared in distilled water (approximately 100,000 urediniospores per 1 ml). For the time course transcriptome analyses, the inoculated leaves on each ramet of the contrasting genotypes were separately harvested at 0, 24, 48, 72, 96, 120, 144, and 168 hpi. The collected leaves were immediately frozen in liquid nitrogen and stored at -80°C .

Target genomic region analysis and sequence assembly. Mapping information for the *Mer* locus was obtained from reports by Yin et al. (2004, 2008) and Zhang et al. (2001), whereas that for *R1* and the two major quantitative trait loci (QTLs) conferring resistance to *M. larici-populina* was derived from reports by Bresson et al. (2011) and Jorge et al. (2005). The positions of the common markers among these mapping studies were determined with the poplar consensus genetic map (Yin et al. 2008). Boundary markers for the target genetic intervals were obtained by integrating the mapping information of the aforementioned studies.

To determine the physical locations of *Mer*, *R1*, and the two major QTLs, *P. deltoides* clone D2-2 (i.e., offspring with a full-sib pedigree for which T-120 is the maternal parent) underwent a de novo whole-genome sequencing analysis with the PacBio, Hi-C, and genetic map data (unpublished data). The de novo assembly of the PacBio long raw reads for the *P. deltoides* clone was completed with the following three assemblers: WTDBG (<https://github.com/ruanjue/wtdbg>), the Canu pipeline (Koren et al. 2017), and Falcon (Chin et al. 2013), with different parameters to enhance the consistency and continuity of genome assembly sequences. Assemblies were merged with quickmerge (Chakraborty et al. 2016) to optimize the base calling of contigs. The *P. deltoides* Hi-C libraries were constructed as described by Lieberman-Aiden et al. (2009). The Hi-C data were mapped to the PacBio assembled contigs based on the Burrows–Wheeler Aligner (BWA) maximum exact match method (Li 2014). The uniquely mapped data were further clustered and extended to pseudochromosomes with LACHESIS software (Burton et al. 2013). The Hi-C assemblies were assessed with the HiC-pro software (Servant et al. 2015), and the placement and orientation errors for each pseudochromosome revealed by obvious chromatin interaction patterns were manually adjusted.

With the assembled target genomic region sequence as a reference, the BLASTN algorithm (Camacho et al. 2009) was used to align the boundary markers to confine the physical locations of the resistance gene loci. Genes in the target genomic region with homologs in *P. deltoides* and *P. trichocarpa* were identified with the BLASTP algorithm (Camacho et al. 2009) (e-value cutoff of $1e-5$), and a comparison of the synteny between these two species was conducted with MCScanX (Wang et al. 2012). Collinearity charts were drawn using Circos (Krzywinski et al. 2009).

The genes in the target genomic region were functionally annotated with the BLAST algorithm (Altschul et al. 1997) (e-value cutoff $\leq 1e-10$) and the NCBI nucleotide databases and nonredundant protein database (Deng et al. 2006), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa et al. 2004), the homologous Protein family (Pfam) database (Finn et al. 2013), the Swiss-Prot protein database (UniProt Consortium 2016), the Cluster of Orthologous Groups database (Tatusov et al. 2000), and the Gene Ontology (GO) database (Ashburner et al. 2000). The annotated resistance genes in the target genomic region were subjected to a domain-based BLAST search against the Pfam (<http://pfam.xfam.org/search/sequence>) (Finn et al. 2013) and SMART (<http://smart.embl-heidelberg.de/>) (Letunic and Bork 2017) databases to characterize the conserved and functional domains. Moreover, the MEME program (<http://meme-suite.org>) (Bailey et al. 2006) was used to elucidate the consensus motifs in these sequences encoding the toll-interleukin 1-receptor (TIR), NBS, and C-terminal leucine-rich repeat (LRR). Because coiled coil (CC) domains cannot be identified with conventional domain search tools, Paircoil2 (<http://cb.csail.mit.edu/cb/paircoil2/paircoil2.html>) (McDonnell et al. 2005) was used to detect the coiled coil domains (p-score cutoff of 0.03).

Transcriptome profiling and analysis. For each time point, the three inoculated leaves from each ramet were pooled, and total RNA was extracted as described by Chang et al. (1993) and then digested with DNase (Takara) at 37°C for 30 min to remove any residual DNA. The purified RNA concentration and quality were determined with the Agilent 2100 Bioanalyzer (Agilent Technologies) and by 1% agarose gel electrophoresis. The messenger RNA-seq libraries were constructed with the Illumina TruSeq RNA Library Prep Kit (Illumina Inc.) and quantified with a NanoDrop spectrophotometer (NanoDrop Technologies). A total of 16 complementary DNA (cDNA) libraries were constructed, corresponding to the samples collected at 0, 24, 48, 72, 96, 120, 144, and 168 hpi for the two contrasting genotypes (T0, T24, T48, T72, T96, T120, T144, T168 and D0, D24, D48, D72, D96, D120, D144, D168). The libraries were sequenced (paired end reads) with the Illumina HiSeq 2000 platform following the manufacturer's standard and sequencing protocols.

