Mapping Gene Markers for Apple Fruit Ring Rot Disease Resistance Using a Multi-omics Approach

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ABSTRACT Apple fruit ring rot (FRR), caused by Botryosphaeria dothidea, is a worldwide disease that impacts Asian apple production regions. However, no substantial progress has thus far been made toward the mapping of candidate genes or the development of effective genetic makers. In this five-year study, the resistance of 1,733 F1 hybrids from the cross ‘Jonathan’ × ‘Golden Delicious’ was phenotyped by non-wounding inoculation with four B. dothidea isolates. We first conducted systematic comparison of different analytic strategies for bulk segregant analysis by re-sequencing (BSA-Seq) and obtained suitable one for outbreeding species such as Malus. Forty-six quantitative trait loci (QTL) for resistance/susceptibility to the four isolates, including one QTL ‘hotspot’ on chromosome 14, were identified via BSA-Seq. Using integrated multi-omics strategies including RNA-sequencing, parental re-sequencing, BSA-Seq and meta-analysis of RNA-sequencing, fifty-seven candidate genes and corresponding functional mutations from the QTL were predicted. Functional mutations located on the candidate genes were validated using competitive allele-specific PCR in hybrids and Malus germplasm accessions with extremely resistant/susceptible phenotypes. Ten effective markers for apple ring rot were developed. The results provide an example of rapid candidate gene mapping for complex traits in outbreeding species.
copy number variation in a gene cluster encoding endopolygalacturonase, which mediates flesh texture and stone adhesion, was identified in peach using re-sequencing and RNA-Seq technology following traditional QTL mapping analysis (Gu et al. 2016). The biosynthesis, regulation, and domestication of bitterness in cucumber were determined using integrated approaches, including genome-wide association studies (GWAS), re-sequencing, and transcriptome analysis (Shang et al. 2014).

In this study, we integrated whole genome parental re-sequencing, BSA-Seq, and RNA-Seq meta-analysis to identify QTL and screen candidate genes for FRR resistance/susceptibility. Markers in the regions of the candidate genes were validated by competitive allele-specific PCR (KASP).

**MATERIALS AND METHODS**

**Plant material**

*Malus* germplasms (60) and biparental cross hybrids (1,773) (‘Jonathan’ × ‘Golden Delicious’) were used as segregating populations. The hybrid cross was performed in 2002 and the seedlings were planted in 2003. All plant materials were subjected to conventional cultivation management practices.

**Phenotyping and sampling**

In the years of 2008, 2009, 2014, 2015, and 2016, phenotyping for FRR resistance was undertaken under room temperature conditions using the ripened fruit and mycelia of four *B. dothidea* isolates (Zz026, Ls1, Lw023, and Lw048) (Guan et al. 2015; Zhuang et al. 2011).

**Re-sequencing of ‘Golden Delicious’ and ‘Jonathan’ and data processing**

Genomic DNA was extracted from the leaves of ‘Golden Delicious’ and ‘Jonathan’ using a Genomic DNA Isolation Kit (TianGen, Beijing, China). The Illumina sequencing libraries were constructed using NEBNext DNA Library Prep Master Mix. Paired-end sequencing was performed using the Illumina HiSeq2500 sequencer (Illumina, San Diego, CA). Burrows-Wheeler Aligner (BWA) was used to map clean reads to the apple double haploid (DH) genome (Li and Durbin 2009; Daccord et al. 2017). SNP and InDel calling were conducted using SAMtools (Li et al. 2009; Li 2011). SNP and InDels with a lowest phred-scaled quality of 30 and a minimum read depth of 10 were kept. Structure variation calling was conducted using the SpeedSeq pipeline (Chiang et al. 2015). InterproScan5.21 was used to conduct functional domain analysis of all the genes in the apple genome (Zdobnov and Apweiler 2001). cis-acting regulatory elements in upstream of the genes were analyzed using PlantCARE web tools (Rombauts et al. 1999). Finally, all the variations were annotated with ANNOVAR based on the functional domain, cis-acting regulatory elements, and gene model information (Wang et al. 2010).

**DNA pooling and BSA data processing**

The resistant/susceptible bulks were constructed using 1,773 hybrids of ‘Jonathan’ × ‘Golden Delicious’ (Guan et al. 2015; Zhuang et al. 2011). Based on the lesion length data after fruit non-wounding inoculation with four isolates for five years, hybrids exhibiting the non-incidence phenotype in at least two years were included in the resistance (R) bulk, while incident hybrids with random lesion lengths in at least two years were chosen as the susceptible (S) bulk. Genomic DNA was extracted from the leaves of the selected hybrids using a Genomic DNA Isolation Kit (TianGen). Eight DNA libraries were constructed after DNA pooling based on the group information. The method of Genomic DNA extraction and the DNA library construction were the same as above. Paired-end sequencing was performed using the Illumina HiSeq X Ten platform (Illumina). The clean reads were mapped to the apple DH genome using BWA, and only uniquely mapped reads with a minimum phred-scaled map quality score of 20 were kept.

The SNP’s showing polymorphisms between parents or which were double-heterozygous in the two parents were subdivided into three subsets: J-type, whereby the genotype of the markers is homozygous in ‘Golden Delicious’ and heterozygous in ‘Jonathan’; G-type, whereby the genotype of the markers is heterozygous in ‘Golden Delicious’ and homozygous in ‘Jonathan’; and H-type, whereby the genotype of the markers is heterozygous in both parents.

Read counts with different genotypes from extremely resistant and susceptible pools were extracted and separated based on the G-, J-, and H-type subsets, respectively. Thus, for each isolate, three files (G, J, and H) with read counts of the coupled pools were generated.

The allele frequency difference (AFD) between two coupled pools and G-value in each site were calculated based on the read counts. Nadaraya-Watson kernel regression was used as a smoothing function, and the G-value at each site within the 1 Mbp sliding window size was calculated (Magwene et al. 2011). The significance threshold of G was estimated using an empirical approach proposed by Magwene et al. (2011). Significant regions with false discovery rate (FDR) <0.01 were selected as candidate QTL.

The QTL analysis was performed three times using each of the three subsets of data. The QTL could be mapped to the pollen, maternal, or both parents using the marker data subset G, J, or H, respectively. QTL with peak G-value exceeding 30% of the significant threshold were defined as major QTL.

