

Construction of a High-Density SNP Genetic Map and QTL Mapping of Cold Tolerance with Candidate Gene Screening in Peach

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Abstract

Background Cold tolerance is a critical trait that constrains peach cultivation and production, directly determining the overwintering safety, regional adaptability and stable yield of peach trees. Clarifying the genetic regulatory mechanism of peach cold tolerance and mining core resistance genes are the key prerequisites for breaking the regional cultivation restriction of high-quality peach varieties.

Results To investigate its genetic basis, a high-density genetic map was constructed from an F_1 population of 150 seedlings generated by crossing the cold-tolerant cultivar 'Donghe No.1' with the cold-sensitive cultivar '21st Century'. Specific locus amplified fragment sequencing (SLAF-seq) generated 2,601 high-quality SNP markers mapped to eight linkage groups, spanning 761.55 cM with an average interval of 0.29 cM and 99.76% marker integrity; 99.29% of intervals between adjacent markers were ≤ 5 cM. Phenotypic data collected over three consecutive years (2022–2024) were used for QTL mapping of five cold-hardiness-related traits—relative electrical conductivity (REC), soluble sugar content (SS), proline content (Pro), starch content (St), and differential thermal analysis (DTA)—in one-year-old dormant shoots. A total of 12 significant QTLs were identified, with most located on linkage groups Pp05 and Pp08, and these explained 6.68%–16.38% of the phenotypic variance. Integration of the QTL intervals with transcriptomic profiles of both parents under -25°C cold stress prioritized nine key candidate genes based on differential expression and functional annotation.

Conclusions This study provides valuable genetic resources and candidate genes for investigating the molecular mechanisms of cold tolerance and advancing marker-assisted breeding in peach.

Introduction

The peach (*Prunus persica*) is native to China and has been cultivated for over 4,000 years. It is widely grown between 25° and 45° north latitude and is a typical temperate deciduous fruit tree [1, 2]. Low-temperature frost damage and late-winter cold snaps are major abiotic stresses that limit peach tree growth and yield formation; therefore, cold tolerance is directly associated with overwintering survival, geographic distribution, and yield stability [3]. Therefore, developing peach cultivars with high cold tolerance and elucidating the molecular genetic mechanisms underlying peach cold tolerance remain major research priorities in peach breeding.

Low-temperature stress disrupts plant cell membrane structure and increases membrane permeability and fluidity, subsequently triggering intracellular ion leakage, metabolic imbalance, and excessive accumulation of reactive oxygen species (ROS), ultimately leading to programmed cell death or even plant death [4, 5]. To cope with low-temperature stress, plants employ complex adaptive mechanisms at three major levels: morphological and structural adjustment, physiological and biochemical regulation, and molecular regulation [6–8]. At the physiological level, plants maintain cellular osmotic balance under low-temperature stress through the accumulation of osmotic regulators such as soluble sugars (SS) and proline (Pro), and by enhancing antioxidant enzyme activity—including superoxide dismutase (SOD) and

peroxidase (POD)—to scavenge ROS [9, 10]. At the molecular level, cold signals are perceived and transduced through pathways involving plasma membrane calcium channels and histone modifications, ultimately inducing cold-tolerance-related genes and activating defense responses [11–13].

Early studies on peach cold tolerance mainly focused on germplasm evaluation and physiological measurements. Cold-tolerant peach germplasm was screened based on changes in electrolyte permeability and malondialdehyde (MDA) content under low-temperature conditions. With rapid advances in omics technologies, research on peach cold tolerance has progressed from physiological analysis toward investigation of molecular regulatory mechanisms. During cold signal transduction, calcium signaling proteins interact with ROS-generating proteins and antioxidant enzymes to maintain ROS homeostasis and regulate peach responses to cold stress [14]. Several core transcription factors associated with peach cold tolerance have been identified in recent years. Previous studies showed that the transcription factor *PpWRKY58* is specifically and strongly induced by low temperature and positively regulates peach cold tolerance [15]. Transcriptomic analysis of peach flower stems further revealed that genes encoding U-box domain proteins, CBL-interacting protein kinases, NAC transcription factors, and dehydration-responsive element-binding proteins are significantly upregulated under cold stress and contribute to enhanced cold tolerance in peach [16].

Cold tolerance in peach is a typical complex quantitative trait controlled by multiple genes, and its phenotypic expression is strongly influenced by genotype-environment interactions [17]. Combining genetic linkage maps with quantitative trait locus (QTL) detection provides an effective approach for elucidating the genetic architecture of this complex trait [18]. Traditional genetic linkage maps, developed mainly using molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment-length polymorphism (AFLP), and simple-sequence repeat (SSR), often had insufficient marker density and low mapping resolution, limiting accurate detection of QTLs associated with cold tolerance [13, 19–21]. The emergence of next-generation sequencing (NGS) enables high-throughput identification of single-nucleotide polymorphism (SNP) markers and construction of high-density genetic maps. These advances substantially improve QTL-mapping resolution and facilitate the identification of causal candidate genes associated with peach cold tolerance [22, 23]. Such mapping approaches have been widely applied to dissect multiple agronomic and stress-adaptation traits in plants, including freezing tolerance in grapevine, wheat, and rice [24–26].

Nevertheless, despite substantial improvements in genetic map density and QTL interval resolution brought by high-throughput technologies, precise mapping of QTLs for peach cold tolerance remains challenging. As a polygenic quantitative trait strongly influenced by environmental variation, cold tolerance in peach tends to yield large and poorly defined QTL regions. Although high-density genetic mapping has become a major research frontier in peach genetics, only a limited set of cold-tolerance-related QTLs have been documented to date, and reliable fine-mapping efforts for this trait remain scarce. In this study, SLAF-seq technology was used to fine-map QTLs associated with peach cold tolerance and was combined with transcriptomic analysis of parental lines after low-temperature stress to screen candidate genes. The aim was to identify key genes regulating cold tolerance in the 'Donghe

No. 1' and '21st Century' varieties, thereby providing theoretical support and a technical basis for molecular breeding of cold-tolerant peaches.

Materials and methods

Plant materials and growth conditions

'Donghe No. 1', a semi-cultivated variety with high cold tolerance in cold regions and tolerance to temperatures as low as -30°C , was used as the female parent, whereas '21st Century', a widely cultivated variety in Hebei Province with relatively low cold hardiness, was used as the male parent. The cross was performed in 2019, and 150 F_1 -generation plants were obtained in 2020. The parental lines and hybrid progeny were planted at the Kongzhuang Base Resource Orchard of the Changli Fruit Tree Research Institute, Hebei Academy of Agricultural and Forestry Sciences ($39^{\circ}45'\text{N}$, $119^{\circ}12'\text{E}$), in a strip planting pattern with a spacing of $0.8\text{ m} \times 1.5\text{ m}$. The plot had sandy loam soil; the parental cultivars and the F_1 progeny were maintained under the same cultivation regimen throughout the growing period.