To obtain high-quality clean reads for the transcript analysis, the raw reads for each sample were filtered with FastQC (Andrews and Fast 2015) and Trimmomatic (Bolger et al. 2014) to discard low-quality reads, trim adapter sequences, and eliminate low-quality bases. The clean reads were then aligned to the reference genome with the default parameters of the universal RNA-seq aligner HISAT2 (Kim et al. 2015). Raw counts for each gene were derived from the read alignments, and they were normalized to fragments per kilobase of transcript per million fragments mapped (FPKM) values. On the basis of pairwise comparisons of the libraries (T0 versus D0, T24 versus D24, T48 versus D48, T72 versus D72, T96 versus D96, T120 versus D120, T144 versus D144, and T168 versus D168), the DEGs in each comparison were identified with the EBseq R package (Leng et al. 2013). Specifically, the DEGs were identified according to the following criteria: false discovery rate < 0.01 and a $\log_2$ expression fold change ≥ 2 .

A weighted gene coexpression network of DEGs was constructed using the weighted gene correlation network analysis (WGCNA) R package (Langfelder and Horvath 2008). The classification of DEGs was performed with GO enrichment analysis using the clusterProfiler R package (Yu et al. 2012) based on the hypergeometric distribution model. We performed a pathway enrichment analysis with the KEGG pathway database (<https://www.genome.jp/kegg>) to identify enriched metabolic or signal transduction pathways associated with the DEGs. The classification of biological terms and the analysis of the enrichment of DEG clusters in the KEGG pathways were completed with the clusterProfiler R package (Yu et al. 2012). The enriched KEGG pathways among the DEGs were integrated and visualized with the pathway R package (Luo and Brouwer 2013).

Allelic variations analysis. To reconstruct the allelic haplotypes, the contrasting genotypes underwent a DNA resequencing analysis with the Illumina HiSeq platform (Illumina Inc.), and the resulting sequence reads for each genotype were separately mapped to the *P. deltoides* reference sequences with the Burrows–Wheeler Aligner

software (Li 2014). The uniquely mapped reads were used for identifying single-nucleotide polymorphisms (SNPs) with the SAMtools (Li et al. 2009) and FreeBayes (Garrison and Marth 2012) analytical packages. The detected SNPs were used to reconstruct the allele haplotypes for the candidate *R* genes and the regulatory sequences (1 kb upstream of the coding gene) with HapCUT2 (Edge et al. 2017). To analyze the allelic variations, the HapCUT2 output was transformed to a .vcf file with an in-house python script, after which a .fasta file was built with VCFtools (Danecek et al. 2011) based on the .vcf file for each haplotype. Allelic variations for each of the candidate genes and regulatory sequences were subsequently determined by aligning multiple sequences with the Clustal Omega software (Sievers and Higgins 2014). Finally, the predicted loss of function of the detected allelic variations was analyzed with ANNOVAR (Wang et al. 2010) and a manual examination.

Phytohormone analyses. The SA content of poplar leaves was quantified at eight time points with the same poplar clones as described for the transcriptome analysis. Experiments were repeated at least three times. For each measurement, approximately 1 g of fresh leaves were ground in a precooled mortar containing liquid nitrogen and then, lyophilized. Then, 50-mg samples were transferred to a 2-ml tube for a phytohormone extraction according to a modified protocol (Pan et al. 2010). After adding 500 μ l of 2-propanol/ H_2O /concentrated HCl (2:1:0.002 vol/vol) solvent, the mixture was incubated for 30 min at 4°C on a rotary shaker (100 rpm). Each sample was spiked with 1 ml of dichloromethane and shaken at 4°C for an additional 30 min prior to a centrifugation at $13,000 \times g$ for 5 min at 4°C. Approximately 900 μ l of solvent from the lower phase was collected and dried under a stream of nitrogen gas. The dried residues were resolved in 0.1 ml of acidified methanol. The SA was quantified by analyzing 5- μ l resuspended samples in an Acquity UPLC high-performance liquid chromatography system (Waters) coupled to a triple quadrupole detector equipped with an electrospray ionization source. The chromatographic separation involved an Acquity BEH C18 column (100 $\times$ 2.1 mm, 1.7 μ m) at 40°C, with two mobile phases consisting of 0.05% formic acid in (A) water and (B) acetonitrile at a flow rate of 0.3 ml/min. Phytohormones were detected under the multireaction monitoring mode, with the electrospray ionization source of the mass spectrometer in the negative ion mode. The transition from deprotonated molecules to characteristic product ions was monitored at m/z 137 to 93. Commercial SA (Sigma Aldrich and Olchemim Ltd.) was used as the external standard.