**Estimating the impact of the reference genome on QTL detection**

To investigate the impact of the reference genome on QTL detection, the clean sequencing reads from the two bulked DNA pools (resistant/susceptible to isolate Zz26) were processed to detect QTL using two versions of the apple reference genome (diploid version 1.0p and DH) (Velasco et al. 2010; Daccord et al. 2017). The DNA sequences without gaps (Ns) of the QTL were obtained from the corresponding genome (diploid version 1.0p or DH) using SAMtools software (Li et al. 2009; Li 2011). The LASTZ sequence alignment program was used to align the sequences against the genome, and dot plots were visualized in the R statistical environment (Harris 2007). To verify the reliability of the QTL, 3-5 markers were selected from each QTL region, and all individuals in the two bulks were genotyped using the KASP assay. Chi-square tests of the genotypic frequency of a certain marker indicating significant differences (P-value <0.05) between the two bulks were considered reliable.

**Comparison of different statistical methods for QTL identification**

To determine the best statistical method, we compared the SNP index (SI), Euclidean distance (ED), and the G-value (GV) methods for QTL identification using the phenotype data of resistance/susceptibility to the Zz26 isolate. The absolute value of the ∆SNP index (∆SI) was used for SI (Wang et al. 2017). Where large numbers of SNPs exist, fitting the ED using a Loess curve with a polynomial exponent of one in the MMAPP may be hugely time-consuming and computationally intensive (Hill et al. 2013). Instead, we used Nadaraya-Watson kernel regression as a smoothing function in ED (Magwene et al. 2011). To get a fair decision of the three statistical methods, we adopted the same threshold selection method. The median of all the scores plus three-times the standard deviation (SD) were used as thresholds for SI, ED, and GV.
\[ G' = \sum_{j \in W} k_j G_j \]  

(1)

\[ k_j = \frac{(1-D_j)^3}{\sum_j (1-D_j)^3} \]  

(2)

\[ ED = \sqrt{(f_{A0} - f_{A1})^2 + (f_{R0} - f_{R1})^2 + (f_{A0} - f_{C0})^2 + (f_{A1} - f_{C1})^2} \]  

(3)

\[ (ED^4)' = \sum_{j \in W} k_j (ED^4)_j \]  

(4)

In the GV method, the G-value was calculated at each SNP using the standard G-statistic followed by smoothing using equation 1, where the sum includes all SNPs within the window W bracketing the SNP, and equation 2, where \( D_j \) is the standardized distance, with value 0 at the focal position and value 1 at the edge of the window. Similarly, in the ED method, the Euclidean distance is first calculated at each SNP location using equation 3, where the letters represent the corresponding allele frequency of the bases in each pool. This distance is then raised to four-times the power, following which the data are summed using the same smoothing function.

RNA-Seq library construction, sequencing, and data processing

The fruit of three hybrids randomly chosen from each of the extremely resistant/susceptible bulks were used for the RNA-Seq analysis (as three independent biological replicates). Three ripened apples from each hybrid were inoculated without wounding with the mycelia of the \( B.\) dothidea Zz26 isolate, and healthy flesh from the very edge of the lesion was cut and sampled at 0 h, 24 h, 48 h, and 96 h after inoculation (Guan et al. 2015). Three apples of each non-inoculated hybrid were also sampled as controls.

Total RNA was extracted using the modified CTAB method (Moser et al. 2004). For each sample, 5 \( \mu \)g total RNA was used to isolate mRNA for the preparation of an RNA-Seq library using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer’s protocols. The cDNA library was sequenced (paired-end 150) from both the 5’ and 3’ ends on the Illumina HiSeq X Ten platform (Illumina) according to the manufacturer’s instructions. The RNA-Seq reads were mapped to the apple DH genome with HISAT2 (Kim et al. 2015). StringTie was used to conduct transcript assembly and quantification (Pertea et al. 2015).

DESeq2 software was used to detect differentially expressed genes (DEGs) between the resistant/susceptible hybrids (Love et al. 2014). Blast2GO was used for gene ontology (GO) classification and GO enrichment analysis (Conesa et al. 2005). KOBAS2.0 was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolism annotation and KEGG enrichment analysis (Xie et al. 2011). The bam files from one individual hybrid were merged, followed by genotyping by SAMtools (Li et al. 2009; Li 2011). SNPs and InDels were called and clustered between resistant and susceptible hybrids.

Candidate gene mining From the QTL regions based on multi-omics data

Narrowing down the QTL regions with varied sliding window sizes: By using the BSA-Seq data, all markers located on the gene body or upstream regions of genes were selected from \( \pm 0.5 \) Mb regions of the QTL main peak and sub-peaks, if present. The markers were then filtered, and those markers with a G-value above the G’-value were selected for subsequent analysis. Sliding window analysis with 0.125 Mb, 0.25 Mb, 0.5 Mb, 0.75 Mb, and 1 Mb window sizes was used. The standard deviation and mean of those peaks were calculated to measure the stability of the peak position. Overlapped peak regions with different window sizes were considered to be the defined region

Excluding genes from QTL intervals using the parental re-sequencing data: Too many genes were present in the narrowed-down QTL intervals. To further reduce the gene number, another criterion for candidate functional single nucleotide variants (SNVs) and structural variations (SVs) screening is that all the variations must be consistent with Mendelian inheritance; i.e., if the QTL is located only in the pollen parent, then the genotype of the screened variant in the pollen parent must be heterozygous, the genotype in the maternal parent must be homozygous, and vice versa. By using the parental SNV and SV database, many genes within the narrowed-down QTL intervals were culled, except where upstream (2,000 bp) variations between the two parents may affect cis-element functioning, or where exonic variations between parents may cause stop-gain, frameshift, or domain non-synonymous altering.

Excluding genes from candidates using transcriptome data: Gene transcription is often affected by variations in up-stream regions, especially on the cis-elements (Espley et al. 2009; Kobayashi et al. 2004; Zhang et al. 2017). The exonic variation can be also detected in the corresponding cDNA samples if the gene is transcribed (Han et al. 2018; Piskol et al. 2013). By using transcriptomic data, the unassociated genes can be further culled from the above-mentioned candidate genes, including those absent in the unigene dataset and thus un-transcribable with those variations in upstream cis-elements, but absent in the DEG dataset; and those with exonic variations absent in the unigene mRNA SNP/InDel-calling dataset.

