Comprehensive analysis of Brassica napus nonexpressor of pathogenesis-related genes family and functional analysis of BnaNPR3.A02 in clubroot resistance

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Abstract

Background

Nonexpressor of pathogenesis-related genes (NPR) are key regulators of salicylic acid (SA)-mediated plant immunity, acting as transcription co-regulators. However, a comprehensive genome-wide analysis of the NPR family in *Brassica napus* has not been reported. Therefore, this study aims to comprehensively identify NPR family members in *B. napus* and its diploid progenitors, analyze their evolutionary characteristics and expression patterns, and functionally characterize the role of BnaNPR3.A02 in clubroot resistance.

Results

In this study, we identified 13, 7, and 7 *NPR* genes in *B. napus*, *B. oleracea*, and *B. rapa*, respectively, which were phylogenetically divided into four conserved subfamilies (□–□). Structural characterization revealed conserved domain architectures and variable exon-intron organizations, while spatiotemporal expression profiling indicated distinct tissue-specific patterns. Promoter cis-element analysis and transcriptomic responses to phytohormones and abiotic stresses suggested broad involvement of BnaNPRs in environmental adaptation. Notably, *BnaNPR3.A02* displayed striking down-regulation following *Plasmodiophora brassicae* infection. Functional assays in Arabidopsis demonstrated that overexpression of *BnaNPR3.A02* significantly increased clubroot susceptibility, whereas *AtNPR3* knockout enhanced resistance, confirming its negative regulatory role. Protein-protein interaction network analysis predicted that BnaNPR3.A02 interacts with multiple proteins including BnaNPR3.C02, and co-expression analysis of its Arabidopsis homolog *AtNPR3(AT5G45110)* identified 300 co-expressed genes significantly enriched in defense response and response to stress GO terms, further supporting its participation in immune responses.

Conclusions

These findings provide a foundation for understanding the evolutionary and functional divergence of the NPR family in *B. napus* and identify *BnaNPR3.A02* as a potential target for breeding clubroot-resistant *B. napus* varieties.

Background

Plants must cope with various adverse environmental conditions or stresses, such as high temperature, low light, drought, and salinity. Similarly, they are surrounded by various pathogens including bacteria, fungi, protists, and insects, and have therefore evolved complex immune systems to defend against biotic stresses. Plant immune responses are regulated by multiple phytohormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), among which SA plays a crucial role in systemic

resistance against biotrophic and hemibiotrophic pathogens [1, 2, 3]. The Nonexpressor of Pathogenesis-Related Genes (NPR) proteins are a unique class of transcriptional co-regulators in plants. As paralogous receptors of the plant defense hormone salicylic acid (SA), they play indispensable roles in hormone-dependent plant immunity [4, 5]. Plant NPR proteins are clustered into four major clades: angiosperm NPR1/2, angiosperm NPR3/4, non-seed plant NPR, and gymnosperm NPR. NPR family members typically contain BTB/POZ domains, ankyrin repeats, and nuclear localization signals, and enhance plant disease resistance by interacting with transcription factors of the TGA family to activate the expression of downstream pathogenesis-related (PR) genes [6].

In the model plant *Arabidopsis thaliana*, the NPR family comprises six NPR proteins: clade I *(*AtNPR1/2), clade II (AtNPR3/4), and clade III (NPR5/BOP2 and NPR6/BOP1) [7]. Clades I/II are primarily involved in defense, while clade III mainly functions in leaf and flower morphogenesis [8]. NPR1 functions as a central regulator of SA signaling and is essential for SA-induced expression of PR defense genes, mediating the activation of systemic acquired resistance (SAR) [9]. *NPR2* is the only paralog that can partially complement the *npr1* mutant [10]. In antagonistic regulation, their paralogs NPR3 and NPR4 act as transcriptional co-repressors that attenuate immune responses by promoting NPR1 degradation, thereby preventing resource wastage caused by excessive immune activation and maintaining immune homeostasis in plants [11, 12, 13]. Studies have shown that SA directly binds NPR3 and NPR4, thereby controlling NPR1 protein stability, revealing a fine-tuned regulatory relationship between NPR1 and NPR3/NPR4 [14, 15].