Validation of gene expression. We did not include biological replicates throughout different time points in the transcriptomic experiments in this study. To amend its limitation for the outcome of transcriptional analysis, we validated the candidate genes expression with biological replicates by performing a real-time quantitative reverse transcription PCR (qRT-PCR) assay using the Applied Biosystems 7500 Fast Real-Time PCR System and the SYBR Premix Ex Taq Kit (Takara). An independent inoculation experiment was performed with the same method and *P. deltoides* clones as described above. In this test, each of the three inoculated leaves was treated as a biological replicate, and total RNAs were separately isolated from each leaves collected at the eight time points. Gene-specific primers were designed with the Primer Premier 5.0 program. Primer details are listed in Supplementary Table S6. The qRT-PCR mixture (20 μ l per reaction) comprised 10 μ l of SYBR Premix Ex Taq (Takara), 6.8 μ l of RNase-free water, 2 μ l of cDNA, 0.4 μ l of ROX Reference Dye II (Takara), and 0.4 μ l of each primer (10 μ M). The PCR program was as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 34 s. Melting curves were generated on completion of the PCR program. All reactions were performed in triplicate for each gene. An ubiquitin gene was selected as a reference control for normalizing data. Relative gene expression levels were calculated according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Results

Analyses of the disease resistance loci and the target genomic region sequence. Seven landscape markers (Supplementary Table

S1) within the target interval were selected to link the mapping information and the poplar genome sequences. Aligning the primer sequences targeting adjacent markers to the *P. trichocarpa* genome sequence resulted in the mapping of the qualitative resistance locus *R1* (inherited from *P. deltoides*) to a genetic interval between markers I_1211 and I_920-3 (Fig. 1). The other qualitative resistance locus, *Mer* (also inherited from *P. deltoides*), was detected downstream of *R1*. To ensure that *Mer* was covered by the target interval, we selected O_206 (the closest simple sequence repeat marker downstream of U_R7) from the poplar consensus map (Yin et al. 2008) as the lower boundary of *Mer*. In addition to the qualitative disease resistance loci, two major QTLs were identified, one of which (*Rus*) was inherited from *P. trichocarpa* and detected upstream of *Mer*, whereas the other major QTL was inherited from *P. deltoides* (designated as *Dqtl* in this study). The QTL confidence interval spanned the genomic region containing the *R1*, *Rus*, and *Mer* loci. Thus, the target interval containing the detected qualitative and major quantitative loci conferring resistance to *M. larici-populina* was confined between markers I_1211 and O_206 (Fig. 1).

The *P. deltoides* genome sequence was assembled with 33.92-Gb PacBio sequences, yielding 4,605 contigs covering 460.2 Mb. After integrating the PacBio data with the Hi-C data, 309.65-Mb genome sequences were anchored and oriented on 19 chromosomes. The pseudomolecule of chromosome 19 covered 20.21 Mb and consisted of 167 contigs. An analysis of synteny revealed a high collinearity for chromosome 19 between *P. trichocarpa* and *P. deltoides* (Supplementary Fig. S2). An alignment with the selected markers indicated that the target interval in the *P. deltoides* genome spanned a 3.5-Mb continuous sequence comprising 219 genes, 53 of which were annotated as *R* genes.

Global analysis of RNA-seq data. The transcriptome sequencing for a complete infection cycle generated 438,387,241 clean paired end reads (88.55 Gb) for the two contrasting genotypes (Fig. 2A). Mapping the obtained reads revealed that the mapping rate varied with a similar trend among the various time points when the *P. deltoides* or *P. trichocarpa* genome was used as a reference (Fig. 2B and C). However, the mapping rate was higher at each time point when sequences were aligned with the *P. deltoides* genome sequence rather than the *P. trichocarpa* genome sequence (Supplementary Table S2), indicating that the *P. deltoides* genome was a more appropriate

reference sequence. Moreover, a small fraction of the clean reads was also successfully mapped to the *M. larici-populina* genome (Duplessis et al. 2011a), with the highest mapping rate at 24 hpi (Fig. 2D). The expressed transcripts derived from the *M. larici-populina* genome uncovered 3,262 predicted genes, accounting for 16.69% of the total number of genes, and the number and expression of genes reached the highest at 24 hpi, of which 91 genes (2.8%) were identified as encoding small secreted proteins (Fig. 3A).