Excluding genes from candidates with functional alterations in the incorrect bulk pool: We attempted to assemble haplotype blocks using paired-reads. The Hidden Markov Model-based algorithm integrated in SAMtools was adopted to assemble haplotype blocks from the DNA sequence reads (Li et al. 2009; Li 2011). The haplotype blocks from the resistant/susceptible bulks were considered as main haplotype blocks representing the haplotype shared by most of the samples in the bulks. BSA markers were used as ‘anchor markers’ to assign the allele frequency to the haplotype. Thus, the haplotype blocks could be determined as enriched in resistant or susceptible pools based on the BSA makers.

Marker validation and gene expression analysis

To examine the RNA-Seq data, randomly selected genes were validated using real-time quantitative (RT-q)PCR, and the gene-specific primers were designed with primer premier software (version 5.0) (Premier Biosoft Interpaers, Palo Alto, CA) (Table S1). One microgram of total RNA was used in the reverse transcription in a total volume of 20 \( \mu \)L in the presence of 6-mer random primer and oligo primer according to the protocol of the TaKaRa kit (TaKaRa Biotechnology Co., Ltd., Japan). The PCR reactions were run in three replicates in a Bio-Rad Sequence Detection System (Bio-Rad Life Science Research, Hercules, CA, USA). Actin was chosen as an internal control gene for normalization. Quantifying the relative expression of the genes at the three sampling time points was performed using the delta-delta Ct method (Livak and Schmittgen 2001). All data were expressed as the mean \( \pm \) SD after normalization.
Validation of SNPs and InDels

Primers were designed using Primer-blast software (NCBI, Maryland, USA) based on the 200-bp sequences flanking the SNPs and InDels to be validated. Then, monoclonal PCR products comprising SNP loci of interest were amplified in both parents and sequenced using Sanger sequencing (BGI, Beijing, China) to verify the SNPs obtained from the re-sequencing data between the parents.

KASP genotyping assay and statistical analysis

A total of 110 DNA samples, including 20 and 30 individuals in the bulks and resistant and susceptible to isolate Zz26, respectively, and also 60 Malus germplasm accessions were genotyped using the KASP assay (LGC Genomics, Beverly, MA, USA). The primers were designed based on the 200-bp sequence flanking the SNPs and InDels (Table S2). The DNA of 'Jonathan' and 'Golden Delicious' was used as control. Then the genotypes were validated by Sanger sequencing. Data were analyzed using the "Endpoint Genotyping" method of the Light Cycler 480 Software (release 1.5.0).

The segregation biases of markers between resistant and susceptible bulks were tested by Chi-square test. Chi-square test was run twice, the first, allele A was supposed to be completely/partially dominant on B, and the next, allele B completely/partially dominant on A. The scenario which Chi-square value was statistically significant was accepted.

Data Availability

The RNA-sequencing data and the whole genome re-sequencing data of the pooled DNA from the bulks and un-pooled DNA from the parental cultivars have been deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA392908. Gene expression data and other additional datasets and codes are also available at Figshare and File 'Readme.txt' contains detailed description of all the additional datasets. File S1 contains all the detailed descriptions of supplemental materials. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7819688.

RESULTS

Bulk construction and whole genome re-sequencing

Based on the phenotypes over five years (2008, 2009, 2014, 2015, and 2016), eight bulks were developed, each including 20–37 hybrids with extremely resistant or susceptible phenotypes to each of the four Botryosphaeria dothidea isolates (Zz26, Ls1, Lw023, and Lw048). A total of 199 hybrids were included in the eight bulks, of which 17 and 21 exhibited extreme resistance or susceptibility to at least two pathogen isolates, respectively (Figure 1 and Table S3).

The whole genome re-sequencing data of the pooled DNA from the bulks and un-pooled DNA from the parental cultivars, 'Jonathan' and 'Golden Delicious', have been deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA392908. A total of 163,934,426 and 172,856,412 clean reads were obtained by parental re-sequencing for the DH apple genome (Table S3). For all the reads (937,097,528) from the eight bulks, 97.89% (917,367,968) were mapped to the reference genome and 72.8% (682,727,645) were uniquely mapped (Table S3).

After filtering, the sum of the remaining J-, G-, and H-type SNPs ranged from 1,693,771 to 3,507,347, and the corresponding SNP density across the genome ranged from 2601/Mb to 5387/Mb in the extreme bulks of the four isolates (Table 1). The distribution of SNPs in the genome, shown as the number of SNPs per Mb with a 10 kbp sliding window, was not even, and almost all regions in the genome excluding the gaps were covered by the sliding window analysis (Figure S1–S4).

Comparison of statistical methods for QTL identification

The profiles of the corresponding major QTL (J06.1, J08.1, J10.1, J10.2, J10.3, G11.1, G15.1, H03.1, H14.1, H14.2, and H16.1) identified through SI, ED, and GV coincided or overlapped well, indicating that the effects of the major QTL were relatively robust. For all statistical methods, the effects of noise were effectively reduced and AFD was measured between the two extreme pools (Figure S5, Figure S6 and Figure S7). However, the QTL by SI covered broader regions and were less significant than by ED or GV (Figure S5, Figure S6, Figure S7 and Table 2), implying that the QTL sensitivity of SI was comparatively lower. As a consequence, one reliable minor QTL (G02.1-GV) was missed in SI (Figure S7 and Table 2). Distinct unbiasedness and low false positives in the QTL were achieved using GV, especially for H-type QTL. The QTL intervals by GV, (2,551,554 bp) were much narrower than that by ED (2,618,442 bp) (Table 2). Additionally, some false positive QTL were found in ED (Figure S6 and Table S11). Collectively, GV was the best statistical method and was thus explored subsequently.

The impact of the reference genome on QTL detection

By using the diploid version (1.0p) and DH genomes, 14 and 12 significant QTL were identified, respectively (Figure 2 and Table S10). The seven reliable QTL in the DH genome coincided or overlapped precisely with nine reliable QTL from the diploid genome (Table S13). However, five reliable QTL (G02.1, J08.1, H16.1, H14.1, and H14.2) in the DH genome did not match any QTL from the diploid genome (Table S13). Conversely, two reliable QTL (G09.1 and G09.2) identified by using the diploid genome were missing from the results of the DH genome (Table S13). Based on the sequence alignment, gaps were clearly detected in two QTL regions between the two genome versions.
Table 1 Numbers of different types of markers used in bulk segregant analysis

| Isolate | J-type | G-type | H-type | Sum |
|---------|--------|--------|--------|-----|
| J vs. G | 1126610 | 500397 | 3098152 | 4725159 |
| Zz26   | 1475502 | 545025 | 1272274 | 3292801 |
| Ls1    | 703321  | 257515 | 732935  | 1679771 |
| Lw023  | 1013974 | 381292 | 961013  | 2356279 |
| Lw048  | 1538283 | 535547 | 1433517 | 3507347 |

Notes: G-type: the genotype of the marker is heterozygous in ‘Golden Delicious’ and homozygous in ‘Jonathan’; J-type: the genotype of the marker is homozygous in ‘Golden Delicious’ and heterozygous in ‘Jonathan’; H-type: the genotype of the marker is heterozygous in both parents; J vs. G: all the numbers of the three types of markers based on the genotype of ‘Golden Delicious’ and ‘Jonathan’.

