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Exploring the Candidate Genes Responsible for Pear Scab Resistance Using Whole-Genome Re-Sequencing of Dna from Two Bulked Segregate Populations

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Abstract: Pear is one of the most important fruits globally; pear scab remains one of the most serious diseases afflicting this plant. However little work has been carried out on study on resistance mechanism of this disease in pear compared with other plants. Candidate genes responsible for pear scab resistance were explored in this study via whole-genome resequencing from two bulked segregate populations in BC1 group Yali × 03-04-034. Bioinformatic analysis of mine hub genes significantly related to pear scab resistance was used. The results indicated that seven hub genes including LOC103967201, LOC103938487, LOC103931625, LOC103929296, LOC103948157, LOC103947768 and LOC103932408 are related significantly to pear scab resistance, which is described as Nucleolar GTP-binding protein 1-like, Probable copper-transporting ATPase HMA5, CEN-like protein 2, Kinesin-like protein KIN-7F, Chaperone protein ClpB1-like, Polygalacturonase inhibitor-like and Haloacid dehalogenase-like hydrolase domain-containing protein 3. It is clear that these genes might contribute to scab resistance by participating in three pathways including protein processing in the endoplasmic reticulum as well as ribosome and protein export. The hub genes distribute within four key modules and collaborate with each other; this observation might explain our previous conclusion that pear scab resistance is controlled by four loci with recessive homozygous genotypes.

Key words: Pear Scab · Bulked Segregate Analysis · Whole-Genome Resequencing · Resistant Genes

INTRODUCTION

Pear is one of the most important fruits globally. This group comprises the third most abundant fruit species in China after apple and citrus; pears grown in China account for over 60% of global cultivated area and production [1]. At the same time, however, pear scab (*V. nashicola*) remains one of the most serious diseases afflicting this fruit. Pear scab has a worldwide distribution, often attacks leaves and fruits and causes the fall of these tissues; this reduces yields and quality in a given year as well as potential yields in subsequent years [2-6]. The application of chemicals remains the primary method for controlling this disease during the cultivation phase [7, 8]. This is not ideal, however, as frequent applications of fungicide can lead to rapid pathogen variations and therefore new issues in disease control. High levels of fungicide residue remaining on fruits can also pollute the environment, damage ecological systems and hinder the sustainable development of agriculture [9-11]. These issues mean that as consumers become more food-conscious and concerned about safety, reductions in or the overall elimination of fungicide use in fruit production have become increasingly important. It is therefore critical both economically and ecologically to study the mechanisms of pear scab resistance in order to better control disease occurrence and spread. Research in these areas will also lead to reductions in the application of fungicide and will provide clear benefits for farmers.

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Research on plant disease resistance mechanisms has emphasized resistance signal transduction pathways [12-14] as well as gene [15-19] and protein interactions [20-25]. A limited body of work has been conducted on resistance mechanisms to pear scab. In one example, workers were able to show that at the cytological level, this pathogen can penetrate the cuticle of all pear varieties be they susceptible, resistant, or immune. This process completes the infection process such that the pathogen forms vegetative hyphae within gaps between epidermal cells before then continuing to grow within pectin layers but not infecting cytoplasm [26, 27]. It is also clear that at the physiological level, resistance to pear scab might involve the extracellular matrix, leucine-rich repeat receptor-like protein kinases, catalase, superoxide dismutase, cutinase, pectinesterase and polygalacturonase-inhibiting proteins (PGIPs) [28-34]. Although this resistance might also be regulated by the salicylic acid (SA) signal transduction pathway [35], the degree of correlation and interactions amongst these factors remain unclear. A linkage map for pear scab resistance was initially constructed using RAPD technology for Japanese pear *P. pyrifolia* Kinchaku and *P. pyrifolia* Kosui [36] before a more detailed alternative was constructed for *P. pyrifolia* Hosui and *P. Communis* Barlett using Amplified Fragment Length Polymorphisms (AFLPs) and Single Sequence Repeat (SSR) technology, respectively. Numerous NBS-LRR genes were located in this iteration that might be related to pear scab resistance [37-39]. Subsequently, five AFLP, one SSR and five Sequence-tagged Site (STS) markers have been shown to be significantly linked to pear scab resistance genes; of these, two STS markers (STS-OPW2 and STS-OPO9) have been successfully applied in assisted selection pear scab resistance breeding [40-42]. A number of major effective loci related to scab resistance are also present in 1, 2, 4, 5, 7, 10, 1 and 7 linkage groups [43-46]. Researchers were then able to combine these observations with reports of scab resistance in apples and other near-source species, cloning several genes including *Vnp1* [47], *Rvp1* [48] and *Pbzsremorin* [49] from *P. bretschneideri* Zaosu as well as the gene *Hcrp* [50] from *P. bretschneideri* Cuiguan. The functions of these genes remain unknown. In sum, research on pear scab resistance has so far been limited to determining approximate genetic locations as well as the recovery of a few clones that might be related to resistance. The functions of these genes have not been verified. It is clear that the mechanisms underlying pear scab resistance remain unknown; research in this area lags behind that focused on other plant disease resistance mechanisms.

The research presented in this paper was initiated due to the successful breeding of super line 03-04-034, a lineage that is stable and highly resistant to pear scab [51]. This lineage was selected from 6, 902 available hybrids of *P. ussuriensis* Jingbaili and *P. bretschneideri* Yali. This approach is important because an extreme outbreak of pear scab took place in Changli, China, in 2005. Almost all hybrids were severely influenced by this outbreak which caused early leaf fall; just the 03-04-034 individual retained its vigor, was not infected at all and has remained highly resistant to pear scab over the next ten years. As the parent cultivars (Jingbaili and Yali) of 03-04-034 were both highly susceptible to pear scab, the generation of a stable and highly resistant hybrid had remained a remote possibility unless the group was large enough. It is clear that 6, 902 individuals were enough to meet this criteria; 03-04-034 is therefore very precious to our research on disease resistance inheritance and underlying mechanisms. This cultivar was then subsequently also back crossed with its parents to generate two BC1 groups including Yali × 03-04-034 and Jingbaili × 03-04-034. The genetic law of disease resistance means that we can utilize these two BC1 groups to analyze this phenomenon; it is likely that resistance is controlled by four pairs of recessive homozygous genes [51].

Candidate genes responsible for pear scab resistance were explored in this study by whole-genome resequencing from two bulked segregate populations in the BC1 group Yali × 03-04-034. This approach has enabled us to enrich our understanding of the pear scab resistance gene pool and provides a basis for future research on both mechanisms and gene utilization.

MATERIALS AND METHODS

Sample Collection and Preparation: This experiment was carried out in the Kongzhuang Basement and Central Laboratory of the Changli Institute for Pomology, Hebei Academy of Agriculture and Forestry Science, Changli County, China. This county (118°45'–119°20' E, 39°25'–39°47' N) is located in the northeastern part of Hebei Province, a monsoonal region within the warm temperate zone of eastern China that experiences a semi-humid continental climate. The annual average temperature of this region is 11°C; the frost-free period lasts for 186 days, accumulated temperature above 0? is 4, 231? and accumulated temperature above 10? is 3, 814?. The average annual precipitation of this region is 638.33 mm while the soil is a deep cinnamon color with a light texture and good permeability.

