

Identification of NADPH oxidase family members associated with cold stress in strawberry

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NADPH oxidase is encoded by a small gene family (Respiratory burst oxidase homologs, *Rbohs*) and plays an important role in regulating various biological processes. However, little information about this gene family is currently available for strawberry. In this study, a total of seven *Rboh* genes were identified from strawberry through genome-wide analysis. Gene structure analysis showed the number of exons ranged from 10 to 23, implying that this variation occurred in *FvRboh* genes by the insertion and distribution of introns; the order and approximate size of exons were relatively conserved. *FvRbohC* was predicted to localize to the thylakoid membrane of the chloroplast, while other members were computed to localize to the plasma membrane, indicating different functions. Amino acid sequence alignment, conserved domain, and motif analysis showed that all identified *FvRbohs* had typical features of plant *Rbohs*. Phylogenetic analysis of *Rbohs* from strawberry, grape, Arabidopsis, and rice suggested that the *FvRbohs* could be divided into five subgroups and showed a closer relationship with those from grape and Arabidopsis than those from rice. The expression patterns of *FvRboh* genes in root, stem, leaf, flower, and fruit revealed robust tissue specificity. The expression levels of *FvRbohA* and *FvRbohD* were quickly induced by cold stress, followed by an increase in NADPH oxidase activity, leading to O_2^- accumulation and triggering the antioxidant reaction by the transient increases in SOD activity. This suggested these two genes may be involved in cold stress and defense responses in strawberry.

NADPH oxidase, an enzyme complex that catalyzes the NADPH-dependent one-electron reduction of molecular oxygen to the superoxide anion O_2^- , is also responsible for the accumulation of reactive oxygen species (ROS) associated with an abrupt rise in oxygen consumption as the respiratory burst oxidase [1–3]. The oxidase complex comprises a membrane-bound heterodimer, called flavocytochrome *b558*, consisting

of gp91^{phox} and p22^{phox}, and four cytosolic components (p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase Rac) in phagocytes. Once the cell is stimulated, cytosolic components will interact with flavocytochrome *b558* to activate NADPH oxidase and then generate O_2^- , subsequently ending with the secondary production of other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) [4–6]. ROS plays an important

Abbreviations

APX, ascorbate peroxidase; CAT, catalase; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; H_2O_2 , hydrogen peroxide; MDA, malondialdehyde; O_2^- , superoxide anion; $OH\cdot$, hydroxyl radical; POD, peroxidase; *Rbohs*, respiratory burst oxidase homologs; ROS, reactive oxygen species; SOD, superoxide dismutase.

role in immunity and cell growth, but excessive accumulation of ROS can cause cellular damage and might be toxic [7,8]. Still, some evidences have shown that ROS at low concentration could be the 'alarm' signal to activate defense responses in plants [9,10]. A balance between generation and elimination of the free radicals can keep cellular ROS homeostasis. Plants have evolved complex antioxidant defense systems including nonenzymatic (low molecular weight antioxidant compounds) and enzymatic (SOD, CAT, POD, and APX) components that scavenge excessively accumulated ROS under stress conditions [11,12].

In plants, NADPH oxidase is known as respiratory burst oxidase homologs (Rbohs), which encodes a homolog of mammalian phagocyte *gp91^{phox}*. It was proved that plant Rbohs are plasma membrane enzymes. The main structure of Rbohs contains six conserved transmembrane domains with C-terminal FAD and NADPH hydrophilic domains, two heme groups, and two N-terminal Ca^{2+} -binding EF-hand motifs which account for being regulated by Ca^{2+} [1]. *Rbohs*, a small gene family, have been identified and isolated in a wide range of plants. *Arabidopsis* has ten members (*AtRbohA–J*), with a tissue-specific expression pattern: *AtRbohA–G* and *AtRbohI* in the roots, *AtRbohH* and *AtRbohJ* in pollens, and *AtRbohD* and *AtRbohF* throughout the plant, suggesting that differential expression profile and functions are formed in plant growth [1,13,14]. NADPH oxidase has not only been linked with plant development, but also responds to different abiotic or biotic stress conditions mainly by adjusting the ROS generation [15]. ROS signals derived by orthologs of two *AtRbohs* (*AtRbohH* and *AtRbohJ*) are involved in pollen tube growth in tobacco [16]. Interestingly, the same function was not testified in *Arabidopsis thaliana*. Moreover, Rboh-dependent ROS generation has been associated with root and hypocotyl elongation, stomatal movement, seed germination, and fruit ripening [17,18]. Several studies have evinced that NADPH oxidases mediate an oxidative burst to respond to stress. The tomato *SlRboh1* (homologous to *AtRbohF*) expression level increased, followed by much ROS accumulation, to adapt to the higher CO_2 concentration environment [19], and it was also related to the regulation of stomatal movements in endurance to high temperature stress mediated by the phytohormones, abscisic acid (ABA), and brassinosteroid (BR) [20,21]. *AtRbohD* could affect H_2O_2 accumulation and involved in the release from suppression of root elongation by ethylene signaling during hypoxic stress [22]. In most instances, an inhibition of NADPH oxidase contributes to the decrease in ROS production, then leading to the change of reaction to cell death and stress resistance.

For example, DPI (diphenylene iodonium) inhibited NADPH oxidase activity, which decreased the production of H_2O_2 so as to lower ethylene-induced cell death rates in rice [23] and resulted in diminishment of expression of several defense-related genes in response to abiotic stress, such as wounding, oligosaccharides, systemin, and methyl jasmonate in *Lycopersicon esculentum* [24].

Strawberry is a perennial herb belonging to berry fruit crops. It is a model plant with high economic and nutritional value in *Rosaceae* genomics research. However, strawberry is often subjected to extreme sporadic chilling injury in the short term, which leads to huge economic loss because insulation measures are not strictly applied. Based on the significance of NADPH oxidase in the regulation of plant growth, development, and adaption to the environment, in this study, we identified NADPH oxidase family members from strawberry and analyzed their conserved motif, homology, and phylogenetic relationship with other plants. Enzyme activity and expression profiles in response to cold stress are also presented to provide scientific and theoretical basis for strawberry cultivation.

Materials and methods

Plant materials and treatments

Different tissues (root, stem, leaf, flower, and ripe fruit) of strawberry (*Fragaria × ananassa* cv. Toyonaka) were collected to verify NADPH oxidase family genes' tissue-specific expression. Meanwhile, the strawberry seedlings from current-year stem tip were grown in 12 cm × 10 cm pots filled with a 1 : 1 (v/v) mixture of soil and perlite, subjected to two-month routine management from March 2014 in the greenhouse of Sichuan Agricultural University. Subsequently, the potted seedlings with vigorous and uniform growth status were used in the following experiment. They were moved to growth chambers (RXZ-260B) with controlled environmental conditions (25 ± 1 °C, $108 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\cdot 16 \text{ h}\cdot\text{d}^{-1}$, and $70 \pm 5\%$ relative humidity) for 2 weeks. After that, the plants were subjected to cold stress at 4 °C. The samples (leaves) were collected at 0, 6, 12, 24, 48, 72, and 96 h after cold stress treatment and prepared in triplicate.

Identification and annotation of FvRboh homologs in the strawberry genome

Ten protein sequences of *Arabidopsis AtRbohA–J* obtained from The *Arabidopsis* Information Resource (<https://www.arabidopsis.org/>) and simultaneously the Hidden Markov Model (HMM) profile of NADPH_Ox (PF08414) downloaded from the Pfam protein family database (<http://pfam.sanger.ac.uk>) were used as queries to search against the strawberry

genome v.1.0 hybrid gene proteins. Subsequently, protein, gene, and cDNA sequences were all retrieved and examined using the National Center for Biotechnology Information (NCBI) BLAST tool with the default cutoff parameters.