Identification of QTL for FRR resistance

For resistance/susceptibility to isolate Zz26, 12 significant QTL were obtained, six of which (J06.1-Zz26, J10.1-Zz26, J10.2-Zz26, J10.3-Zz26, H14.1-Zz26, and H14.2-Zz26) were considered as major QTL (Figure 2 and Table S10). For resistance/susceptibility to B. dothidea isolate Ls1, Lw023, and Lw048, 14, 8, and 12 significant QTL were detected, respectively. Of these QTL, seven (H14.1-Ls1, G15.1-Ls1; G02.1-Lw023, G13.1-Lw023, G14.2-Lw023, H14.1-Lw048, and J15.2-Lw048) constituted major QTL (Table S14 and Figures 2, 3, 4, and 5).

Of the significant QTL, five QTL regions coincided or overlapped with resistance/susceptibility to more than two isolates. The QTL G02.1-Zz26/G02.1-Ls1, G15.1-Ls1/H15.1-Lw023 and G06.1-Ls1/G06.1-Lw048 coincided, while the QTL regions of J10.2-Zz26/G10.2-Lw023 and H14.2-Zz26/H14.1-Ls1/H14.1-Lw048 overlapped (Table S14 and Figure 6). H14.2-Zz26/H14.1-Ls1/H14.1-Lw048 were consistently mapped to the same region along with four previously reported major gene loci (sf-g14-ls1, sf-g14-lw048, Rb-g14-lw048, and Rb-g14-zz26) (Figure S11) (Cui et al. 2014).

Narrowing down the QTL intervals with varied sliding window sizes

By using varied sliding window sizes, the 46 QTL for FRR resistance to the four pathogen isolates were significantly reduced (Table S15). The spanning regions of the 13 major QTL were narrowed down from 1,685,139 bp to 743,971 bp on average, and thus the significance of the average GV scores was also increased from 5.80 to 6.25 (Table S15). Several broader QTL intervals were split into two or more narrower sub-peaks; e.g., the QTL J06.1-Zz26 was spliced into two sub peaks, J06.1_sub1-Zz26 and J06.2_sub2-Zz26, respectively (Table S15). As a result of this splicing, the QTL interval was narrowed down from 4,800,068 bp to 1,000,000 bp and 500,000 bp, and the average GV scores increased from 8.93 to 8.34 and 9.71, respectively (Table S15).

Table 2 Comparison of overlapped QTL for apple (Jonathan × Golden Delicious) resistance/susceptibility to Botryosphaeria dothidea isolate Zz26, detected by three statistic methods

| Statistic method | QTL | Chromosome | Origin | QTL start | QTL end | QTL region | Peak position | Peak (SI/G/ED) | Threshold | Percentage exceeding threshold | KASP confirmed (YES/NO) |
|------------------|-----|------------|--------|-----------|---------|------------|---------------|---------------|-----------|-------------------------------|-------------------------|
| GV               | H03.1 Chr03 H | 33952320 | 35776974 | 1824654  | 34822605 | 5.2269 | 3.8659 | 35.21% | YES |
| SI               | H03.1 Chr03 H | 33397901 | 35869112 | 2471211  | 4799846  | 0.2034 | 0.1500 | 115.00% | YES |
| ED               | H03.1 Chr03 H | 30404631 | 35889232 | 1847601  | 35389232 | 0.017 | 0.0121 | 40.43% | YES |
| GV               | J06.1 Chr06 J | 1501802  | 7049918  | 5548161  | 4799846  | 0.2276 | 0.1500 | 51.71% | YES |
| SI               | J06.1 Chr06 J | 1668863  | 7529246  | 5860383  | 4799846  | 0.2276 | 0.1500 | 51.71% | YES |

Notes: G-type: the genotype of the marker is heterozygous in ‘Golden Delicious’ and homozygous in ‘Jonathan’; J-type: the genotype of the marker is homozygous in ‘Golden Delicious’ and heterozygous in ‘Jonathan’; H-type: the genotype of the marker is heterozygous in both parents; J vs. G: all the numbers of the three types of markers based on the genotype of ‘Golden Delicious’ and ‘Jonathan’.

Notes: Statistic method, GV: G value method, SI: SNP index method, ED: Euclidean distance method.
Candidate gene mining from QTL regions based on multi-omics data

Excluding genes from the QTL intervals using parental re-sequencing data: Of the 3,058 genes scattered on the 49 narrowed-down QTL regions including sub peaks (Table S15), 1,346 genes were excluded using the parental SNV and SV databases, and 24,977 SNVs and 253 SVs were distributed in the 1,712 remaining candidate genes (Table S16).

Excluding genes from candidates using transcriptome data: The RNA-Seq data were deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA392908. After removing low-quality reads and those derived from rRNA, the total reads input for mapping was 285.86 M, of which 207.9 M (80.63%) were aligned to the apple DH genome (Table S17). Overall, 136,983 transcripts from 86,808 genes (63,538 referenced and 23,270 non-referenced) were assembled.

The Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values of three representative genes, MD05G1236300, MD05G1261100, and MD08G1128800, were closely correlated with their relative expressions by RT-qPCR ($R^2 = 0.921-0.962, P < 0.01$) (Figure S12). The correlation analysis and hierarchical cluster analysis revealed a close grouping of the same stage in both the extremely susceptible and resistant hybrids (Figure S13).

In total, 1,570 DEGs were detected between the extremely susceptible and resistant hybrids across the sampling time points (Figure S14). SNVs, including 503,067 SNPs and 37,402 InDels, were identified in 36,796 genes. Following functional annotation, 103,510 non-synonymous SNVs were identified and were distributed across 24,533 genes.