A BC1 group of Yali × 03-04-034 (comprising 2, 372 individuals) was assembled and planted in the Kongzhuang Basement between 2009 and 2010 at a density of 2 m × 0.4 m. A total of 20 one-year old *P. betulifolia* seedlings were then planted in the same place on March 1st, 2010; these seedlings were then cut to 10 cm above the ground on March 25th, 2010 and grafted with ten Yali scions and ten 03-04-034 scions. Over the period between 2010 and 2015, the disease resistance of this BC1 group was then evaluated (six consecutive years); totals of 162 stable non-susceptible and 65 highly susceptible individuals (disease index ≥ 65.0%) were identified.

DNA was extracted from fresh leaves collected from the two parents as well as from 30 non-susceptible (HR_bulk) and 30 highly susceptible (HS_bulk) individuals with the same potential vigor on May 2nd, 2018. This was done using via the Column CTAB Method for Large-scale Plant Genome Extraction Kit (SENO, SN0204, Zhangjiakou, China). The degradation and contamination of DNA was monitored using 1% agarose gels, while purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, California, United States). Concentrations of DNA were measured using a Qubit® DNA Assay Kit in a Qubit® 2.0 Fluorimeter (Life Technologies, CA, United States).

Library Preparation: A total of 1.5 μg DNA per sample was used as input material for sample preparations. Sequencing libraries were then generated using a Truseq Nano DNA HT Sample preparation Kit (Illumina, TG-202-1003, California, USA) following the manufacturer's recommendations while index codes were added to attribute sequences to each sample. Samples of DNA were fragmented by sonication to a size of 350 base pairs (bp); these fragments were then end-polished, A-tailed and ligated using the full-length adapter for Illumina sequencing for further PCR amplification. PCR products were then purified (AMPure XP system) and libraries were analyzed for their size distribution using an Agilent2100 Bioanalyzer and quantified using real-time PCR.

Illumina Sequencing: The libraries constructed above were then sequenced on an Illumina HiSeq2500 platform; this led to 125-bp paired-end reads with insert sizes around 350 bp.

Data Analysis

Quality Control: In order to ensure that the reads generated here were reliable and lacked artificial bias (including low quality paired reads which were resulted mainly from base-calling duplicates and adapter contamination), raw data (raw reads) in fastq format was initially processed through a series of quality control (QC) procedures using in-house C scripts.

The QC standards applied here involved the removal of:

- Reads with ≥ 10% unidentified nucleotides (nt).
- Reads with > 50% bases with phred quality < 5.
- Reads with > 10 nt aligned to the adapter allowing for ≤ 10% mismatches.
- Putative PCR duplicates generated by PCR amplification in the library construction process (cases where read 1 and read 2 of two paired-end reads are completely identical).

Reference Genome Mapping: Burrows-Wheeler Aligner (BWA (v0.7.10) was used [52] to align clean reads in each sample versus the reference genome *P. communis* genome assembly v2.0 (GDR) (settings: mem -t 4 -k 32 –M -R).

Alignment files were then converted to BAM files using the software SAM tools (v0.1.19) [53] (settings: –bS –t) and potential PCR duplications were removed using the 'rmdup' command. In cases where multiple read pairs possessed identical coordinates on genome reference, we retained those with the highest mapping accuracy.

SNP/InDel Detection and Annotation: Variant calling procedures were performed for all samples using the Unified Genotyper function in the software GATK (v3.3-0) [54]. The SNP function was utilized via the Variant Filtration parameters in GATK (settings: --filter Expression 'QD < 4.0 || FS > 60.0 || MQ < 40.0', -G_filter 'GQ <20', --cluster Window Size 4), while InDels were filtered using the Variant Filtration parameters (settings: --filter Expression 'QD < 4.0 || FS > 200.0 ||Read Pos Rank Sum < -20.0 || In breeding Coeff < -0.8 '). The software ANNOVAR (v2013Aug23) [55] was then used to annotate SNPs or InDels based on GFF3 files of the reference genome.

The SNP-Index: Different SNPs between two parents were extracted from vcf files. Thus, read depth information for homozygous SNPs in offspring pools were extracted in order to calculate the SNP-Index [56]. We then used the different SNPs of two parents as a reference to read statistical information for the different SNPs and others in the offspring pools. We then calculated the ratio of the number of different reads in total, the SNP-Index of base sites and filtered out those where the value was less than 0.3 in both pools. The sliding window method was then

Table 1: Seven candidate gene primers utilized for absolute qRT-PCR

Gene	Sense sequence	Sense sequence position	Antisense sequence	Antisense sequence position	Product length
LOC103967201	GAAGCAATGAAGACAGTGAT	951	TCTCTGATTCAACAACCTCT	1, 088	138
LOC103938487	CTATCGCTAAGGAAGTTGGA	932	AGTGCTGGTGAGTCATTG	1, 070	139
LOC103931625	GGAGAGTTATTGGAGATGTTG	35	GATGGTTACTGAGGAAGGAA	153	119
LOC103929296	AGCAGACAGGAGATAAGGA	2, 453	TCAGAGGAAGAAGGAGATTG	2, 588	136
LOC103948157	AGTTGCTCGGAGATTGAAT	2, 124	GCATCGTTAGAAGAGTTGAA	2, 260	137
LOC103947768	CTCTTCTCCTCCGTCCTAA	93	TACCAATCACAACAGTCAGT	235	143
LOC103932408	AGCAGCATTACTTGACACT	2, 191	TTCTCCACACCTTCTACAC	2, 338	148

used to evaluate a SNP-Index for the entire genome; this means that the average of all SNP-Index values for each window was then used in individual cases. We used a window size of 1 Mb and step size of 10 Kb as our default settings, the difference in SNP-Index values for the two pools was then calculated and reported as the Delta SNP-Index.

Bioinformatic Mining: Candidate Genes and a Co-expression Network: Co-expressions of candidate genes were mined using the Search Tool in the Retrieval of Interacting Genes (STRING, http://string –db.org) resource to construct a protein-protein interaction network (PPI) and analyze modules and pathways. Hub genes related to pear scab resistance in this PPI network were then clustered using the Molecular Complex Detection (MCODE) function in the software Cytoscape v3.7.2. The selection criteria for this step were MCODE scores > 5, degree cut-off = 2, node score cut-off = 0.2, max depth =100 and k-score = 2.