All peach materials, including cultivated varieties and preserved wild peach germplasm, are legally conserved resources maintained in the germplasm resource nursery of Changli Institute of pomology, Hebei Academy of Agriculture and Forestry Sciences.

Field investigation of cold tolerance

Freezing injury in the hybrid progeny was assessed for three consecutive years from 2022 to 2024. Freezing injury was graded as follows: Grade 1: No browning in xylem, phloem, and cambium; Grade 2: Slight browning in xylem and phloem; Grade 3: Obvious browning of xylem and phloem tissues; Grade 4: Aboveground parts died, while roots survived and could regenerate suckers; Grade 5: Complete death of both aboveground and underground parts.

Cold tolerance identification of F population

Beginning in mid-December each year during 2022–2024, 150 individual plants from the segregating F_1 population derived from the cross 'Donghe No. 1' $\times$ '21st Century', along with both parents, were selected for the experiment. One-year-old dormant shoots with consistent growth, diameter, and maturity were collected. The shoots were repeatedly washed with tap water and distilled water, and were then sealed in fresh-keeping bags for subsequent use.

Treatments included a normal-temperature control treatment at 4°C and cold-stress treatment at -25°C , with all shoot samples treated for 12 h. After treatment, cold tolerance-related physiological and biochemical indicators, including differential thermal analysis parameters (DTA), relative electrical conductivity (REC), soluble sugar (SS), starch (St), and free proline content (Pro), were determined. The

subordinate function method was used to comprehensively evaluate cold tolerance in the F_1 progeny. Detailed results and data were reported previously [27].

Library Construction and Illumina Sequencing

Whole-genome resequencing was used to genotype the two parental lines, whereas the 150 F_1 individuals were genotyped with an established modified SLAF-seq protocol [28]. Young leaves were collected from new shoots of the 150 F_1 segregating progeny and their parents, and genomic DNA was extracted from each sample using the CTAB method [29]. DNA integrity and purity were evaluated by agarose gel electrophoresis. In silico restriction digestion was performed using basic genomic information for *P. persica* (L.) Batsch, including genome size and GC content. The peach reference genome v2.0.a1 is available at ftp://ftp.bioinfo.wsu.edu/species/Prunus_persica/Prunus_persica_v2.0.a1/.

Following in silico double-digestion simulation using the peach reference genome, RsaI + HaeIII was selected as the optimal restriction-enzyme combination. Target SLAF tags were defined as fragments ranging from 264 bp to 314 bp, and a total of 100 999 SLAF tags were predicted. Qualified genomic DNA samples were double-digested with RsaI and HaeIII. The resulting products underwent end repair, 3'-A tailing, sequencing-adaptor ligation, and PCR amplification. Fragments of the expected size (264–314 bp) were excised from gels and purified to obtain valid SLAF tags. After quality-control assessment of the library, paired-end PE150 sequencing was performed on an Illumina platform (Illumina Inc., San Diego, CA, USA). Sequencing depths of 20-fold and 5-fold were used for the two parents and F_1 progeny, respectively. Raw sequencing reads were quality-filtered, and SNP calling was performed according to a previously described workflow [28]. Polymorphic SNP loci remaining after filtering were used to construct the genetic linkage map.

Construction and quality assessment of genetic maps

The genetic map was constructed using the double pseudo-testcross method [30]. Polymorphic SNP markers were subjected to the following filtering criteria to ensure map quality: (1) Markers with parental sequencing depth below 10× were removed to ensure accurate progeny genotyping. (2) Offspring genotype correction: SNP genotypes with progeny depth below 2× were treated as missing. (3) Completeness filtering: markers genotyped in at least 75% of the progeny were selected. (4) Segregation distortion marker filtering: polymorphic markers showing severe segregation distortion were filtered (Chi-square test $P < 0.001$). Pairwise MLOD values were calculated among the screened SNP markers for grouping, and markers with MLOD values below 3 relative to other SNP markers were removed. The retained markers were assigned to eight linkage groups. HighMap software was then used for each linkage group to determine the linear marker order, estimate genetic distances between adjacent markers, and construct a high-density genetic map [31].

QTL mapping analysis and candidate gene screening

Based on the high-density peach genetic linkage map constructed in this study and shoot cold-tolerance phenotypic data collected continuously across three years from 2022 to 2024, QTL mapping of cold tolerance in one-year-old peach shoots was conducted using the composite interval mapping method in MapQTL 6.0 software [32]. The mapping results were visualized using the ggplot2 package. The genome-wide logarithm of odds (LOD) threshold for QTL significance was estimated using 1000 permutation tests, which were performed separately for each trait under each treatment in each year. QTLs with maximum LOD values exceeding the corresponding permutation-derived threshold were regarded as statistically significant. The phenotypic variance explained (PVE) by each significant QTL locus was calculated at the LOD peak position. The mapped cold-tolerance QTL intervals were aligned against the physical map of the peach v2.0.a1 reference genome, and all gene annotation information within each QTL interval was extracted to support candidate gene screening. Genes within the QTL intervals were identified by marker mapping, and functional annotation was performed using the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Candidate genes were predicted according to their annotated biological functions.

Transcriptomic DEG analysis and qRT-PCR validation

The parental cultivars 'Donghe No. 1' and '21st Century' were subjected to transcriptome analysis after low-temperature (-25°C) stress, with three biological replicates included for each treatment. Differential expression analysis was performed using edgeR. Genes meeting the criteria $|\text{Log}_2(\text{Fold Change})| \geq 1$ and $\text{FDR} < 0.05$ were identified as significantly differentially expressed genes (DEGs).

Total RNA was isolated from shoots with the Universal Plant Total RNA Kit (TransGene, Beijing, China). First-strand cDNA synthesis was performed using the HiScript® II Q RT SuperMix for qPCR (+ gDNA wiper) kit (Cat. No. R223-01, Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative real-time PCR was carried out with ChamQ Universal SYBR qPCR Master Mix (Cat. No. Q711-02, Vazyme Biotech Co., Ltd., Nanjing, China). Primer sequences for the target and reference genes are provided in Table 1. Relative expression values were obtained by normalizing gene expression to the peach reference gene *PpActin* (PPA022840). The 25 μL qPCR reaction mixture contained 12.5 μL of 2 $\times$ ChamQ Universal SYBR qPCR Master Mix, 1 μL cDNA template, 0.5 μL forward primer, 0.5 μL reverse primer, with ddH₂O added to a final volume of 25 μL . RT-qPCR analysis included three biological replicates. For each biological replicate, three technical replicates were run to ensure precision. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [33]. Fold change was defined as the ratio of gene expression levels between the two cultivars.

