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Identification of *VcRBOH* genes in blueberry and functional characterization of *VcRBOHF* in plant defense

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Abstract

Reactive oxygen species (ROS) serve as signal molecules in plant defense responses, and the respiratory burst oxidase homolog (RBOH) enzyme plays a crucial role in their production. Although numerous RBOH family members have been identified in various plants, little is known about the RBOH genes in blueberries. In this study, we identified six *VcRBOH* genes from the blueberry genome. Phylogenetic analysis revealed that these *VcRBOH* genes can be classified into three subgroups. Conserved domain and motif analysis demonstrated high sequence similarity among VcRBOH proteins. Analysis of cis-acting elements suggested that *VcRBOH* genes may be involved in stress, light, and phytohormone responsiveness. Based on transcriptome data, we observed low expression levels of *VcRBOHB*, *VcRBOHC*, and *VcRBOHE* during the flower_at_anthesis stage. In contrast, VcRBOHA and VcRBOHD showed relatively high expression levels in various tissues. The reverse-transcription quantitative PCR (RT-qPCR) analysis indicated rapid induction of *VcRBOHF* by flg22 and chitin treatments. Notably, overexpression of VcRBOHF in Arabidopsis promoted PTI responses, including increased expression of marker genes, ROS production, and callose deposition. Moreover, the overexpression of VcRBOHF resulted in enhanced disease resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 infection. These findings provide valuable insights into the roles of *VcRBOHF* genes in plant defense responses and lay the groundwork for a more comprehensive understanding of the molecular mechanisms underpinning blueberry disease resistance.

Keywords Reactive oxygen species (ROS), RBOH family, Blueberry, Disease resistance

Introduction

Plants, as with all living organisms, are continually exposed to a variety of pathogens and environmental stresses, and have evolved intricate defense mechanisms for self-protection. Among these mechanisms, plant immunity plays a pivotal role in the recognition and response to pathogen attacks. Two main branches of plant immunity are known as pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) [1]. PTI is the first line of defense in plants, triggered by the recognition of pathogen-associated molecular patterns (PAMPs) such as flg22 derived from bacterial flagellin and chitin derived from cell walls of fungi or the

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exoskeletons of insects. On the other hand, ETI is a more specific and robust defense mechanism that is activated upon the recognition of pathogen effectors by plant resistance (R) proteins [2]. The signaling events that regulate PTI and ETI are complex and involve the activation of multiple signaling pathways, including mitogen-activated protein kinase (MAPK) cascades [3], hormonal changes, and the production of reactive oxygen species (ROS) [4]. Understanding these signaling pathways is essential for developing strategies to enhance plant immunity and protect plants against to pathogens.

The role of ROS in plant defense is primarily manifested in three aspects: firstly, ROS exhibits toxicity towards pathogens and can directly inhibit their invasion [5]; secondly, it induces the cross-linking of glycoproteins and synthesis of callose, thereby modifying cell wall composition and forming intracellular barriers to impede pathogen expansion [6, 7]; thirdly, ROS also serves as messengers to transmit signals for various cellular defense responses that restrict pathogen infestation, such as stomatal closure [8] and the expression of defense genes [9]. Cellular responses triggered by ROS as signaling molecules involve complex signal transduction. It was found that ROS-induced calcium signature can be recognized by calcium-dependent protein kinase 12 and transmitted to the nucleus via a phosphorylation cascade reaction to activate downstream gene expression [10]. In addition to aerobic metabolic pathways, ROS are mainly produced through the catalytic activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), which are also known as respiratory burst oxidase homologs (RBOHs) in plants [11, 12]. They encode a homologue of NADPH oxidase gp91^{phox}, which was first identified in mammalian phagocytes [13]. Apart from sharing conserved domains with the NOX gene family including NADPH oxidase domain, ferric reductase-like transmembrane, FAD-binding domain, and NAD binding domain, the RBOH gene family has also been characterized with two EF-hand domains that associate with Ca^{2+} binding and phosphorylation [14, 15].

Currently, RBOH family genes have been identified in many plant species such as Arabidopsis with 10 members named *AtRBOHA- AtRBOHJ* [14], *Nicotiana tabacum* with 14 members [16], *Glycine max* with 17 members [17], *Brassica rapa* with 14 members [18], pepper with 7 members [19], *Citrus sinensis* with 7 members [20], and *Triticum aestivum* with 40 members [21]. Consistent with the important roles of NOX-dependent ROS in cellular signal transduction, accumulated research has suggested that RBOH genes are involved in plant defense. For example, the Arabidopsis *AtRBOHD* is involved in ROS production in PTI and ETI [22, 23], where stomatal closure due to PTI is essential for limiting the invasion of pathogenic bacteria *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 [24]. *AtRBOHD* has also been reported to be directly involved in resistance to different pathogens [25, 26], and is sometimes functionally redundant with *AtRBOHF*, as only in *rbohD rbohF* double mutants are shown to be more sensitive to necrotrophic pathogen *Plectosphaerella cucumerina* [27]. Moreover, *AtRBOHF* is involved in the defense against *Pst* DC3000 and *Magnaporthe oryzae* in Arabidopsis through ROS production and salicylic acid (SA) accumulation, respectively [27, 28]. In *Nicotiana tabacum*, *NbRBOHB*-mediated ROS production has a negative effect on disease resistance to *Botrytis cinerea* and red clover necrotic mosaic virus [29, 30]. However, *SlRBOHB* in tomato has been reported to positively regulate disease resistance to *Botrytis cinerea* [31]. In addition, ectopic expression of cassava *MeRBOHs* enhanced the resistance of Arabidopsis against *Pst* DC3000 [32].

The blueberry (*Vaccnium corymbosum* L.) is an important horticultural crop that many consumers love for its high nutritional value and health benefits. With the expansion of the blueberry planting area [33], the disease has brought great challenges to the yield and quality of blueberries. Therefore, researching blueberry disease resistance is important. Publication of the *V. corymbosum* cv. Draper genome database provides the foundation for genome-wide analysis of defense-related gene resources [34]. Based on prior knowledge that the *RBOH* genes are key enzymes in the production of ROS and may play an important role in plant defense, we used domain-scan analysis in this study to identify VcRBOH members in blueberry. We also analyzed the chromosomal location, gene structure, protein motif, and promoter cis-acting elements of *VcRBOH* genes in blueberry, and phylogenetic relationships of *RBOH* genes in blueberry, Arabidopsis, tomato, and rice. The expression patterns of *VcRBOH* genes in different tissues and response to PAMPs were analyzed based on transcriptome sequencing data and RT-qPCR respectively. Furthermore, Overexpressing *VcRBOHF* in Arabidopsis protoplasts and plants enhanced PTI responses, which correlated well with increased resistance to *Pst* DC3000 in *VcRBOHF* overexpressor plants. Our results indicated that *VcRBOHF* might be an important candidate for improving blueberry disease resistance.