Expression Analysis of Candidate Resistant Genes Revealed by Quantitative Real-Time PCR: We investigated the expression patterns of genes LOC103967201, LOC103938487, LOC103931625, LOC103929296, LOC103948157, LOC103947768 and LOC103932408 using absolute fluorescence quantification real-time PCR (qRT-PCR). This analysis also incorporated tissue cultured seedlings from three highly susceptible (03-19-99, 03-20-15 and Jingbaili) and three highly resistant cultivars (D3, D8 and *P. bretschneideri* Huangguan), all cultured for 30 days. Thus, 03-19-99 and 03-20-15 both comprised super lines selected from Yali and Jingbaili combinations while D3 and D8 comprised dwarf pear stocks, shown to be highly resistant to pear scab via successive 5 years. Fresh leaves from each cultivar with the same potential were collected in triplicate for RNA extraction. Samples of total RNA were then extracted using the Polysaccharide and Polyphenol Plant Total RNA Extraction Kit with the Genomic DNA Eraser (SENO, SN0305-AD, Zhangjiakou, China) and reversely transcribed to complementary deoxyribonucleic acid (cDNA) with a PrimeScriptTM RT reagent Kit and a gDNA Eraser (Takaka, Dalian, China). Expression of these seven candidate genes was then assessed using absolute quantitative reverse transcription-PCR (qRT-PCR) in a SYBRGreen 2xqPCR Mix (SENO, DF0203, Zhangjiakou, China) and with an ABI 7500 Fast Real-time PCR system (Thermo Fisher Scientific, Waltham, United States). Primers were designed using Primer Premier 6 (Table 1) and were all synthesized by Sangon Biotech (Shanghai, China). Resultant cDNA was then amplified using the conventional PCR approach described by Lee *et al*.[57]; product concentrations were measured using an Ultra-micro spectrophotometer (BIO-DL, Micro Drop, Shanghai, China) while gene standard curves were drawn using the method outlined by Lee *et al*. [56].

RESULTS

Whole-Genome Resequencing Using Bulked Segregate Analysis (BSA) and BLAST Results: The whole-genome resequencing principle applied in this study is summarized in Figure 1. In this approach, two parents with extremely contrasting phenotypic values are crossed to generate a progeny population segregated for a given trait value. A progeny with a desired trait is then selected for self-crossing, multi-generational self-crossing, or parental backcrossing to obtain F2, RIL, or BC1 groups that are still segregated for a particular trait value. Two bulks composed of extremely contrasting phenotypes from these groups were then used to mine related candidate genes via whole-genome resequencing.

The stable and highly resistant line 03-04-034 was backcrossed in this study with its highly susceptible parent (Yali) in order to construct a BC1 group which directly reflects the distribution ratio of F1 generation gametes. A total of 30 non-susceptible and 30 highly susceptible individuals from this BC1 group were therefore designated as either a highly resistant bulk (HR_bulk) or highly susceptible bulk (HS_bulk). These two bulks were then further utilized to mine resistant candidate genes. As a result of the BLAST process, we determined the blasting ratio between the two bulks and the *P.* × *bretschneideri* genome assembly v1.1 (Genome Database for Rosaceae, GDR) to be about 60%.

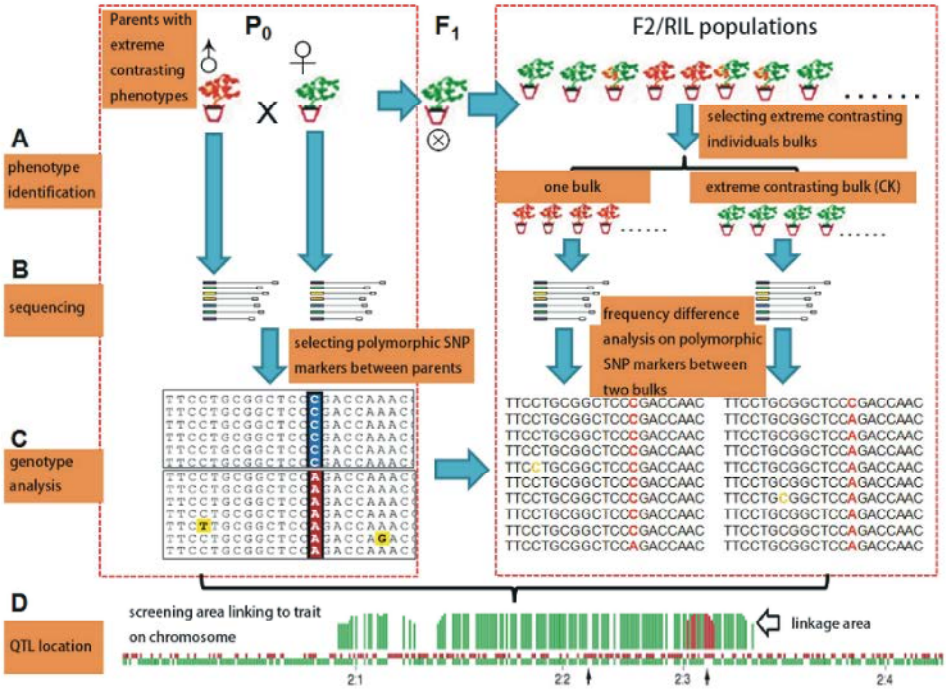

Fig 1. The principle and flow of BSA whole-genome resequencing

Table 2: Summary of sequencing data quality

Sample	Raw bases (bp)	Clean base (bp)	Effective rate (%)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
HSP	6, 406, 293, 600	6, 099, 727, 500	95.21	0.01	96.09	90.47	37.20
HRP	5, 912, 591, 400	5, 744, 384, 400	97.16	0.02	95.77	89.75	37.34
HS_bulk	12, 072, 844, 200	11, 807, 556, 900	97.80	0.02	95.61	89.46	37.33
HR_bulk	12, 949, 963, 500	12, 535, 923, 900	96.80	0.01	96.19	90.64	37.25

Table 3: Sequencing depth and coverage statistics

Sample	Mapped reads	Total reads	Mapping rate (%)	Average depth (X)	Coverage at least 1X (%)	Coverage at least 4X (%)
HSP	36, 465, 700	40, 664, 850	89.67	10.27	91.70	78.39
HRP	34, 023, 644	38, 295, 896	88.84	9.69	91.27	76.38
HS_bulk	70, 493, 322	78, 717, 046	89.55	19.09	96.79	90.38
HR_bulk	74, 775, 330	83, 572, 826	89.47	19.90	96.84	90.79

In contrast, the BLAST ratio between the two bulks and the *P. communis* genome assembly v2.0 (GDR) reached as high as between 77% and 80%, far higher than the earlier result (*P.* × *bretschneideri*).

The basic information recorded in the reference genome evaluated in this study was shown in Table 2. Effective rates were 95.21%, 97.16%, 97.80% and 96.80%, respectively, while error rates were 0.01%, 0.02%, 0.02% and 0.01%, respectively.

Sequencing depth and coverage statistics from this analysis are summarized in Table 3.