In addition to the transcriptomic data in this study, the RNA-Seq data of 25 transcriptome bio-projects and 432 samples from the SRA database were downloaded (Table S18). All the reads were mapped to the DH reference genome to quantify the expression of each gene. The
expression data of the genes were also used to assist in the screening of organ-specific or non-expressive genes (Eide et al. 1996).

Using the transcriptomic data, 56 non-expressed genes and 46 non-DEGs without variations in their CDS were culled from the 503 candidates in the QTL intervals for resistance/susceptibility to isolate Zz26 (Table S16). In the QTL regions for resistance/susceptibility to isolate Ls1, Lw023, and Lw048, 221 of the 1,209 genes were excluded owing to non-expression in any apple fruit samples (Table S16).

Excluding genes from candidates by functional annotation: One thousand and one of the 1,389 candidate genes were successfully assigned with Gene Ontology (GO) terms. Based on the detailed gene annotations, 386 genes were excluded due to the inconsistency of their organ/tissue/sub-cellular localization, developmental dynamics, and physiological pathway annotations, after which 1,003 genes remained (Table S16).

Excluding genes from candidates based on their functional alteration appearing in the incorrect bulk: The allele frequency of the screened functional variations in the extremity pools can aid candidate gene prediction. However, only small parts of the SNPs used in the BSA analysis can be directly assigned with allele frequency. Here, we phased the variations and constructed 740,329 haplotype blocks in the two parents. BSA markers were used as 'anchor markers' to assign the AFD between two bulks to the haplotype. For the pollen parent ‘Golden Delicious’, 5,505,264 SNPs were phased into 364,768 haplotype blocks, and after anchoring, 162,184 (44.46%) haplotype blocks were assigned an AFD between bulks. For the maternal parent ‘Jonathan’, 6,122,520 SNPs were phased into 375,561 haplotype blocks, and 196,220 (52.25%) haplotype blocks were assigned an AFD to the bulk pools. Considering meiotic crossover, the haplotypes of the male and female parent were referenced to each other. As for resistance/susceptibility to isolate Zz26, in this study, 2,051 of the 3,211 variations in
278 candidate genes were assigned an AFD, and the 42 genes with unexpected AFD values between the two bulk pools were excluded. For example, a stop-gain variation was screened in the CDS of MD15G1419300 in QTL G15.1, which is annotated as 'mitogen-activated protein kinase kinase kinase' gene; however, the stop-gain variation was enriched in the R pool, which is inconsistent with its function. On the contrary, a stop-gain variation in the CDS of MD10G1288400 in J10.3, a serine/threonine-protein kinase-like protein, was enriched in the S pool, which was expected.

Ultimately, 57 candidate genes from 46 significant QTL associated with resistance/susceptibility to four B. dothidea isolates were selected for further experimental validation (Table 3 and Table S14).

**Experimental validation of the candidate genes**

The presence/absence of variations in the 14 candidate genes from 11 major QTL linked to resistance to FRR isolate Zz26 was confirmed by Sanger sequencing (Table S19 and Table S20). The segregation of the variants was then validated by KASP genotyping in hybrids with extreme phenotypes, and all the variants exhibited significant associations between genotypes and phenotypes ($P < 0.05$) (Table S21). The presence/absence of the markers was then verified in each 30 Malus germplasm accessions highly resistant/susceptible to FRR isolate Zz26 (Table S22). Ten KASP markers from nine QTL exhibited significant associations with the phenotypes (Table S23).

Of the 14 candidate genes, five genes (MD03G1259600, MD16G1236700, MD10G1169200, MD10G1288400, and MD10G1288500), located in four QTL (H03.1, H16.1, J10.1, and J10.3), constituted protein kinases or kinase-like proteins (Table S19). The variations were validated by Sanger sequencing in the cis-element or transcription factor binding site of the promotors of MD03G1259600, MD16G1236700, and MD10G1169200 (Table S20), which is highly consistent with the finding that the expressions of these genes in fruit from the resistant
bulk 48 h after inoculation were significantly higher than that in the susceptible bulk (Figure S15). Frame-shift deletions and stop-gain mutants were located in the CDS of the other two protein kinase genes, MD10G1288400 and MD10G1288500, respectively, which may cause functional loss of the genes (Table S20).

From the major QTL J06.1, MD06G1037600, annotated as ‘NDR1/HIN1-like 8’ was ultimately screened as a candidate gene (Table S19). Re-sequencing and Sanger sequencing confirmed the variations in the cis-element and transcription factor (TF) binding site of this gene between parents (Table S20). The KASP analysis of the marker (KASP100) located in the promoter of MD06G1037600 exhibited significant association with the phenotype in the bulks ($\chi^2 = 7.4108, P = 0.006480 < 0.05$) and the 60 accessions with extreme phenotypes ($\chi^2 = 5.93, P = 0.0146 < 0.05$) (Table S21, Table S23, and Table S20). The expression of this gene in the fruit from the resistant bulk 48 h after inoculation was consistently significantly higher than that in the susceptible bulk (Figure S15).

Three pentatricopeptide repeat-containing genes, MD02G1213800, MD08G1120500, and MD08G1120700, were identified as candidate genes from QTL G02.1 and J08.1 (Table S19). Nonsynonymous SNVs were distributed in the functional domain of MD02G1213800, and the KASP analysis of one functional variation marker (KASP146) exhibited significant association with the phenotype of the two extreme pools ($\chi^2 = 9.736, P = 0.001807 < 0.05$) (Table S18 and Table S21). Stop-gain and frame-shift variations were detected and validated in both MD08G1120500 and MD08G1120700 (Table S19 and Table S20). Furthermore, KASP analysis confirmed that the stop-gain mutation A (KASP107), enriched in the susceptible bulk, exhibited significant AFD values between the two pools ($\chi^2 = 5.333, P = 0.02090 < 0.05$) and the 60 extreme accessions ($\chi^2 = 4.0074, P = 0.0453 < 0.05$) (Table S20, Table S21, and Table S23). At the same time, frame-shift variations (KASP117) were also significantly enriched in the susceptible bulk ($\chi^2 = 3.8595, P = 0.04950 < 0.05$) and extremely susceptible accessions ($\chi^2 = 4.0074, P = 0.0453 < 0.05$) (Table S20, Table S21, and Table S23).
In the region of QTL G15.1, a disease-resistance gene (MD15G1416500) was detected with the largest G’ value (Table S19). A functional SNP (C to A) causing stop-gain was present in its CDS, and allele A was enriched in the susceptible bulk (Table S19). Furthermore, the corresponding KASP maker (KASP66) was significantly associated with the phenotype in both the resistant/susceptible pools ($\chi^2 = 9.145, P = 0.0025$) and 60 extreme accessions ($\chi^2 = 5.079, P = 0.0242 < 0.05$) (Table S20, Table S21, and Table S23).

