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Research Article

Comparative *In Silico* Analysis of *Respiratory Burst Oxidase Homologs* (*Rboh*) Gene Family in Economically Significant Dicot Crop Plants

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ABSTRACT

Reactive oxygen species (ROS), including superoxide and hydrogen peroxide, play a crucial role in the early defense mechanisms of plants against both biotic and abiotic stresses. Among the key contributors to ROS production are *respiratory burst oxidase homologs* (*Rboh*), plant-specific NADPH oxidases that regulate stress responses, signaling, development, and programmed cell death. While *Rboh* gene families have been extensively studied in model plants such as *Arabidopsis thaliana*, their characterization in other dicot species remains limited. Here, we conducted a genome-wide analysis of *Rboh* genes in four agriculturally important dicot species: *Brassica oleracea*, *Daucus carota*, *Helianthus annuus*, and *Capsicum annuum*. Using *Arabidopsis Rboh* sequences as references, we identified 38 *Rboh* genes across these species, which were compared with the 10 known *Arabidopsis Rboh* genes. Chromosomal mapping, gene structure, conserved domain, and motif analyses revealed that *Rboh* genes are evolutionarily conserved, with the number of genes aligning closely with those in previously studied plants. Phylogenetic analysis grouped these genes into two major clades and six subgroups, reflecting shared evolutionary lineages and potential functional similarities. Variations in exon-intron structures and unique domain duplications or deletions indicated possible functional divergence among specific genes. Chromosomal mapping showed uneven distribution of *Rboh* genes within each genome, consistent with patterns observed in other plant species. This study enhances the current understanding of the *Rboh* gene family in dicots, providing a foundation for future research on their functional roles and potential applications in improving plant resilience through genetic engineering or breeding strategies.

Keywords: *Rboh* gene family, Reactive oxygen species (ROS), Dicot crop plants, Genome-wide analysis, Plant stress resilience

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INTRODUCTION

The increased production of ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), is primarily associated with the initial response of plants against biological and environmental stresses (Kaur and Pati 2016). ROS function as secondary messengers in cellular responses and play fundamental roles in modulating protein activity, regulating growth, delaying development,

and transducing signals under various biotic and abiotic stress conditions (Song et al. 2014; Mhamdi and Van Breusegem 2018; Qi et al. 2018). However, intermittent ROS production during stress can lead to oxidative damage to vital biomolecules, including DNA, RNA, proteins, and lipids, ultimately accelerating the aging process in plants (Kesari et al. 2020; Künstler et al. 2020).

Plants have evolved various mechanisms to mitigate the damaging effects of ROS, primarily through the production of antioxidants such as superoxide dismutase, glutathione reductase, ascorbate peroxidase, and catalase (Apel and Hirt 2004). In addition to their role in damage control, ROS are also central to plant defense mechanisms against pathogens (Torres 2010). Specifically, oxidases and peroxidases mediate ROS production, triggering an apoplastic oxidative burst that directly eliminates pathogens (Baker and Orlandi 1995; Lamb and Dixon 1997; Bolwell 1999). This initial rapid phase of ROS production is followed by a prolonged second phase, which is closely associated with the hypersensitive response (Levine et al. 2003).

Among the ROS-producing enzymes, NADPH oxidase has been extensively studied for its role in mediating stress responses across various organisms, including plants (Segal et al. 2012; Yamauchi et al. 2017). These oxidases, which transfer electrons from cytosolic NADPH to apoplastic oxygen, are integral plasma membrane-localized proteins known as *Rbohs* in vascular plants (Suzuki et al. 2011). NADPH oxidase is a multigenic enzyme complex that catalyzes the transfer of electrons to molecular oxygen, generating superoxide anion radicals (O_2^-) (Lambeth 2004). The superoxide anions subsequently initiate a cascade of reactions that produce various ROS, which are critical for plant defense against bacterial and fungal pathogens (Tiwari et al. 2020). Beyond their role in pathogen defense, *Rbohs* are involved in various physiological processes, such as facilitating pollen tube growth via ROS production (Potocký et al. 2007) and regulating symbiotic nodule formation in *Medicago truncatula* through *RbohA* activity (Marino et al. 2011). In *A. thaliana*, *RbohD* and *RbohF* have been shown to function in pathogen defense as well as in abscisic acid (ABA) signaling (Torres et al. 2002; Kwak et al. 2003).

The *Rbohs* are plant homologs of the catalytic subunit of mammalian NADPH oxidase, known as $gp91^{phox}$ (Groom et al. 1996). These proteins are characterized by the presence of conserved domains, including an NADPH_Ox domain at the N-terminus, a ferric reductase-like transmembrane component, FAD-binding and NAD-binding domains, and several calcium-binding EF-hand motifs (Sumimoto 2008; Kaur et al. 2017). Since the first characterization of an *Rboh* gene in rice (*Oryza sativa*) by Groom et al. (1996), the number of *Rboh* homologs has been found to vary across plant lineages. Genome-wide analyses have identified *Rboh* genes in several plant species, including *Vitis vinifera*, *Triticum aestivum*, *A. thaliana*, *Fragaria ananassa*, *Hevea brasiliensis*, *Manihot esculenta*, *M. truncatula*, and *Brassica rapa* (Sagi and Fluhr 2006; Cheng et al. 2019; Li et al. 2019; Liu et al. 2019; Navathe et al. 2019; Zhao and Zou 2019; Zou et al. 2019; Abdelgawad et al. 2020). However, compared to the extensive studies conducted on *A. thaliana*, research on *Rboh* genes in other plants remains limited.

In this study, we focused on the identification, characterization, chromosomal positioning, phylogenetic relationships, conserved motif analysis, gene structure analysis, and conserved domain analysis of *Rboh* genes in four economically and agriculturally significant dicot plants: *B. oleracea*, *D. carota*, *H. annuus*, and *C. annuum*. Our findings revealed varying numbers of *Rboh* genes in these plants. This research provides insights into the structure and function of the *Rboh* gene family in these dicot plants.