Results

Genome-wide identification of *VcRBOH* genes in blueberry

A total of six blueberry *VcRBOH* genes were identified and named *VcRBOHA* to *VcRBOHE* according to the nomenclature on NCBI (Table S1). Due to the incomplete chromosomal assembly of the blueberry genome, genes can only be located on chromosome scaffolds. The *VcRBOH* genes were evenly distributed across seven chromosome scaffolds in the blueberry (Fig. 1). The

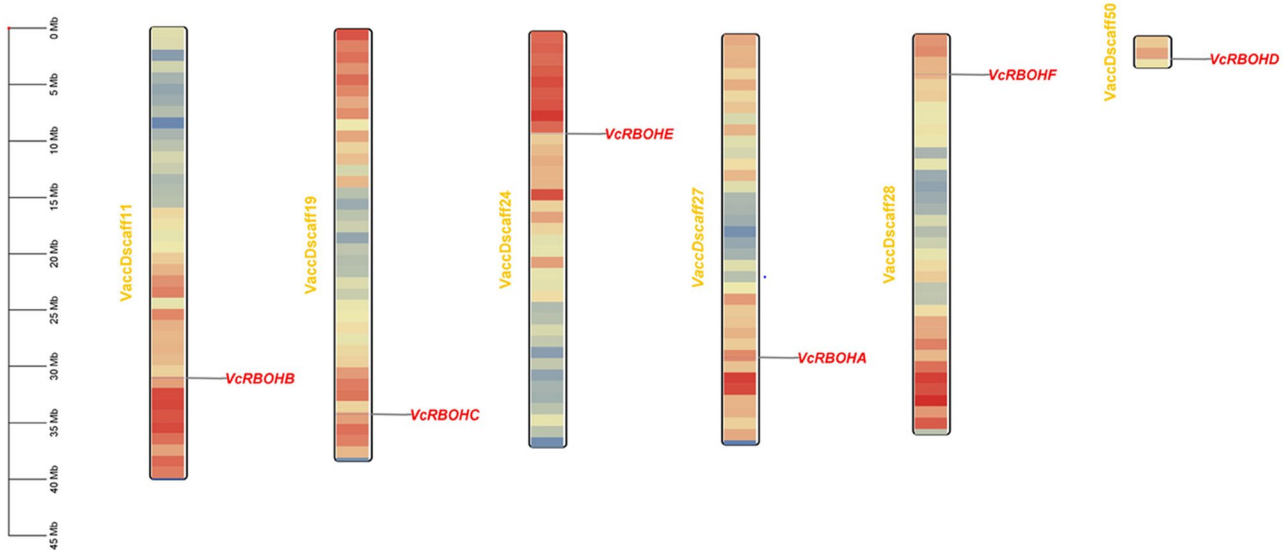

Fig. 1 The location of *VcRBOH* genes on the pseudochromosomes. The left bar indicates the length of each pseudochromosomes in megabytes (Mb). The chromosomal color reflects gene density, with cool colors for low density and warm colors for high density

lengths of the predicted amino acids varied widely, with VcRBOHE being the shortest, containing 804 amino acids, and VcRBOHB being the longest, containing 1634 amino acids. Analysis of the physicochemical properties of proteins by ProtParam showed that the molecular weight of the VcRBOH proteins varied from 92.76 to 187.68 kDa, and the theoretical isoelectric point of the protein ranges from 8.92 to 9.13. For the instability index, all VcRBOH protein sequences had values higher than 40, indicating that they were predicted to be unstable. Subcellular localization predictions showed that all VcRBOH proteins were located on the plasma membrane.

Phylogenetic and evolutionary analysis of VcRBOH proteins

To investigate the evolutionary relationship of VcRBOH proteins, the amino acid sequences of *RBOH* genes in blueberry, Arabidopsis, tomato, and rice were constructed using the Mega11 software (Fig. 2). The VcRBOH proteins were divided into three evolutionary branches, each containing RBOH members from the four species. The VcRBOHB, VcRBOHC, and VcRBOHE were clustered into group I, VcRBOHA and VcRBOHD were clustered into group II, and VcRBOHF was clustered into group III, respectively. Except for VcRBOHF, which is genetically closest to AtRBOHF, the other members of the VcRBOH proteins are genetically closest to SlRBOH members. The results suggest that the RBOH proteins of blueberry are more closely related to the RBOH proteins of tomato in terms of evolutionary relationship compared to its relationship with Arabidopsis and rice.

Domain, motif composition and gene structure analysis of VcRBOH members

To investigate the domain composition of VcRBOH proteins, conserved domains were detected using NCBI-CDD program based on their evolutionary correlation. VcRBOH members contain multiple conserved domains, including NADPH_Ox, Ferric_reduct, FAD_binding_8, NAD_binding_6 and EF hand binding domain (Fig. 3A), which is consistent with RBOH proteins in other plant species [19, 32]. The amino acid sequence alignment of VcRBOHs showed that these domains were similar to RBOH of Arabidopsis thaliana, rice, and tomato (Fig. S1), indicating that these regions were conserved in different plants. Moreover, these conserved domains appeared in the same order from the N-terminal region to the C-terminal region, indicating similarity in the composition of the VcRBOH protein sequence. Interestingly, both VcRBOHB and VcRBOHC contain two NADPH_Ox, NAD_binding_6, and Ferric_reduct domains, suggesting they may be functionally similar.

The conserved motifs of VcRBOH proteins with default parameter settings were analyzed by MEME. Fifteen conserved motifs were uncovered in VcRBOH proteins (Fig. 3B). Except that VcRBOHE lacks motif 14 and VcRBOHF lacks motif 13, all other VcRBOH proteins contain all motifs. The arrangement of the motifs is entirely consistent, which suggests a high conservation of protein sequences among VcRBOH proteins. Among these motifs, motif 11 and motif 10 was included in the NADPH_Ox domain, motifs 3, 8, 9, and 4 constituted the Ferric_reduct domain, motifs 15, 2, and 5 constituted the FAD_binding_8 domain, and motifs 5, 1, 7, and 14 constituted the NAD_binding_6 domain (Fig. 3C).