Two F-box/kelch-repeat proteins (MD06G1017400 and MD10G1255900) were detected in the QTL J06.1_sub1 and J10.2, respectively (Table S19). This protein is a component of SCF (ASK-cullin-F-box) E3 ubiquitin ligase complexes, which may mediate the ubiquitination and subsequent proteasomal degradation of target proteins (Zhu et al. 2017). Protein ubiquitination processes play an important role in plant–pathogen interactions (Lee et al. 2011). Re-sequencing and Sanger sequencing confirmed the SNPs and InDels in the cis-element and TF binding site of MD06G1017400 between the parents (Table S18). One SNP (KASP140) located in the TF binding site was significantly associated with the phenotype in the two pools ($\chi^2 = 5.718, P = 0.0168 < 0.05$) and 60 extreme accessions ($\chi^2 = 5.880, P = 0.0153 < 0.05$) (Table S20, Table S21, and Table S23). The expression of this gene in the fruit of hybrids from the resistant bulk 48 h after

Figure 6 Circular overview of the QTL for apple resistance/susceptibility to fruit ring rot (*Botryosphaeria dothidea*) isolates Ls1 (red), Lw023 (orange), Lw048 (green), and Zz26 (blue) across the genome. The outer circle: QTL from ‘Golden Delicious’ (G); intermediate circle: QTL from ‘Jonathan’ (J); the inner circle: QTL from both ‘Golden Delicious’ and ‘Jonathan’. The colored dots in the central map indicate the geographical origin of the pathogen isolates.
| Isolate | Chromosome | QTL  | gene ID       | R/S | Mutation type           | Gene annotation                                      |
|---------|------------|------|---------------|-----|-------------------------|------------------------------------------------------|
| Lw048   | Chr02      | H02.1| MD02G1036600  | S   | frame-shift insertion   | Protein kinase superfamily protein                   |
| Lw048   | Chr02      | H02.1| MD02G1040200  | R   | CREs & nonsynonymous SNV| Transcriptional corepressor LEUNIG                  |
| Lw048   | Chr02      | H02.2| MD02G1085900  | —   | frame-shift deletion    | Ankyrin repeat family protein                        |
| Lw048   | Chr02      | H02.3| MD02G1170800  | R   | CREs                    | AP2/B3-like transcriptional factor family protein   |
| Lw048   | Chr02      | H02.3| MD02G1174000  | S   | CREs & nonsynonymous SNV| Probable receptor-like serine/threonine-protein kinase|
| Lw048   | Chr02      | H02.3| MD02G1191600  | S   | stop-gain               | Pathogenesis-related protein 1C                      |
| Lw048   | Chr03      | H03.1| MD03G1067300  | S   | nonsynonymous SNV       | NB-ARC domain-containing disease resistance protein  |
| Lw048   | Chr06      | G06.1| MD06G1173700  | S   | stop-gain               | Serine/threonine-protein kinase ATM                  |
| Lw048   | Chr08      | J08.1| MD08G1229700  | —   | frame-shift insertion   | Protein kinase family protein                        |
| Lw048   | Chr08      | J08.1| MD08G1239000  | —   | frame-shift insertion   | Disease resistance protein                           |
| Lw048   | Chr08      | J08.1| MD08G1241800  | —   | nonsynonymous SNV       | Leucine-rich receptor-like protein kinase family protein|
| Lw048   | Chr08      | J08.1| MD08G1244800  | S   | stop-loss               | Protein kinase superfamily protein                   |
| Lw048   | Chr04      | J04.1| MD04G1066000  | S   | stop-gain               | CTR1_ARATH Serine/threonine-protein kinase CTR1     |
| Lw048   | Chr14      | H14.1| MD14G1162500  | —   | CREs                    | Unknown                                              |
| Lw048   | Chr15      | J15.3| MD15G1282900  | S   | stop-gain               | Leucine-rich receptor-like protein kinase family protein|
| Ls1     | Chr06      | G06.1| MD06G1173700  | S   | stop-gain               | Serine/threonine-protein kinase ATM                  |
| Ls1     | Chr10      | G10.1| MD10G1006400  | S   | stop-gain               | WRKY transcription factor 19                        |
| Ls1     | Chr10      | G10.1| MD10G1007300  | —   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr10      | G10.1| MD10G1007600  | S   | nonsynonymous SNV       | disease resistance protein                           |
| Ls1     | Chr10      | G10.1| MD10G1006500  | —   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr10      | G10.1| MD10G1006600  | S   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr10      | G10.1| MD10G1006800  | —   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr10      | G10.1| MD10G1006900  | —   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr10      | G10.2| MD10G1021000  | S   | frame-shift insertion   | Disease resistance protein                           |
| Ls1     | Chr13      | H13.1| MD13G1191200  | —   | nonsynonymous SNV       | PLAT/LH2 domain-containing lipoxygenase family protein|
| Ls1     | Chr13      | J13.1| MD13G1210700  | —   | CREs & nonsynonymous SNV| HR-like lesion-inducing protein-related              |
| Ls1     | Chr14      | H14.1| MD14G1162500  | —   | CREs                    | Unknown                                              |
| Ls1     | Chr15      | G15.1| MD15G1103400  | —   | nonsynonymous SNV       | Transcriptional corepressor LEUNIG                  |
| Ls1     | Chr15      | G15.1| MD15G1104000  | S   | nonsynonymous SNV       | Disease resistance protein                           |
| Ls1     | Chr15      | G15.1| MD15G1103200  | —   | nonsynonymous SNV       | AIG2-like (avirulence induced gene) family protein   |
| Ls1     | Chr15      | G15.2| MD15G1129100  | —   | frame-shift deletion    | TOPLESS-related 1                                    |
| Ls1     | Chr17      | J17.2| MD17G1028400  | —   | frame-shift deletion    | Protein TPR2                                         |
| Ls1     | Chr17      | J17.2| MD17G1028700  | S   | stop-gain               | Ankyrin repeat-containing protein NPR4               |
| Ls1     | Chr17      | J17.2| MD17G1029100  | S   | stop-gain               | Ankyrin repeat family protein                        |
| Ls1     | Chr17      | J17.2| MD17G1029200  | —   | frame-shift deletion    | Ankyrin repeat family protein                        |
| Ls1     | Chr02      | G02.1| MD02G1213800  | S   | nonsynonymous SNV       | Pentatricopeptide repeat-containing protein          |
| Ls1     | Chr17      | J17.2| MD17G1054100  | —   | nonsynonymous SNV       | WRKY DNA-binding protein 3                           |
| Lw023   | Chr02      | G02.1| MD02G1229600  | S   | upstream                | Two-component response regulator ORR22             |
| Lw023   | Chr02      | G02.1| MD02G1230200  | —   | upstream                | Seven transmembrane MLO family protein               |

(continued)
inoculation was consistently significantly higher than that of the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15). While for another gene, MD10G1255900, a functional SNP (G-to-A) (KASP87) causing stop-gain was present in its CDS, and an enriched allele A was also present in the susceptible bulk (Figure S15).