MATERIALS AND METHODS

Identification, characterization, and nomenclature of *Rboh* genes

Four dicot plants—*B. oleracea*, *D. carota*, *H. annuus*, and *C. annuum*—were selected for the genome-wide identification and characterization of the *Rboh* gene family. To identify candidate genes, previously identified *A. thaliana Rboh* genes were used as query sequences for BLASTp searches with a standard e-value threshold of 1^{e-10} against plant genome databases available on the Ensembl Plants platform (https://plants.ensembl.org/index.html). The BLASTp results were manually screened to remove redundant transcripts corresponding to the same gene. The resulting protein sequences were collected for further analysis.

To confirm the presence of conserved domains characteristic of the *Rboh* gene family, the identified protein sequences were subjected to domain-based screening using the Conserved Domain Database (CDD) available on NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The analysis revealed the presence of five conserved domains: NADPH_Ox (pfam08414), NAD_binding_6 (pfam08030), NOX_Duox_like_FAD_NADP/Ferric_reduct (cd06186/pfam01794), FAD-binding_8 (pfam08022), and EF-hand (cd00051), which are hallmark features of the *Rboh* gene family. In total, 10 *Rboh* genes were confirmed in *A. thaliana* (previously known), along with 12 genes in *B. oleracea*, nine genes in *D. carota*, 12 genes in *H. annuus*,

and five genes in *C. annuum*. The identified genes were renamed based on their corresponding plant species and chromosomal positions, following the renaming method described by Marino et al. (2011). The genomic DNA (gDNA), coding DNA sequences (CDS), and protein sequences of all identified *Rboh* genes were retrieved in FASTA format from the Ensembl Plants database (https://plants.ensembl.org/index.html). Additional gene features, such as chromosomal location, strand type, number of exons, protein length, CDS and gDNA lengths, and isoelectric points (pI), were also obtained from the same database and are summarized in Table 1. However, for *A. thaliana Rboh* genes, all sequence data and associated features were retrieved from The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/).

Phylogenetic analysis

A phylogenetic tree of 48 Rboh protein sequences was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0. Protein sequences were aligned using the MUSCLE algorithm with default parameters: gap opening penalty -2.9, gap extension penalty 0, hydrophobicity multiplier 1.2, and UPGMA clustering. The aligned data were analyzed using the Neighbor-Joining method, with 1,000 bootstrap replications, partial gap deletion, and a 95% site coverage cutoff. The optimal tree, with a total branch length of 8.10875193, was constructed from a final dataset of 493 positions. Evolutionary distances were calculated using the Poisson correction model and are expressed as the number of amino acid substitutions per site.

Conserved motif and domain analysis

To identify conserved motifs in Rboh protein sequences, a motif analysis was conducted using the online MEME Suite tool (http://meme-suite.org/). Conserved domains within the identified motifs were analyzed using specific parameters. For motif discovery, the following settings were applied: a maximum of 7 motifs, a minimum motif width of 60, and a maximum motif width of 210. Protein sequences of all *Rboh* genes were organized based on their clustering in the phylogenetic tree. Consequently, the discovered motifs were aligned and displayed alongside their respective genes for better visualization and comparison. Additionally, conserved domain analysis was performed using the online SMART tool (http://smart.embl-heidelberg.de/) with default parameters to validate and annotate the conserved regions further.

Chromosomal mapping of different *Rboh* genes

Chromosomal positioning was performed in four plants: *B. oleracea, D. carota, H. annuus,* and *C. annuum,* excluding *A. thaliana,* which has already been performed in different genome-wide studies such as Kaur and Pati (2016). For the positioning of the genes on their respective chromosomes, MapChart software was used with default parameters.

Gene structure analysis

Gene structure analysis was performed to analyze the intron-exon pattern of all 48 *Rboh* genes. For this purpose, an online available tool named Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/) was used. The gDNA and CDs of all genes were arranged according to the groups formed by phylogenetic analysis. The arranged gDNA and CDs were used to map untranslated regions, the number of exons, and the number of introns across the length of all sequences.

RESULTS

***In silico* characterization of the *Rboh* genes**

A genome-wide *in silico* analysis was performed to identify and characterize the *Rboh* gene family in four dicot plants: *B. oleracea*, *D. carota*, *H. annuus*, and *C. annuum*. A total of 38 *Rboh* genes were identified across these species. The loci encoding these putative Rboh proteins are listed in Table 1, along with their locus names and other characteristics. The analysis revealed significant variation in the genomic DNA (gDNA) lengths of the identified genes, ranging from 2,561 bp (*BoRbohD*) to 13,291 bp (*CaRbohA*). Similarly, the coding sequence (CDS), protein length, molecular weight, and isoelectric point (pI) also displayed considerable variation. The *BoRbohB* gene exhibited the longest CDS (3,894 bp), the largest protein length (1,297 amino acids), and the highest molecular weight (148,094.50 Da). In contrast, *BoRbohD* had the shortest CDS (846 bp), the smallest protein length (281 amino acids), and the lowest molecular weight (31,336.27 Da) and isoelectric point (6.7588 pH). On the other hand, *CaRbohA* was found to have the highest isoelectric point, measuring 9.9693 pH. Across all 48 *Rboh* genes analyzed (including those previously reported), the average gDNA length was 6,151.7 bp, with an average CDS length of 2,633.8 bp and an average protein length of 878.7 amino acid residues. The average molecular weight of these proteins was determined to be 99,769.6 Da, while the average isoelectric point value was calculated as 9.1 pH.