Beyond Arabidopsis, the *NPR* family has been identified and functionally characterized in various crops. In wheat, 17 *NPR* genes were identified genome-wide using bioinformatics approaches. These genes exhibit a conserved evolutionary distribution pattern on chromosomes, their promoter regions contain SA-related cis-regulatory elements, and some members show induced expression under biotic stress [16]. Targeted degradation of TaNPR3 in wheat can break immune balance and confer broad-spectrum disease resistance, providing a new strategy for disease resistance breeding [17]. In rice, alternative splicing of *OsNPR3* is promoted by the splicing regulator OsRBP11, which is targeted by bacterial TAL effectors. The resulting truncated OsNPR3 protein variant compromises defense gene activation by sequestering OsNPR1, thereby enhancing susceptibility to pathogens; natural variation in *OsRBP11* can restore OsNPR1 defense function and enhance rice resistance to different bacterial strains [18]. In maize, NPR1-like proteins are highly conserved across species, and their transcript abundance changes significantly under abiotic stresses such as cold, heat, salt, and drought, as well as under pathogen treatment. Overexpression of *ZmNPR1* enhances maize resistance to smut fungus [19]. In peanut, eight *AhNPR* genes were identified and characterized. Promoter and expression analyses indicated their important roles in growth, hormone signaling, and stress responses. Overexpression of *AhNPR3A* and *AhNPR4A* significantly enhanced cold tolerance in Arabidopsis, while VIGS-mediated silencing of these genes led to increased cold sensitivity [20]. Furthermore, overexpression of *AtNPR1* in citrus enhanced tolerance to Huanglongbing (HLB) with a balanced immune response [21]. Expression of *AtNPR1* in chickpea activated SAR and enhanced resistance to Fusarium wilt [22].

NPR genes not only play central roles in biotic stress responses but also exhibit important functions in various abiotic stress responses. Studies have shown that overexpression of *AtNPR1* in Arabidopsis enhances resistance to fungal and bacterial pathogens but simultaneously increases sensitivity to abiotic stresses, reflecting a trade-off between immune activation and growth-development [23]. In Arabidopsis, the translocation of NPR1 from chloroplasts to the nucleus activates tolerance to salt stress, and chloroplast-targeted NPR1 overexpression enhances stress tolerance and photosynthetic capacity [24]. These findings reveal the cross-regulatory functions of *NPR* genes in biotic and abiotic stress responses, positioning them as key hubs integrating multiple stress signals.

Brassica napus is a globally important oil crop widely cultivated worldwide. However, during its growth, it faces multiple environmental and biotic challenges. Among these, clubroot disease caused by the protist *Plasmodiophora brassicae* seriously impairs the growth, development, and yield of rapeseed: infected fields typically suffer yield losses of 20–30%, and in severe cases losses can exceed 80% or even lead to total crop failure, causing enormous economic losses [25, 26]. The disease has now spread to more than 80 countries worldwide [27]. Traditional disease control measures are ineffective, and breeding and planting resistant varieties is the most effective approach. Mining disease resistance-related genes can provide a theoretical basis for resistance breeding. Although the *NPR* gene family has been systematically identified and characterized in various plants including Arabidopsis, wheat, rice, maize, soybean, and peanut, a genome-wide systematic identification and functional analysis of the *NPR* gene family in *Brassica napus* has not yet been reported. This study aims to identify NPR family genes in the cruciferous plants diploid *Brassica rapa* (AA), *Brassica oleracea* (CC), and allotetraploid *Brassica napus* (AACC) through genome-wide analysis, and to systematically analyze their gene structures, conserved domains, phylogenetic relationships, as well as the expression patterns of *BnaNPRs* in different tissues, under different hormone treatments, under abiotic stresses, and under biotic stress with *Plasmodiophora brassicae*. Furthermore, this study heterologously overexpresses *BnaNPR3.A02* in Arabidopsis and performs functional complementation analysis using the Arabidopsis *npr3* mutant to evaluate its role in clubroot resistance. This study lays a foundation for in-depth exploration of the biological functions of BnaNPRs in the growth, development, and stress responses of *B. napus*, and provides a theoretical basis for breeding stress-resistant *B. napus* varieties.

Materials and methods

Identification of NPR family genes in Brassica spp.

The amino acid sequences of the AtNPR family were obtained from the TAIR database (https://www.arabidopsis.org/) [28]. The amino acid sequences for *B. napus* (Dar_V5/), *B. rapa* (Chiifu_V3.0/), and *B. oleracea* (HDEM_V1.0/) were retrieved from the Brassicaceae database (http://brassicadb.cn/#/Download/) [29]. The AtNPR family amino acid sequences were used as seed sequences to perform BLASTP search in Brassica species [30]. Subsequently, HMMER search program (v3.3) was utilized to identify non-redundant hits in Brassica spp. with an E-value threshold of < 1e-5, based on the Hidden Markov Model (HMM) profile (PF00651) retrieved from the Pfam database

(https://www.ebi.ac.uk/interpro/entry/pfam/) [31]. Genes identified by both methods were retained. To further validate the NPR family genes, we conducted a search using the NCBI Conserved Domain Search (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to confirm the presence of the Broad-Complex, Tramtrack and Bric a brac (BTB) and Pox virus and Zinc finger (POZ) domain [32].