Table 1
Primer sequences

Gene ID	Forward (5'--3')	Reverse (5'--3')
Prupe.4G056600_v2.0.a1	CACATGGCAAATGTGGGTGG	CTCCTCCCCAAGACAGCAAG
Prupe.4G057300_v2.0.a1	AGCACAACATTTCATCGCCG	CTCGTTTGCTCCAACAGGTG
Prupe.8G051700_v2.0.a1	TCACCCAAATCGGAGAAGGC	GGTGAATCCTCCGACAGCAA
Prupe.8G050900_v2.0.a1	TAGAAATCGTGCAGCAGCCA	CTTTTCACTGCCACAGCCAC
Prupe.8G053800_v2.0.a1	ACTGCGCCGGAATTTTGAAC	CCCAACGAGAGACGTGCGAAA
PpActin	GATTCCGGTGCCCAGAAGT	CCAGCAGCTTCCATTCCAA

Results and analysis

Sequencing data analysis

Sequencing of the SLAF-seq library on the Illumina platform generated a total of 267,179,514 raw reads (Table 2). Across all samples, the mean Q30 score and GC content were 93.89% and 41.15%, respectively, with a normal GC distribution. The maternal parent 'Donghe No. 1' generated 40,817,877 reads, with an average Q30 of 91.33% and an average GC content of 38.83%; the paternal parent '21st Century' generated 34,219,259 reads, with an average Q30 of 90.34% and an average GC content of 40.11%. Each progeny sample generated an average of 1,280,949 reads, with a mean Q30 of 93.93%, an average GC content of 41.17%, and a normal GC distribution. Overall, the sequencing data were of high quality and suitable for subsequent analyses.

Table 2
Sequencing data for the parents and F₁ progeny

Item	Total reads	Bp Total bases	Q30 percentage/%	GC percentage/%
Donghe No. 1	40 817 877	6 122 681 550	91.33	38.83
21st Century	34 219 259	5 132 888 850	90.34	40.11
Offspring	1 280 949	192 142 350	93.93	41.17
Control	179 781	26 967 150	93.37	42.75
Total	267 179 514	40 076 927 100	93.89	41.15
Note: Total Clean Reads: number of reads after filtering; Total Clean Bases: number of bases after filtering; Q30: percentage of bases with a sequencing quality value higher than 30. The same definitions apply below.				

SNP Tag Development and Genotyping Screening

Sequencing reads were mapped to the peach reference genome using BWA, and variant calling was performed with the GATK toolkit for the parents and progeny. As shown in Tables 3, 558,496 and 477,153 SNP markers were detected in the maternal cultivar ‘Donghe No. 1’ and the paternal cultivar ‘21st Century’, with heterozygosity rates of 75.98% and 27.57%, respectively. The average number of SNPs detected in the segregating F₁ progeny was 110,887, with an average heterozygosity rate of 32.88% (Table 3). In total, 978,613 SNP markers were obtained across all samples. Markers with missing parental genotypes, parental sequencing depth less than 4×, and non-polymorphic markers between parents were removed first. The remaining 680,934 markers were classified into eight segregation types for the CP (double pseudo-testcross) population. Because the aa×bb segregation type is applicable to inbred populations rather than the CP population, markers of aa×bb type were excluded, leaving 479,738 candidate polymorphic markers (Table 4, Fig. 1).

Table 3
Statistics of sequencing data alignment and SNP information

Sample ID	Mapped reads/%	Properly mapped reads/%	Number of SNPs	Heter ratio/%
Donghe No. 1	96.29	88.38	558 496	75.98%
21st Century	92.71	87.55	477 153	27.57%
Offspring	96.18	86.68	110 887	32.88%

Table 4
Statistics of SNP classification

Type	Number	Type	Number	Type	Number
Total markers	978 613	aa×bb	201 196	hkxhk	59 210
Missing parental markers	219 210	ab×cc	442	lmxll	350 544
Depth below 4x	8 188	ab×cd	2	nnxnp	68 972
Non-polymorphic markers	70 281	cc×ab	120	/	/
Remaining markers	680 934	ef×eg	448	/	/

Construction of Genetic Linkage Map

Additional stringent filtering criteria, including parental sequencing depth ≥ 10×, genotype integrity ≥ 75% in progeny, and removal of markers with severe segregation distortion (Chi-square test, P < 0.001) were applied. A total of 3,961 high-quality SNP markers were retained for genetic map construction. Pairwise MLOD values were calculated for the 3,961 selected SNP markers. The minimum and maximum marker numbers per linkage group were specified, together with a predefined MLOD value range. Markers were ordered in ascending order according to their MLOD values. Those showing the highest pairwise MLOD values were assigned to the same linkage group. Markers with MLOD values below 3 relative to all other

SNP markers were removed. Ultimately, eight linkage groups comprising 2,601 mapped markers were constructed, with a total map distance of 761.55 cM (Fig. 2).

Genetic Map Evaluation

The constructed genetic map contained 2,601 markers across 8 linkage groups, with an average of 325 markers per linkage group. The average genetic distance between adjacent markers was 0.29 cM (Table 5). Of the mapped markers, 292 showed segregation distortion, accounting for 11.23% of all markers. In the integrated genetic map, the number of SNP markers per linkage group (LG) ranged from 242 on LG04 (total length 63.81 cM, average marker interval 0.26 cM) to 482 on LG01 (total length 101.74 cM, average marker interval 0.21 cM). Among all LGs, LG04 was the shortest and harbored the fewest SNP markers, whereas LG06 was the longest, containing 307 markers with a total genetic distance of 132.12 cM and an average marker interval of 0.43 cM. The largest gap differed among LGs, ranging from 5.11 cM on LG04 to 12.00 cM on LG06. Regarding marker density, LG06 was the sparsest, whereas LG01 was the densest, with 482 markers and an average interval of 0.21 cM between adjacent markers.

Table 5
Distribution of Molecular Markers and Fragment-Separated Marker Loci on Linkage Groups

LG ID	MarkerNum	Total Distance/cM	Average Distance/cM	Gaps < = 5	Max Gap/cM	Total BS
Pp01	482	101.76	0.21	99.38%	8.14	22
Pp02	263	94.94	0.36	99.24%	7.48	8
Pp03	320	107.38	0.34	99.69%	6.29	46
Pp04	242	63.81	0.26	99.59%	5.11	0
Pp05	292	74.47	0.26	99.31%	7.51	38
Pp06	307	132.12	0.43	98.69%	12.00	102
Pp07	417	101.35	0.24	99.52%	10.14	0
Pp08	278	85.72	0.31	98.92%	7.44	76
Total	2 601	761.55	0.29	99.29%	12.00	292

Total BS: Number of distorted markers mapped on this linkage group

Sequencing-depth analysis showed average marker depths of 40.27× for the parents and 26.64× for the offspring across linkage groups. These relatively high depths supported the accuracy of the molecular markers (Table 6). Marker-integrity analysis for each individual in the mapping population showed an average integrity of 99.76%, supporting accurate genotyping for genetic map construction (Fig. 3C). Most recombination blocks were successfully identified in the haplotype maps. Larger segments within

each individual retained a consistent origin, indicating the high quality of the genetic map (Fig. 3A). The heatmap also showed strong linkage relationships between adjacent markers within linkage groups (Fig. 3B).