Table 1. Characteristics of respiratory burst oxidase homolog (*Rboh*) genes from five dicot plants

Gene	Locus ID	Chr	Exons	Locus position	Orientation	gDNA	CDs	Protein	MW (Da)	pI
Arabidopsis thaliana										
AtRbohA	At5g07390	5	12	2335895-2339913	Reverse	4019	2709	902	102934.6	9.54
AtRbohB	At1g09090	1	12	2932739-2936586	Forward	3848	2532	843	96389.3	9.59
AtRbohC	At5g51060	5	11	20757284-20762431	Reverse	5148	2718	905	102517.4	9.91
AtRbohD	At5g47910	5	8	19397443-19402061	Forward	4619	2766	921	103907.7	9.68
AtRbohE	At1g19230	1	14	6643942-6649149	Forward	5208	2859	952	107701.6	8.9
AtRbohF	At1g64060	1	14	23769774-23776984	Forward	7211	2835	944	108417.3	9.54
AtRbohG	At4g25090	4	11	12878667-12883805	Reverse	5139	2550	849	96861.7	9.41
AtRbohH	At5g60010	5	11	24160270-24165052	Forward	4783	2661	886	100626.7	9.5627
AtRbohI	At4g11230	4	10	6840473-6845627	Reverse	5155	2826	941	106951	8.58
AtRbohJ	At3g45810	3	13	16832726-16837792	Reverse	5067	2739	912	102936.2	9.9
Daucus carota										
DcRbohA	KZN09289	1	12	22603326-22607935	Forward	4610	2634	877	100307.58	8.52
DcRbohB	KZN07512	2	11	41438129-41444968	Reverse	6840	2787	928	106165.65	8.89
DcRbohC	KZM93080	5	12	2215950-2220822	Forward	4873	2784	927	104327.54	9.07
DcRbohD	KZM95617	5	14	33904298-33908385	Reverse	4088	2667	888	101078.82	9.03
DcRbohE	KZM87673	7	14	19261956-19267499	Reverse	5544	2808	935	106170.57	8.19
DcRbohF	KZM88550	7	12	29123921-29127968	Forward	4048	2817	938	105106.17	8.84
DcRbohG	KZM88551	7	12	29131330-29135150	Forward	3821	2457	818	92504.47	8.90
DcRbohH	KZM88553	7	10	29159147-29163199	Forward	4053	2376	791	88742.06	8.30
DcRbohI	KZM81781	9	12	5927281-5931640	Reverse	4360	2682	893	101808.52	8.93
Helianthus annuum										
HaRbohA	OTG33413	2	12	16964113-16970884	Reverse	7912	2754	913	103467.36	9.31
HaRbohB	OTG29767	4	13	172845308-172850614	Forward	6507	2769	922	104167.21	9.4007
HaRbohD	OTG26183	5	12	191009986-191015013	Reverse	6228	2793	930	104013.12	8.8349
HaRbohC	OTG24190	5	14	25387457-25393798	Forward	7542	2442	813	93380.12	9.0845
HaRbohF	OTG22860	6	14	36866565-36871795	Reverse	6431	2484	827	93539.19	8.5789
HaRbohG	OTG23063	6	13	46526188-46532096	Reverse	7109	2745	914	103680.72	8.8003
HaRbohE	OTG21957	6	14	5219065-5229280	Forward	11416	2784	927	105957.26	9.2038
HaRbohH	OTG18979	8	9	96659546-96663406	Forward	5061	1929	642	73982.83	7.9692
HaRbohI	OTG18983	8	12	96777094-96784911	Forward	9018	2589	862	97774.67	9.0117
HaRbohJ	OTG18986	8	12	96829253-96835421	Forward	7369	2787	928	105390.86	9.3501
HaRbohK	OTG13013	10	12	233456087-233462612	Forward	7726	2742	913	102586.30	9.14
HaRbohL	OTF84878	17	14	2050066-2059112	Forward	10247	2580	859	98349.87	8.8070
Capsicum annuum										
CaRbohA	PHT92305	1	14	2731807-2743957	Forward	13291	2657	878	100479.79	9.9693
CaRbohB	PHT80159	6	6	236294086-236296330	Reverse	3385	1524	507	58253.64	9.6595
CaRbohC	PHT75801	7	14	79061138-79070176	Forward	10179	2817	938	105834.05	8.8555
CaRbohD	PHT62030	8	12	4098-8390	Reverse	5493	2619	872	99354.10	9.2804
CaRbohE	PHT62968	8	12	221190-222516	Forward	6823	2622	873	99415.84	9.6244
Brassica oleracea										
BoRbohA	Bo2g025190	2	13	46966679-46971312	Forward	6278	2655	983	111856.62	9.6366
BoRbohB	Bo3g004310	3	19	1591281-1598783	Reverse	8703	3894	1297	148094.50	9.6851
BoRbohC	Bo3g004330	3	12	1603763-1607752	Reverse	5190	2613	870	98560.64	8.9596
BoRbohD	Bo3g019810	3	5	6852142-6853502	Reverse	2561	846	281	31336.27	6.7588
BoRbohE	Bo3g023910	3	10	8770715-8775557	Forward	6043	2733	910	103050.63	9.8473
BoRbohF	Bo3g130140	3	13	47606356-47612223	Reverse	7068	2637	878	99480.86	9.3974
BoRbohG	Bo5g027260	5	14	9442589-9448199	Forward	6811	3099	1032	116336.97	9.4572
BoRbohH	Bo7g088940	7	8	34330494-34334985	Forward	5692	2769	922	103881.30	9.3877
BoRbohI	Bo7g109970	7	10	43551148-43554548	Reverse	4601	1860	619	70085.27	9.2188
BoRbohJ	Bo8g102860	8	13	36291834-36296904	Reverse	6271	2901	966	109701.12	9.0315
BoRbohK	Bo9g033770	9	14	11449908-11455531	Reverse	6824	2847	948	108108.80	9.4994
BoRbohL	Bo9g175450	9	12	52021168-52025037	Reverse	5070	2727	908	103369.83	9.7152

Where gDNA=genomic DNA, CDs=Coding sequence, MW=molecular weight, pI=Isoelectric point