Bioinformatics analysis

The exon–intron structure of the *FvRbohs* was identified using the online Gene Structure Display Server v.2.0 (<http://gsds.cbi.pku.edu.cn/>) based on alignments of their coding sequences with corresponding genomic sequences. The protein molecular weight, theoretical pI, instability, aliphatic index, and grand average of hydropathicity (GRAVY) index were obtained using ExpPASy ProtParam tool (<http://web.expasy.org/protparam/>). Putative signal peptides and subcellular location were predicted in the SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and ProtComp v.9.0 (<http://linux1.softberry.com/berry.phtml?topic=procomppl&group=programs&subgroup=proloc>). DNAMAN v.8.0 software was used to perform multiple sequence alignment of *FvRboh* protein amino acid sequences. Additionally, SMART program (<http://smart.embl-heidelberg.de>), NCBI CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>), and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) were applied to speculate EF-hands, transmembrane domains (TMs), and conserved binding sites for flavin adenine dinucleotide (FAD), NAD pyrophosphate, and NADP ribose. Phylogenetic relationship of *Rbohs* between strawberry and other species was constructed using Clustal X v.2.0 and MEGA v.6.0 software with the neighbor-joining (NJ) method under the Poisson model, and 1000 bootstrap test replicates would evaluate the reliability of interior branches. The basic sequence information for bioinformatics analysis was described in Appendix S1.

NADPH oxidase extraction and assays

A two-phase aqueous polymer partition system was used to isolate the leaf plasma membranes. The purity of the plasma membrane was assessed by assaying the activities of marker enzymes (vanadate-sensitive ATPase). A total of one milliliter reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 0.5 mM XTT, 100 μ M EDTA, 15–20 μ g membrane protein, and 100 μ M NADPH. After the addition of 100 μ M NADPH, the reaction was monitored at 470 nm with absorbance coefficient of 21.6 $\text{mm}^{-1}\cdot\text{cm}^{-1}$. The NADPH oxidase activity was assayed based on the reduction of XTT by O_2^- radicals [25,26]. Corrections were made for background production in the presence of 50 units of SOD.

Expression profiles of *FvRbohs*

Total RNA was extracted from samples using the improved CTAB method [27]. After quality assessment, 1 μ g of total

RNA was reverse-transcribed into the complementary DNA (cDNA) with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan) according to the manufacturer's instructions. The expression level of NADPH oxidase genes in different tissues and cold stress was determined by semiquantitative RT-PCR and quantitative RT-PCR, respectively. All quantitative real-time PCRs were performed using SYBR Green Premix Ex Taq™ (Takara, Japan) on the CFX96 real-time PCR system (Bio-Rad, USA) in triplicate of each sample. Total 20 μ L reaction contained 0.6 μ L of each primer (10 μ M), 10 μ L SYBR Premix (Takara, Japan), 2 μ L cDNA (10 ng) template, and 6.8 μ L of RNase-free water. Reaction protocol was set with two-step cycling conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Fluorescence was monitored at the end of the annealing step each cycle. Melting curve was inserted, ramping from 65 °C to 95 °C (increment 0.5 °C/5 s) after the final cycle. The relative expression level was analyzed with the $2^{-\Delta\Delta\text{Ct}}$ method. *FaActin* was used as the reference gene to standardize the raw data. Primers for *Rbohs* and *Actin* genes are listed in Table 1.

Determination of O_2^- production rate and SOD enzyme activity

A total of 0.1 g of the leaf powder was homogenized in 1 mL ice-cold potassium phosphate buffer (50 mM, pH 7.8) containing 1% (w/v) of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10 000 $\times g$ for 10 min at 4 °C. The supernatant fraction was prepared for determination of O_2^- content and SOD activity. Total soluble protein contents of the extracts were determined according to Bradford [28], and bovine serum albumin was used as a standard.

Superoxide anion (O_2^-) was determined according to the method of Cai *et al.* [29]. The supernatant (0.5 mL) was mixed with 0.5 mL of 50 mM potassium phosphate buffer (pH 7.8) and 1 mL of 1 mM hydroxylamine hydrochloride and then incubated at 25 °C for 1 h. After incubation, 1 mL of 17 mM *p*-aminophenylsulfonic acid (in glacial acetic acid/ H_2O (3 : 1)) and 1 mL of 7 mM α -naphthylamine (in glacial acetic acid/ H_2O (3 : 1)) were added into the mixture for a further 20 min at 25 °C, followed by immediate measurement of absorbance at 530 nm. A standard curve with NO_2^- was used to calculate the production rate of O_2^- from the chemical reaction of O_2^- and hydroxylamine hydrochloride.

Total SOD (EC 1.15.1.1) activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium chloride (NBT) [30,31]. The 3 mL of reaction mixture was comprised of 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μ M NBT, 2 μ M riboflavin, 100 μ M EDTA, and 100 μ L of enzyme extract. Subsequently, the reaction mixtures were illuminated under

Table 1. Primers for semiquantitative RT-PCR and quantitative RT-PCR.

Gene name	Forward primers (5' to 3')	Reverse primers (5' to 3')	Product size (bp)
<i>FvRbohA</i>	CTCGTCCAATAGTAGAATCC	ATTATTCTGAGAAGCAATCG	172
<i>FvRbohB</i>	GGATATGAGACAGTGAAGAT	GAAGTAATTGAGAACGGATG	164
<i>FvRbohC</i>	CCAGAAGATCATATCGGAGAAG	CTCGTTGTCGGAGTGATACT	75
<i>FvRbohD</i>	TGTTGATGACCATAGCATTC	AGGAGAGCGTAGACTATAAC	142
<i>FvRbohE</i>	TATAATGCTGAGTGCTTCTG	TGGTCTGCTATAGTCTATGTAA	172
<i>FvRbohF</i>	TGGCGACGAGCATGGATAGTTT	AGGGTTTCAGCAGCACCTTTGG	143
<i>FvRbohH</i>	TGCACGGTCTGCGCTTATTA	TCCGGCTTTGTGAGACAACA	80
<i>FvActin</i>	TTACAGAGACCACCTATAACTC	GCTCATCCTATCAGCGATT	122

Table 2. List of *FvRbohs* identified in strawberry.

Gen name	Gene ID	Chromosome	Location	Gene length (bp)	ORF length (bp)	Amino acid length (aa)
<i>FvRbohA</i>	gene31855	chr5	1932913–1942293	9381	3084	1027
<i>FvRbohB</i>	gene22214	chr6	5063705–5067964	4260	2661	886
<i>FvRbohC</i>	gene01814	– ^a	– ^a	12025	5598	1865
<i>FvRbohD</i>	gene00215	chr5	6658520–6662290	3771	2808	935
<i>FvRbohE</i>	gene12928	chr1	7411220–7415355	4136	2649	882
<i>FvRbohF</i>	gene26084	chr5	7980576–7984305	3730	2376	791
<i>FvRbohH</i>	gene14024	– ^a	– ^a	4296	2598	865

^aUnplaced scaffold.

light intensity of 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 25 °C for 15 min. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

Lipid peroxidation

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) contents in the leaves using the thiobarbituric acid method [32,33]. A total of 0.2 g of leaf samples was homogenized in 4 mL of 10% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 $\times g$ for 10 min. An aliquot of 2 mL supernatant was mixed with 2 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA) made in 10% TCA. The mixture was boiled at 100 °C for 10 min and then quickly cooled on ice. Samples were centrifuged at 10 000 $\times g$ for 10 min. The supernatant absorbance was monitored at 450, 532, and 600 nm, respectively, and MDA concentration was expressed as $\mu\text{mol}\cdot\text{g}^{-1}$ fresh weight.

Results

Identification and gene structure of *Rboh* homologs in the strawberry genome

A total of seven *Rboh* genes were identified and then named *FvRbohA*, *FvRbohB*, *FvRbohC*, *FvRbohD*, *FvRbohE*, *FvRbohF*, and *FvRbohH* according to conserved domain and multiple sequence alignment with

Arabidopsis (Table 2). As shown, five genes except for *FvRbohC* and *FvRbohH* were all mapped to a specific chromosome (1, 5, and 6, respectively). The length of 9381-bp *FvRbohA* and 12025-bp *FvRbohC* genes was much longer than that of other members. In addition, the open reading frame length ranged from 2376 to 5598 bp and deduced protein sequence lengths varied from 791 to 1865 amino acids.

The unrooted phylogenetic tree showed that *FvRboh* genes were placed in two well-resolved clades. Five members including *FvRbohA*, *FvRbohB*, *FvRbohC*, *FvRbohD*, and *FvRbohE* shared one clade, which indicated they might share a common evolutionary history. The other clade only contained *FvRbohH* and *FvRbohF* (Fig. 1A). The number of exons ranged from 10 in *FvRbohD* to 23 in *FvRbohC*. *FvRbohF* and *FvRbohH* owned 14 exons, while other genes had different number of exons. Although the order and approximate size of exons among the *FvRbohs* were relatively conserved, the length of introns was variable, which lead to a diversity of gene structures. In particular, *FvRbohC* and *FvRbohA*, respectively, contained one and four long introns consistent with their big size (Fig. 1B).