A pairwise comparison of the contrasting genotypes identified >3,500 DEGs at each time point. A distinct difference was observed between the contrasting genotypes regarding the number of up- and down-regulated DEGs in response to the *M. larici-populina* infection. The proportion of up-regulated DEGs was generally higher in the resistant genotype than in the susceptible genotype across the various time points, except at 168 hpi (Fig. 3B and C). In contrast, the proportion of down-regulated DEGs was higher in the susceptible genotype than in the resistant genotype throughout the analyzed period, implying that the rust infection differentially affected the gene expression in the contrasting genotypes. To analyze the expression pattern of DEGs between resistant and susceptible genotypes during rust inoculation, we performed WGCNA of all DEGs. In total, 16,544 DEGs were assigned to 16 gene modules that contained between 376 and 2,301 genes (Supplementary Fig. S3A). To reveal the direction and magnitude of gene expression patterns between each module in infection progression, the relative average FPKMs of module eigengenes were calculated and displayed in the histogram (Supplementary Fig. S3B). The histogram results showed that the genes in the red module were significantly higher expressed at 24 hpi, whereas the genes in the green, pink, and midnight blue modules were significantly higher expressed at 48 hpi (Supplementary Fig. S3B). It is thus hypothesized that genes in these modules highly expressed at the early stage of infection are related to disease resistance. In addition, the genes in the yellow-green module are highly constitutively expressed in the resistant genotype at all time points, and it is reasonable to hypothesize that a series of complex constitutively expressed resistance genes in this module activates the induction of defense responses (Supplementary Fig. S3B). According to GO enrichment analysis of genes in the five modules, most of the genes are involved in immune system process, immune effector process, response to SA, defense response to fungus, and systemic

Fig. 1. Integration of the mapping information for the reported genetic loci conferring resistance to *Melampsora larici-populina*. The physical positions are derived from alignment of mapped markers to the sequence assembly of chromosome 19 in *Populus deltoides*. *P. trichocarpa*, *Populus trichocarpa*.

acquired resistance (Supplementary Fig. S3C), all of which have been implicated in defense response in other plants (Fu and Dong 2013; Hacquard et al. 2011b; Ullah et al. 2019).

Identifying *R* genes conferring resistance to *M. larici-populina*. All of the detected DEGs were mapped to the *P. deltoides* genome, and 112 DEGs were located in the target genomic region. Of these DEGs, 43 were *R* genes (Supplementary Table S3), with 4 in the *R1* locus, 13 in the *Mer* locus, and 26 in the intermediate regions covered by *Dqtl* (Fig. 4A). An examination of the expression patterns of the 43 *R* genes in the target region indicated that a TIR-NBS-LRR gene (*EVM0026813*) and a CC-NBS-LRR gene (*EVM0025825*) were constitutively expressed in the resistant genotype throughout the analyzed period. However, these genes were not expressed or were expressed at very low levels in the susceptible genotype at all time points (Fig. 4B). Furthermore, an *R* gene encoding a G-type lectin S receptor-like serine/threonine kinase (GsSRK; *EVM0004305*) was not expressed in either examined genotype before the inoculation, but the expression levels were up-regulated in response to the rust infection.

In the *P. trichocarpa* genome, the homologs of *EVM0004305*, *EVM0026813*, and *EVM0025825* were identified as *Potri.019G011800*, *Potri.019G052000*, and *Potri.T026900*, respectively (Supplementary Table S3). The expression of the three candidate genes was further examined with microarray data for various poplar tissues, including the roots, stems, leaves, seedlings, and catkins, available from the PopGenIE online resource (<http://popgenie.org/>). The *EVM0026813* and *EVM0025825* genes were constitutively expressed in various poplar tissues (Fig. 4C). In contrast, *EVM0004305* expression was undetectable based on the available microarray data, suggesting that *EVM0004305* expression may be induced specifically by a rust infection. We further examined the *R* genes outside the target region and observed a lack of distinct differences in their expression patterns between the contrasting genotypes (Supplementary Table S4).

Allelic variation of the candidate *R* genes. To analyze the allelic variation of the candidate *R* genes, the DNA resequencing data for the contrasting genotypes were mapped to the *P. deltoides* reference genome. In each genotype, two allelic haplotypes were constructed

Fig. 2. Mapping results of the time course transcriptomic data. **A**, The rust disease symptom on resistant clone T-120 and susceptible clone D-896 of *Populus deltoides* leaves. **B**, The mapping rate for transcripts from the resistant and susceptible genotypes at different time points by using the *P. deltoides* genome as reference. **C**, The mapping rate for transcripts from the contrasting genotypes at different time points by using the *Populus trichocarpa* genome as reference. **D**, The mapping rate for transcripts from the contrasting genotypes at different time points by using the *Melampsora larici-populina* genome as reference.

Fig. 3. The statistics of expressed genes in the time course transcriptomic data. **A**, The dynamic gene expression of *Melampsora larici-populina* fungi. **B**, The rate of up-regulated differentially expressed genes (DEGs) in poplar responding to *M. larici-populina* infection between the contrasting genotypes. **C**, The rate of down-regulated DEGs in poplar responding to *M. larici-populina* infection between the contrasting genotypes. dpi, days postinoculation.

for each gene as well as for the corresponding 1,000-bp upstream regulatory sequences. Aligning the four allelic haplotypes in the contrasting genotypes revealed the structural changes for each gene (Table 1 and Supplementary Datasets S1 to S3).