Whole-Genome Resequencing Based on Parental Background Identified One Candidate Gene Controlling Scab Resistance: The results of SNP tests using parental comparisons as a control were annotated and genotyped. A total of 4, 619 polymorphic markers were selected and the SNP-index of the two bulks within these marker loci were calculated and analyzed. The calculation method used for this step was the same SNP-index designated 0 while the markedly different SNP-index was designated as 1. Further, in order to mitigate the impacts of sequencing and BLAST errors, polymorphic loci following SNP-index calculations were filtered using two standards; the first of these was that loci with SNP-index characteristics of both bulks less than 0.3 and SNP depths were less than 7 were filtered out, while the other was that loci with missing SNP-index values were removed. A total of 2, 926 polymorphic marker loci were then obtained following this filtering step; these were distributed respectively on the

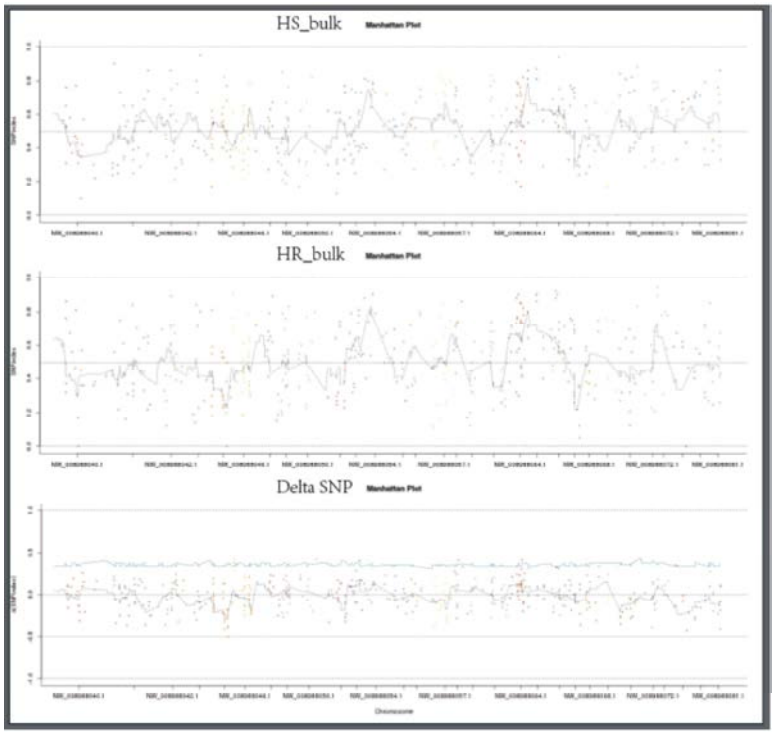

Fig. 2: SNP-index values for HS_bulk and HR_bulk alongside the Delta SNP index for the whole re-sequenced genome following parental comparisons. The x-axis of this graph shows the position of 19 chromosomes while the y-axis shows the SNP-index with a 1-Mb window at a 1-Kb step.

Table 4: Annotation of polymorphic marker loci with significant differences

	Category	Number of SNPs with parental background	Number of SNPs without parental background
Exonic	Stop gains	0	0
	Stop losses	0	0
	Synonymous	0	2
	Non-synonymous	0	4
	Upstream	1	10
	Intronic	0	0
	Splicing	0	14
	Downstream	0	8
	Upstream/downstream	0	0
	Intergenic	7	193
	ts	8	175
	tv	1	62
	ts/tv	8	2.82
	Total	9	237

chromosomes of the two bulks (Figure 2). Values of ? (SNP-index) were then calculated based on difference between the two bulks (HR_bulk SNP-index - HS_bulk SNP-index); This difference was permutation tested 1, 000 times and a 95% confidence level was applied as the screening threshold.

Results show that when a parental comparison was used as the control in this analysis, obtained Delta SNPs comprised additional selected SNPs with significant differences between the two bulks. The selected standard was therefore used to extract loci close to 0.7 in the HR_bulk sample and close to 0.3 in the HS_bulk. A total of nine polymorphic marker loci were selected (Table 4). Annotation conducted using ANNOVA enabled genes causing stop losses, stop gains, non-synonymous mutations and splicing to be preferentially selected as candidate genes. On this basis, a single candidate gene was selected (Table 5), LOC103932408 (haloacid dehalogenase-like hydrolase domain-containing protein 3).

Table 5: Candidate genes related to pear scab resistance

	TransID	Gene symbol	Variant	Chromosome	Pos	Ref	Alt	Description
Parental background present	gene32532	LOC103932408	upstream	NW_008988597.1	85657	C	T	Haloacid dehalogenase-like hydrolase domain-containing protein 3
Parental background absent	gene2662	LOC103967201	nonsynonymous	NW_008988049.1	495635	G	A	Nucleolar GTP-binding protein 1-like
	gene2920	LOC103929296	nonsynonymous	NW_008988050.1	818682	C	T	Kinesin-like protein KIN-7F
	gene8678	LOC103947768	nonsynonymous	NW_008988089.1	194372	G	A	Polygalacturonase inhibitor-like
	gene39085	LOC108866609	nonsynonymous	NW_008989080.1	23685	G	A	Uncharacterized mitochondrial protein AtMg00810-like
	gene6632	LOC103945513	upstream	NW_008988072.1	493050	T	C	TMV resistance protein N-like
	gene9046	LOC103948157	upstream	NW_008988092.1	448146	A	G	Chaperone protein ClpB1-like
	gene16665	LOC103956565	upstream	NW_008988179.1	326988	C	T	TMV resistance protein N-like
	gene19637	LOC103959836	upstream	NW_008988221.1	541167	T	C	Uncharacterized protein LOC103959836
	gene31006	LOC103930728	upstream	NW_008988521.1	340919	G	T	Histone H2A
	gene31813	LOC103931625	upstream	NW_008988556.1	28302	A	C	CEN-like protein 2
	gene32428	LOC103932294	upstream	NW_008988589.1	265283	A	G	Translation initiation factor IF-2-like
	gene33531	LOC103933489	upstream	NW_008988642.1	242182	A	G	Frataxin, mitochondrial-like
	gene38114	LOC103938487	upstream	NW_008989979.1	33018	T	C	Probable copper-transporting ATPase HMA5

Whole-Genome Resequencing Without Parental Background Identified 13 Candidate Genes Controlling Scab Resistance: In our subsequent analysis which parental comparisons were not used as a control, 5, 115, 942 polymorphic marker loci were obtained after annotating, genotyping, calculating and filtering. These loci were distributed on the chromosomes of two bulks (Figure 3). Thus, Delta SNPs were then further selected as SNPs exhibiting significant differences between the two bulks and a selecting standard was used to pick out loci close to 0.85 from the HR_bulk and close to 0.15 from the HS_bulk. A total of 237 polymorphic marker loci were therefore selected (Table 4). On the basis of ANNOVA annotation, genes causing stop losses, stop gains, non-synonymous mutations and splicing were then preferentially selected as candidate genes; this led to the selection of a total of 13 candidate genes related to pear scab resistance (Table 5), including LOC103967201 (nucleolar GTP-binding protein 1-like), LOC103929296 (kinesin-like protein KIN-7F) and LOC103947768 (polygalacturonase inhibitor-like), LOC103930728 (histone H2A).