Ankyrin repeat-containing genes participate in the processes of signal transduction and protein kinase activity and thus may play an important role in the plant immune system (Mou et al. 2013; Ngaki et al. 2016). In the QTL of G11.1, an ankyrin repeat-containing gene (MD11G116700) was identified in the peak (Table S19). The expression of this gene in the fruit of hybrids from the resistant bulk 48 h after inoculation was significantly higher than that of the susceptible bulk (Figure S15). The significant difference in expression was possibly caused by the validated mutants in the promoter region of this gene between parents (Figure S15 and Table S20). Furthermore, the KASP marker KASP126, developed in the candidate functional InDels, was significantly associated with the phenotype in the two pools ($\chi^2 = 5.966$, $P = 0.02255 < 0.05$) and the 60 extreme accessions ($\chi^2 = 9.72$, $P = 0.001823 < 0.05$) (Table S19, Table S20, Table S21, and Table S23).

**DISCUSSION**

The efficiency of the BAS-Seq strategy for QTL mapping in an outbreeding population

The double pseudo-testcross hypothesis was successfully applied to BSA mapping by separating the three types of markers. Data from either BSA-Seq or parental re-sequencing revealed three types of marker polymorphisms. The J- and G-type markers exhibit allelic heterozygosity and resemble the test cross for the maternal and paternal parent, respectively (Grattapaglia and Sederoff 1994). H-type markers exhibiting dual allelic heterozygosity are quite similar to the F2 progeny in inbreeding species (Ries et al. 2012b; Takos et al. 2006). The functional variations in the candidate genes controlling the target traits are also expected to possess the three types of polymorphisms (Bai et al. 2012b; Takos et al. 2006). Here, 13 major QTL were detected, four of which were from G, five of which were from J, and four of which were from the H polymorphism types (Table S14 and Figure 2-5). The predicted and validated functional mutations in the candidate genes also exhibited J-, G-, and H-type polymorphisms, which facilitates further gene exploration (Table S19 and Table S20). The QTL detected by using 3-type method exhibited the narrowest QTL intervals or the highest G’ value (File S2).

### Table 3, continued

| Isolate | Chromosome | QTL | gene ID | R/S | Mutation type | Gene annotation |
|---------|------------|-----|--------|-----|---------------|-----------------|
| Lw023   | Chr10      | G10.1| MD10G1111400 | S   | CREs & nonsynonymous SNV | Basic form of pathogenesis-related protein 1 |
| Lw023   | Chr10      | G10.2| MD10G1243000 | S   | nonsynonymous SNV | WRKY transcription factor 14 |
| Lw023   | Chr10      | G10.2| MD10G1243400 | R   | upstream | Receptor-like serine/threonine-protein kinase ALE2 |
| Lw023   | Chr14      | G14.2| MD14G1236800 | —   | nonsynonymous SNV | HCP-like superfamily protein |
| Lw023   | Chr14      | G14.2| MD14G1236600 | —   | nonsynonymous SNV | Prostaglandin E synthase 2 |
| Lw023   | Chr15      | H15.1| MD15G1106800 | S   | stop-gain | Leucine-rich repeat protein kinase family protein |
| Lw023   | Chr15      | H15.1| MD15G1103700 | S   | stop-loss | Disease resistance protein |
| Lw023   | Chr15      | H15.1| MD15G1104000 | S   | frame-shift deletion | Disease resistance protein |
| Lw023   | Chr15      | H15.1| MD15G1106600 | —   | CREs & nonsynonymous SNV | WRKY DNA-binding protein 11 |
| Zz26    | Chr02      | G02.1| MD02G1213800 | S   | nonsynonymous SNV | Pentatricopeptide repeat-containing protein |
| Zz26    | Chr11      | G11.1| MD11G116700 | S   | CREs | Ankyrin repeat-containing protein |
| Zz26    | Chr15      | G15.1| MD15G1416500 | S   | stop-gain | Disease resistance protein |
| Zz26    | Chr03      | H03.1| MD03G1259600 | —   | CREs | Serine/threonine-protein kinase |
| Zz26    | Chr14      | H14.2| MD14G1162500 | —   | CREs | Unknown |
| Zz26    | Chr16      | H16.1| MD16G1236700 | R   | CREs | Serine/threonine-protein kinase |
| Zz26    | Chr06      | J06.1_sub1 | MD06G1017400 | S   | CREs | F-box/kelch-repeat protein |
| Zz26    | Chr06      | J06.1_sub2 | MD06G1037600 | S   | CREs | NDR1/HIN1-like 8 |
| Zz26    | Chr08      | J08.1| MD08G1120500 | S   | stop-gain | Pentatricopeptide repeat-containing protein |
| Zz26    | Chr08      | J08.1| MD08G1120700 | S   | frame-shift | Pentatricopeptide repeat-containing protein |
| Zz26    | Chr10      | J10.1| MD10G1169200 | —   | CREs | Kinase-like protein TMKL1 |
| Zz26    | Chr10      | J10.2| MD10G1255900 | S   | stop-gain | F-box/kelch-repeat protein |
| Zz26    | Chr10      | J10.3| MD10G1288400 | —   | frame-shift deletion | Serine/threonine-protein kinase |
| Zz26    | Chr10      | J10.3| MD10G1288500 | —   | nonsynonymous SNV | Prostaglandin E synthase 2 |

**Notes:** Enriched pool (R/S/—): the enriched pools of the candidate functional variations, R: extremely resistant pools, S: extremely susceptible pools; ——: unknown; CREs: cis-acting regulatory elements.
the full utilization of polymorphic markers as well as the direct mapping of QTL on certain parent(s).