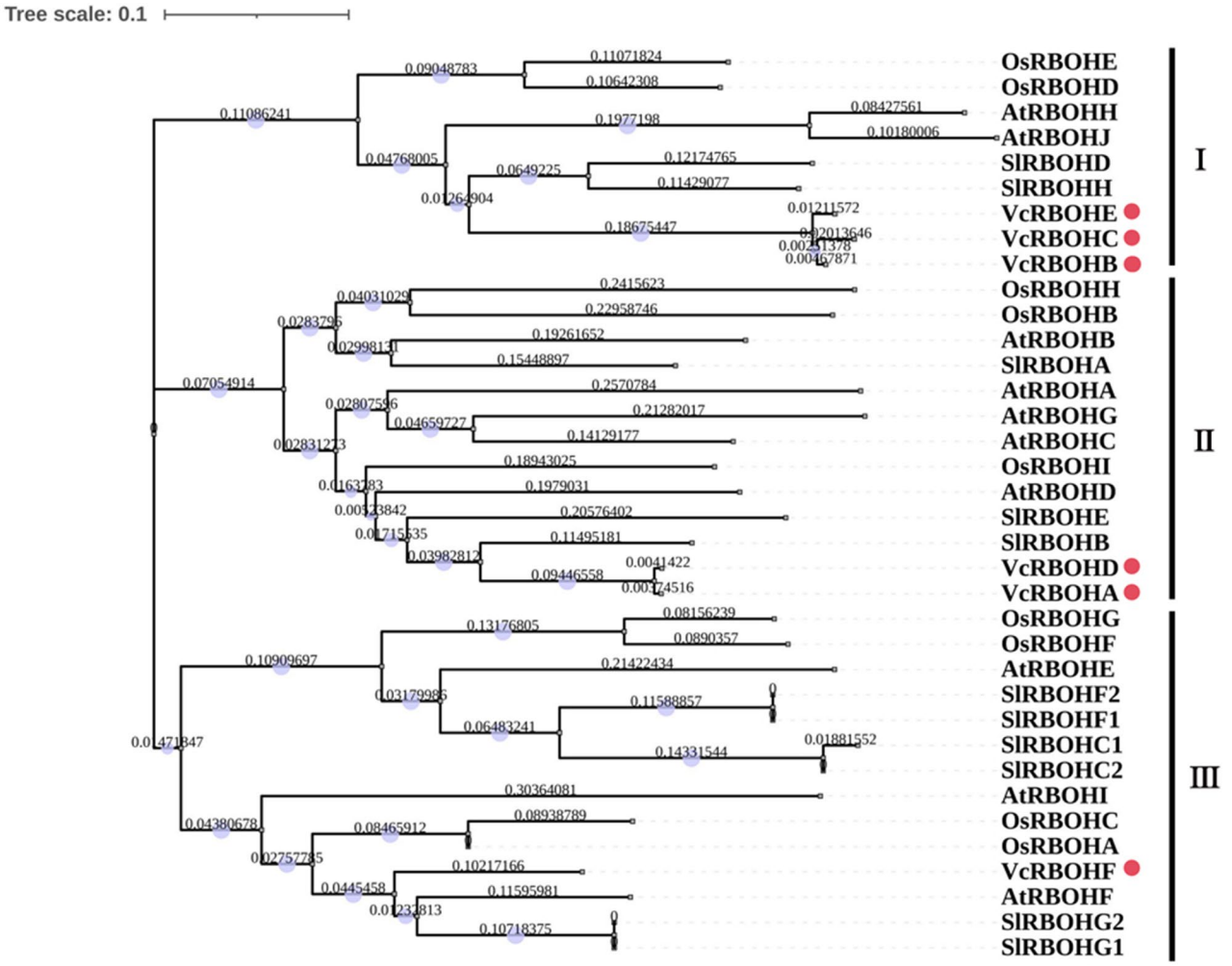

Fig. 2 Phylogenetic analysis of the RBOH proteins from blueberry, Arabidopsis, rice, and tomato. A total of 36 RBOH proteins from different plants were analyzed using the Neighbor-joining method. VcRBOH proteins of blueberry were labeled with red circles

To further explore the structural characteristics of the *VcRBOH* genes in blueberry, exon-intron structures were obtained based on their genomic information. As shown in Fig. 3D, the coding sequence (CDS) and untranslated region (UTR) of members exhibited variability in number and length. The distribution of exons and introns in genes located in the same clade is similar, such as *VcRBOHA* and *VcRBOHD*, which may be closely related to the evolution of gene families. The *VcRBOHA*, *VcRBOHD*, and *VcRBOHC* contain 5'-terminal and 3'-terminal UTR structures, whereas *VcRBOHB*, *VcRBOHF*, and *VcRBOHE* lack 5'-terminal UTR structures. In addition, *VcRBOHB* contains more exon structures than the other members, which may be related to gene duplication and divergence events during evolution.

Cis-element analysis of the *VcRBOH* genes

To better understand the functions of *VcRBOH* genes, cis-elements analysis was performed using their promoter sequence 2000 bp upstream from the initiation codon. Thirteen representative types of cis-elements containing a total 158 number of elements were identified (Fig. 4A). These cis-elements were categorized into three groups based on their functions: stress responsiveness, light responsiveness, and phytohormone responsiveness. For the elements related to stress, cis-acting elements involved in defense and stress responsiveness were present in the promoter regions of *VcRBOHA*, *VcRBOHC*, and *VcRBOHF* genes (Fig. 4B), cis-acting elements involved in low-temperature responsiveness were found in *VcRBOHA*, *VcRBOHD*, and *VcRBOHF* genes, while the MYB binding site involved in drought-inducibility and cis-acting regulatory element essential for the anaerobic induction were widespread in all *VcRBOH* genes. A total of 56 (35.4%) elements related to light responsiveness were identified, encompassing four subcategories: cis-acting element involved in light responsiveness, cis-acting regulatory element involved in light responsiveness,

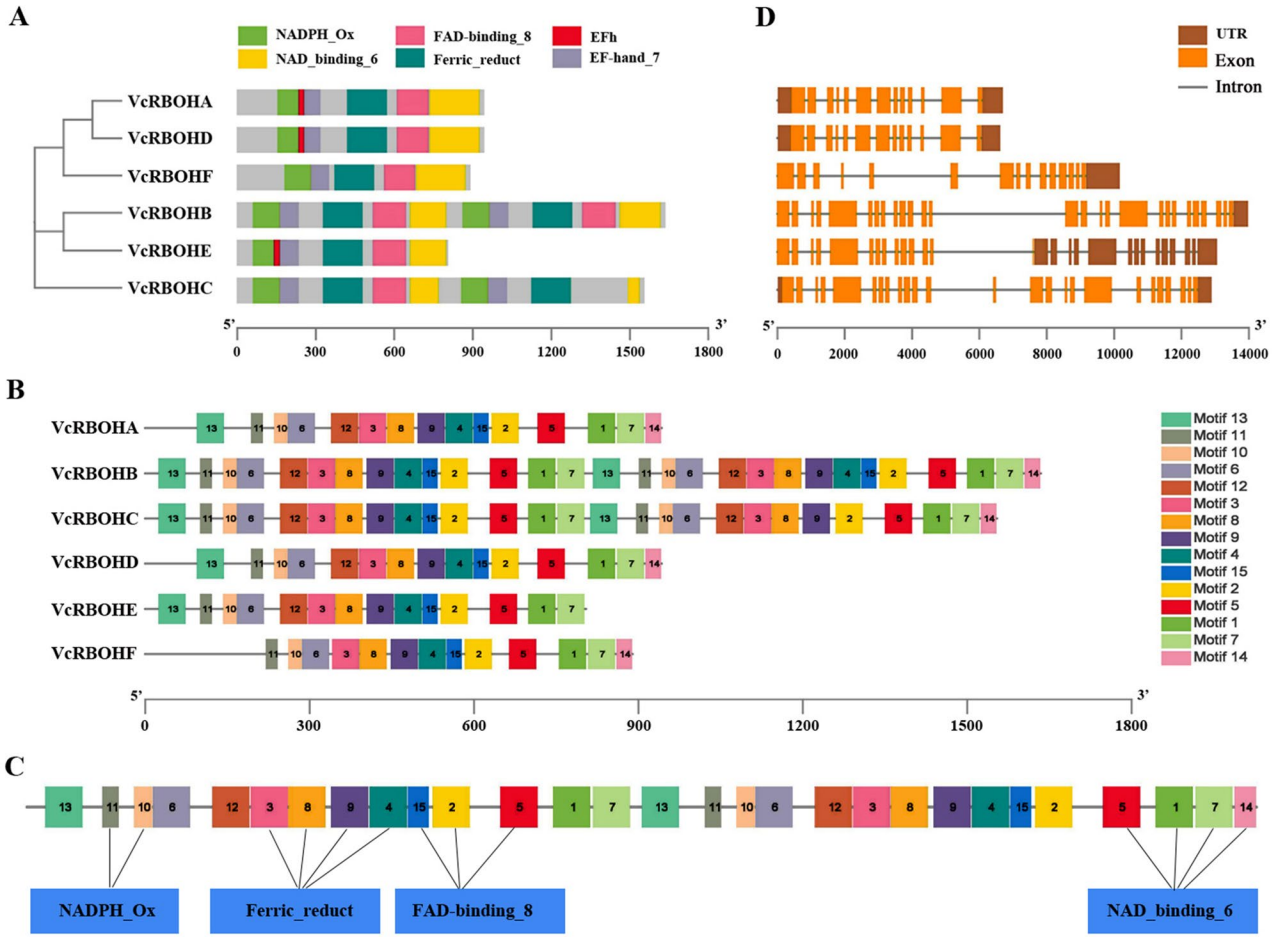

Fig. 3 Conserved domain, motif, and gene structure analysis of VcRBOHs. (**A**) Phylogenetic relationship and conserved domains, and (**B**) motifs distribution on VcRBOH proteins. The scale bar at the bottom represents the protein sequence. (**C**) The Constituent relationship between motif and conserved domain in VcRBOH proteins. (**D**) Structures of *VcRBOH* genes. The UTR, Exon, and Intron were marked with brown rectangles, orange rectangles, and black lines, respectively. The scale bar at the bottom represents the length of the gene sequence

light responsive element, and part of a light responsive element. These elements related to light responsiveness were also identified in all *VcRBOH* genes. The phytohormone responsiveness cis-elements were the most numerous, accounting for 39.2% of the total elements. The number of cis-elements responding to abscisic acid and methyl jasmonate (MeJA) was higher than auxin, SA, and gibberellin-responsive elements. Furthermore, *VcRBOHA*, and *VcRBOHD* promoter sequences contain the most cis-acting elements involved in the abscisic acid responsiveness, and *VcRBOHF* promoter sequences contain the most cis-acting regulatory elements involved in the MeJA-responsiveness. Previous studies have shown that phytohormones play an important role in plants facing disturbances from biotic or abiotic factors [35, 36]. Thus, these results suggest that *VcRBOH* genes may function and have different expression levels in response to various biotic and abiotic stresses.