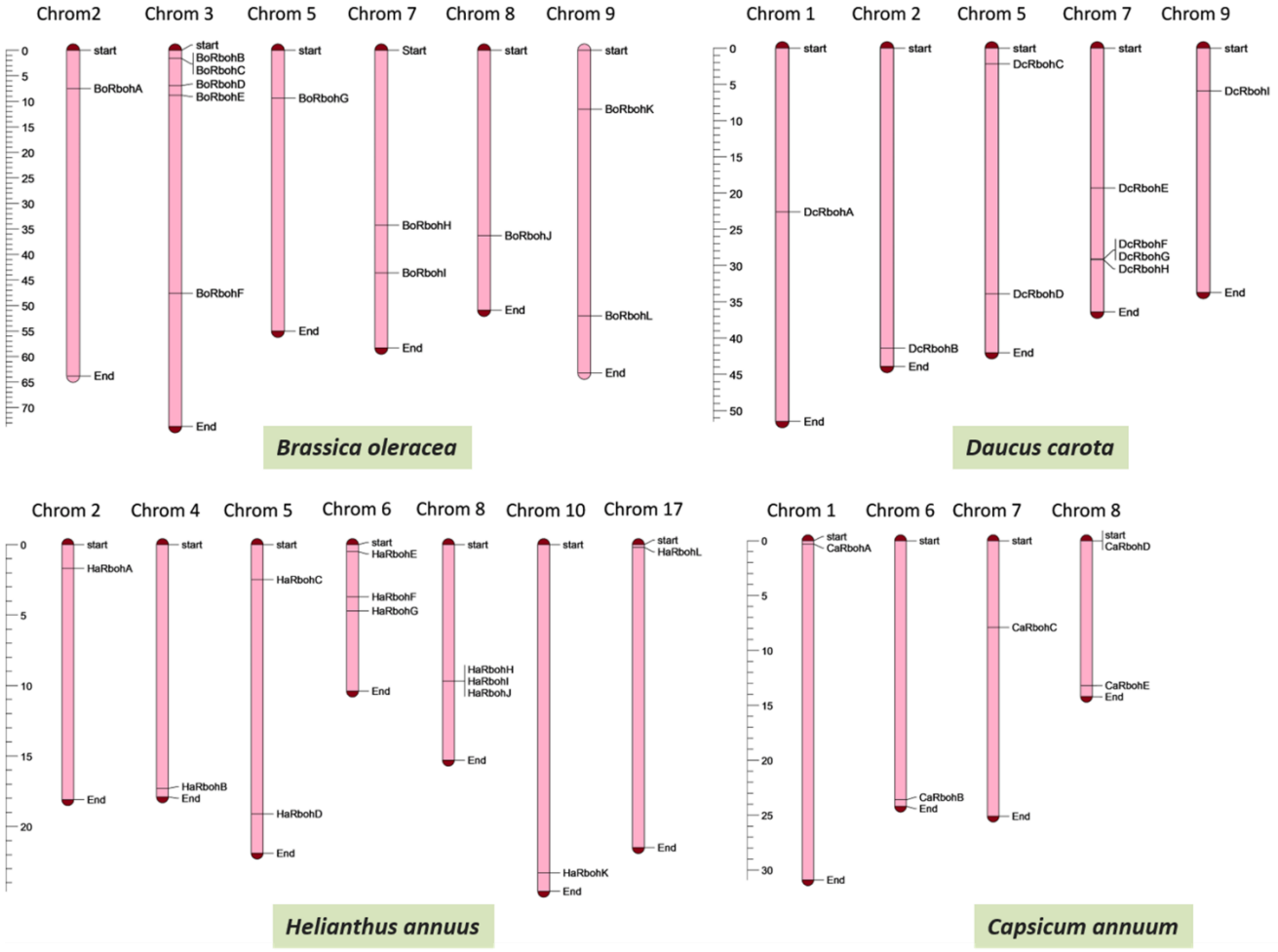

Figure 1. Localization of *Rboh* genes on the chromosomes of different dicots. MapChart software was used to localize the *Rboh* genes on chromosomes.

Table 2. Different attributes of motifs predicted in Rboh protein sequences

Motif	E-value	Sites	Width	Consensus sequence
1.	4.8^{e-3584}	46	117	WYSHHLFVIVYVLLIVHGYFLYLTKEWYKKTTWMYLAVPVLLYAGERLJRAF RSSIKSVKILKVAVYPGNVLALHMSKPQGFKYKSGQYMFVNCPAVSPFEW HPFSITSAPGDDYLS
2.	2.7^{e-3204}	45	94	VMBEVAEMDKNGVIEMHNYCTSVYEEGDARSALITMLQSLNHAKNGVDIV SGTRVRTHFARPNWRKVFKRIAVKHPNSRIGVFYCGAPALVKEL
3.	4.8^{e-3029}	47	92	ITKEZLKEFWEQISDQSFDSRLQIFFDMVDKBADGRJTEEEVKEIISLSASAN KLSNJKKQADEYAALIMEELDPDNLGYIELEQLETLLLQ
4.	2.1^{e-2616}	47	76	LKYFVLDNWKRIWVLALWJGIMAGLFTWKFIQYKNRAAFQVMGYCVCTAK GAAETLKLNMALILLPVCRNTITWLR
5.	1.8^{e-2054}	47	82	FDDNJNFHKVIAVAIAIGVGLHAGSHLACDFPRLJHATEEEYEPMEQYFGG QKPPYYWFLVGVEGGTGGIMMVLMMIAFTLA
6.	1.1^{e-1851}	45	75	SEVCPPPESGKSGLLAADEENQPSFPKLLIDGPYGAPAQDYKKYDVLLLVG LGIGATPFISILKDJLNNIKAKEE
7.	2.3^{e-1149}	47	82	SKRRPQRRLRRSKSAAAGALKGLKFISKNDGGAGWAWVEKEFRFLTLATB GLLPRSKFGECIGMKDSSEFAAEVFDALLRRR