Collinearity analysis was performed by comparing marker positions on the genome and the genetic map, and the Spearman correlation coefficient between each linkage group and the physical map was calculated. The closer the Spearman coefficient was to 1, the better the collinearity between the genetic and physical maps (Fig. 3D, Table 7). In this study, the order of most markers on each linkage group was consistent with the genome, indicating good collinearity and high accuracy of the calculated genetic recombination rates. These findings confirm that this genetic map is suitable for subsequent QTL mapping analyses.

Table 6
Statistics of marker sequencing depth

Item	MarkerNum	Total Depth	Average Depth
Donghe No. 1	2,601	100,071	38.47
21st Century	2,601	109,388	42.06
Offspring	2,497	66,528.17	26.64

Table 7
Correlation Coefficients of Each Linkage Group

LG ID	Spearman	LG ID	Spearman
Pp01	0.92	Pp05	0.86
Pp02	0.93	Pp06	0.91
Pp03	1.00	Pp07	0.95
Pp04	0.96	Pp08	0.92

QTL Mapping of Cold-Tolerance Traits in Peach and Identification of Candidate Genes

QTL mapping of peach cold-tolerance-related traits was performed using MapQTL software and physiological indicator data collected continuously from 2022 to 2024. 12 QTL loci significantly associated with peach cold tolerance were identified and distributed across chromosomes 3, 4, 5, 6, 7, and 8 (Table 8, Fig. 4). The maximum LOD values ranged from 2.20 to 5.59, with phenotypic variance explained (PVE) values ranging from 6.68% (*qREC08-2*) to 16.38% (*qDTA06-1*). Among these QTLs, two QTLs governing proline and starch contents, *qPro03-1* (2023) and *qSt03-2* (2024), were co-localized within the same genetic interval (38.73–38.73 cM) on chromosome 3, both containing 11 candidate genes. Notably, a prominent cold-tolerance-related QTL cluster was identified on chromosome 8,

including three loci detected across different years: *qSS08-3* (75.07 cM, 2023), *qREC08-1* (76.13–76.48 cM, 2022), and *qREC08-2* (79.32–82.32 cM, 2023), among which *qREC08-1* showed a notably high phenotypic contribution, with a PVE of 14.80%. These closely distributed loci control soluble sugars and relative electrical conductivity, indicating that this genomic region is strongly associated with peach cold tolerance. In addition, *qDTA06-1* on chromosome 6 showed the strongest phenotypic effect among all detected QTLs, with a maximum PVE of 16.38%.

In total, 1,363 genes were initially annotated across the genomic intervals of the 12 detected cold-tolerance QTLs. After removing redundant genes from overlapping QTL regions, a final set of 1,290 unique genes was obtained, which lays a solid foundation for the subsequent screening and identification of candidate genes associated with cold tolerance in peach.

Table 8
Positions and Phenotypic Variance Explained of QTLs for Cold Hardiness in the Peach F₁ Population

LG	Year	^a QTL	Genetic position /cM	Marker number	Maximum LOD	PVE(%)	Gene number
Pp03	2023	<i>qPro03-1</i>	38.73–38.73	2	3.54	10.69	11
Pp03	2024	<i>qSt03-2</i>	38.73–38.73	2	2.69	8.25	11
Pp04	2024	<i>qPro04-1</i>	53.25–53.95	4	3.13	9.53	214
Pp04	2022	<i>qDTA04-2</i>	42.73–44.13	5	3.08	9.34	296
Pp05	2023	<i>qSt05-1</i>	38.87–38.87	2	4.64	13.76	13
Pp05	2022	<i>qREC05-1</i>	61.66–61.66	23	2.20	6.78	147
Pp05	2024	<i>qREC05-2</i>	19.12–19.12	5	2.70	8.27	0
Pp06	2024	<i>qDTA06-1</i>	6.33–6.33	4	5.59	16.38	8
Pp07	2023	<i>qDTA07-1</i>	4.04–4.04	8	4.40	13.13	207
Pp08	2022	<i>qREC08-1</i>	76.13–76.48	4	4.31	14.80	69
Pp08	2023	<i>qREC08-2</i>	79.32–82.32	13	2.16	6.68	172
Pp08	2023	<i>qSS08-3</i>	75.07–75.07	7	4.44	13.25	226

LG, linkage group; ^aQTL names represent the traits, linkage group, and order of the QTLs.

Transcriptome Differential Gene Analysis and Screening of Cold-Tolerance Candidate Genes

RNA was extracted from one-year-old dormant shoots of 'Donghe No. 1' and '21st Century' under normal-temperature and -25°C cold-stress conditions, respectively. mRNA was purified and reverse-transcribed into cDNA for differentially expressed gene (DEG) analysis. Under normal-temperature conditions, 1 509 stable DEGs were identified between the two parents, comprising 768 upregulated genes (50.89%) and 741 downregulated genes (49.11%). Under -25°C cold-stress conditions, 1 252 stable DEGs were detected between the two parents, including 716 upregulated genes (57.19%) and 536 downregulated genes (42.82%) (Fig. 5a).

Randomly selected DEGs were validated by qRT-PCR to assess the reliability and accuracy of the transcriptome sequencing data. Expression trends of the tested genes were highly consistent between the qRT-PCR and transcriptome datasets, supporting the reliability of subsequent differential expression analysis and candidate gene screening (Fig. 5b).

A Venn diagram analysis was performed using genes within QTL intervals and DEGs identified under normal-temperature and cold-stress conditions (Fig. 5c). Forty-two genes were common to the QTL-interval gene set, normal-temperature DEGs, and cold-stress DEGs. Thirty-one genes were located within QTL intervals and were differentially expressed only under normal-temperature conditions, whereas 19 QTL-located DEGs responded specifically to cold stress. In addition, 852 DEGs were detected under both normal-temperature and cold-stress conditions but were located outside the QTL regions; these genes were excluded from subsequent screening because they lacked genetic positional evidence.

All QTL-located DEGs were classified into two categories for comprehensive screening of cold-tolerance candidate genes. The first category consisted of 42 overlapping genes that were differentially expressed between the two parents under both normal and cold conditions within the cold-tolerance QTL intervals, and were considered core candidates with stable genotypic expression variation. The second category consisted of 19 cold-specific DEGs located within QTL intervals; these genes showed no differential expression under normal-temperature conditions but were significantly differentially expressed under cold-stress conditions. Importantly, the expression divergence of these 19 genes was induced specifically by low-temperature stress rather than by inherent genetic differences between the parents, indicating that they are important functional candidates for cold response and cold-tolerance regulation in peach (Fig. 5c).