Tissue-specific analysis of the *VcRBOH* genes in blueberry

The expression patterns of *VcRBOH* genes in different tissues at different time points were analyzed using transcriptome sequencing data. As shown in Fig. 5, a tissue-specific expression heatmap was drawn based on $\log_2$ (FPKM) values. The *VcRBOHB*, *VcRBOHE*, and *VcRBOHC* have similar expression patterns, with low expression in flower_at_anthesis and almost no expression in other tissues, suggesting that they may have specific roles at this development stage in blueberry. In contrast, *VcRBOHD* and *VcRBOHA* were detected in various tissues, and their expression levels were relatively higher in flower_bud and shoot than in other tissues. For *VcRBOHF*, low expression levels were observed in flower_bud, green_fruit, petal_fall, and shoot. These results suggest that *VcRBOH* genes with the same evolutionary branch or homology are similar in tissue expression patterns and that *VcRBOHA* and *VcRBOHD* may play important roles in the regulation of blueberry development.

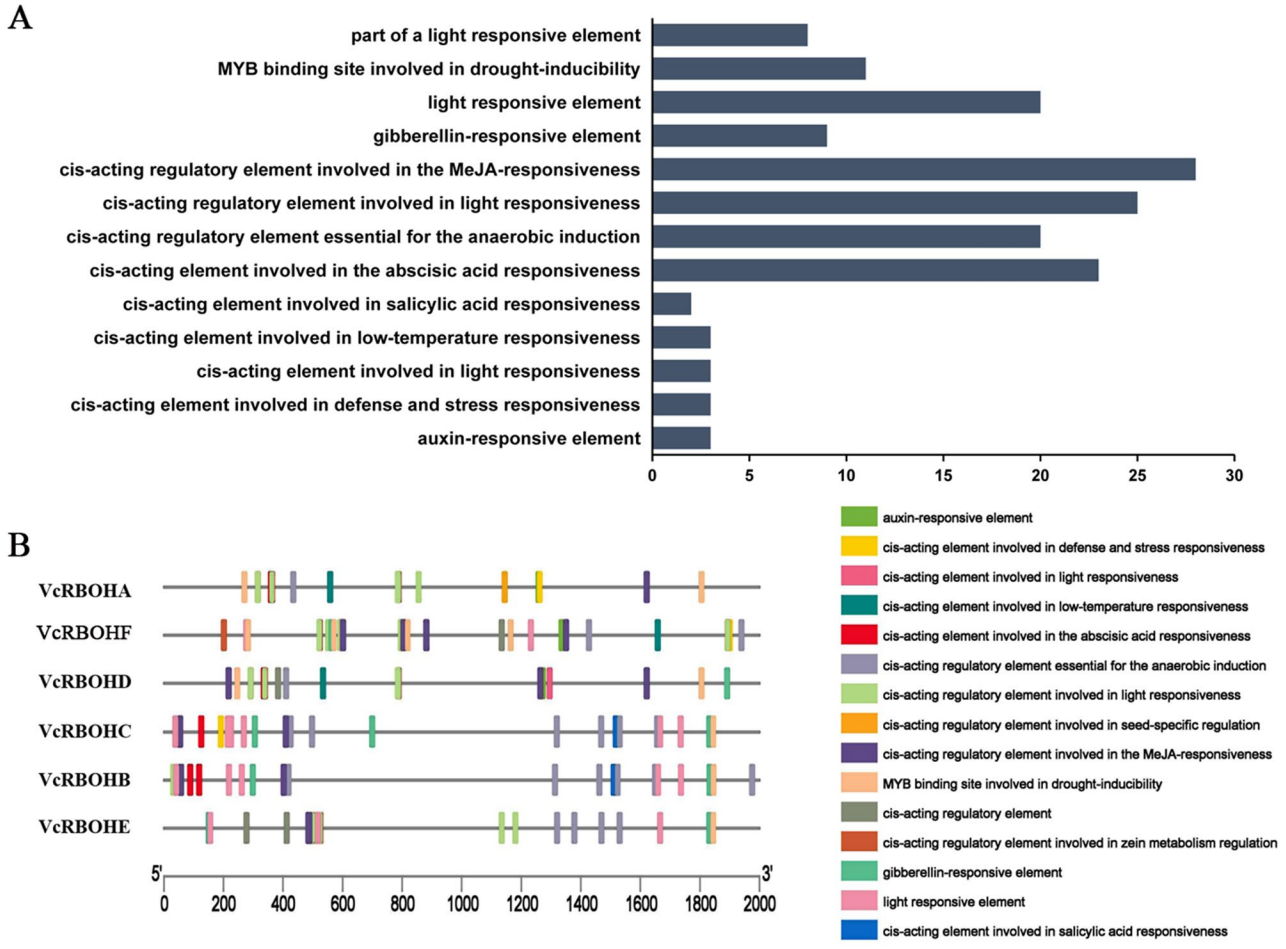

Fig. 4 Cis-acting element analysis of promoter sequences of *VcRBOH* genes. (**A**) The number of different cis-acting elements of *VcRBOH* genes. (**B**) Positions of cis-acting element on the *VcRBOH* gene promoters. The scale bar at the bottom represents the promoter sequence, and the colored blocks indicate different cis-elements

Expression analysis of *VcRBOH* genes in response to PAMPs

Considering that the production of ROS in response to PTI mainly depends on the RBOH pathway [37], we examined the expression level of the *VcRBOH* gene in flg22 and chitin-treated leaves using RT-qPCR. The *VcRBOH* genes exhibited different expression patterns in response to flg22 at different hours post treatment (hpt) (Fig. 6A). The relative expression of *VcRBOHA* and *VcRBOHD* was not significantly different from that of the control group (0 hpt, without PAMP treatment) at all-time points after flg22 treatment, and *VcRBOHB*, *VcRBOHC*, and *VcRBOHF* were all induced to be up-regulated at 1 hpt. In contrast, the expression of *VcRBOHE* was suppressed at 0.5 hpt. Notably, the expression of *VcRBOHF* was highly upregulated at all-time points after flg22 treatment. Regarding chitin treatment (Fig. 6B), the expression of *VcRBOHF* were induced at 0.5 and 1 hpt, while *VcRBOHA* and *VcRBOHB* were suppressed at 1 pht. Interestingly, *VcRBOHD* expression was suppressed at 0.5 hpt and highly induced at 1 hpt, suggesting that its expression may be precisely controlled. Taken together, *VcRBOHF* was rapidly and significantly up-regulated by flg22 and chitin treatment, indicating that *VcRBOHF* could play an important role in PTI.