Chromosomal positioning of *Rboh* genes

The chromosomal positioning of 4 plants (excluding *A. thaliana*) was performed using MapChart desktop-based software. As a result, 12 genes from *B. oleracea,* nine genes from *D. carota,* 12 genes from *H. annuus,* and five genes from *C. annuum* were positioned onto their corresponding chromosomes. In the case of *B. oleracea,* one gene (*BoRbohA*) on chromosome 2, five genes (*BoRbohB-F*) on chromosome 3, one gene (*BoRbohG*) on chromosome 5, two genes (*BoRbohH* and *BoRbohI*) on chromosome 7, one gene (*BoRbohJ*) on chromosome 8, and two genes (*BoRbohK* and *BoRbohL*) on chromosome 9 were positioned (Figure 1). In the case of *D. carota*, *DcRbohA*, *DcRbohB*, *DcRbohC, DcRbohD, DcRbohE-H, and DcRbohI* were mapped onto chromosomes 1, 2, 5, 7, and 9, respectively. *H. annuus Rboh* genes, including *HaRbohA*, *HaRbohB, HaRbohC and D, HaRbohE-G, HaRbohH-J, HaRbohK*, and *HaRbohI, were localized on chromosomes* 2, 4, 5, 6, 8, 10, and 17, respectively. Similarly, *CaRbohA*, *CaRbohB, CaRbohC*, and *CaRbohD and E* genes of *C. annuum* were mapped on chromosomes 1, 6, 7, and 8, respectively.

Phylogenetic and conserved motifs analyses of the *Rboh* genes

Phylogenetic analysis clustered all Rboh protein sequences into two major clades, designated as Clade 1 and Clade 2. Clade 1 was further divided into Group A and Group B, with Group A subdivided into Subgroups A1 and A2. Subgroup A1 contained 13 *Rboh* genes, of which 11 exhibited all seven conserved motifs. However, two exceptions were observed in this subgroup: *HaRbohH*, which lacked motifs 2 and 6, and *DcRbohH*, which lacked motif 1. Subgroup A2 exhibited greater variability in motif patterns among its nine members. For instance, *BoRbohI* contained only four motifs (1, 3, 4, and 7), while *BoRbohD* had just two motifs (3 and 7). The remaining members of Subgroup A2 displayed all seven motifs, except for *BoRbohB*, which, in addition to the seven conserved motifs, showed duplication of motifs 1, 2, 5, and 6 at the C-terminus of its polypeptide chain. In Group B, four members—*AtRbohB*, *CaRbohE*, *DcRbohA*, and *DcRbohI*—exhibited all seven motifs in a consistent pattern across their protein sequences. Clade 2 was divided into Group C and Group D. Group C consisted of six members—*AtRbohF*, *BoRbohK*, *CaRbohA*, *DcRbohB*, *HaRbohL*, and *HaRbohE*—all of which displayed seven conserved motifs, except *AtRbohI*, which lacked motifs 3 and 7 at its N-terminus. Group D was further subdivided into Subgroups D1 and D2, containing seven and eight members, respectively. All members of Subgroup D1—*HaRbohB*, *HaRbohG*, *DcRbohE*, *CaRbohC*, *BoRbohJ*, *AtRbohE*, and *BoRbohG*—presented all seven conserved motifs in their peptide sequences. Similarly, all seven motifs were identified in the members of Subgroup D2, including *DcRbohD*, *HaRbohC*, *HaRbohF*, *AtRbohJ*, *BoRbohF*, *AtRbohH*, and *BoRbohA*. However, *CaRbohB* from Subgroup D2 displayed only four motifs (3, 4, 5, and 7) at its N-terminus. Comprehensive details about motif widths, sites, E-values, and consensus sequences are provided in Table 2.

Gene structure analysis of *Rboh* genes

Gene structure analysis of the 48 *Rboh* genes was conducted to compare the conserved intron-exon patterns within their genomic DNA. The phylogenetic groups clustered from the analysis revealed variations in the number of introns, exons, and the positioning of untranslated regions (UTRs). For example, all genes in Subgroup A1 exhibited UTRs at both the 5' and 3' ends of their DNA sequences (Figure 3). However, *HaRbohI* displayed comparatively longer intron lengths than other genes in this subgroup. In Subgroup A2, *AtRbohG* had a UTR only at its 3' end, while all other members showed UTRs at both ends. The maximum number of exons (19) was observed in *BoRbohB*, whereas the minimum number of exons (5) was found in *BoRbohD*. In Group B, nearly all members contained 12 exons of varying lengths with UTRs at both ends; however, *AtRbohB* lacked a 5' UTR. In Group C, five genes—*AtRbohF*, *BoRbohK*, *CaRbohA*, *HaRbohL*, and *HaRbohE*—shared a common exon count of 14. Among these, *CaRbohA* and *HaRbohE* had significantly longer introns compared to the others. The remaining two genes in Group C, *DcRbohB* and *AtRbohI*, contained 11 and 10 exons, respectively. In Subgroup D1, three members (*HaRbohB*, *HaRbohG*, and *BoRbohJ*) exhibited 13 exons, while four genes (*DcRbohE*, *CaRbohC*, *AtRbohE*, and *BoRbohG*) had 14 exons. Similarly, members of Subgroup D2 showed varied exon counts, ranging from 6 to 14, with UTRs consistently present at both the 5' and 3' ends.

Prediction of conserved domains in *Rboh* genes

Conserved domain analysis was performed on *Rboh* genes that exhibited structural variations in their characteristic domains. This analysis identified seven genes—*BoRbohB*, *DcRbohD*, *AtRbohI*, *BoRbohI*, *DcRbohB*, *HaRbohH*, and *BoRbohD*—with distinct domain structures (Figure 4). *BoRbohB* displayed duplication of the Ferric Reductase, FAD-Binding_8, and NAD-Binding_6 domains at its C-terminus. Similarly, *DcRbohD* exhibited a duplicated EF-hand motif in addition to all the characteristic domains of the *Rboh* family. The remaining genes exhibited various domain patterns, with *BoRbohD* being notable for containing only the NADPH oxidase domain due to its short protein sequence.