Amino acid sequence and conserved motif analysis of *FvRbohs*

The relative molecular mass of the seven predicted *FvRboh* proteins ranged from 90.20 KDa (*FvRbohF*)

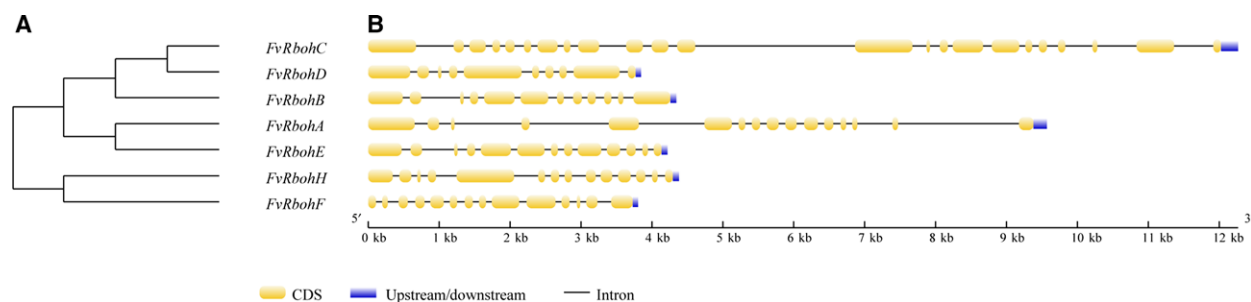


Fig. 1. Phylogenetic tree (A) and exon–intron structures (B) of *FvRboh* genes. A phylogenetic tree of *FvRboh* genes was constructed with the neighbor-joining method with 1000 bootstrap test replicates. Exons, introns, and untranslated regions (UTRs) are indicated by yellow boxes, gray horizontal lines, and blue boxes, respectively. The scale bar represents 12 kb.

to 206.97 kDa (FvRbohF), however, with similar corresponding isoelectric point around 9.0. Instability indexes higher than 40 showed that all family members of FvRbohs were unstable protein. Aliphatic indexes were predicted between 85.84 and 92.10. FvRbohs are inclined to be hydrophilic because grand average of hydropathy (GRAVY) negative values varied from -0.117 to -0.275 . These proteins had no signal peptides, but contained six transmembrane helices (TMHs). Subcellular localization results indicated that most of the FvRbohs were localized to the plasma membrane, while only FvRbohC was computed to reside in the chloroplast thylakoid membrane (Table 3).

In addition, multiple sequence alignment of the seven FvRboh proteins was performed (Fig. 2), which demonstrated that these sequences were highly conservative and contained typical conserved domains of NADPH oxidase including two putative Ca^{2+} -binding EF-hands and six transmembrane domains (TM1–6) in the N-terminal region, and flavin adenine dinucleotide (FAD), NAD pyrophosphate, and NADP ribose conserved binding sites in the C-terminal region. To further reveal the structural diversity and function prediction of FvRboh proteins, a total of eight conserved motifs were identified using online MEME tool

and annotated based on Pfam, NCBI CDD, and PROSITE databases (Fig. 3). All seven FvRbohs shared eight motifs once, except for motif 3 in FvRbohH. Moreover, the order and distribution of the eight motifs in these proteins were almost the same as described above (Fig. 3A). Motif 1 was annotated as FAD-binding domain; motifs 2, 6, and 8 were annotated as NAD-binding region; and motif 5 was annotated as EF-hands (Fig. 3B).

Phylogenetic analysis of Rbohs in different plants

To analyze phylogenetic relationship of NADPH oxidase family members in strawberry, grape, Arabidopsis, and rice, the amino acid sequences of 33 Rboh proteins, including 7 from strawberry, 7 from grape, 10 from Arabidopsis, and 9 from rice, were aligned and used to construct an unrooted phylogenetic tree with the neighbor-joining method (Fig. 4). The results indicated that 33 Rbohs could be assigned to five distinct subgroups (I, II, III, IV, and V). FvRbohC and FvRbohD were divided into the subgroup I. FvRbohF and FvRbohH were classified into the subgroup V. FvRbohA, FvRbohB, and FvRbohE belonged to subgroups IV, II, and III, respectively. Notably, FvRbohs were clustered together with grape or Arabidopsis

Table 3. Protein properties of FvRbohs.

Protein	MW (kDa)	pI	Instability index	Aliphatic index	GRAVY	SignalP	TMHs	Location
FvRbohA	116.25	8.94	50.16	86.83	-0.255	No	6	Plasma membrane
FvRbohB	101.02	8.93	40.47	92.10	-0.170	No	6	Plasma membrane
FvRbohC	206.97	8.88	46.45	88.53	-0.240	No	6	Chloroplast thylakoid membrane
FvRbohD	105.46	9.14	40.47	85.84	-0.275	No	6	Plasma membrane
FvRbohE	100.03	8.63	48.85	87.61	-0.174	No	6	Plasma membrane
FvRbohF	90.20	8.74	50.13	85.94	-0.117	No	6	Plasma membrane
FvRbohH	98.37	8.93	43.79	88.81	-0.159	No	6	Plasma membrane