Bioinformatic Mining for Candidate Genes: Co-expression genes for 14 candidates were mined. These were then assembled into co-expression networks; 11 candidate genes were found to be co-expressed (Figures 4 and 5), including LOC103967201 (XP_009378751.1), LOC103929296 (XP_009336747.1), LOC103947768 (XP_009356992.1), LOC103948157(XP_009357430.1), LOC103959836 (XP_009370480.1), LOC103930728 (XP_009338378.1), LOC103931625(XP_009339416.1), LOC103932294 (XP_009340130.1), LOC103933489 (XP_009341459.1),LOC103938487(XP_009346763.1)and LOC103932408 (XP_009340276.1). All candidates had 11 co-expression genes, of which LOC108866609 was not found in the *P.* × *bretschneideri* gene pool. In contrast, LOC103945513 and LOC103956565 were present but had no co-expression genes.

A network of co-expressed genes for all 11 candidates was constructed (Figure 6). This network has 121 nodes, 661 edges, an average node degree of 109 and a PPI enrichment p-value < 1.0e-16. This graph also exhibits significantly more interactions than expected; enrichment analysis indicates that genes resistant to pear scab as well as those that are co-expressed fare enriched in PFAM and SMART protein domains (Table 6), while KEGG pathway analysis showed that these genes are mainly enriched in endoplasmic reticulum protein processing, the sulfur relay system, thiamine metabolism and N-Glycan biosynthesis (Table 6).

A total of eight critical modules (including 69 hub genes) were extracted from the co-expression network using the software Cytoscape (Figure 7). These 69 hub genes were then further utilized to construct a hub gene co-expression network as well as for key pathway analysis (Figure 8 and Table 7). Information about this network includes a total of 69 nodes, 393 edges, an average node degree of 11.4 and a PPI enrichment p-value < 1.0e-16. This network also exhibits significantly more interactions than expected; enrichment analysis indicates that genes resistant to pear scab as well as their co-expressed counterparts are enriched in PFAM and SMART protein domains (Table 7), while KEGG pathway analysis reveals genes are mainly enriched in endoplasmic reticulum protein processing as well as ribosome and protein export (Table 7). Results reveal that seven candidate genes related to pear scab resistance are involved in these modules, specifically LOC103967201 (XP_009378751.1) (Figure 7A), LOC103938487 (XP_009346763.1) (Figure 7A), LOC103931625(XP_009339416.1) (Figure 7B), LOC103929296 (XP_009336747.1) (Figure 7C), LOC103948157(XP_009357430.1) (Figure 7E), LOC103947768(XP_009356992.1) (Figure 7E) and LOC103932408(XP_009340276.1) (Figure 7E). It is clear that LOC103967201 and LOC103938487 collaborated in the same module (Figure 7A), as did LOC103932408, LOC103948157 and LOC103932408 (Figure 7F).

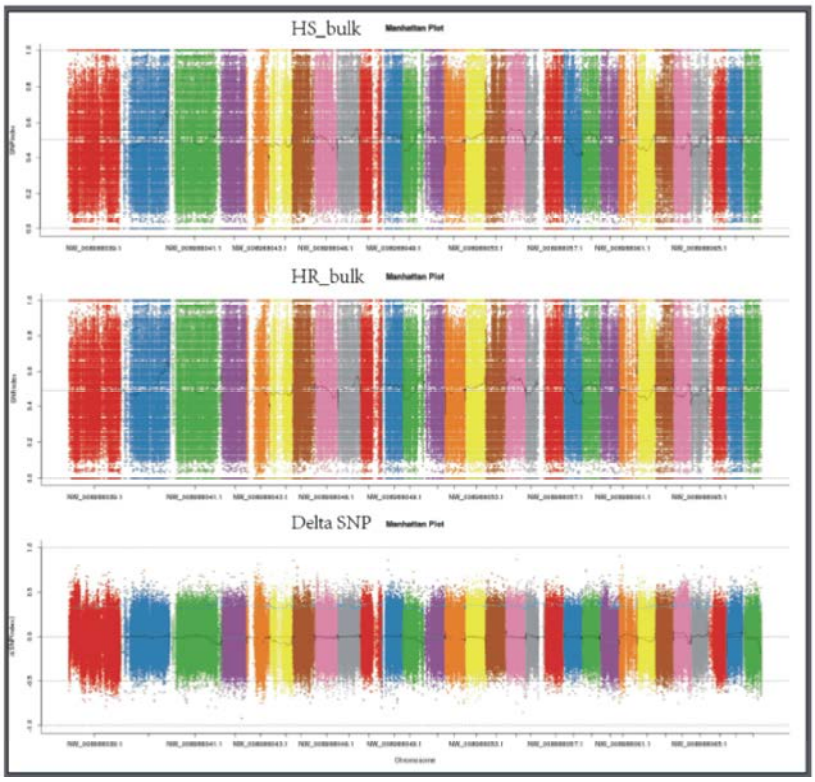

Fig. 3: SNP-index values for HS_bulk and HR_bulk samples as well as Delta SNP index values for the whole-genome resequencing analysis reported here given a non-parental background. The x-axis in this case shows the coordinates of 19 chromosomes while y-axis shows the SNP-index with a 1-Mb window and at a 1-Kb step

Table 6: GO and KEGG pathway enrichment analysis of genes resistant to pear scab alongside co-expressed examples

Term	Description	Observed gene count	Background gene count	False discovery rate (FDR)
PFAM Protein domains				
PF01212	Beta-eliminating lyase	6	12	3.00E-09
PF00266	Aminotransferase class-V	7	33	4.63E-09
PF01041	DegT/DnrJ/EryC1/StrS aminotransferase family	6	16	4.63E-09
PF00011	Hsp20/alpha crystallin family	7	57	7.20E-08
PF03345	Oligosaccharyltransferase 48 kDa subunit beta	4	4	2.11E-07
……	……	……	……	……
SMART protein domains				
SM00298	Chromatin organization modifier domain	4	21	4.60E-05
SM01349	XMAP215/Dis1 proteins, such as Alp14 and XMAP215, increase microtubules dynamic polymerization rates by recruiting soluble tubulin via conserved TOG domains to polymerizing microtubule plus ends.	2	3	0.0017
SM00879	The Brix domain is found in a number of eukaryotic proteins including SSF from yeast and humans, the *Arabidopsis thaliana* Peter Pan-like protein and in several hypothetical proteins.	2	10	0.0073
SM00838	Elongation factor G C-terminus	2	17	0.014
KEGG analysis				
pxb04141	Protein processing in endoplasmic reticulum	16	296	3.95E-13
pxb04122	Sulfur relay system	6	14	7.03E-10
pxb00730	Thiamine metabolism	6	35	5.13E-08
pxb00510	N-Glycan biosynthesis	4	59	0.00041
pxb03060	Protein export	4	58	0.00041
pxb03010	Ribosome	7	365	0.0014
pxb00860	Porphyrin and chlorophyll metabolism	3	64	0.006
pxb04145	Phagosome	3	103	0.0192
pxb01100	Metabolic pathways	17	2553	0.0199