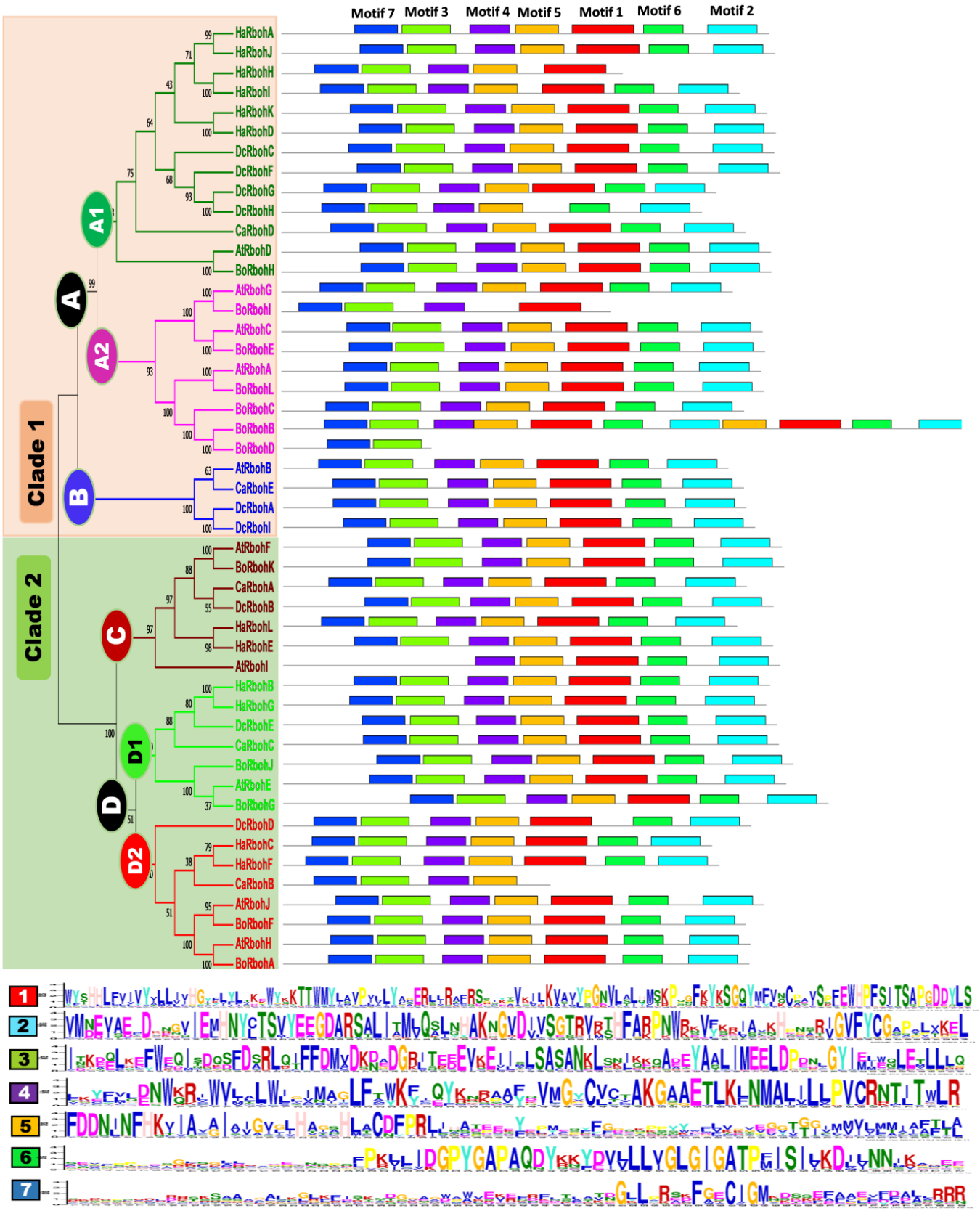

Figure 2. Phylogenetic and conserved motif analyses of the *Rboh* gene family. The phylogenetic tree is divided into Clade 1 and Clade 2, distinguished by different background colors. Each clade is further classified into groups and subgroups, labeled with specific letters. Conserved motifs (numbered 1 to 7), identified using the MEME Suite tool, are displayed along with their positions in the protein sequences. The height of each motif represents the significance of the match, while the motif width indicates the number of amino acids in the respective motif. Sequence logos for all motifs are shown at the bottom, where the height of each residue reflects its degree of conservation.