FvRbohA	MRGHQRRWASDVPDKTIASGTTSPGTESTSGEEFVEVLDLQDDNTIVLRVFEATVNVNIPDDGEATFASSSRSSIRSSSSNRIRQFSQELKAEAVAKA	102
FvRbohBMEIEENPLPEKESIRVGG.....HSGPLVIGSKTRSNLSAR.....FKEDDCQCVFVLDVDCDDNTFVLR	62
FvRbohCMKETAPEDHGEAATAGVAFPLQVHSDNELFAPFKMSGAGYVAFPLSGTLNKRGTG.....SKRTNHNHEEYVLDVSDVSSVK	81
FvRbohDMGSRRDQELSSDSVEMARTEHAFSG.....FLGFSSSGAWNRKSLDRKRTSTST.....ENDDEAVETPDLDDVSEAVHS	77
FvRbohEMLPFLNDLHNHNDQLVEVTLNNDNDIVVCSVAFVAGTSEEEETGFGMLGRS.....	56
FvRbohFSSSSCTNDNIMLPSFSSVWTESD.....KTEPLQAVNQE.....QAPFMSDGLRFRMEL.....TANGGMREDVWDR	11
FvRbohHDVPSSSENVLGTNNIVRRRAG.....FHGN.....VPSGTGVPEAR.....GGGGGGGSLRGLN.....IKGSEZAPRPMERK	27
ConsensusMNISENE.....GREDEVSKVGLQNDIDEMV.....	
FvRbohA	KQFSQELKAEKRFWSHGNSRVLAAS.....SAIASQNNGGGAGSYHDSALAAALRRQRAQLDRTSQAQKALRG.....FISNCKSISNGLDANNEVESN	199
FvRbohB	NIR.GADEFGSTASRIEGRFSLASQLSF.....KLRKVSHELK.....RMKSSLSFKVVDQF.....ARFQVARALKGRHMT.....RNVCSQWLEEMR	143
FvRbohC	FLFRGDANNIDSLTLGGDDFPQKSNNDQSFVRTASVRIK.....QELKRLTSEFKQQLPQRRFRDRMQSAAAFALRGKRPIS.....KIDGNAPMPAVEKR	174
FvRbohD	VQAAGGGGHEDPQLLAKKTLLEGKKSASFSSLLRSTSHIRQVSQLKRLASFWRNSGAGRRIDRTSATAHALKSRPITA.....KASAANGASVEKR	176
FvRbohELSATSRLRRTFGLIRSTSRRTAAS.....SNSS.....ELPFLPREARRLRKLRTRSGAQRALNRRISKTTGFSDAEERKQVEAR	138
FvRbohFSSSSCTNDNIMLPSFSSVWTESD.....KTEPLQAVNQE.....QAPFMSDGLRFRMEL.....TANGGMREDVWDR	77
FvRbohHDVPSSSENVLGTNNIVRRRAG.....FHGN.....VPSGTGVPEAR.....GGGGGGGSLRGLN.....IKGSEZAPRPMERK	96
ConsensusMNISENE.....GREDEVSKVGLQNDIDEMV.....	
EF-hand 1		
FvRbohA	YKILAND.....GNLYRALFACIYGMK.....DSKEFFLELFDALGRRRLKVKDR.....ISKDEYEEFSQISDCSFDSDICFFDMVDRNIGIATEVEEKELIMS	293
FvRbohB	FDELAVD.....GTLPKSVFACIYGMK.....ESSEFFCEVFDALARRRGITSC.VTKEDREEREQISDHSFNARICFFDMVDRNADGRISEEVKELIMS	237
FvRbohC	FKKITGST.....NGMLPRAAFEGCIYGMK.DCKEFFGKLFDAARHKGIGDS.INKEDKEMATQVSECGFNRIQFFDMVDRKDGGRISVEEVKELIMS	271
FvRbohD	ESDITANS.....NGLINSSIFEGCIYGMK.EKSEFFGELFRALARRRISGDA.INKACREEREQISDSESDSDICFFDMVDRKDGGRISVEEVKELIMS	273
FvRbohE	FSEIAYD.....GLIARADFGCIYGMK.DSEFFVGIFFTLARRRQNIITK.ISKEEDRDFRSQISDCSFDSDICFFDMVDRNIGIATEVEEKELIMS	232
FvRbohF	FDRIISITIGTEFVIRKSDIGLIGME.....QSEFFNQILFARRRSMD.RNFG.IFKAEICQSVRSDITDFCSDFSDICFFDMVDRNIGIATEVEEKELIMS	175
FvRbohH	FRQVAVE.....DRLSRDDIETIGGGRESKEEFGQLEDAICRRRKLCAADGITIIVEFLRLEDITSEDTESLQFFDMVDRNIGIATEVEEKELIMS	193
Consensusf c i g m e f a i w r q f f d m d g g i s	
EF-hand 2		
FvRbohA	FCANRSLRKEAEYAAIMBELLPERGYTELWQFEDILQK.DTYLNSYQALSYSQALSQNLQGLRRRSFIRISSKCIYQLQENRRRIVLWVCI	394
FvRbohB	ASANRSLRKEAEYAAIMBELLHNLGYIDLHNBEMLLSGFTQSSNLETDSHQSRLLSEKLVFTKEYNEIRWRRLRSYFVEDNRWRIVLWVCI	379
FvRbohC	ASANRSLRKEAEYAAIMBELLSPNLYMIDSDPILYGFPEETVARGKESRNLKMLSKLRFVREINFLTRWRANFLFEDNRWRIVLWVCI	373
FvRbohD	ASANRSLRKEAEYAAIMBELLSPNLYMIDSDPILYGFVHSHVTVSDSRVLSQLSKLRFQENNEIKRQVHKTFFLLDNRWRIVLWVCI	375
FvRbohE	ASANRSLRKEAEYAAIMBELLDPNGYIELWQFEDILQK.DVYIDYSRPLSAVSVAVSQNLSSFRPKNLLRRLNRSNCLLIDNRWRIVLWVCI	333
FvRbohF	ATNIRLITPEADYAAIMBELLTEKRGVIELQFEDILQK.....LSKASFHNHLSIRHDFYHNFQVCRFNSRINILFRYWRWRIVLWVCI	270
FvRbohH	ASANRSLRKEAEYAAIMBELLDPHGYTELWQFEDILQK.REMVMVETAEKFKISKRTQLAKAMIFRVRNEMSKFTSTIIELVYENRWRIVLWVCI	295
Consensus	a n l a y a l m e l d g y i e l w q e l q k n t r q l a k a m i f r v r n e m s k f t s t i i e l v y e n r w r i v l w v c i	
TM1		
FvRbohA	MICFAWFEYQNRKNSFCIQIGYCLLARGAETLRNMAALDIPVCRNITLRL.NTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	495
FvRbohB	MICFAWFEYQNRHREVYHVMGVQVCIARGCAETLRNMAALDIPVCRNITLRLNTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	441
FvRbohC	MALFAWFEYQNRNRAAYHVMGVQVCIARGCAETLRNMAALDIPVCRNITLRLNTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	475
FvRbohD	VSPFAWFEYQNRKNSFCIQIGYCLLARGAETLRNMAALDIPVCRNITLRLNTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	477
FvRbohE	MALFAWFEYQNRKNSFCIQIGYCLLARGAETLRNMAALDIPVCRNITLRLNTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	434
FvRbohF	DHVFEGQTLAARFGY.HQSEFFELITKEVASGIGVIMAVASFLARFRGSGFS.LKRSVRNVMGNFVWSEHFLFIPVHLAGNLCACSESLIKV	372
FvRbohH	NVGFHWYFCQKNSLRYKMGKCCARGCAETLRNMAALDIPVCRNITLRLNTRVGVVFPFDNINFRHSFAAIVHGVHLAGNLCACSESLIKV	396
Consensus	l f k q y c a k a e t l r n m a l d i p v c r n i t l r l n t r v g v v f p f d n i n f r h s f a a i v h g v h l a g n l c a c s e s l i k v	
TM2		
FvRbohA	PESVVEEYLHDDST.HRFDLIDLVKRGASVIGILMVICITIAFLALPKWFRSLIK.LKRPFRNLKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	595
FvRbohB	TNDQYDK.LKFPFGQRPRDWNWFKGTSEWGVVWADLVAIYLAQCFWFRNRLRLNLRKLRKLGKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	542
FvRbohC	DSVTYAF.MRQYGE.QPSNWFVFKVSEVIGIMVWVITLALTPAFCFRGKLNKLPKLRKLGKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	575
FvRbohD	TECVES.MIPVGEQDPDWNWFKVGEDELTGGMVWVITLALTPAFCFRGKLNKLPKLRKLGKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	577
FvRbohE	SPEEFAP.ISATFHG.KKFEFELTGFPEELTGLMVENASFLASRFRNMVR.LPAFENKLGKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	533
FvRbohF	DHVFEGQTLAARFGY.HQSEFFELITKEVASGIGVIMAVASFLARFRGSGFS.LKRSVRNVMGNFVWSEHFLFIPVHLAGNLCACSESLIKV	472
FvRbohH	PHDFMDIAGPFRY.KQSEMDLVKSIPIGICIFVWVAESFLANSLER.SIN.LKGLKNWIKGNFVWSEHFLFIPVHLAGNLCACSESLIKV	495
Consensus	f y g v m l a r c l p g n f w y s h l y l h l k	
TM3		
FvRbohA	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	655
FvRbohB	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	643
FvRbohC	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	635
FvRbohD	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	637
FvRbohE	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	593
FvRbohF	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	533
FvRbohH	ITIMNLSVFLVLAGERLIRVHRSGFSSVRLR.....VALYHGVLVHMSKQCFVYHSGVM	555
Consensus	t t m y p y e d v k g v m l a r c l p g n f w y s h l y l h l k	
FAD		
FvRbohA	FVCGPAPFEEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....EPP.LAGSGLLRADETT.....KKS.LPKRLIGGY	733
FvRbohB	YINGCAIPFCWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....QFP.SIEHSGLLRADIEQP.....NIHTRNPKRLIGGY	724
FvRbohC	FVCGPAPFEEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....QFP.FSGNSGLLRADCMQS.....NDLN.PBRVLIIGGY	715
FvRbohD	FVCGPAPFEEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....QFS.AAGSGLLRADCMQE.....NNFS.PBRVLIIGGY	717
FvRbohE	FVCGPAPFEEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....HSISIVHQASVITAMDQRR.....LFN.QRLELIGGY	672
FvRbohF	FVCGPAPFEEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....LSSLN.....S.PBRVLIIGGY	597
FvRbohH	FLKFDLNEWHPPFSITSPGDDLSVHIRGWDWICELKRVFEAC.....TTELRNIESKVSQVTTQTTRKGLVRLTRVRFPTVDEVEDFKLEEFPQVFRVLIIGGY	657
Consensus	c s whpfs ts p d lsvhir gdw ic el k r v f e a c e p p l a g s g l l r a d e t t k k s l p k r l i g g y	
NAD pyrophosphate		
FvRbohA	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....LSDFSRNSLSSGSDTSFNKLNPK.....RKTKLTKTIVYVTRQCSBFWRG	829
FvRbohB	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....LQMLVGVKRRKST.....FKKRRVYVTRQCSBFWRG	804
FvRbohC	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....SQNALESAGTAGSGSFPVLSHNS.....FKKRRVYVTRQCSBFWRG	801
FvRbohD	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....NNSLNKNELEDEYAVLVDLESGRAGNINNSSSNGKSNKNGKGFTRQAYVYVTRQCSBFWRG	819
FvRbohE	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....NTEISDLSLSSFTSCLIPND.....KKSQRITVYVTRQCSBFWRG	766
FvRbohF	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....SSHCDHSGCRECSSR.....KSPLRVYVTRQCSBFWRG	674
FvRbohH	GAFQDYRNEDVLLHGLGIGATFISIVRHNIVKMEQADS.....SGHVRRSSSLGKLLSGDHVEVN.....TKTPEYVYVTRQCSBFWRG	749
Consensus	g a p q y l g l g i g a t p k d n a q k g d s h f p n w n g a w t r e s f w f	
NADP ribose		
FvRbohA	VMNEVADQ.QRQVIBMNYITSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	930
FvRbohB	VMNEVAEN.DLDGLIPIHNYCTSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	886
FvRbohC	AMNEVAEL.DHNRVIFHNYCTSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	902
FvRbohD	LINEVAEM.DKGVIFHNYCTSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	920
FvRbohE	VMNEVAEN.DHKGIVHNYITSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	867
FvRbohF	IMKEISDSKQCSLIVMESHITSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	776
FvRbohH	VMDIIEAC.QRQVIBMNYITSVVEGDARSLLITMVALNEARGLDIVSGTRVRFHFRFNRKRVFSKFCSEYGARIGVYCGAFVLAELSKICYEF	850
Consensus	e h tsvy egdars ll i t m v a l n e a r g l d i v s g t r v r f h f r f n r k r v f s k f c s e y g a r i g v y c g a f v l a e l s k i c y e f	
FvRbohA	NQKTHWAEDSLEKSETEEGSLDLWDESATPPSHRVTAATAAVLHHPATLYTGGFNGSLILRRNSCVHIASVYILCMWFRCCARANLAICDPIAV	1026
FvRbohB	886
FvRbohC	NHKTSTREFFHKENF.....	917
FvRbohD	SHRISTREFFHKENF.....	935
FvRbohE	SKRISTREFFHKENF.....	882
FvRbohF	STRISTREFFHKENY.....	791
FvRbohH	SINSSTREFFHKENF.....	865
Consensus	