VcRBOHF promotes the PTI responses in Arabidopsis

Given the rapid response of *VcRBOHF* to flg22 and chitin induction, we used the Arabidopsis protoplast transient expression system to test whether it could promote the PTI response. *FRK1*, as a marker gene of PTI, can be induced and up-regulated by flg22 [38]. Therefore, we transferred the *FRK1* promoter luciferase reporter gene vector and *VcRBOHF* overexpression vector into Arabidopsis protoplasts to test luciferase activity after flg22 treatment. Compared with the empty control, the LUC/REN ratio was significantly increased when the *FRK1pro*-LUC reporter was cotransformed with 35 S-VcRBOHF (Fig. 7A). To detect flg22-induced ROS burst and callose deposition, we generated the transgenic Arabidopsis expressing *VcRBOHF* with the 35 S promoter

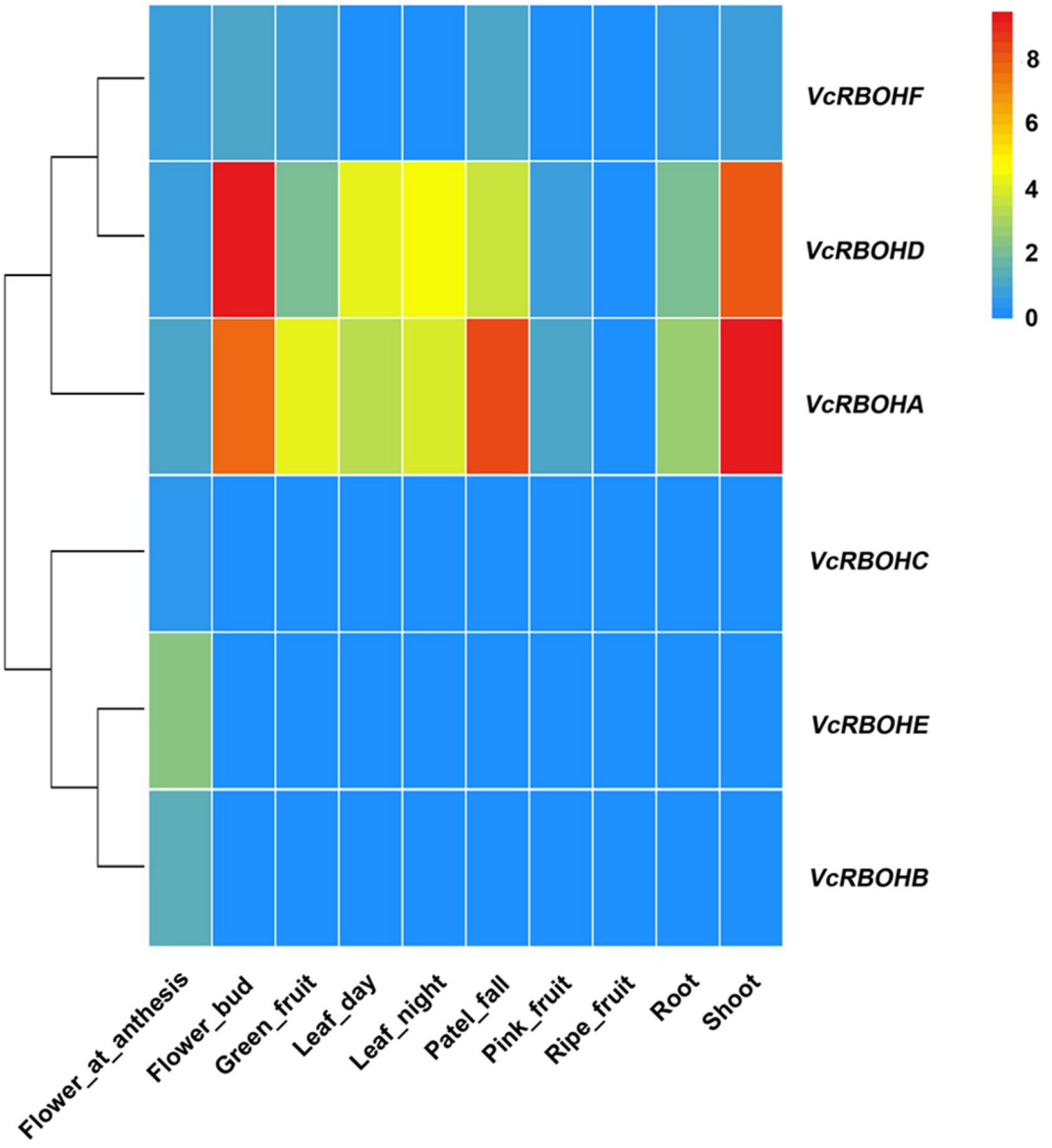

Fig. 5 Heatmap illustration of tissue expression patterns of *VcRBOH* genes based on FPKM (fragments per kilobase per million measure) values. The log2 transformation of FPKM values and visualization were performed by R software v3.6.3. The color scale indicates the levels of gene expression

(designated OE-VcRBOHF). Reverse transcription (RT)-PCR showed that *VcRBOHF* transcripts were detectable only in the four OE-VcRBOHF transgenic lines, but not in wild-type Col-0 plants (Fig. S2). OE-VcRBOHF-1 and OE-VcRBOHF-2 lines with higher expression levels were selected for further study. As shown in Fig. 7B, the kinetics of ROS accumulation were similar in Col-0, OE-VcRBOHF-1, and OE-VcRBOHF-2 plants. However, compared with Col-0 plants, the level of ROS was increased in two VcRBOHF overexpression plants. Similarly, the two overexpression lines exhibited more callose deposition compared to Col-0 plants (Fig. 7C). Taken together, these results indicate that transgenic expression of VcRBOHF can promote PTI responses induced by flg22 in Arabidopsis.

VcRBOHF increases disease resistance in Arabidopsis

To further explore whether VcRBOHF plays a role in disease resistance, the VcRBOHF overexpression and Col-0 lines were dip-inoculated with virulent bacteria pathogen *Pst* DC3000, and disease symptoms and bacterial numbers were evaluated. As shown in Fig. 8, Col-0 displayed typical chlorosis symptoms and aggressive multiplication bacteria at 3 days post-inoculation (dpi), while OE-VcRBOHF-1 and OE-VcRBOHF-2 exhibited milder symptoms and contained fewer bacterial titers at