The GV method with a sliding window can realize noise reduction and unbiased statistical analysis for the three marker types. Three methods, namely SI, ED, and GV, are commonly used in BSA-Seq to reduce the noise and to measure the AFD between two extreme pools (Hill et al. 2013; Magwene et al. 2011; Takagi et al. 2013). In this study, 11 of the 12 significant QTL detected using the three methods coincided perfectly, indicating comparable effectiveness (Figure S5, Figure S6, Figure S7, and Table 2). However, the QTL profiles detected by GV were both narrower and more significant than those detected by SI (Table 2) and exhibited better unbiasedness and a lower false-positive ratio than that of ED (Table S11).

Sliding window analysis using different window sizes significantly narrowed down the QTL intervals. Fine-mapping based on large natural or pedigree segregation populations has been widely used but is both costly and labor-intensive/time-consuming (Bai et al. 2012a; Laurens et al. 2018; Terakami et al. 2016). It is very difficult to create recombinant inbred lines or near isogenic lines in outbreeding woody perennials, such as apple, pear, and peach, which greatly hinders fine mapping in these species (Di Pierro et al. 2016). Here we showed that a large amount of markers derived from the deeper re-sequencing of the two DNA pools made it possible to narrow down the QTL intervals with varied sliding window sizes analysis, e.g., for resistance/susceptibility to isolate Zz26, the marker density is close to 1059/Mbp on average (Table 1 and Figure S1), which is almost 200 times more than conventional MapQTL methods (Gardner et al. 2014; Sun et al. 2015). By using varied sliding window analyses, the spanning region of the QTL was narrowed down significantly from 1,639,342 bp to 707,246 bp, the averaged GV score increased from 5.55 to 6.03, and the stability of the QTL could also be evaluated (Figure S16 and Table S15).

Compared with the previous studies using the same segregating population, more QTL with much narrow interval were detected and a large number of previously reported QTL or major loci were reproduced in this study (Figure S11) (Cui et al., 2014; Zhuang et al., 2011). H14.1-Ls1, H14.1-Lw048 and H14.2-Zz26 coincided with Sf-g14-Ls1, Sf-g14-Lw048 (Table S14 and Figure S11) (Cui et al., 2014). Sf-j10-zz26 was split into three narrower QTL, J10.1-Zz26, J10.2-Zz26 and J10.3-Zz26, showing that the accuracy on QTL identification is higher by BSA-seq than MapQTL based on low density linkage maps (Cui et al., 2014). The pathogen isolate Mx1 was not used in this study because its weak pathogenicity to apple fruit (Zhuang et al., 2011). Therefore, a few QTL or major loci reported by Cui et al. (2014) and Zhuang et al. (2011) were not presented.

**Multi-omics enables gene culling from candidates in QTL regions**

Deep re-sequencing indicated a lot of genetic variation between the two parental cultivars, which is consistent with previous reports (Lee et al. 2016; Xing et al. 2016; Zhang et al. 2014). In the QTL intervals, genes lacking potential function-altering variations can be excluded. Transcriptomes can simultaneously indicate DEGs and genetic variations in CDS (Balan et al. 2018; Bonnet et al. 2017; Hill et al. 2013). In this study, 1,570 DEGs and 103,510 SNVs in the CDS of 24,533 genes were identified between the resistant and susceptible hybrids (Figure S14). These data, combined with large online transcriptome datasets, assisted the efficient excluding of many genes from the QTL intervals (Balan et al. 2017; Balan et al. 2018; Guo et al. 2017).

**The polygenetic signature of apple resistance to FRR provides evidence for the pathogenic variation among isolates**

In this study, phenotyping was performed for five years, and hybrids with year-long robust resistance/susceptibility were selected for bulk creation. A total of 46 QTL, including 13 major QTL, were ultimately detected for resistance/susceptibility to the four Botryosphaeria dothidea isolates (Table S14). At least three major QTL for each pathogen isolate were found. These QTL demonstrated the apparent polygenic signature of apple resistance to FRR, which corroborates previous reports (Cui et al. 2014; Zhuang et al. 2011). A variety of factors influence apple resistance to FRR, such as the size and density of the lenticels, the thickness of the wax layer, and the polyphenol content (Bai et al. 2015; Bénarouf and Parsi 2000; Fan et al. 2017; Li et al. 2013; Yu et al. 2014; Guan et al. 2015). Protein kinase plays an important role in signal transduction and pathogen recognition in the plant immune system (Park et al. 2012). Receptor-like protein kinases (RLPs) are widely reported to participate in plant-pathogen interactions (Krujit et al. 2005). The NDR1 gene was once reported to confer resistance to Pseudomonas syringae pv. tomato DC3000 and plays an important role in defense against bacteria and viruses, and in the response to salicylic acid (Varet et al. 2003; Varet et al. 2002). MD06G1037600 (MdNDR1), MD10G1288500 (RLP), MD10G1288400 (Serine/threonine-protein kinase-like protein), and MD10G1169200 (Kinase-like protein TMKL), which are associated with the activation of disease resistance, pathogen recognition, and disease resistance signaling, were predicted as the most likely candidates determining resistance/susceptibility to FRR (Table 3 and Table S19).

Only four of the 46 QTL for resistance/susceptibility to the four isolates coincided or overlapped with others (Table S14). The pathogenicity of B. dothidea differs significantly among isolates (Liu et al. 2011; Zhuang et al. 2011). The biological characteristics, pathogenicity, and internal transcribed spacer sequences differ significantly between pathogen strains of FRR (Lv et al. 2012). The diversity in resistance/susceptibility of apple to varied pathogen isolates supports the genetic differentiation of the pathogen according to the gene-for-gene hypothesis (Keen 1990; Van der Biezen and Jones 1998).

For a complex trait like apple ring rot disease resistance, large number of QTL each capture only a small proportion of the total genetic variance, and the pathogenicity varies dramatically among isolates (Cui et al., 2014). The 46 QTL identified in this study can be developed into QTL based genomics-assisted breeding or can be used as diagnostic markers among high density SNP arrays, which enables empirical genomic selection (Bianco et al., 2014; Bianco et al., 2016). In addition, the candidate genes can help further research on the molecular mechanism of apple FRR resistance.

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**ETHICAL STANDARDS**

The experiments were performed in accordance with all relevant Chinese laws.

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