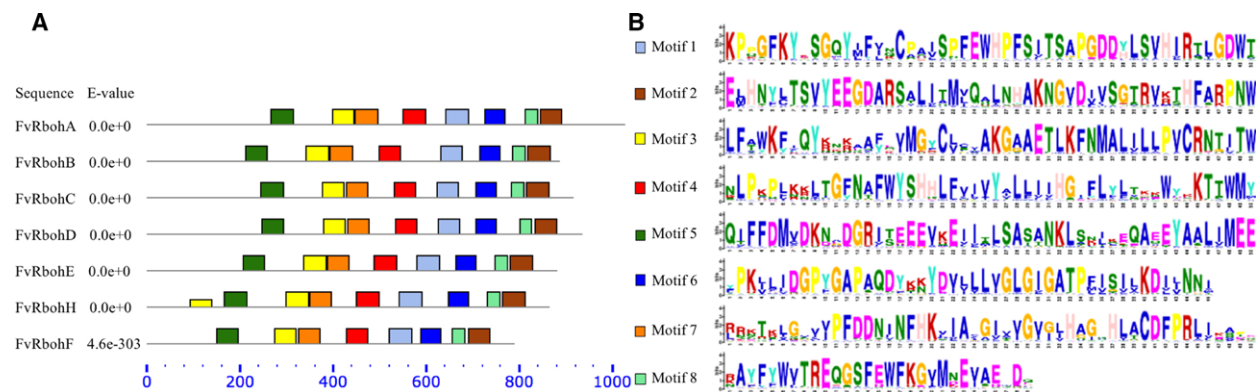


Fig. 3. Conserved motifs distribution (A) and sequence (B) in *FvRboh* proteins. Motif of the *FvRboh* family members in strawberry was analyzed using the MEME web server. Different color boxes represent eight putative motifs, respectively. Names of all family members and *E*-values are shown on the left side. The blue dotted line scale represents the length of amino acid. The height of a letter indicates its relative frequency at the given position in the motif.

Rbohs first, suggesting that strawberry *Rboh* proteins were more closely related to those from grape and *Arabidopsis* than to those of rice, which was consistent with the fact that strawberry, grape, and *Arabidopsis* are eudicots and diverged more recently from a common ancestor than from the lineage leading to monocots.

NADPH oxidase activity and expression patterns of *FvRbohs* in response to cold stress

Expression of *Rbohs* in plant as previously reported was tissue-specific. In our study, the results indicated *FvRbohA*, *FvRbohC*, *FvRbohD*, and *FvRbohF* could be detected in root, stem, leaf, flower, and fruit. *FvRbohB* and *FvRbohE* were expressed in root, stem, flower, and fruit, while *FvRbohH* was only observed in flower and fruit (Fig. 5A). Exposure to cold stress, NADPH oxidase activity in strawberry leaves showed a tendency: increased quickly and peaked at 48 h, and then had a decrease. Although the enzyme activity decreased in the late phase of treatment, it was still higher than that of the initial phase (25 °C). The relative expression levels of four members (*FvRbohA*, *FvRbohC*, *FvRbohD*, and *FvRbohF*) that could be detected in strawberry leaves were examined to explore their response to cold stress by real-time qPCR analysis. As shown, *FvRbohA* and *FvRbohD* were keeping high expression levels, while *FvRbohC* and *FvRbohF* were difficult to be tested during cold treatment. Generally, the expression

patterns of *FvRbohA* and *FvRbohD* were observed to be M-shaped. The transcript abundances of *RbohA* and *RbohD* were highly induced and reached the maximum value after the first 6-h low-temperature exposure (Fig. 5B), so the peak value showed up earlier than that of NADPH oxidase activity.

O₂⁻ production rate, SOD enzyme activity, and lipid peroxidation

O₂⁻ production rate increased slowly at the initial stage of cold stress and then followed a transient burst. After 72 h, a slight decrease in O₂⁻ production rate was almost coinciding with NADPH oxidase activity. SOD activity had a quick increase and reached the peak value at 24 h, which inhibited the O₂⁻ production. However, at the late stage of cold stress, SOD activity began to decrease (Fig. 6B). The MDA accumulation was often used as an indicator of lipid peroxidation. In general, the successive cold stress for 96 h caused a linear increase in MDA content (Fig. 6C).

Discussion

It was well known that ROS was toxic to biological organisms by oxidizing lipids, proteins, DNA, and carbohydrates, resulting in breakdown of normal cellular, membrane, and reproductive functions [34], but ROS

Fig. 2. Protein sequence multi-alignment and domain structure of the *Rbohs* from strawberry. Conservative residues are highlighted by black shadings, and a lower level of conservations is indicated by lighter shadings. EF-hands and conserved binding sites for FAD, NAD pyrophosphate, and NADP ribose are represented by straight lines. Transmembrane domains are indicated by boxes.