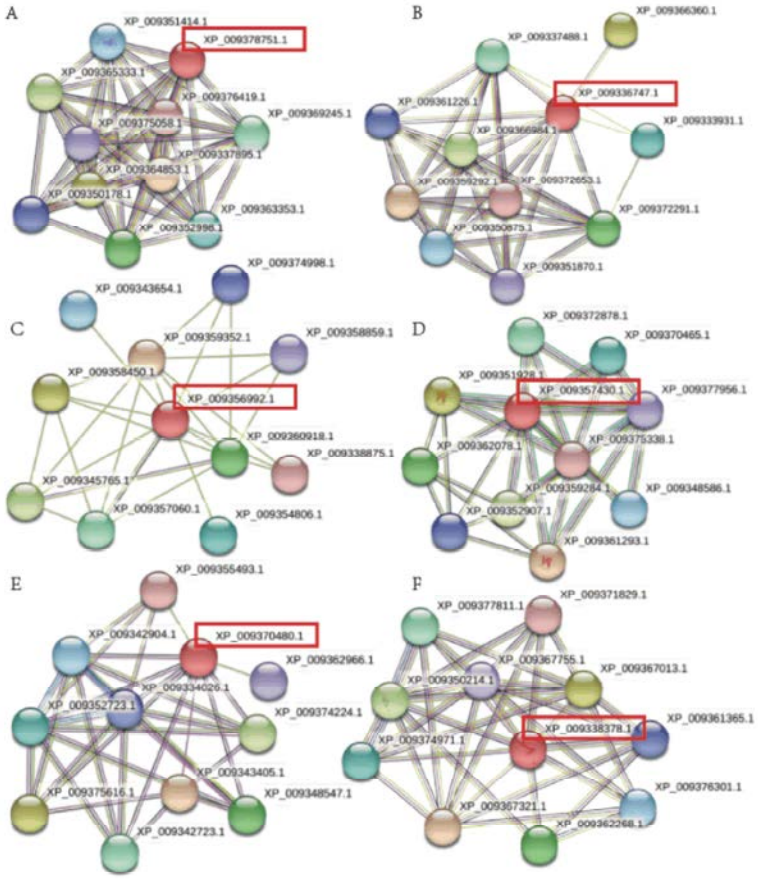

Fig. 4: Co-expression genes and networks for six candidate genes. A, co-expression genes and network for LOC103967201 (XP_009378751.1). B, co-expression genes and network for LOC103929296 (XP_009336747.1). C, co-expression genes and network for LOC103947768 (XP_009356992.1). D, co-expression genes and network for LOC103948157 (XP_009357430.1). E, co-expression genes and network for LOC103959836 (XP_009370480.1). F, co-expression genes and network for LOC103930728 (XP_009338378.1)

Table 7: Hub gene GO and KEGG pathway enrichment analysis

Term	Description	Observed gene count	Background gene count	FDR
PFAM Protein domains				
PF00573	Ribosomal protein L4/L1 family	4	5	1.07E-07
PF00011	Hsp20/alpha crystallin family	5	57	4.54E-06
PF10236	Mitochondrial ribosomal death-associated protein 3	3	3	4.54E-06
PF06068	TIP49 C-terminus	3	7	1.35E-05
PF01201	Ribosomal protein S8e	3	14	6.07E-05
……	……	……	……	……
SMART protein domains				
SM01349	XMAP215/Dis1 proteins, such as Alp14 and XMAP215, increase microtubules dynamic polymerization rates by recruiting soluble tubulin via their conserved TOG domains to polymerizing microtubule plus ends.	2	3	0.00064
SM00879	The Brix domain is found in a number of eukaryotic proteins including SSF proteins from yeast and humans, the Arabidopsis thaliana Peter Pan-like protein and in several hypothetical proteins	2	10	0.0021
KEGG analysis				
pxb04141	Protein processing in endoplasmic reticulum	9	296	9.76E-08
pxb03010	Ribosome	7	365	4.73E-05
pxb03060	Protein export	3	58	0.00088

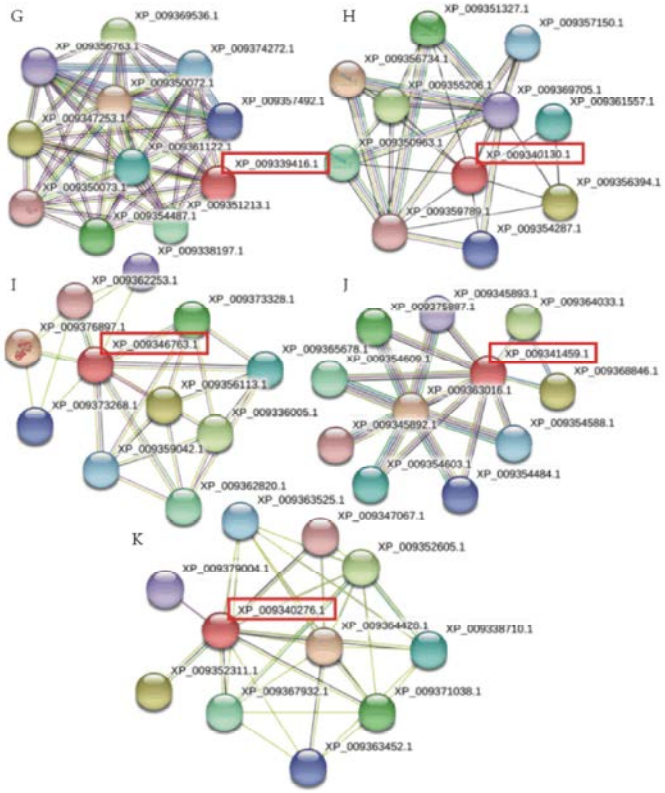

Fig. 5: Co-expression genes and networks for five candidate genes. G, co-expression genes and network for LOC103931625 (XP_009339416.1). H, co-expression genes and network for LOC103932294 (XP_009340130.1). I, co-expression genes and network for LOC103933489 (XP_009341459.1). J, co-expression genes and network for LOC103938487 (XP_009346763.1). K, co-expression genes and network for LOC103932408 (XP_009340276.1)