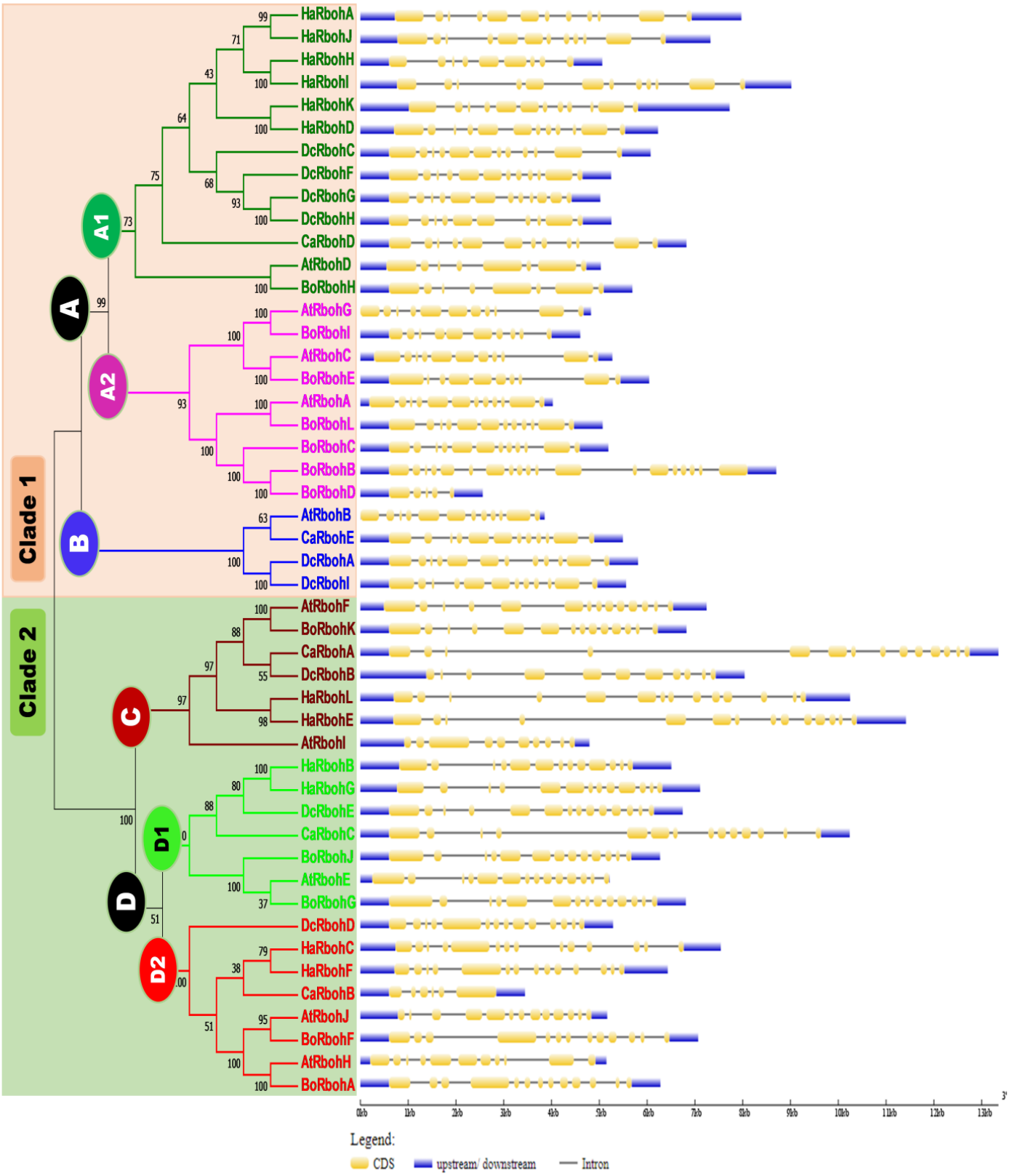

Figure 3. Phylogenetic analysis, combined with exon-intron structure analysis of *Rboh* genes. The phylogenetic tree is divided into Clade 1 and Clade 2, differentiated by distinct background colors. Each clade is further subdivided into groups and subgroups, labeled with specific letters. The scale at the bottom of the figure represents gene lengths in 1,000 base pairs (Kbp). Exons, untranslated regions (UTRs), and introns are depicted by golden lines, blue lines, and thin grey lines, respectively, highlighting their positions within the gene structure.