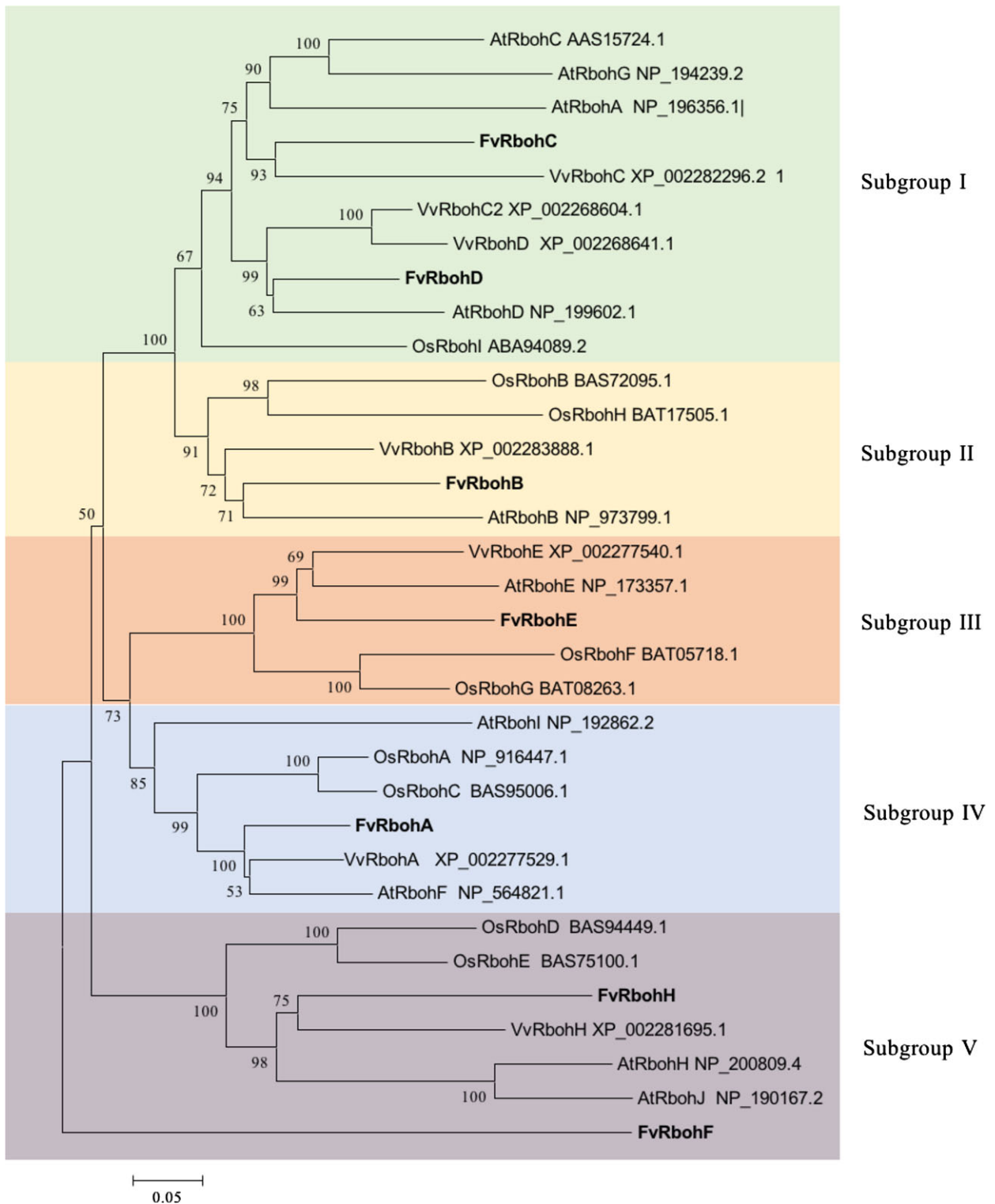


Fig. 4. Phylogenetic analysis of Rbohs in strawberry, grape, Arabidopsis, and rice. The phylogenetic tree was constructed with the neighbor-joining method based on the Poisson model. Tree reliability was assessed using 1000 bootstrap replicates. The numbers indicated for each clade represent bootstrap support values given as percentages. Five subgroups are shown as I, II, III, IV, and V.

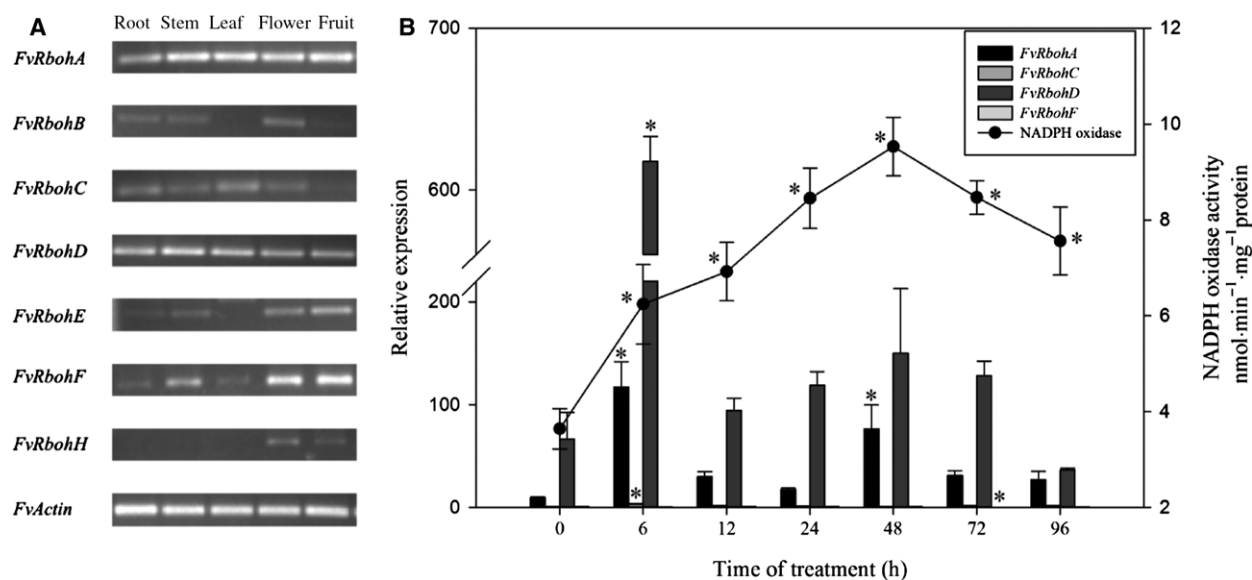


Fig. 5. Expression patterns of *FvRbohs* in different tissues (A) and response to cold stress (B). The left and right Y-axis is the scale of the relative transcript abundance level and NADPH oxidase activity, respectively. The X-axis is the time course of 4 °C cold treatment. Values represent means \pm standard error from three biological experiments ($n = 3$). Asterisks indicate significant differences based on one-way ANOVA in SPSS 23.0 followed by the Dunnett t -test (time 0 as the control, $P < 0.05$).

when its concentration is in the appropriate range can act as a signal molecule to influence cell growth, organ development, and defense responses. In plant, NADPH oxidase-catalyzed conversion of the superoxide anion (O_2^-) to other ROS, such as hydrogen peroxide, hydroxyl radicals, and perhydroxyl radicals, was the major source of ROS production [26]. NADPH oxidase was coded by a small gene family of *Rbohs*. Benefiting from the availability of whole-genome sequence in recent years, so far, a few model and crop plant NADPH oxidase families have been identified at the genomewide level, including 10 in *Arabidopsis* [1], 9 in rice [35], 6 in barley [36], 9 in apple [37], and 7 in grape [38]. However, very little is known about this family in strawberry.

This study comprehensively identified and characterized seven *FvRboh* genes from strawberry at the genomewide level, which are mainly distributed in chromosomes 1, 5, and 6. Gene structure analysis showed *FvRbohs* included at least ten exons; *FvRbohC* had the most exons of 23, which was greatly surpassed that in other plants. Actually, the majority of *Rbohs* harbored 10–14 exons in *Arabidopsis*, rice, barley, and grape, except that *AtRbohD* contained eight exons and *OsRbohD*, *VvRbohB*, and *VvRbohD* had 15 exons in their coding DNA sequence (CDS) [39]. The large structural variation of *Rboh* genes implied the significant genomic change during their evolutionary history, possibly as a result of highly diverse distribution and

insertion of intronic regions amid the exonic sequences. In addition, *FvRbohC* protein was predicted to localize to the thylakoid membrane of the chloroplast, while other members were computed to localize to the plasma membrane, indicating the different functions. This case was also reported in the grape [38], in contrast to *Arabidopsis* and rice, where all the *Rbohs* were predicted to localize to the plasma membrane. The alignment of seven *FvRbohs* clearly showed the presence of two EF-hands, six TMs, FAD, and NAD-binding sites, which were known to be present in *Rbohs* from other plant species. EF-hand regions that were absent from the mammalian phagocyte gp91^{phox} protein can bind Ca^{2+} , accounting for the direct regulation of plant *Rbohs* by Ca^{2+} [1,14,40]. Thus, the regulation way of NADPH oxidases in plant might be different from that in mammalian phagocytes. Subsequently, the characterization and distribution of conserved motifs furtherly emphasized the importance of these structures. The phylogenetic tree of *Rboh* proteins from different plant species showed *FvRbohs* had a closer correlation with *VvRbohs* and *AtRbohs* than those from monocotyledonous rice, indicating the genes were established prior to the divergence of the corresponding taxonomic lineages. Moreover, those homologs clustered in the same group were possible to be involved in similar functions; however, further experimental analyses are necessary to confirm this.