The *EVM0004305* gene encodes a conserved serine/threonine kinase domain (Supplementary Fig. S4A). An examination of the allelic variation of *EVM0004305* revealed a synonymous SNP in the exonic region (chromosome 19: 14666996) of the resistant genotype and an SNP in the upstream region (chromosome 19: 14672032) of the susceptible genotype (Supplementary Dataset S1). Moreover, an intergenotype SNP was detected in the upstream regulatory region of chromosome 19: 14672140 (homozygous C in T-120 and homozygous T in D-896) (indicated with a red rectangular frame in Supplementary Dataset S1). This intergenotype SNP may be important for the differential induction of *EVM0004305* expression in the contrasting genotypes.

The *EVM0025825* gene encodes NBS and LRR domains (Supplementary Fig. S4B). The upstream regulatory sequences were identical in the four allelic haplotypes of *EVM0025825*. In contrast, many intra- and intergenotype nucleotide variations were detected in the intronic and exonic regions of this gene. In the exonic region, one of the detected SNPs introduced a stop codon in an alternate allele of the susceptible genotype, and three SNPs resulted in nonsynonymous mutations between the contrasting genotypes (Table 1 and Supplementary Dataset S2). These analyses suggested that the functional deficiency of *EVM0025825* is likely related to the nonsynonymous mutations or the introduced stop codon in the exonic region of the susceptible genotype.

The *EVM0026813* gene includes sequences encoding the TIR and two LRR domains (Supplementary Fig. S4C). The sequence of this gene varied considerably among the constructed allelic haplotypes (Table 1 and Supplementary Dataset S3). Additionally, an introduced premature stop codon was detected in one of the alternate alleles in the contrasting genotypes. We also detected six SNPs and three insertion/deletions in the regulatory sequence of this gene between

the contrasting genotypes. Moreover, 10 intergenotype nonsynonymous mutations were identified in the exonic region of *EVM0026813*.

Expression of defense signaling genes in response to a rust infection. The quantification of SA content indicated that the phytohormone accumulated more in T-120 than in D-896 throughout the analyzed period (Fig. 5A). In the susceptible interaction, the fungal biomass substantially increased during the intermediate infection stage (Figs. 2D and 3A). The SA contents sharply increased after 48 hpi in T-120 (Fig. 5A), thereby activating the defense response in the incompatible interaction. To further elucidate the complex regulatory networks controlling the expression of SA-mediated defense signaling genes in response to a rust infection, a KEGG pathway enrichment analysis was used to identify the SA-related DEGs. The KEGG pathways most affected by the rust infection were plant-pathogen interaction and plant hormone signal transduction (Fig. 5B). Several genes, including those encoding a mitogen-activated protein (MAP) kinase, nonexpressor of pathogenesis-related protein (NPR)-like protein 3, RLK-Pelle_LRR-II, NBS domain-containing disease resistance protein, chitin elicitor receptor kinase 1, and pathogenesis-related protein 1 (PR-1), were highly expressed in T-120 at 48 hpi (Supplementary Table S5). Of these genes, *PR-1* expression increased the most at 48 hpi in the resistant interaction. Specifically, in T-120, the *PR-1* FPKM value increased from 35.4 before the rust infection to 653.5 at 48 hpi (Fig. 5C and Supplementary Table S5). A similar change in expression was not observed for D-896. An examination of the changes in the SA concentration and the *PR-1* expression level indicated that *PR-1* expression is responsive to SA accumulation. Moreover, *PR-1* seems to be the critical gene for inhibiting the growth and reproduction of rust pathogens.

Gene expression validation. To validate gene expression based on RNA-seq data, qRT-PCR was performed for the three candidate *R* genes and the *PR-1* gene through the inoculation process. It was proved that the qRT-PCR expressions were consistent with the RNA-seq expressions for these genes (Figs. 4D and 5D). In detail,

Fig. 4. *R* genes in the target region covered by the resistant loci in the *Populus deltoides* genome. **A**, The left vertical bar shows the physical boundaries of *R1*, *Rus*, *Mer*, and *Dgtl*. This region contained a total of 219 genes, and 112 of them were differentially expressed genes (DEGs) that included 43 *R* genes. The right vertical bar shows the physical distribution of the 43 *R* genes. The three *R* genes found to confer resistance to *Melampsora larici-populina* infection are labeled in red. **B**, RNA-sequencing expression levels of *EVM0026813*, *EVM0025825*, and *EVM0004305* across the eight time points in the contrasting genotypes. FPKM, fragments per kilobase of transcript per million fragments mapped. **C**, The heat map shows the expression of *EVM0026813*, *EVM0025825*, and *EVM0004305* in the online microarrays and transcriptomic sequences for poplar roots, stems, leaves, seedlings, and catkins. The deeper color indicates higher expression, and the lighter color indicates lower expression. **D**, Quantitative reverse transcription PCR (qRT-PCR) validation of candidate *R* genes of *P. deltoides* on *M. larici-populina* infection. Total RNA was extracted from poplar leaves in the contrasting genotypes inoculated with *M. larici-populina* at eight different time points. The expression levels of the three candidate *R* genes were normalized to ubiquitin expression. All reactions were performed in triplicate for each gene, from which the means and standard errors were calculated.