Physicochemical properties and subcellular localization of NPR proteins

The physicochemical properties of NPR proteins from *B. napus*, *B. rapa* and *B. oleracea* were analyzed using the ProtParam tool on the ExPASy server (http://prosite.expasy.org/) [33]. The subcellular localization of NPR proteins in Brassica spp. was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/), with the highest-scoring prediction being selected [34].

Phylogenetic analysis of the NPR family in Brassica spp.

The NPR family protein sequences were subjected to multiple sequence alignment using the MAFFT online server (v7.520, https://mafft.cbrc.jp/alignment/server/) [35]. Maximum likelihood (ML) phylogenetic trees were constructed using IQ-TREE software (v3.0.1) [36]. The best-fit model was automatically determined using the ModelFinder function implemented in IQ-TREE (with the -m MFP parameter). Branch support was assessed using 1,000 replicates each of ultrafast bootstrap (UFBoot) and SH-like approximate likelihood ratio test (SH-aLRT).

Analysis of conserved motifs, domains and gene structure of NPRs in Brassica spp.

Conserved motifs in NPR proteins were predicted using the MEME suite (http://meme-suite.org/) [37], with the number of motifs set to 20 and motif length ranging from 6 to 50 amino acids. Conserved domains were annotated using the InterPro database (https://www.ebi.ac.uk/interpro/) [31]. Additionally, gene structure analysis was conducted based on gene annotation information. Gene structures, conserved motif distributions, and domain architectures were visualized using ggtree v3.14.0 in RStudio [38].

Gene duplication, and Ka/Ks analyses of NPR genes in Brassica spp.

The chromosomal localization of *NPR* genes in Brassica species was extracted from the GFF files and visualized using TBtools [39]. Intra-species and inter-species synteny analyses were performed using MCScanX to identify segmental duplications and tandem duplications [40]. Gene density, GC content, and non-coding sequence ratio were calculated via sliding window analysis and visualized using TBtools for *B. napus*, *B. rapa* and *B. oleracea*. The non-synonymous (Ka) and synonymous (Ks) substitution ratios between duplicated gene pairs were calculated using TBtools.

Cis-regulatory element analysis in the promoter region of NPR genes in Brassica spp.

The 2,000 bp upstream sequences from the ATG start codon were extracted and submitted to the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for cis-regulatory element identification [41]. The distribution of promoter elements was visualized using heatmaps generated in RStudio.

Expression profiling of BnaNPRs across tissues and in response to abiotic stresses and phytohormones in B. napus

The spatiotemporal and stress-responsive expression patterns of BnaNPRs in *B. napus* cultivar ZS11 were analyzed using transcriptomic data from the BnIR database (https://yanglab.hzau.edu.cn/BnIR) [42]. For abiotic stress treatments, seedlings were exposed to salt (200 mM NaCl), drought (air-drying), temperature extremes (-4°C freezing, 4°C cold, 38°C heat), or osmotic stress (300 mM mannitol). Root and leaf samples were collected at 0.25, 0.5, 1, 3, 6, 12, and 24 h post-treatment. Phytohormone responses were treated with 10 μM auxin (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), gibberellin (GA), abscisic acid (ABA), trans-zeatin (TZ), jasmonate methyl ester (JA-Me), and brassinolide (BL), with sampling at 0.5, 1, 3, and 6 h after application. Data normalization and heatmap visualization were performed using TBtools [39].

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from *B. napus* line “1603” root tissues using the TIANGEN RNAprep Pure Plant Kit (DP432) according to the manufacturer's instructions. Genomic DNA was removed and first-strand cDNA was synthesized using the TransGen EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. qRT-PCR was performed on a Life Technologies QuantStudio 7 system using the UniPeak U + One Step qPCR SYBR Green Kit (Vazyme, Q226-01). *BnaActin*, *AtActin7*, and *PbActin* were used as reference genes, respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [43]. Three biological replicates were set for each sample, and three independent replicate experiments were performed. The primer list is provided in Supplementary Table S1.