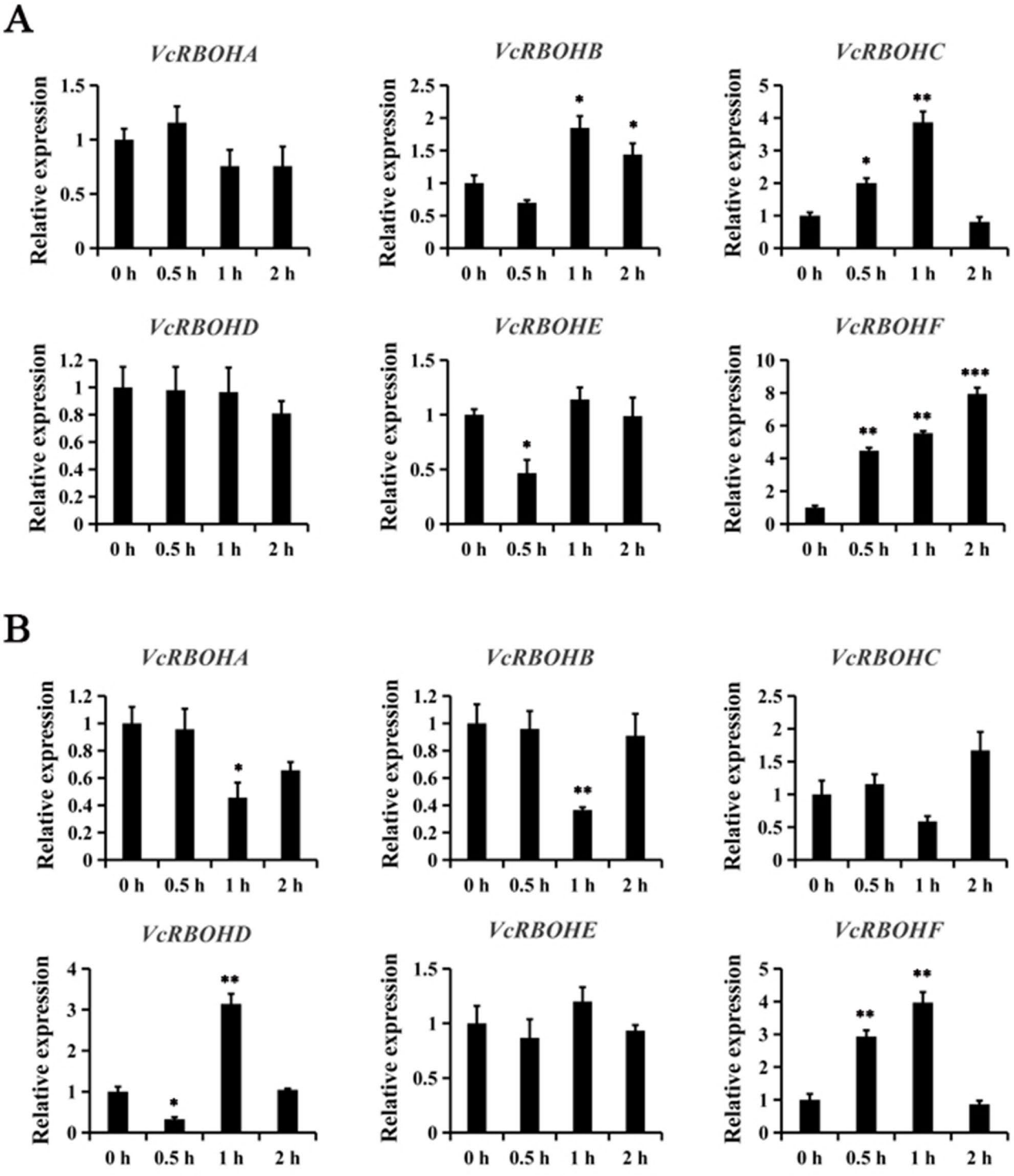

Fig. 6 Expression analysis of *VcRBOH* genes in response to flg22 and chitin. (**A**) Relative expression of *VcRBOH* genes in response to flg22. (**B**) Relative expression of *VcRBOH* genes in response to chitin. Expression levels were normalized to *VcGAPDH*. Values represented as mean ± SD of three biological replicates. Statistically significant differences were analyzed at different time points (Student's t-test): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

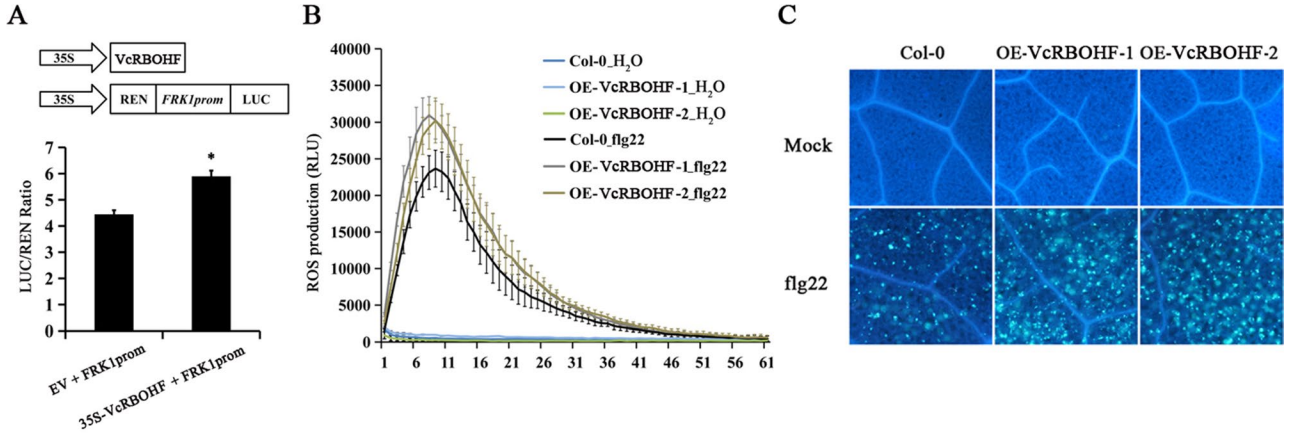

Fig. 7 VcRBOHF promotes the PTI responses in Arabidopsis. (**A**) Transient expression of *VcRBOHF* in the protoplasts of Arabidopsis increased the expression of *FRK1* in response to flg22 treatment. The LUC/REN ratio represents the expression level. Asterisks indicate significant differences between *FRK1* promoter reporter vectors cotransformed with 35 S-VcRBOHF or empty vectors (EV) by Student's t-test (*, $P < 0.05$). Values are given as mean ± SD of three biological replicates. (**B**) Luminescence induced by H2O or flg22 in Col-0 and VcRBOHF overexpression lines and expressed as relative light units (RLUs). (**C**) Microscope images of callose deposition induced by H_2O (Mock) or flg22 in Col-0 and VcRBOHF overexpression lines

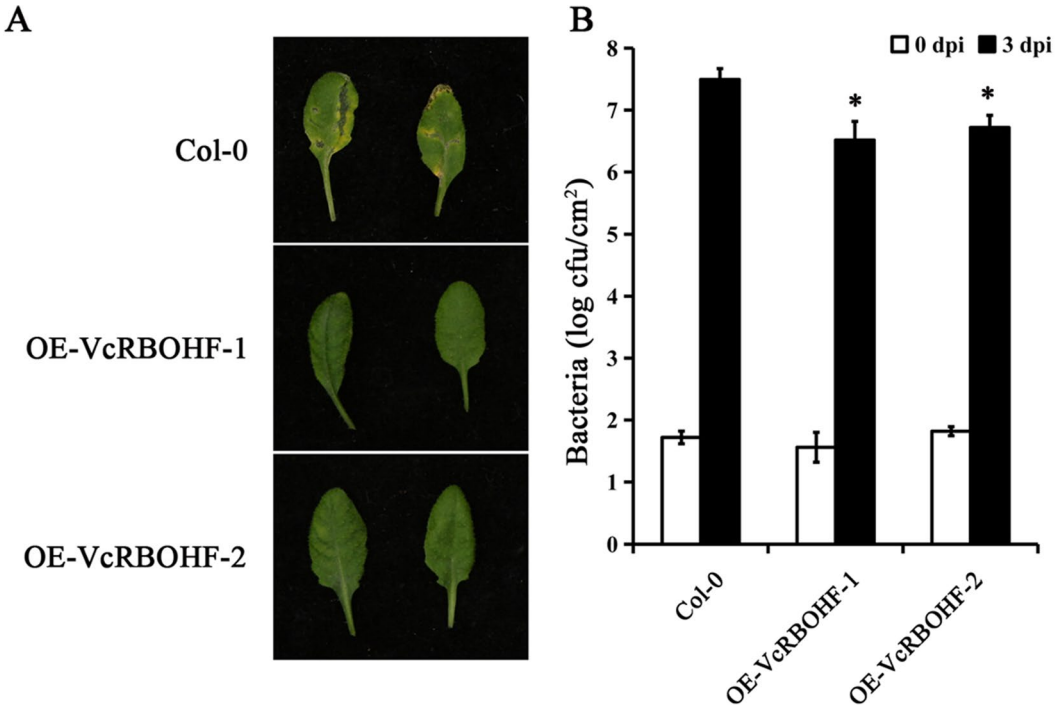

Fig. 8 VcRBOHF enhances the disease resistance against *Pst* DC3000. (**A**) Disease symptoms were in the Col-0, OE-VcRBOHF-1 and OE-VcRBOHF-2 lines after dip-inoculation with *Pst* DC3000 (1×10^7 cfu/ml) at 3 dpi. (**B**) Bacterial growth in Col-0, OE-VcRBOHF-1, and OE-VcRBOHF-2 lines. Error bars indicate SD ($n = 3$). Asterisks indicate a significant difference from Col-0 and OE-VcRBOHF lines at 3 dpi based on Student's t-test (*, $p < 0.05$)

3 dpi. These results indicate that transgenic expression of VcRBOHF increases disease resistance in Arabidopsis.