A total of 61 QTL-based DEGs (42 core DEGs and 19 cold-specific DEGs) formed the primary candidate pool and were subsequently annotated using the NR and Swiss-Prot databases. Genes encoding unknown or hypothetical proteins and those functionally unrelated to stress responses were excluded. Genes associated with responses to abiotic stress, calcium signal transduction, transcriptional regulation, lipid metabolism, and cell-wall biosynthesis were retained. Transcriptome profiles were then used to prioritize genes with clear expression differences between the two parental genotypes under

cold stress. This multistep screening identified nine key candidate genes associated with peach cold tolerance, including NAC family transcription factors, calcium signaling components, lipid metabolism-related proteins, and cell-wall synthesis-related proteins. Among them, *Prupe.8G051400.1_v2.0.a1* encodes a GDSL esterase involved in epidermal lipid remodeling, while *Prupe.8G051700.1_v2.0.a1* encodes the LRR receptor kinase PSKR1, which plays a vital role in Ca²⁺-dependent cold signal transduction (Table 9).

Table 9
Cold-Tolerance Candidate Gene Screening Results

Gene ID	Gene description
<i>Prupe.5G090500_v2.0.a1</i>	Dehydration-responsive element-binding protein 1F (DREB1F)
<i>Prupe.8G035400.1_v2.0.a1</i>	NAC domain-containing protein 78 T32M21.10
<i>Prupe.8G035100.1_v2.0.a1</i>	Cellulose synthase A catalytic subunit 2 T22F8.250
<i>Prupe.8G085500.1_v2.0.a1</i>	Cold shock protein 1 T19K4.150
<i>Prupe.8G051400.1_v2.0.a1</i>	GDSL esterase/lipase At1g29670
<i>Prupe.8G067800.1_v2.0.a1</i>	Mitochondrial uncoupling protein PUMP4
<i>Prupe.7G007800.1_v2.0.a1</i>	Cold signal transduction, MAPK cascade MEKK1/MAPKKK1
<i>Prupe.5G027700.1_v2.0.a1</i>	CSLD3 cellulose synthase-like protein
<i>Prupe.8G051700.1_v2.0.a1</i>	LRR receptor-like serine/threonine-protein kinase PSKR1 At2g24230

Discussion

High-density genetic map and QTLs for peach cold tolerance

A high-density genetic linkage map is an important prerequisite for fine mapping of quantitative trait loci (QTL) in fruit trees, candidate-gene identification, and analysis of genetic mechanisms [34–37]. Compared with traditional SSR and AFLP markers, SLAF-seq can efficiently generate high-density SNP markers with stable genotyping and is therefore suitable for precise genetic analysis of complex quantitative traits in fruit trees [38, 39]. In this study, the ‘Donghe No.1’ × ‘21st Century’ F₁ hybrid population of peach served as the mapping population, and a peach genetic linkage map containing 2,601 high-quality SNP markers across eight linkage groups was constructed based on SLAF-seq technology. The total map length was 761.55 cM, with an average marker interval of 0.29 cM and marker integrity of 99.76%. Based on these indicators, the quality of this map exceeds that of most previously reported genetic maps for peach cold tolerance, effectively overcoming the limitations of traditional molecular markers in resolution and marker resources. The map enriches molecular marker resources

for the peach genome and provides a reliable genomic platform for high-precision genetic analysis of cold tolerance and other horticultural quantitative traits in peach.

Peach cold tolerance is a complex quantitative trait controlled by multiple genes, with trait expression jointly influenced by genetic background and field factors such as temperature, light, precipitation, and microenvironment [40, 41]. Phenotypic values vary among years, which increases the difficulty of identifying stable cold-tolerance loci [42]. Using several years of continuous cold-tolerance phenotyping data together with composite interval mapping, this study identified eight reproducible QTLs associated with cold tolerance across two linkage groups: Pp03 and Pp08. Among them, linkage group Pp08 was enriched with multiple major-effect cold-tolerance QTLs, with the core locus qREC08-1 explaining 14.80% of the phenotypic variation, making it an important target region for molecular breeding of cold-resistant peaches. Current QTL research in peach mainly focuses on horticultural traits such as fruit quality and tree architecture, whereas high-density fine-mapping studies of cold-tolerance traits remain relatively scarce. The present results help address this gap in peach cold-tolerance genomics. QTL regions that are stably co-located across multiple years represent conserved functional regions involved in low-temperature responses in peach, providing precise targets for development of cold-tolerance functional markers and identification of core genes.

Integrating QTL-interval gene annotation with differential transcriptome analysis of the parents under low-temperature stress identified nine core candidate genes for peach cold tolerance, primarily associated with transcriptional regulation, stress signal transduction, cell membrane lipid metabolism, and low-temperature defense responses. NAC transcription factors, cold shock proteins, and ASR1 proteins can enhance plant cold tolerance through activation of downstream cold-responsive genes and maintaining cell membrane stability [43–46]. DREB1F and DREB3/TINY2 transcription factors also positively regulate plant cold tolerance: DREB1F improves low-temperature tolerance by modulating ROS homeostasis and cold-responsive signaling pathways, whereas DREB3/TINY2 mediates crosstalk between cold and drought stress responses to balance plant stress tolerance and growth [47, 48]. In addition, GDSL esterase/lipase proteins and LRR receptor kinases can mediate adaptive responses to low-temperature stress in plants by regulating cell membrane lipid remodeling and calcium signaling pathways [49–51]. Although some candidate genes showed no significant correlation between transcriptomic expression and cold-tolerance phenotypes, possibly because of sampling time, stress gradients, or differences in sequencing material, their potential roles in peach cold tolerance require further validation.

The cold-tolerance QTLs identified in this study explained 6.68%–16.38% of phenotypic variation, consistent with the genetic characteristics of quantitative traits in perennial fruit trees, which are typically controlled by many minor-effect genes with additive effects. Most cold-tolerance QTLs showed year-to-year variation in detection, consistent with QTL mapping patterns reported for quantitative traits in fruit trees such as grape and European plum [52–54]. Because peach is a perennial deciduous fruit tree grown under field conditions, QTL expression effects are substantially influenced by annual variation in temperature, precipitation, and field microenvironments, resulting in few stable major-effect cold-

tolerance loci and limited functional marker resources for breeding. Traditional fine mapping of fruit tree QTLs requires construction of large mapping populations, which involves long experimental cycles and substantial workload; by integrating QTL mapping with transcriptome analysis, this study circumvented some labor-intensive steps of large-scale population screening, shortened the candidate-gene discovery period, and provided an efficient and feasible research approach for screening abiotic stress-related genes in perennial fruit trees.

This study has several limitations. The functions of candidate genes were inferred primarily from gene annotation and transcriptome expression differences, without in vivo or in vitro functional validation; therefore, their regulatory mechanisms of cold tolerance remain unclear. The functional effects of SNP variations within QTL intervals and gene regulatory interaction networks also require systematic analysis. Future work will include SNP genotyping of candidate genes in natural and hybrid populations to identify functional molecular markers closely associated with cold tolerance; the biological functions of core genes will be elucidated through gene overexpression and knockout techniques to clarify molecular pathways involved in the low-temperature response of peach; based on stable major-effect QTLs, practical molecular markers will be developed to improve the peach marker-assisted breeding system, providing theoretical support and gene resources for developing new cold-resistant high-quality peach varieties and innovating cold-resistant germplasm.