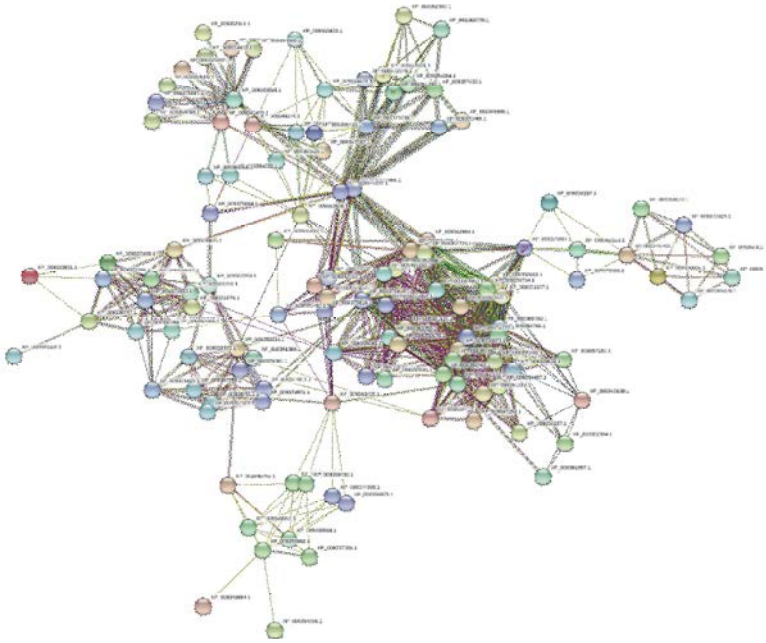

Fig. 6: A co-expression network for all candidates and co-expressed genes. This network was constructed using the P. x bretschneideri gene pool

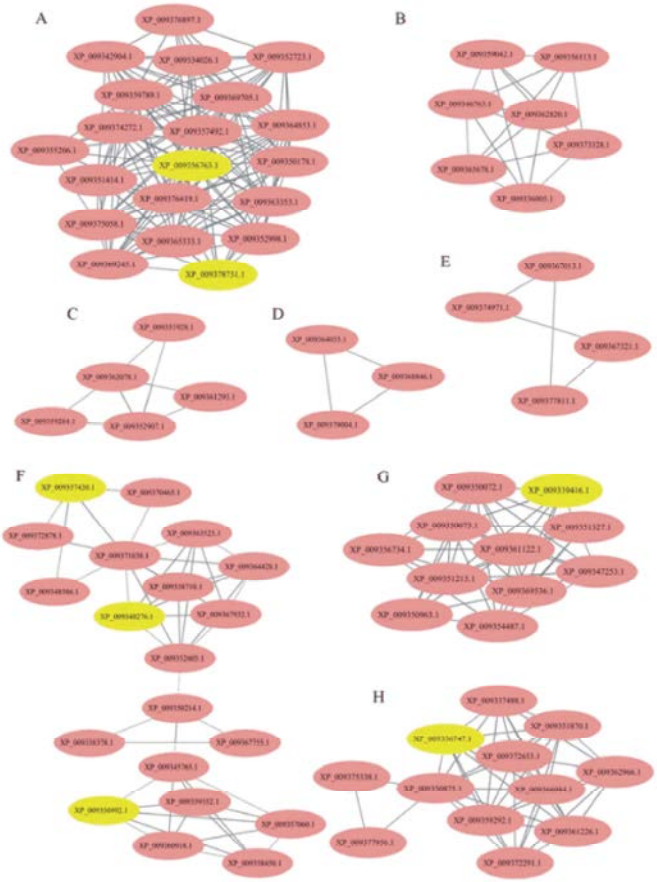

Fig. 7: Hub gene modules selected from the network resistant to pear scab and their co-expressed counterparts. Genes in yellow are candidates related to scab resistance

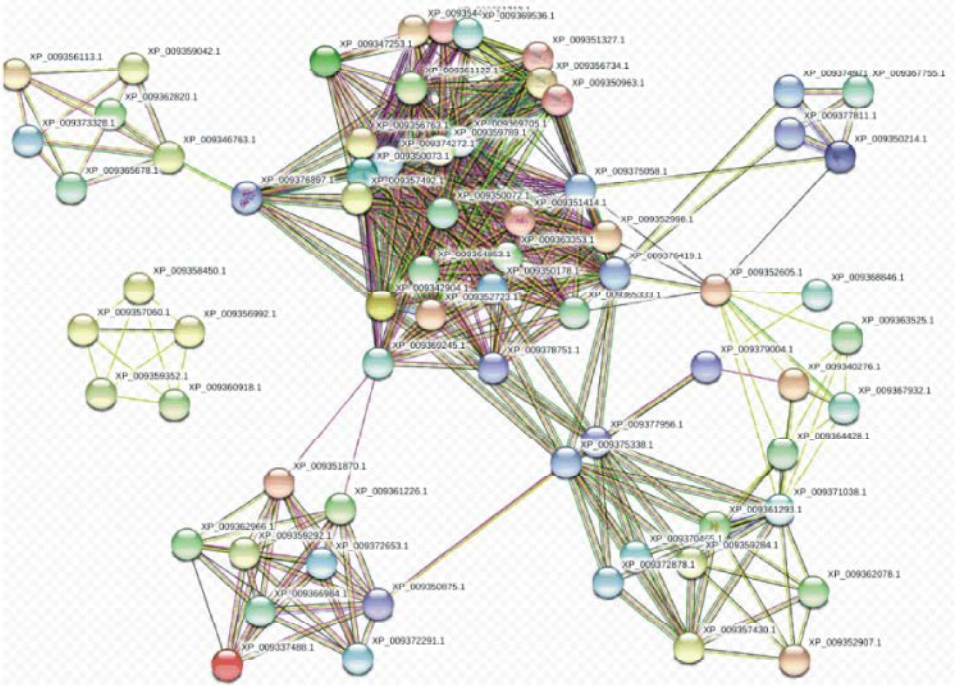

Fig. 8: Co-expression network for hub genes. This network was constructed for the P. x bretschneideri gene pool