Materials and methods

Identification and chromosomal localization of *VcRBOH* genes in blueberry

The protein sequences of RBOH from Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa* L.) were downloaded from the NCBI database, which was used as query sequences against the blueberry genome database (GDV, https://www.vaccinium.org) to identify the VcRBOH protein sequences. Then, the candidate protein sequences were confirmed by Hidden Markov Model (HMM) search using NADPH oxidase domain (PF08414) and CD-search (https://www.ncbi.nlm.nih.gov/cdd/) as previously described [39]. Finally, the VcRBOH proteins were obtained by removing redundant and structure domain incomplete sequences. The physicochemical

properties of the VcRBOHs were analyzed on the Expasy website (https://web.expasy.org/protparam/), and the subcellular localization was predicted using WOLFSORT (https://wolfpsort.hgc.jp). Chromosomal location analysis of the *VcRBOH* gene was performed using the blueberry genome annotation file under the Gene location visualize function of Tbtools software.

Phylogenetic analysis

The identified blueberry RBOH protein sequences were subjected to multiple sequence alignment using the MUSCLE program in MEGA11 software, along with the RBOH protein sequences of Arabidopsis thaliana, rice, and tomato obtained concerning previous studies [19]. The phylogenetic tree was constructed using the Neighbour-Joining (NJ) method with the bootstrap value set to 1000. The resulting phylogenetic tree file was visualized using the iTol website (https://itol.embl.de/).

Conserved domain, motif, and gene structure analysis of the VcRBOH members

Conserved domains including NADPH_Ox, Ferric_reduct, FAD_binding_8, and NAD_binding_6 were identified using CD-search and SMART online programs. The motifs in the VcRBOH sequences are recognized using the MEME Suite v 5.5.5 website (http://meme-suite.org/tools/meme) with the number of motifs set to 15. The gene structures of *VcRBOH* members were visualized via the Tbtools software using a genome annotation file downloaded from the GDV.

Cis-acting element analysis of VcRBOH genes promoter

The upstream 2000 bp sequences from the initiation codons of *VcRBOH* genes were obtained from the blueberry database and predicted for cis-acting elements via the online PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html). The resulting file was visualized using the Tbtools software.

Expression pattern analysis of *VcRBOH* genes

The expression profiles of the VcRBOH gene from the blueberry cultivar 'Draper' were obtained from the BioProject database in the NCBI repository (accession number: PRJNA494180) for various organs, including flower buds, flowers at anthesis, petal fall, green fruit, ripe fruit, roots, shoots, and leaves during day and night. Transcript abundance was estimated using log2-transformed FPKM (fragments per kilobase per million measure).

PAMPs treatments and quantitative RT-qPCR analysis

The northern highbush blueberry 'Bluecrop' plants from tissue culture were grown in a growth chamber at 25 °C with 70% relative humidity under a 16-h-light/8-h dark photoperiod. Three leaves each of four six-month seedlings were collected following spray with flg22 (1 μM), or chitin (10 μM) for 0.5 h, 1 h, or 2 h, using samples collected at time 0 (without PAMP treatment) as control. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA was synthesized using the kit PrimeScript™ RT Master Mix (TaKaRa, Japan), and the RT-qPCR analysis was performed as previously described [40]. The blueberry glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (*GAPDH*) was used as the reference. Primers used for RT-qPCR are listed in Supplementary Table S2. Three independent biological replicates were performed in all experiments.

Generation of transgenic Arabidopsis plants

The full-length *VcRBOHF* coding sequence was cloned into a pCNGF vector to generate *35 S-VcRBOHF* construct. Then, the construct was transformed into the *Agrobacterium tumefaciens* GV3101 strain. Transformation of plants was achieved by the floral-dip method. Transgenic plants were selected on MS medium containing 25 mg/ml kanamycin, and the presence and expression of the *VcRBOHF* were confirmed by PCR and RT-PCR analyses.

PTI responses analysis in Arabidopsis

For *FRK1* expression, the coding sequence of *VcRBOHF* was cloned into the pGreenII 62-SK vector to generate the effector. The empty vector (EV) was used as a negative control. *FRK1* (Gene locus: AT2G19190) promoter sequence was cloned into the pGreenII 0800-LUC vector to generate the reporter. The plasmids were transferred into Arabidopsis protoplasts as previously described [41]. Firefly luciferase (LUC) and Renilla luciferase (REN) activity levels treatment with flg22 (1 μM) were measured using the Dual-Luciferase Assay Kit (Promega) according to the protocol.

For ROS burst, the luminol-based assay was previously described [42]. Arabidopsis leaf discs were placed on 96-well plates floating in 200 ml of deionized water overnight. Then, deionized water was replaced by the buffer (20 mM luminol, 0.02 mg/ml horseradish peroxidase) added with 100 nM flg22 or deionized water to detect the production of ROS. Luminescence was measured with a luminometer and recorded the dynamic process within 1 h.

For callose deposition, leaves were infiltrated with flg22 (1μM) or water (Mock) for 18–24 h. The harvested leaf samples were dipped with 95% ethanol to make them transparent. Aniline blue stained at room temperature in the dark as previously described [43]. Then, the callose deposits were observed under a fluorescence microscope with an UV filter.

Pathogen inoculation in transgenic Arabidopsis plants

Bacterial *Pst* DC3000 culture and cell collection were performed as previously described [44]. Four-week-old plants were dipped with a bacterial suspension containing 10^7 CFU/ml *Pst* DC3000 (OD_{600} = 0.02) with 0.02% Silwet L-77. Disease symptoms were recorded by camera, and bacterial load were determined by serial-dilution assays at 0 and 3 days post-inoculation (dpi).

Discussion

In the face of pathogen infection, RBOH can affect plants' defense response by regulating ROS production. Therefore, the plant *RBOH* gene can be used as a candidate to study plant defense response. Due to the ease of access to genomic data, more and more research has expanded to identify and analyze *RBOH* genes beyond model plants at the genome-wide level. For example, eight RBOH members have been identified in eggplant (*Solanum melongena* L) [39], seven RBOH members in pepper (*Capsicum annuum* L) [19], and eight RBOH members in cassava (*Manihotis esculenta* Crantz) [32]. In this study, we identified a total of six *RBOH* gene members from the genomic data of blueberry and named them *VcRBOHA-F*, which were evenly distributed on 6 chromosomes (Fig. 1). It has been shown that RBOH proteins are mainly located in the membrane and catalyze NADPH in the cytoplasm while transferring electrons to generate ROS [45]. Through transient expression systems, the MeRBOHs and SmRBOHs were localized to the plasma membrane in Arabidopsis protoplasts and tobacco mesophyll cells, respectively [32, 39]. Similar to cassava, all the VcRBOH members in blueberry were predicted to be located on the plasma membrane (Table S1), because multiple transmembrane regions that anchor the protein to the plasma membrane were detected in all VcRBOHs. However, in addition to the plasma membrane, several ZmRBOH proteins in maize were predicted to be located on the nucleus and mitochondria [46]. Therefore, the subcellular localization of VcRBOH proteins needs to be further confirmed by transient expression experiments.

The phylogenetic tree was constructed by the protein sequences of the RBOH family in blueberry, Arabidopsis, tomato, and rice (Fig. 2). Consistent with previous studies, RBOH members were divided into three evolutionary branches [39]. Compared to Arabidopsis and rice, the evolutionary relationship between VcRBOHs in blueberry and SlRBOHs in tomatoes is relatively close except for VcRBOHF (Fig. 2). Genes clustered together based on protein sequences may have similar functions. For example, it was shown that TaNOX12 from wheat (*Triticum aestivum* L.) shares high sequence similarity with AtRBOHF and exhibits a similar expression pattern under stress conditions [47]. A recent study has shown that AtRBOHF plays a crucial role in stomatal immunity and defense against *Pst* DC3000 through complex control of both ROS production and apoplastic pH [48]. We note that VcRBOHF is evolutionarily close to Arabidopsis AtRBOHF, suggesting that it may have a similar function to AtRBOHF (Fig. 2). In addition to functional similarities, genes that are evolutionarily closely related also exhibit structural similarities. Indeed, VcRBOHA and VcRBOHD clustered in the subgroup II, and their protein structural domains and motifs showed a high degree of identity (Figs. 2 and 3). Different conserved domains have different functions, the NADPH_Ox domain is associated with ROS production and the EF-hand domain is involved in calcium ion binding [49]. Among the RBOH members in eggplant, not all genes contained both NADPH_Ox, Ferric_reduct, FAD_binding_8, NAD_binding_6, and EF-hand binding domains [39]. However, our study found that all VcRBOH members contain these conserved domains (Fig. 3), suggesting their integrity in ROS production. In the visual analysis of VcRBOH protein domains, we found that VcRBOHB and VcRBOHC contain two NADPH_Ox, NAD_binding_6 structural, and Ferric_reduct domains (Fig. 3). This suggests that these domains were duplicated and retained in the genome during evolution, resulting in multiple copies. Protein domain duplication is one of the important mechanisms of protein evolution, which provides the basis for the diversity and complexity of protein functions. Through protein domain duplication, organisms can quickly adapt to environmental changes and produce new functions [50]. We have not found this phenomenon in Arabidopsis, rice, and tomato, and whether RBOH protein domain replication is unique in blueberry remains to be further studied.