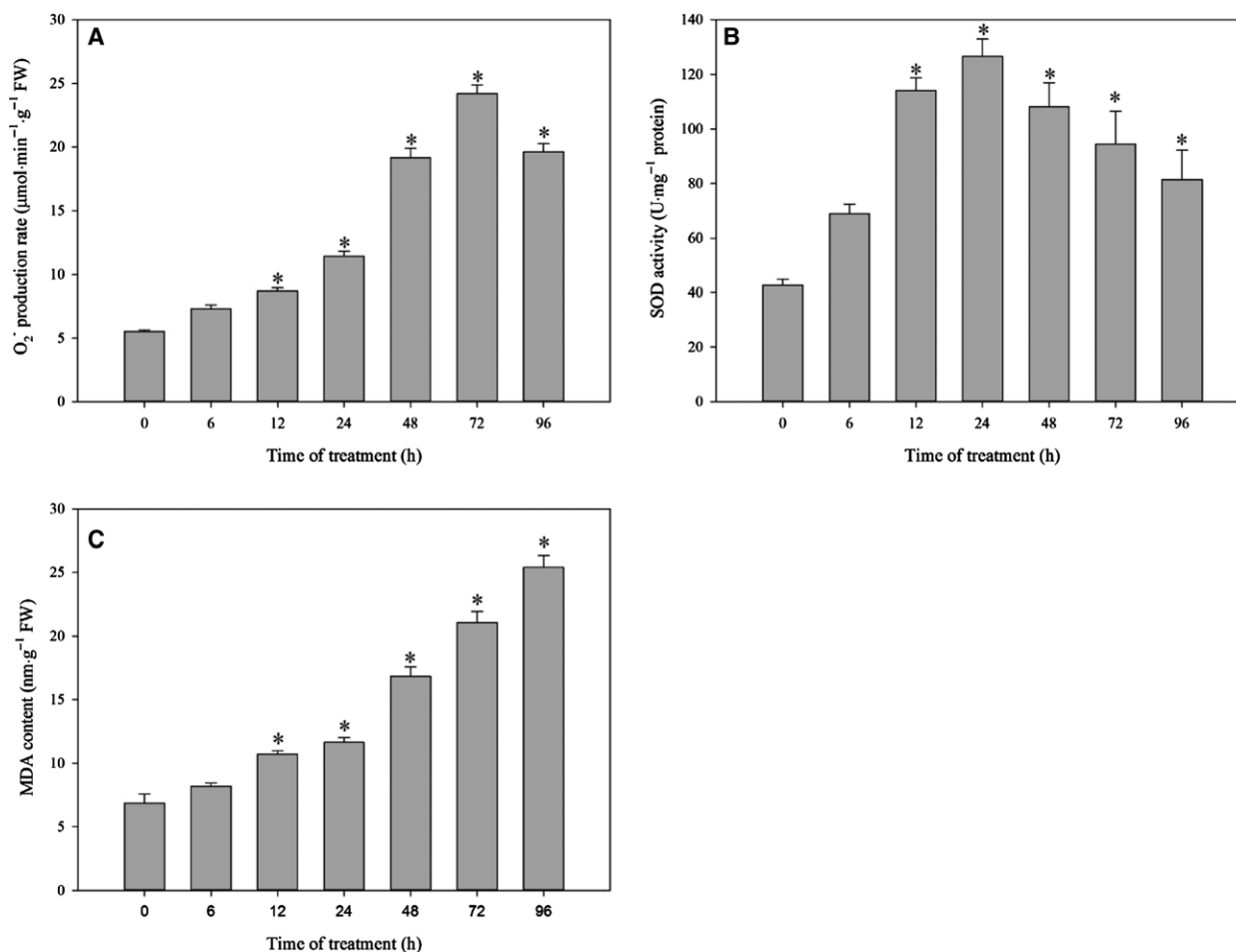


Fig. 6. O₂⁻ production rate (A), SOD enzyme activity (B), and lipid peroxidation (C). Each value represents mean ± standard error in histogram. Values represent means ± standard error from three biological experiments ($n = 3$). Asterisks indicate significant differences based on one-way ANOVA in SPSS 23.0 followed by the Dunnett t -test (time 0 as the control, $P < 0.05$).

The tissue-specific expression patterns of *Rboh* genes have been reported in some species; however, there is no uniform expression pattern for the plant *Rboh* genes reported. In grape, seven *VvRboh* genes could be detected in all tissues involved in young leaves, roots, stems, inflorescences, berries, tendrils, and ovules [38], while four of the six and two of the ten *Rboh* genes had widespread constitutive spatial expression patterns in barley and *Arabidopsis*, respectively [1,36]. In our study, *FvRbohA*, *FvRbohC*, *FvRbohD*, and *FvRbohF* were tested in all tissues; *FvRbohA* and *FvRbohD* were the highly expressed genes. *FvRbohB* and *FvRbohE* had transcript abundances in most of the tissues except for leaves, while *FvRbohH* was expressed only in flower and fruit. These findings might suggest tissue-specific function of these family members.

It has been documented that low temperature, as many other stressful environmental conditions, could

trigger enhanced generation of ROS to disrupt cellular homeostasis. But ROS when its concentration is in the appropriate range can act as a signal molecule to initiate defense responses [41]. Plasma membrane, peroxisomes, chloroplasts, and mitochondria are potential sources of ROS in plant cells [42]. The plasma membrane-located NADPH oxidases have been shown to mainly involve in ROS production and play critical roles in plant development and defense responses [15,43]. Our study showed that NADPH oxidase activity increased sharply at the early stage of strawberry seedlings exposed to cold stress and then had a slight decline, but still kept high level, compared to 0 h (25 °C), accompanied by the production of the superoxide anion. To alleviate the negative effects of stress and balance the ROS level, organism would initiate enzymatic and nonenzymatic mechanisms to maintain the cellular redox homeostasis. SOD was involved in

dismutation of O_2^- to H_2O_2 , and its activity reflected the ability to adapt to stress in plant. In our study, SOD activity increased at the initial treatment and then decreased, which demonstrated that short-term cold stress could induce antioxidative defense mechanism in the plant. However, the longer the stress time was, the more ROS produced, so that antioxidative enzyme activity was impaired and the ROS could not be removed effectively. Subsequently, peroxidation of lipids in the cell membrane resulted in a massive MDA accumulation, which was toxic to plant [44]. Correspondingly, the *Rboh* genes (*FvRbohA*, *FvRbohC*, *FvRbohD*, and *FvRbohF*) which were specifically expressed in leaves were monitored during the cold treatment. *FvRbohA* and *FvRbohD* reacted quickly to cold stress by improving transcript levels, while *FvRbohC* and *FvRbohF* were keeping a low expression level during this process. *CsRbohA* was upregulated by low temperature in short time, which was consistent with our findings. *AtRbohD* in *Arabidopsis thaliana* was constitutively and ubiquitously expressed and showed a high degree of stress responsiveness [45,46]. Recently, it was reported that the expression level of *AtRbohD* had an extremely significant increase at an early stage in the hypoxia response. *AtRbohD*-knock-out mutant was used to further demonstrate that *AtRbohD* played a key role in regulating the transcript abundance of downstream hypoxia-inducible genes at an early stage during hypoxic stress and could be a cross talk in ethylene modulating H_2O_2 signal transduction in the hypoxia response pathway [22]. These facts hinted that *FvRbohA* and *FvRbohD* might be the dominate induction of antioxidant defense system in response to cold stress.

Conclusion

In this study, we totally identified seven *Rboh* genes from the strawberry genome and revealed classification, gene structure, evolution, conserved protein motif, and phylogenetic relationship by systematical bioinformatic analysis. NADPH oxidase was related to initiation of antioxidant system in leaves of strawberry against cold stress by regulating O_2^- production. Expression profile analysis among different tissues (root, stem, leaf, flower, and fruit) showed that *FvRboh* genes had the tissue-specific characteristic. Furthermore, *FvRbohA* and *FvRbohD* maintained the high expression in response to cold stress, implying that they played a crucial role in this process. This information provides some insights into potential functions of strawberry *Rbohs* and increases our understanding of the molecular basis of the acquired cold

tolerance of strawberry and even the adaptability of strawberry to other stress conditions.

Author contributions

YTZ and HRT conceived and designed the experiment. YTZ, YLL, and YWH performed the experiment and analyzed the data. WJH collected the samples. YTZ wrote the manuscript. YZ, XRW, and HRT reviewed drafts of the manuscript. All authors read and approved the final version.