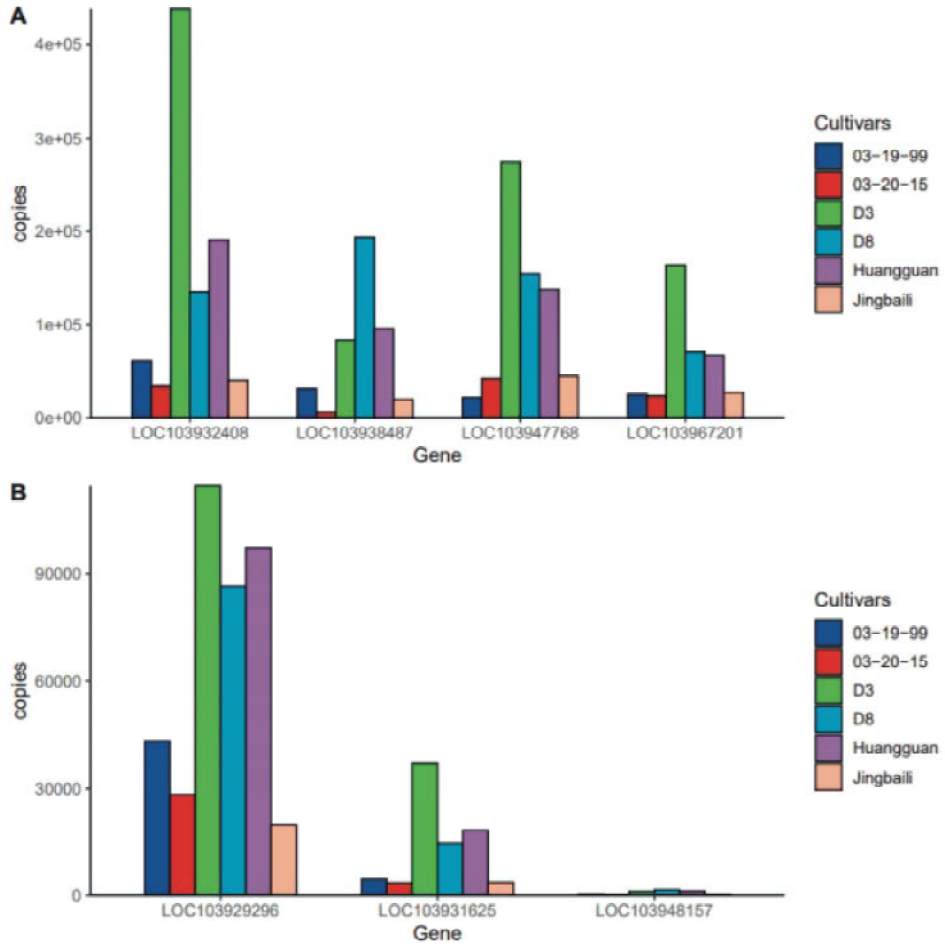

Fig. 9: Identification of seven hub genes using qRT-PCR. Cultivars 03-19-99, 03-20-15 and Jingbaili are highly susceptible to pear scab, while D3, D8 and Huangguan are highly resistant

Expression Analysis of Candidate Resistant Genes Using qRT-PCR: Absolute fluorescence qRT-PCR analysis of seven hub genes from six pear cultivars (including three highly resistant and three highly susceptible varieties) was performed. Results indicate that all seven genes are significantly expressed in highly resistant cultivars but exhibit very low expression levels in highly susceptible cultivars (Figure 9). Expression copies of all seven genes in highly resistant cultivars were twice the levels seen in highly susceptible cultivars, sometimes even as high as five times; indeed, expression copies of LOC103932408 in D3 occurred at a level six times higher than in Jingbaili. All of these seven genes are significantly related to pear scab resistance. Although the expression copies of LOC103931625 and LOC103948157 in cultivars remained low, these nevertheless accorded with the rule of high expression in highly resistant cultivars and low expression in the highly susceptible counterparts.

DISCUSSION

The cultivars Jingbaili and Yali are the parents of 03-04-034, a variety which is stable and highly resistant to pear scab. These plants belong to the species *P. bretschneideri* and *P. ussuriensis*, respectively and so the BC1 group is derived from the cross between Yali and 03-04-034. This latter cross turns out to be more inclined to *P. bretschneideri* and so the reference genome in this case should theoretically also be *P. bretschneideri*; however, during BLAST search, we found that the ratio between the BC1 group and the *P. bretschneideri* genome was about 60%. At the same time, the blasting ratio between the BC1 group and the *P. communis* genome could reach between 77% and 80%, so we used the *P. communis* genome as our reference in this analysis. It is generally the case that the sequencing error rate increases in concert with sequence length and so the error

rate at a single base position should be less than 0.5%. The error rates reported here were all less than 0.02%, an acceptable level.

Sequencing results were then analyzed step-by-step and one candidate gene with a parental background alongside 13 that lacked this background were selected and further subject to bioinformatic analysis to predict their function. Co-expression genes were found and networks were constructed using STRING. Thus, co-expression genes and networks for 11 candidates (including 121 genes) were obtained while the other three genes had no co-expression counterparts. A total of 121 genes were constructed in one co-expression network and further used to analyze related pathways while eight modules including 69 hub genes were finally extracted using Cytoscape. This analysis reveals that seven candidate genes are significantly related to pear scab resistance, distributed respectively within four modules and collaborating with one another (Figure 7AFGH). This result may explain our former conclusion, that pear scab resistance is controlled by four pairs of recessive homozygous genes [51].

The seven hub genes identified here that are significantly related to pear scab resistance include LOC103967201, LOC103938487, LOC103931625, LOC103929296, LOC103948157, LOC103947768 and LOC103932408. Descriptions for these genes are Nucleolar GTP-binding protein 1-like, Probable copper-transporting ATPase HMA5, CEN-like protein 2, Kinesin-like protein KIN-7F, Chaperone protein ClpB1-like, Polygalacturonase inhibitor-like and Haloacid dehalogenase-like hydrolase domain-containing protein 3, respectively. Earlier research has also shown that the nucleolar GTP-binding protein 1-like (LOC103967201) gene plays a novel role in plant innate immunity by regulating guard cell signaling in response to biotic stimuli via jasmonic acid (JA)- and abscisic acid (ABA)-mediated pathways [58]. At the same time, a number of other members of the RHO family of small GTPases and RAB GTPases have also been noted to have important roles in regulating SA- and JA-mediated defense signaling and stomatal immunity [59, 60]. Indeed, NOG1-1 and NOG1-2 in Arabidopsis have been identified as members of the OBG family, regulating ribosome biogenesis in yeast and bacteria. We have also demonstrated that this gene and its counterparts are enriched in the ribosome. The probable copper-transporter ATPase HMA5 has also been reported to be involved in copper tolerance variation mechanisms in Arabidopsis [61], while CEN-like protein 2 is thought to control intercalary meristem activity and phase changes [62, 63]. Kinesin-like proteins also play important roles in regulating respiration during seed germination at low temperatures and may be related to tolerance of this condition [64]. The chaperone protein ClpB has also been reported to be essential to heat-shock responses [65] and the polygalacturonase inhibitor-like gene has also been widely reported as playing an important role in regulating pear scab resistance [30-33]. The haloacid dehalogenase-like hydrolase domain-containing protein 3 has been reported so far just in humans and animals where is has been attributed to post-translational modifications and significant changes following chronic stress [66]. Apart from polygalacturonase inhibitor-like genes, all the others discussed here have not so far been reported to be associated with pear scab resistance. Nevertheless, they all play important roles in defenses against biotic and abiotic stress and so might participate in scab resistance via three possible pathways including protein processing in the endoplasmic reticulum, ribosome and protein export. The resistant genes identified in this paper greatly enriched our understanding of the pear scab resistance gene pool; the discovery of these elements provides valuable experience for mining other resistant genes.

CONCLUSIONS

A total of seven genes are shown here to be significantly related to pear scab resistance, including LOC103967201, LOC103938487, LOC103931625, LOC103929296, LOC103948157, LOC103947768 and LOC103932408. Descriptions for these elements are Nucleolar GTP-binding protein 1-like, Probable copper-transporting ATPase HMA5, CEN-like protein 2, Kinesin-like protein KIN-7F, Chaperone protein ClpB1-like, Polygalacturonase inhibitor-like and Haloacid dehalogenase-like hydrolase domain-containing protein 3. These genes may be attributed to scab resistance via participation in three possible pathways including protein processing in the endoplasmic reticulum, ribosome and protein export. The genes identified here are distributed respectively within four modules and collaborate with one another; this result may explain our former conclusion that pear scab resistance is controlled by four pairs of recessive homozygous genes.

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