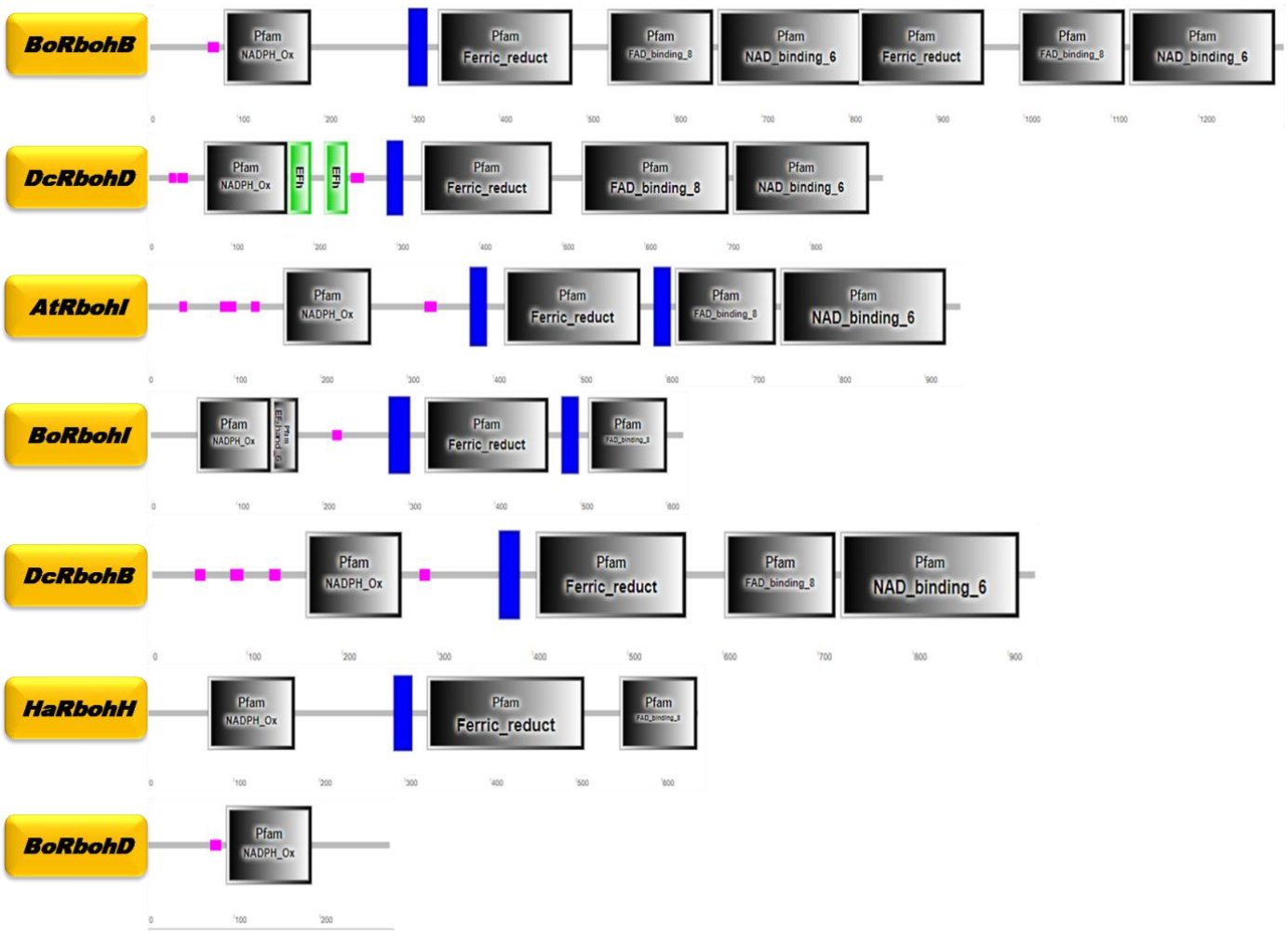

Figure 4. Conserved domains predicted in *Rboh* genes. The scale below each sequence represents the polypeptide length in amino acids. Black and green blocks denote different types of conserved domains, while vertical blue blocks indicate predicted transmembrane regions. Additionally, pink dots represent low-complexity regions, as identified by the SMART domain database.

DISCUSSION

Since the first report of *Rboh* gene identification in *Oryza sativa* (Groom et al. 1996), several experimental and *in silico* genome-wide approaches have been employed to identify *Rboh* genes in various crop plants (Zhao and Zou 2019; Yu et al. 2020). Despite their vital roles in numerous physiological processes, including plant defense against a wide range of pathogens (Kaur et al. 2014; Qu et al. 2017), no comprehensive information has been available about their presence in certain dicot plants, such as *B. oleracea, D. carota, H. annuus*, and *C. annuum*. The availability of full-genome sequences for these four plants provided an opportunity to perform a genome-wide analysis for the identification and characterization of the *Rboh* gene family. As a result, we identified 12 genes from *B. oleracea,* nine from *D. carota,* 12 from *H. annuus,* and five from *C. annuum*.

The number of *Rboh* genes identified in these plants is consistent with the numbers reported in other species. For instance, similar to *D. carota*, 9 *Rboh* genes have been reported in *Sorghum bicolor* (*SbRboh01–09*), *Brachypodium distachyon* (*BdRboh01–09*), *Oryza sativa* (*OsRboh01–09*), and cassava (*Manihot esculenta*) (Koda et al. 2017; Li et al. 2019; Zhao and Zou 2019; Wang et al. 2020). The 5 *Rboh* genes identified in *C. annuum* matched with the number reported in *Physcomitrella patens (PpRboh01–05)* (Li et al. 2019). Similarly, *B. oleracea* and *H. annuus* carried an equal number of 12 *Rboh* genes, suggesting that the gene count may reflect conservation of their ancestral gene complement, as seen in *Jatropha curcas* and *Ricinus communis* (Zhao and Zou 2019). Additionally, the calculated average molecular weight of the 48 Rboh proteins was 99.7 kDa, closely aligning with the 100.41 kDa average molecular weight reported for Rboh proteins in five fruit-producing plants (Cheng et al. 2019). Chromosomal positioning of 38 *Rboh* genes from *B. oleracea, D. carota, H. annuus*, and *C. annuum* was performed, excluding the 10 *Rboh* genes from *A. thaliana*, which have already been mapped in various studies (Kaur and Pati 2016).

Chromosomal mapping revealed an uneven distribution of *Rboh* genes across the genomes of these four plants, a pattern also reported in other plant genomes, including *Brassica rapa* (Li et al. 2019), *J. curcas*, and *R. communis* (Zhao and Zou 2019). Phylogenetic analysis grouped all 48 *Rboh* genes into two major clades, suggesting independent divergence of these genes over time. Within these clades, six subgroups (A1, A2, B, C, D1, and D2) were identified, showing similarities to previously reported phylogenetic lineages of *Rboh* genes in other plants. For instance, in *V. vinifera, A. thaliana, N. benthamiana, N. tabacum, Z. mays, O. sativa, S. tuberosum*, and *H. vulgare*, *Rboh* genes were classified into six subgroups, such as I, II, III, IV, V, and VI (Cheng et al. 2019). Similarly, phylogenetic analysis of 65 Rboh protein sequences from *S. bicolor*, *P. patens*, *A. thaliana*, *S. moellendorffii*, *P. sitchensis*, Z. mays, *P. trichocarpa*, and *V. vinifera* also clustered these proteins into six groups (I–VI) (Wang et al. 2013).

Interestingly, *BoRbohB* from *B. oleracea* exhibited seven conserved motifs, similar to *Rboh* genes in other plants (Cheng et al. 2019; Wang et al. 2013). However, unlike other *Rboh* genes, *BoRbohB* showed duplication of motifs 1, 2, 5, and 6 at the C-terminus of its polypeptide sequence, suggesting a segmental duplication event. Conversely, *BoRbohD* exhibited only two conserved motifs and had a short protein length of 281 residues, indicating a deletion event during its evolution. This study provides significant insights into the structure and characteristics of Rboh genes in four economically and agriculturally important dicot plants. The identification and characterization of these genes will contribute to a better understanding of their roles in plant defense mechanisms against pathogens, offering potential applications in the improvement of crop resistance.

CONCLUSION

This study provides a comprehensive *in silico* characterization of the *Rboh* gene family in four economically important dicot plants, identifying 38 *Rboh* genes and revealing their chromosomal distribution, conserved motifs, gene structures, and phylogenetic relationships. The uneven chromosomal distribution and clustering into two main clades with six subgroups highlight the evolutionary conservation and divergence of these genes. Specific duplication and deletion events suggest functional diversification within the gene family. These findings offer valuable insights into the potential roles of *Rboh* genes in plant stress responses and provide a foundation for future studies aimed at improving plant resilience against pathogens through genetic engineering strategies.

AUTHOR CONTRIBUTIONS

Conceptualization, AZ and ZN; methodology, JUH; software, AZ; validation, DA and AH; formal analysis and investigation, RA, AH; data curation, AZ and ZA.; writing—original draft preparation, ST; writing—review and editing, AZ; supervision. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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