gene *EVM0026813* and gene *EVM0025825* were constitutively expressed in T120, whereas only very low expression was detected during the inoculation process in D896. As for *EVM0004305*, qRT-PCR verified that it expressed in neither of the contrastive genotypes before inoculation, whereas its expression specifically elevated at 24 and 48 hpi in the resistant genotype. When *R* genes recognize the rust pathogen, the signal transduction mediated by the endogenous hormone would ultimately induce the expression of *PR* genes. As the consequence, it is noticeable that the expression of the SA-mediated signal transduction *PR-1* (*EVM0034396*) gene increased sharply at 48 hpi merely in the resistant genotype, which was about 57 times higher than that of susceptible genotype. The significantly higher expression of *EVM0034396* in the resistant genotype lasted until the end of the intermediate stage. The time-sequential changes in *EVM0034396* expression were as expected during the host–pathogen interaction process: this gene expressed extremely low at the early stage (0 and 24 hpi) and then specifically elevated in the resistant genotype during the intermediate stage (48, 72, and 96 hpi), leading to pathogen arrest in the resistant genotype and pathogen proliferation in the susceptible genotype. It was also notable that both the qRT-PCR and RNA-seq data revealed that this gene expressed higher in the susceptible genotype than in the resistant genotype at 144 hpi, which indicated that *EVM0034396* might be involved in the late defense of rust proliferation in the susceptible genotype.

Discussion

Controlled inoculations have enabled the detection of qualitative and quantitative resistance to *M. larici-populina* in poplar, with the major resistance gene loci mapped to the telomeric end of chromosome 19 (Cervera et al. 1996, 2001; Jorge et al. 2005; Yin et al. 2004). In this study, we assembled the *P. deltoides* chromosome 19 sequence, which provided a unique opportunity to convert the target interval with resistance gene loci into confined physical sequences.

A previous study confirmed that the target genomic region is characterized by the presence of an *R* gene supercluster (Bresson et al. 2011). Thus far, two qualitative loci (*R1* and *Mer*) conferring resistance to *M. larici-populina* have been mapped (Bresson et al. 2011; Cervera et al. 2001; Jorge et al. 2005; Yin et al. 2004; Zhang et al. 2001). Bresson et al. (2011) detected the qualitative resistance locus *R1* in a genetic interval between markers I_1211 and I_920-3. The other qualitative resistance locus, *Mer*, was localized with marker U_R7 (Yin et al. 2008), which was developed based on a bacterial artificial chromosomes (BAC) sequence located 0.4 cM from the *Mer* locus (Lescot et al. 2004). Aligning the primer sequences designed for the markers adjacent to *R1* and *Mer* to the *P. trichocarpa* reference genome sequence indicated that *Mer* and *R1* are two distinct loci, with *Mer* located downstream of *R1*. A total of nine QTLs conferring resistance to *M. larici-populina* were detected by Jorge et al. (2005), of which seven had only limited and specific effects,

whereas the other two had large and broad-spectrum effects. Regarding the two major QTLs, one (*Rus*) was inherited from *P. trichocarpa*, and it was detected very close to *R1*. A high-resolution mapping study revealed that *Rus* is located downstream of *R1* in an interval between markers 14N08-F and RGAs135-1 (Bresson et al. 2011). An alignment involving the adjacent markers indicated that *Rus* is located upstream of *Mer*. The other major QTL, *Dqtl*, was inherited from *P. deltoides*, and its confidence interval spanned the genomic region containing *R1*, *Rus*, and *Mer* (Jorge et al. 2005). Finally, we confined the target interval containing the detected qualitative and major quantitative loci conferring resistance to *M. larici-populina* to a 3.5-Mb continuous sequence in the *P. deltoides* genome (Fig. 1).

Differences in the transcriptome profiles of contrasting genotypes during the early to late defense responses of a pathogen–host interaction can provide insights into the host response to pathogens and enhance our understanding of the evolution of disease resistance and pathogenicity (Azaiez et al. 2009; Miranda et al. 2007; Rinaldi et al. 2007). In transcriptomic studies on poplar–rust interactions, the expression levels of many genes were observed to be differentially regulated by the rust infection. For example, a number of genes were differentially expressed in the *P. trichocarpa* × *P. deltoides* hybrid infected by incompatible and compatible *M. larici-populina* pathotypes at 48 hpi (Rinaldi et al. 2007). Miranda et al. (2007) conducted a time course transcriptomic analysis (24, 48, 96, and 144 hpi) of the compatible interaction between the *P. trichocarpa* × *P. deltoides* host and the *M. medusae* leaf rust pathogen. They detected 1,388 up-regulated elements and 1,404 down-regulated elements. In this study, we performed a more intensive time course transcriptomic study involving the incompatible and compatible interactions between *P. deltoides* and *M. larici-populina*. Many DEGs were detected throughout the analyzed period, and the proportions of up-regulated and down-regulated DEGs varied between the resistant and susceptible hosts (Supplementary Fig. S3). These results implied that the molecular mechanisms underlying responses to a rust infection differ substantially between the contrasting genotypes (Supplementary Fig. S3).