References

- Sagi M and Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. *Plant Physiol* **141**, 336–340.
- Quinlan CL, Treberg JR, Perevoshchikova IV, Orr AL and Brand MD (2012) Native rates of superoxide production from multiple sites in isolated mitochondria measured using endogenous reporters. *Free Radic Biol Med* **53**, 1807–1817.
- Brandes RP, Weissmann N and Schroder K (2014) Nox family NADPH oxidases: molecular mechanisms of activation. *Free Radic Biol Med* **76**, 208–226.
- Vignais PV (2002) The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci* **59**, 1428–1459.
- Quinn MT and Gauss KA (2004) Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. *J Leukoc Biol* **76**, 760–781.
- Mizrahi A, Berdichevsky Y, Ugolev Y, Molshanski-Mor S, Nakash Y, Dahan I, Alloul N, Gorzalczany Y, Sarfstein R, Hirshberg M *et al.* (2006) Assembly of the phagocyte NADPH oxidase complex chimeric constructs derived from the cytosolic components as tools for exploring structure-function relationships. *J Leukoc Biol* **79**, 881–895.
- Panday A, Sahoo MK, Osorio D and Batra S (2015) NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell Mol Immunol* **12**, 5–23.
- Sharma P, Jha AB, Dubey RS and Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot* **2012**, 1–26.
- Reczek CR and Chandel NS (2015) ROS-dependent signal transduction. *Curr Opin Cell Biol* **33**, 8–13.
- Foyer CH and Noctor G (2009) Redox regulation in photosynthetic organisms—signaling, acclimation, and practical implications. *Antioxid Redox Sign* **11**, 861–905.
- Del Rio LA (2015) ROS and RNS in plant physiology: an overview. *J Exp Bot* **66**, 2827–2837.

- 12 Liu N and Lin Z (2014) Reactive oxygen species and relative enzyme activities in the development of aerial roots of Chinese Banyan (*Ficus microcarpa*). *J Plant Growth Regul* **33**, 160–168.
- 13 Torres MA and Dangel JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr Opin Plant Biol* **8**, 397–403.
- 14 Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE and Jones JDG (1998) Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (gp91phox). *Plant J* **14**, 365–370.
- 15 Marino D, Dunand C, Puppo A and Pauly N (2012) A burst of plant NADPH oxidases. *Trends Plant Sci* **17**, 9–15.
- 16 Potocký M, Jones MA, Bezvoda R, Smirnov N and Žárský V (2007) Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. *New Phytol* **174**, 742–751.
- 17 Dunand C, Crevecoeur M and Penel C (2007) Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: possible interaction with peroxidases. *New Phytol* **174**, 332–341.
- 18 Zhang J, Chen C, Zhang D, Li H, Li P and Ma F (2014) Reactive oxygen species produced via plasma membrane NADPH oxidase regulate anthocyanin synthesis in apple peel. *Planta* **240**, 1023–1035.
- 19 Yi C, Yao K, Cai S, Li H, Zhou J, Xia X, Shi K, Yu J, Foyer CH and Zhou Y (2015) High atmospheric carbon dioxide-dependent alleviation of salt stress is linked to RESPIRATORY BURST OXIDASE 1 (RBOH1)-dependent H₂O₂ production in tomato (*Solanum lycopersicum*). *J Exp Bot* **66**, 7391–7404.
- 20 Xia XJ, Gao CJ, Song LX, Zhou YH, Shi K and Yu JQ (2014) Role of H₂O₂ dynamics in brassinosteroid-induced stomatal closure and opening in *Solanum lycopersicum*. *Plant, Cell Environ* **37**, 2036–2050.
- 21 Zhou J, Wang J, Li X, Xia XJ, Zhou YH, Shi K, Chen Z and Yu JQ (2014) H₂O₂ mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses. *J Exp Bot* **65**, 4371–4383.
- 22 Yang CY and Hong CP (2015) The NADPH oxidase Rboh D is involved in primary hypoxia signalling and modulates expression of hypoxia-inducible genes under hypoxic stress. *Environ Exp Bot* **115**, 63–72.
- 23 Steffens B and Sauter M (2009) Epidermal cell death in rice is confined to cells with a distinct molecular identity and is mediated by ethylene and H₂O₂ through an autoamplified signal pathway. *Plant Cell* **21**, 184–196.
- 24 Orozco-Cárdenas ML, Narváez-Vásquez J and Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* **13**, 179–191.
- 25 Zhou J, Wang J, Shi K, Xia XJ, Zhou YH and Yu JQ (2012) Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants. *Plant Physiol Biochem* **60**, 141–149.
- 26 Sagi M and Fluhr R (2001) Superoxide production by plant homologues of the gp91phox NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. *Plant Physiol* **126**, 1281–1290.
- 27 Chen Q, Yu HW, Wang XR, Xie XL, Yue XY and Tang HR (2012) An alternative cetyltrimethylammonium bromide-based protocol for RNA isolation from blackberry (*Rubus* L.). *Genet Mol Res* **11**, 1773–1782.
- 28 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- 29 Cai C, Chen K, Xu W, Zhang W, Li X and Ferguson I (2006) Effect of 1-MCP on postharvest quality of loquat fruit. *Postharvest Biol Tec* **40**, 155–162.
- 30 Ali B, Tao Q, Zhou Y, Gill RA, Ali S, Rafiq MT, Xu L and Zhou W (2013) 5-Aminolevulinic acid mitigates the cadmium-induced changes in *Brassica napus* as revealed by the biochemical and ultra-structural evaluation of roots. *Ecotoxicol Environ Saf* **92**, 271–280.
- 31 Beauchamp C and Irwin F (1971) Superoxide dismutase- improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276–287.
- 32 Zheng X and Tian S (2006) Effect of oxalic acid on control of postharvest browning of litchi fruit. *Food Chem* **96**, 519–523.
- 33 Shi H, Ye T and Chan Z (2013) Exogenous application of hydrogen sulfide donor sodium hydrosulfide enhanced multiple abiotic stress tolerance in bermudagrass (*Cynodon dactylon* (L). Pers.). *Plant Physiol Biochem* **71**, 226–234.
- 34 Rodriguez R and Redman R (2005) Balancing the generation and elimination of reactive oxygen species. *Proc Natl Acad Sci USA* **102**, 3175–3176.
- 35 Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T *et al.* (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. *Plant Cell* **19**, 4022–4034.
- 36 Lightfoot DJ, Boettcher A, Little A, Shirley N and Able AJ (2008) Identification and characterisation of barley (*Hordeum vulgare*) respiratory burst oxidase homologue family members. *Funct Plant Biol* **35**, 347–359.
- 37 Cepauskas D, Miliute I, Staniene G, Gelvonauskiene D, Stanys V, Jesaitis AJ and Baniulis D (2015) Characterization of apple NADPH oxidase genes and

- their expression associated with oxidative stress in shoot culture *in vitro*. *Plant Cell Tiss Org* **124**, 621–633.
- 38 Cheng C, Xu X, Gao M, Li J, Guo C, Song J and Wang X (2013) Genome-wide analysis of respiratory burst oxidase homologs in grape (*Vitis vinifera* L.). *Int J Mol Sci* **14**, 24169–24186.
- 39 Kaur G and Pati PK (2016) Analysis of cis-acting regulatory elements of Respiratory burst oxidase homolog (Rboh) gene families in Arabidopsis and rice provides clues for their diverse functions. *Comput Biol Chem* **62**, 104–118.
- 40 Keller T, Damude HG, Werner D, Doerner P, Dixon RA and Lamb C (1998) A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca²⁺ binding motifs. *Plant Cell* **10**, 255–266.
- 41 Mittler R, Vanderauwera S, Gollery M and Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci* **9**, 490–498.
- 42 Gill SS and Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* **48**, 909–930.
- 43 Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD *et al.* (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**, 442–446.
- 44 Luo Y, Tang H and Zhang Y (2011) Production of reactive oxygen species and antioxidant metabolism about strawberry leaves to low temperatures. *J Agr Sci* **3**, 89.
- 45 Suzuki N, Miller G, Morales J, Shulaev V, Torres MA and Mittler R (2011) Respiratory burst oxidases: the engines of ROS signaling. *Curr Opin Plant Biol* **14**, 691–699.
- 46 Liu Y and He C (2016) Regulation of plant reactive oxygen species (ROS) in stress responses: learning from AtRBOHD. *Plant Cell Rep* **35**, 995–1007.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Basic sequence information for bioinformatics analysis.