Conclusion

A high-precision, high-integrity peach SNP genetic linkage map was constructed in this study using SLAF-seq technology, comprising eight linkage groups and 2,601 high-quality SNP markers, with a total map distance of 761.55 cM and an average marker interval of 0.29 cM. Analysis of cold-tolerance phenotypic data collected over 3 years revealed 12 significant QTLs for peach cold tolerance, mainly enriched in linkage group Pp08, with the highest phenotypic variance explained being 14.80%. Further integration of transcriptomic data under low-temperature stress with functional annotation identified 9 core cold-tolerance candidate genes involved in peach low-temperature signal transduction, cell membrane homeostasis regulation, and stress defense. These findings clarify key genetic regions and core functional genes associated with peach cold-tolerance traits and preliminarily reveal the genetic regulatory basis of peach cold tolerance, providing theoretical and resource support for understanding the molecular mechanisms of peach cold tolerance, developing functional molecular markers, marker-assisted breeding, and development of cold-resistant germplasm.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Q.T., writing—review and editing, writing—original draft, validation, visualization, methodology, investigation, funding acquisition, formal analysis, and conceptualization; J.L., Z.T. and Y.W., data curation and reference data collection; Z.W. and G.L., investigation and resources; R.C., manuscript revision; F.W., investigation and formal analysis; Y.L., resources, supervision, funding acquisition, and approval of the final manuscript.

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Data Availability

Raw DNA and RNA sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accessions PRJNA1535944 and PRJNA1535870, respectively. The data will be publicly available upon publication of this article.

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Figures

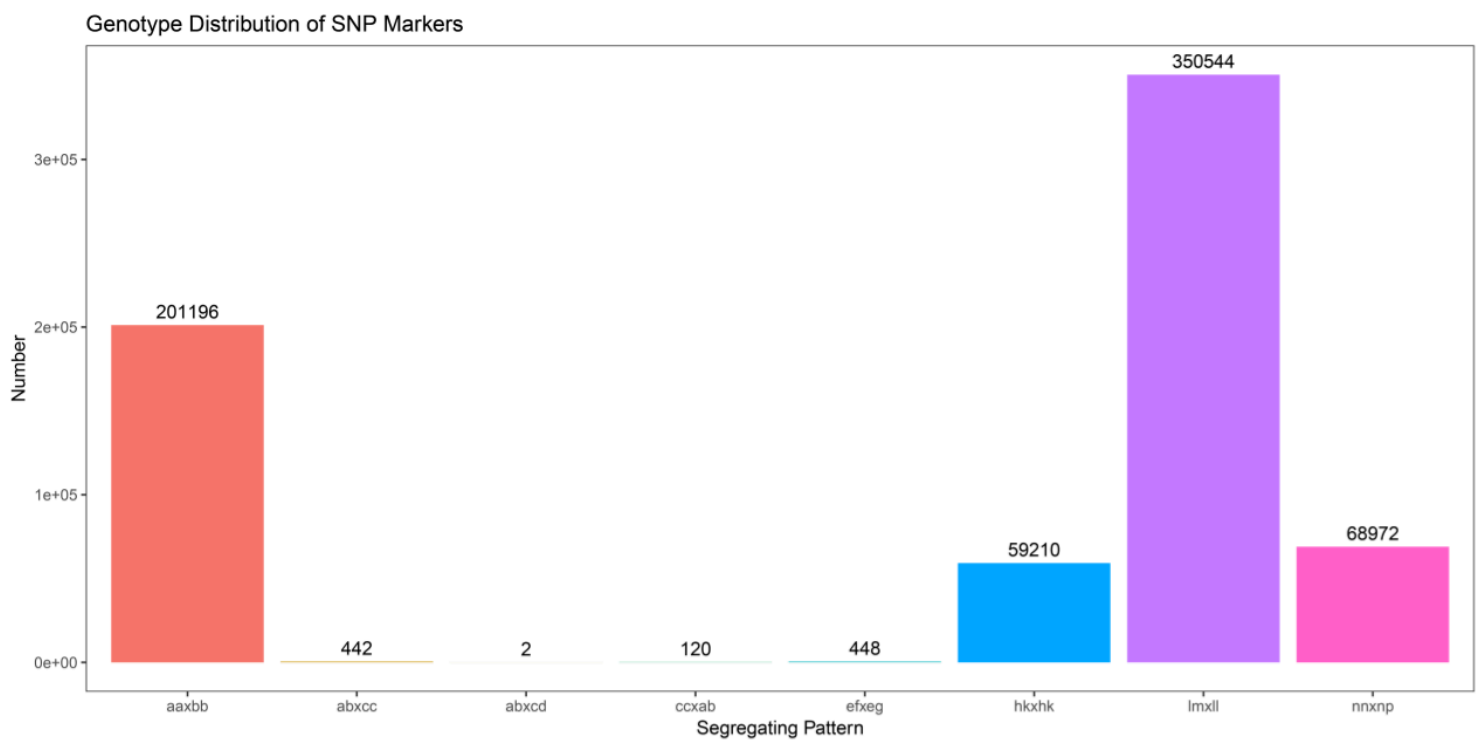


Figure 1

Number of polymorphic SNPs (y-axis) for each of the eight segregation patterns (x-axis). SNPs, single-nucleotide polymorphisms