A previous transcriptome study proved that plant defense responses during the incompatible interaction are induced in the early stage (24 to 48 hpi) of a rust infection (Azaiez et al. 2009; Boyle et al. 2010; Levée et al. 2009; Miranda et al. 2007; Rinaldi et al. 2007). In the intermediate stage, the rust pathogen biomass increases considerably in the susceptible host but not in the resistant host, wherein fungal growth is arrested because of the activation of genes responsive to a rust infection. The induced expression of these genes triggers a specific recognition mechanism, resulting in a highly localized hypersensitive response (Hacquard et al. 2011b; Laurans and Pilate 1999). In this study, we used the *M. larici-populina* genome as a reference and revealed that the rust genes were most highly expressed at 24 hpi (Fig. 2D). In T-120, the SA concentration started to increase sharply at 48 hpi (Fig. 5A), which coincided with when the expression of the downstream *PR-1* gene

Table 1. Summary of single-nucleotide polymorphisms (SNPs)/insertions and deletions (InDels) detected in the three candidate *R* genes^a

Gene and comparison	Upstream region SNPs/InDels	Intron SNPs/InDels	Exon		
			SNPs/InDels	NonsyM	Gain SC
EVM0004305					
Intro T-120	0/0	0/0	1/0	0	0
Intro D-896	1/0	0/0	0/0	0	0
Inter T-120 and D-896	1/0	0/0	0/0	0	0
EVM0025825					
Intro T-120	0/0	146/18	46/0	34	0
Intro D-896	0/0	206/27	101/1	45	1
Inter T-120 and D-896	0/0	125/21	9/0	3	0
EVM0026813					
Intro T-120	58/6	19/1	85/3	43	1
Intro D-896	4/0	0/0	39/1	16	1
Inter T-120 and D-896	6/3	10/2	40/3	10	0

^a Introgenotype comparison detects the SNPs and the InDels between the alternate alleles within T-120 or D-896. Intergenotype comparison detects the SNPs and the InDels homozygous within each genotype but polymorphic between the contrasting genotype. NonsyM, nonsynonymous mutation; SC, stop codon.

increased considerably (Fig. 5C). Our results confirmed that the defense responses of a resistant host are induced during the early stage of a rust infection. Therefore, to identify the *R* genes conferring resistance to *M. larici-populina*, we focused on the genes that were highly expressed during the early stage of the incompatible interaction. The *R* genes differentially expressed in the contrasting genotypes in the late infection stage are probably involved in the late responses to rust proliferation, but they are likely not involved in activating defense responses.

To defend against pathogens, plants have evolved the following two defense mechanisms: pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Specifically, PTI confers nonhost resistance, whereas ETI confers host–pathogen-specific resistance. The interactions between *Melampsora* species and poplar are highly specific. In ETI, the proteins encoded by *R* genes recognize specific pathogen AVR products and trigger an immune response to the pathogen. Some *R* genes are constitutively expressed, whereas others are inducible genes. By examining the DEGs mapped to the target interval, we determined that an inducible *R* gene, *EVM0004305*, was expressed at a relatively high level at 24 and 48 hpi in T-120, but it was relatively highly expressed at a later colonization stage in D-896. The *EVM0004305* gene encodes a GsSRK that contributes to plant defense (Kim et al. 2009, 2010). Additionally, it was previously identified as a defense-related gene that may help regulate *PR* gene expression (Khoza et al. 2019; Muchero et al. 2018; Van Loon and Van Strien 1999). On the basis of its expression patterns in the contrasting genotypes, we propose that *EVM0004305* (GsSRK) is involved in the early defense responses of the incompatible interaction, whereas it may influence the later responses to rust proliferation in the compatible interaction. Furthermore, we observed that *EVM0026813* (TIR-NBS-LRR) and

EVM0025825 (CC-NBS-LRR) were constitutively expressed in the resistant host. These genes were identified as candidates for the gene(s) conferring resistance to *M. larici-populina*. Thus, in the target genomic interval, the constitutively expressed *EVM0026813* and *EVM0025825* and the inducible *EVM0004305* are likely the *R* genes conferring resistance to *M. larici-populina*. The identified nucleotide variations in the regulatory sequence and the exonic region may contribute to the functional deficiency of these candidate *R* genes in the susceptible genotype. Moreover, no other *R* gene on chromosome 19 was differentially expressed between the contrasting genotypes, implying that they are not associated with the differences in the susceptibility to the rust pathogen.