Cis-regulatory element and tissue-specific analysis can provide clues for gene function studies. Analysis of the promoter cis-acting elements of 6 *VcRBOH* genes revealed that they were attributed to stress responsiveness, light responsiveness, and phytohormone responsiveness, suggesting that *VcRBOH* genes may be involved in these biological processes. Previous studies have found that *RBOH* genes do play a role in response to various stresses and phytohormones. For example, maize *ZmRBOHB-α* was reported to be involved in various abiotic stresses such as wounding, cold, and salt [51]. BoRBOHD and BoRBOHF were responses to heavy metal stress in cabbage [52]. The cis-acting elements involved in defense and stress responsiveness were found in the promoter regions of *VcRBOHA*, *VcRBOHC*, and *VcRBOHF* genes, suggesting that these genes may participate in plant defense. It was found that *SlRBOH1* improves tomato tolerance by enhancing RBOH enzyme activity in response to ABA induction [53]. Arabidopsis *AtRBOHD* is involved in ROS production regulated by ethylene (ETH) and SA [54]. In our study, the cis-element involved

in ABA and MeJA responsiveness was widespread in all the *VcRBOH* gene promoters (Fig. 4), suggesting that the VcRBOH-dependent ROS production in blueberry may be regulated by ABA and MeJA. Since binding sites can occur randomly in the genome, assessing their enrichment in the promoter regions of unrelated genes can more accurately predict the biological processes involving *VcRBOHs*. This complex issue demands systematic analysis and additional research. For tissue-specific analysis, Arabidopsis *AtRBOHD* and *AtRBOHF* are expressed throughout the plant, whereas *AtRBOHH* and *AtRBOHJ* are only present in pollen tubes [55]. Correspondingly, it has been shown that *AtRBOHD* and *AtRBOHF* are indeed involved in multiple functions in Arabidopsis, whereas *AtRBOHH* and *AtRBOHJ* play a role in pollen tube growth [14, 56]. Thus, *VcRBOHA* and *VcRBOHD*, expressed in various tissues, may be equally multifunctional. In contrast, *VcRBOHB* and *VcRBOHE* expressed only in flower_at_anthesis, may be associated with flower growth and development.

PAMPs can induce ROS production and defense gene expression [4]. We noticed that some *VcRBOH* genes exhibit transient inhibition after PAMPs treatment, such as *VcRBOHB* and *VcRBOHE*, which are downregulated and then upregulated after flg22 treatment. We did not find studies on the expression patterns of other species' RBOH genes in response to PAMPs, so we are unclear whether the transient downregulation of some RBOH genes is a common feature. However, research shows that *GmCRK77* in soybeans is significantly downregulated at 30 min and upregulated at 90 min after flg22 treatment [57]. Gene expression in plant cells is a dynamic balancing process. Under external environmental stimulation, the initial downregulation may be to quickly adjust the cell state to adapt to external stimuli, while subsequent upregulation is to activate defense mechanisms. This dynamic balance may also involve feedback regulation mechanisms to ensure the appropriateness and timeliness of gene expression [58]. Our results showed that both flg22 and chitin can rapidly induce the expression of *VcRBOHF* (Fig. 6), suggesting that *VcRBOHF* may be involved in the PTI response of blueberry. To this end, the expression levels of the PTI marker gene *FRK1* were tested in Arabidopsis protoplast. After flg22 treatment, the overexpression of VcRBOHF in protoplasts significantly enhanced the expression of *FRK1* (Fig. 7), indicating that VcRBOHF can promote PTI response. Due to the challenges of genetically transforming blueberries, we introduced *VcRBOHF* into Arabidopsis to create transgenic plants, enabling us to directly assess the impact of *VcRBOHF* on ROS production. As expected, flg22-induced ROS levels were higher in OE-VcRBOHF-1 and OE-VcRBOHF-2 than in Col-0 plants (Fig. 7), directly supporting the role of VcRBOHF in ROS production. Similar to previous studies, the ectopic expression of cassava *MeRBOHB* and *MeRBOHF* in Arabidopsis enhances the production of ROS [32]. Upon recognition of flg22, the receptor flagellin-sensitive 2 (FLS2) interacts with Brassinosteroid Insensitive 1 (BRI1)-associated kinase receptor 1 (BAK1) to form a complex that activates and phosphorylates the downstream receptor Botrytis-induced kinase (BIK1) [59]. Activated BIK1 directly interacts with RBOHD, leading ROS production in Arabidopsis and strawberry [60, 61]. Therefore, whether VcRBOHF-enhanced ROS production is related to BIK1 interactions needs further investigated. PAMPs and ROS can both trigger callose deposition in plants [62]. Flg22-induced callose deposition also increased in VcRBOHF overexpressing plants, paralleling the results of ROS production (Fig. 7). These results suggest that VcRBOHF may play a role in plant defense via the PTI pathway. To confirm VcRBOHF's role in plant defense, lines overexpressing VcRBOHF were challenged with *Pst* DC3000. The results indicated that these lines had increased disease resistance compared to Col-0 plants (Fig. 8). Our study showed that VcRBOHF is involved in plant defense and provides candidate genes for further elucidating the disease resistance mechanism of blueberry.

Conclusions

In this study, we identified six *VcRBOH* genes from the blueberry genome. The phylogenetic relationship, motif, and conserved domain analysis showed that VcRBOHs have high similarity in protein sequences and functions. Cis-acting elements analysis suggested *VcRBOH* genes may be involved in stress, light, and phytohormone responsiveness. According to RNA-seq data, the tissue-specific analysis suggested that *VcRBOHA* and *VcRBOHD* may play important roles in regulating blueberry development. In addition, RT-qPCR showed that *VcRBOHF* was rapidly and significantly up-regulated by flg22 and chitin treatment. Notably, overexpression of *VcRBOHF* in Arabidopsis promoted PTI responses and enhanced plant disease resistance. Our findings provide valuable insights into the roles of *VcRBOHF* genes in plant defense responses.

Abbreviations

ETI	Effector-triggered immunity
ETH	ethylene
NADPH	Nicotinamide adenine dinucleotide phosphate
PAMPs	Pathogen-associated molecular patterns
PTI	Pattern-triggered immunity
RBOH	Respiratory burst oxidase homolog
ROS	Reactive oxygen species
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SA	salicylic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11303-8.

Supplementary Material 1

Supplementary Material 2

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Not applicable.

Author contributions
Z.S. and C.C. carried out bioinformatics analysis. D.L. designed this research. Z.S. wrote the manuscript. T.Y., Y.Z., Y.W., and D.X. analyzed the data. H.D. revised the manuscript. All authors reviewed the manuscript.

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Data availability
All data and materials used in this study are publicly available. The raw sequencing data from this study were obtained from the NCBI repository (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA494180/) for tissue-specific expression. The other datasets supporting the conclusions of the article is included within the article (and its additional files).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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