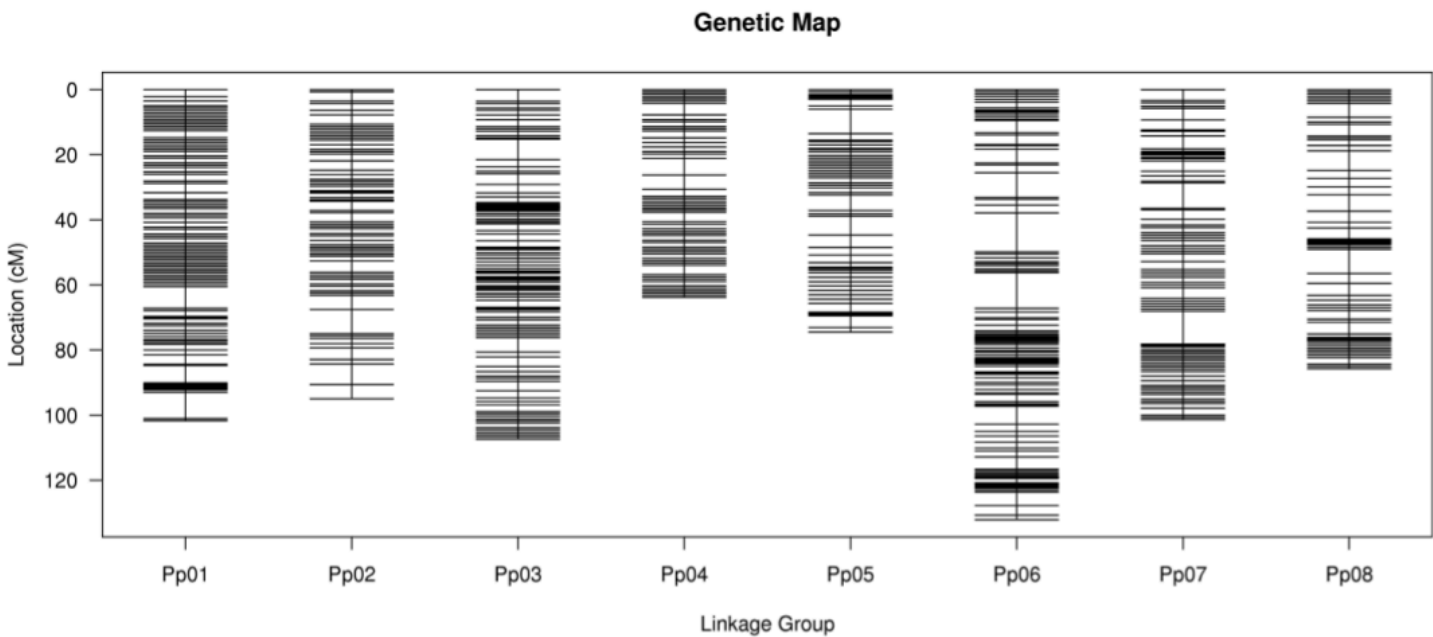


Figure 2

‘Donghe No. 1’ × ‘21st Century’ High-density genetic map. The black lines represent molecular markers. The x-axis shows the linkage groups, and their genetic positions are indicated on the y-axis. SNPs, single-nucleotide polymorphisms.

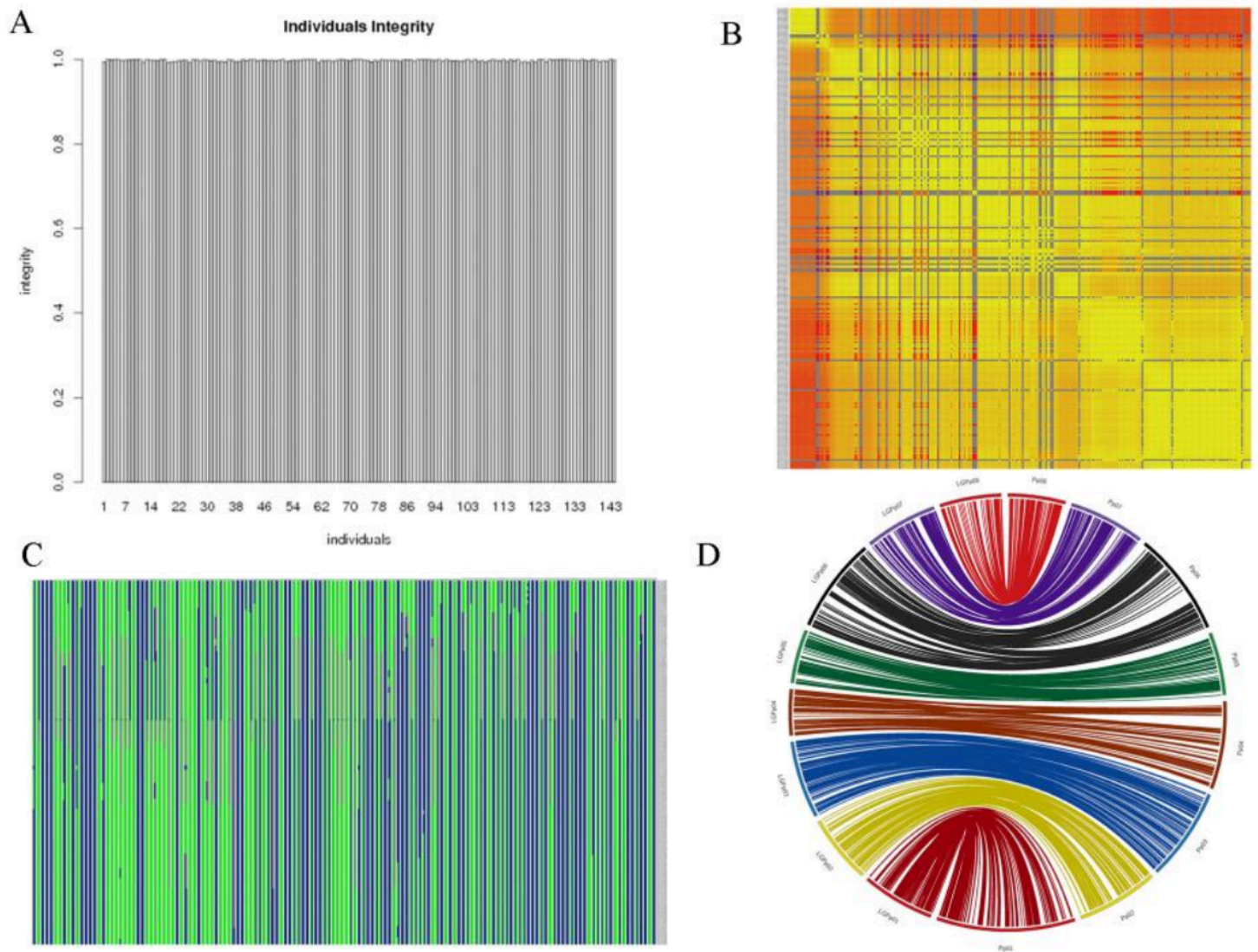


Figure 3

Evaluation of the genetic map. (A) Average integrity of mapping markers. (B) Haplotype maps for each individual. (C) Heatmap of integrated group LG8. (D) Collinearity analysis of marker positions on the linkage map and physical map.