The *R* gene-mediated plant defense response involves a complex signaling pathway. When the proteins encoded by the *R* genes specifically recognize the pathogen AVR factors that inhibit host resistance, an endogenous hormone activates a series of disease resistance signals and ultimately induces the expression of *PR* genes (Bari and Jones 2009; Johnson et al. 2003; Lyons et al. 2013). In poplar, SA is a fundamental component of the signal transduction pathway that triggers defense responses against the rust pathogen (Shah 2003; Vlot et al. 2009). Systemic acquired resistance is mainly achieved through the SA-mediated signaling pathway. The infection of a plant by biotrophic pathogens causes SA to accumulate, which is the essential cellular signal for activating plant resistance to pathogens. An earlier investigation confirmed that SA up-regulates the expression of nine *PR* genes in tobacco and regulates *PR* protein synthesis at the transcript level (Bol 1988). In this study, we detected 1,058 SA-mediated genes, of which 162 were highly expressed in T-120 in the early infection stage. Many plant proteins interact with SA, including NPR1, which is encoded by a key gene in *Arabidopsis thaliana*, rice, tomato, and wheat (Chern et al. 2005; Fitzgerald et al.

Fig. 5. Salicylic acid (SA)-mediated defense signaling genes. **A**, SA concentration in leaves of the resistant and susceptible genotypes at different inoculation times. Data were analyzed by Student's *t* test. The vertical bar at each time point indicates the mean $\pm$ standard error. **P* $\leq$ 0.05; ***P* < 0.01. **B**, The significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways involving the SA-mediated up-regulated differentially expressed genes in T-120 at 48 h postinoculation. MAPK, mitogen-activated protein kinases; VEGF, vascular endothelial growth factor. **C**, RNA-sequencing expression levels of the *PR-1* (*EVM0034396*) gene across the eight time points in the contrasting genotypes. FPKM, fragments per kilobase of transcript per million fragments mapped. **D**, Quantitative reverse transcription PCR (qRT-PCR) validation of *PR-1* genes of *Populus deltoides* on *Melampsora larici-populina* infection. Total RNA was extracted from poplar leaves in the contrasting genotypes inoculated with *M. larici-populina* at eight different time points. All reactions were performed in triplicate for each gene, from which the means and standard errors were calculated.

2004; Lin et al. 2004; Makandar et al. 2006). An analysis of the *A. thaliana npr1* mutant in a previous study revealed the lack of expression of a PR-encoding gene and a failure to activate systemic acquired resistance, implying that *NPR1* is critical for systemic acquired resistance (Cao et al. 1997). Furthermore, *NPR1* expression is maintained at a very low level in the nucleus (Mou et al. 2003). In this study, we determined that the FPKM value of the *NPR1* gene (*EVM0016789*) was very low in both genotypes, but it peaked at 48 hpi in T-120. Poplar has abundant defense-related genes, including the basic PR protein (PR-1), β -1,3-glucanase (PR-2), chitinase (PR-3 and PR-4), and thaumatin-like protein (PR-5), which likely represent the poplar PR protein families (Duplessis et al. 2009). The *PR-1* gene is associated with systemic disease resistance and defense responses (Mitsuhashi et al. 2008), and it is a key gene for SA-mediated signal transduction (Kinkema et al. 2000). The expression of *PR-1* (*EVM0034396*) increased sharply at 48 hpi in T-120, which may be related to the strong defense response of the resistant host. Consequently, *PR-1* expression is likely important for the induction of plant defenses against *M. larici-populina*.

In this study, we proved that a rust infection profoundly affects poplar molecular mechanisms. By combining genetic mapping, genome sequencing, and time course transcriptome profile data, three *R* genes conferring resistance to *M. larici-populina* were identified, which recognized the specific rust AVR genes and subsequently activated the SA accumulation in the resistant host. In consequence, increase in SA concentration ultimately activated the expression of *PR* genes and led to the defense response to rust infection. Among the *PR* genes, *PR-1* was identified as the most significantly affected member. Our results could be applied to many practical aspects of rust disease diagnosis and management. In this study, the primer sequences and the experimental protocol for qRT-PCR quantification of *PR-1* expression were provided, which can be applied to diagnose the host resistance to *M. larici-populina* infection. Through several decades, many elite *P. deltoides* cultivars have been bred worldwide, but the resistant genotypes to rust infection are scarce. The traditional breeding program for poplar rust resistance can be accelerated significantly through marker-aided selection. This study provides the desirable genes resource for developing a genetic toolkit applicable for a marker-aided selection program in breeding new poplars exhibiting resistance to *M. larici-populina*. The genetic toolkit will also be useful for protecting the copyrights of the new breeding cultivars. Moreover, a previous study indicated that the presence of qualitative resistance genes frequently affects the quantitative resistance of poplar to leaf rusts (Dowkiw and Bastien 2007). Each of the identified *R* genes in this study may confer a specific qualitative resistance. Therefore, these *R* genes are useful to test whether the combination of individual *R* gene can induce durable quantitative resistance to rust disease in future studies, which is the theoretical basis for pyramiding the detected *R* genes for durable resistance by gene engineering.

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