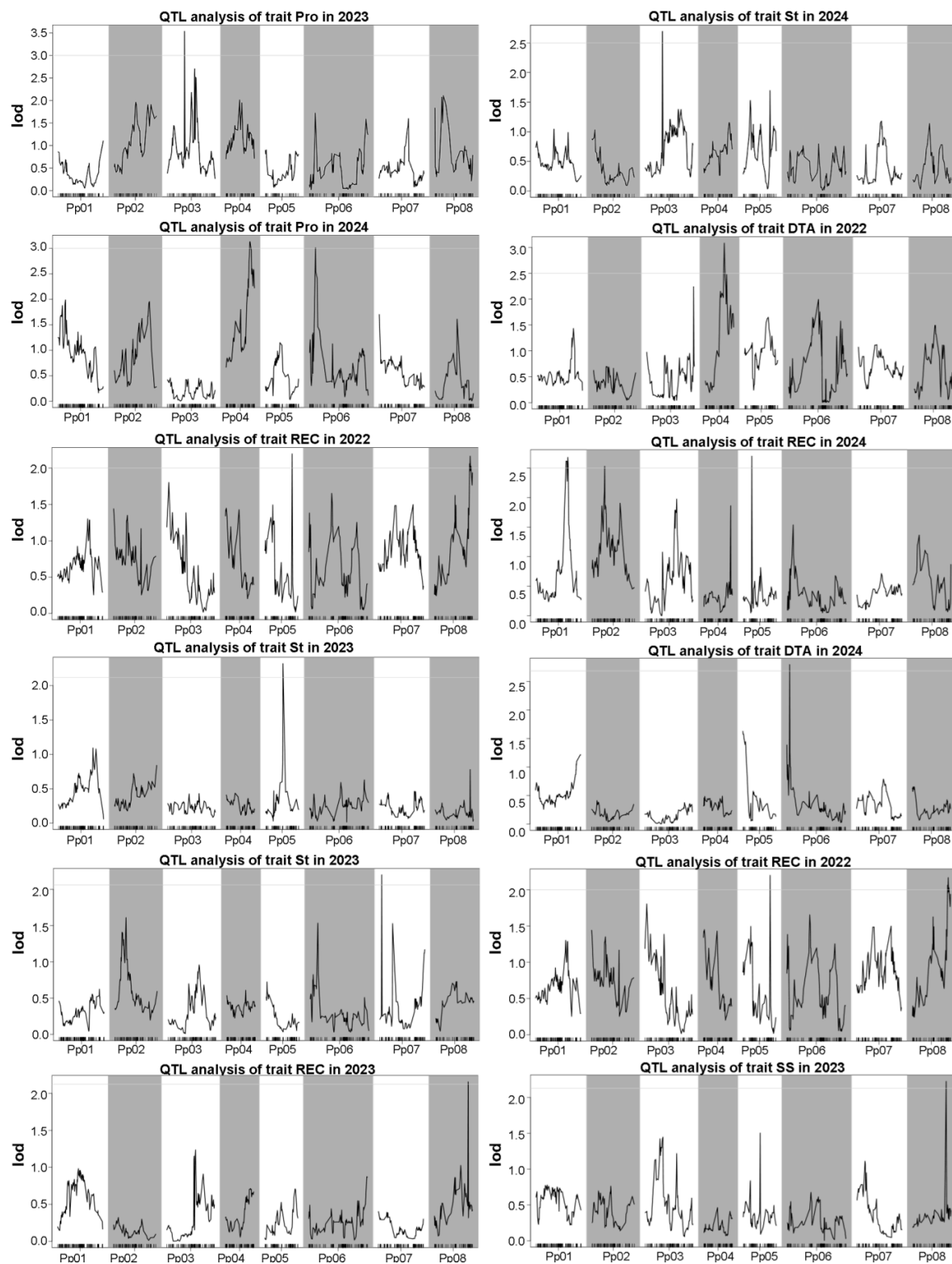


Figure 4

QTL analysis of cold tolerance genes in peach

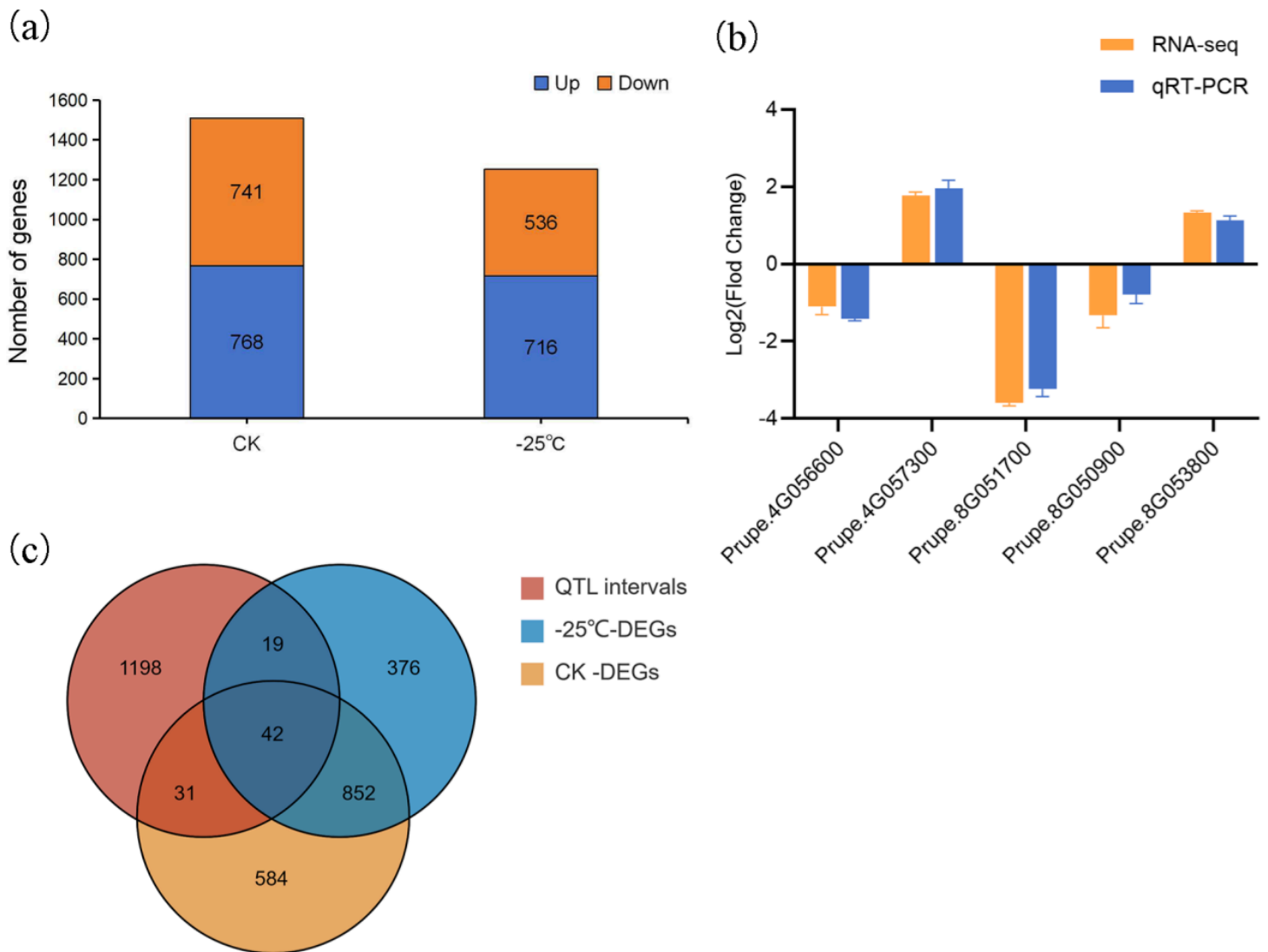


Figure 5

Overview of differentially expressed genes (DEGs) under cold stress in peach. (a) Bar graph showing DEGs under CK and -25 °C conditions. (b) Comparison of transcriptome sequencing data and qRT-PCR results. (c) Venn diagram of CK-DEGs, -25 °C-DEGs, and genes within QTL intervals.