Construction of expression vector and genetic transformation

cDNA from *B. napus* line “1603” was used as template to clone the *BnaNPR3.A02* gene. The overexpression vector pCAMBIA1300-eGFP was digested with BglII and XbaI. The target fragment was then ligated into the digested vector using the ClonExpress II One Step Cloning Kit (Vazyme, C112-01). The primers used for cloning are listed in Table S1. For CRISPR/Cas9-mediated gene editing, single guide RNA (sgRNA) targeting *AtNPR3* was designed using CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) [44]. Complementary oligonucleotides were synthesized to construct the knockout vector. The target fragment was amplified from the pcbc-c1 vector using specific primers (Table S1) and inserted into the BsaI-digested PBSE vector via homologous recombination.

After verifying the positive clones by sequencing, the recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101, which was subsequently introduced into *A. thaliana* plants using the floral dip method [45]. Transgenic plants were selected on 1/2 Murashige and Skoog (MS) medium containing 25 μg/mL hygromycin until homozygous lines were obtained.

Plant growth conditions, P. brassicae inoculation, and phenotypic analysis

Seeds of *Arabidopsis thaliana* and *Brassica napus* line '1603', obtained from the rapeseed breeding research group of Northwest A&F University, were sown separately in pots containing a substrate mixture of soil: vermiculite: perlite = 3:1:1. The plants were then grown in a growth chamber at 24°C under a photoperiod of 16 h light (20°C) / 8 h dark. Inoculation was performed using a *P. brassicae* suspension prepared according to the method of Williams [46]. Root tissues of *B. napus* line '1603' were collected at 0, 7, 14, 21, and 28 days post-inoculation (dpi), and root tissues of *A. thaliana* were collected at 0 and 28 dpi. All samples were immediately frozen in liquid nitrogen and stored at − 80°C.

At 28 dpi, the roots were carefully pulled out, washed, and observed for tumor size and distribution on the roots. The severity of clubroot disease was classified into four grades:

Grade 0 – no disease symptoms;

Grade 1 – no tumor on the main root, few and small galls on lateral roots;

Grade 2 – slight infection on the main root or severe galls on lateral roots;

Grade 3 – large galls on the main root, with almost no lateral root formation.

The disease index was calculated according to the method described by Liu et al. [47]. The quantification of *P. brassicae* biomass was performed according to the method described by Wang et al. [48].

GO annotation of BnaNPRs and identification of BnaNPR3.A02 co-expressed genes

GO annotation of the BnaNPRs was performed using the BnIR database GO enrichment program (https://yanglab.hzau.edu.cn/BnIR/GO) [42]. To identify co-expressed genes of the *A. thaliana* homolog *AtNPR3*, the ATTED-II v13 database (https://atted.jp/) was used [49]. GO enrichment analysis of the co-expressed genes was conducted in TAIR database (https://v2.arabidopsis.org/tools/go_term_enrichment.jsp) [28].

Statistical analysis

Statistical analyses and data visualization were performed using R software (https://www.r-project.org/). Data are presented as mean ± standard deviation (SD) from three biological replicates. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used. For comparisons between two groups, an unpaired two-tailed Student's t-test was applied.

Results

Identification of NPR family genes in Brassica spp.

To elucidate the evolutionary history of the NPR family, we performed a systematic genome-wide identification and analysis. Using *AtNPR1-4* homologs and the Pfam conserved domain model (PF00651) as queries, BLASTP and HMMER searches were conducted against the proteomes of *B. napus*, *B. rapa*, and *B. oleracea*. After stringent filtering, 13, 7, and 7 *NPR* genes were identified in *B. napus*, *B. oleracea*, and *B. rapa*, respectively (Table S2). These genes were named based on their sequence similarity to Arabidopsis *NPR* homologs. Although *B. napus* (AACC) originated from hybridization and polyploidization of its diploid progenitors *B. rapa* (AA) and *B. oleracea* (CC), the total number of NPR family members in *B. napus* was lower than the sum of those in the two diploid parents.

Physicochemical properties of NPR proteins from Arabidopsis and Brassica species were analyzed. The results showed that the amino acid lengths ranged from 434 to 1022 aa (average 605 aa), molecular weights from 48.91 to 114.71 kDa (average 67.84 kDa), and isoelectric points (pI) from 4.59 to 8.07 (average 5.58). Notably, the instability index of all 31 NPR proteins was greater than 40, indicating that both Arabidopsis and Brassica NPR proteins are unstable. Subcellular localization prediction revealed diverse intracellular distributions: 12 proteins were localized in the nucleus, 7 in chloroplasts, 10 in the cytoplasm, one (BnaNPR1.C08) in both cytoplasm and nucleus, and one (BolNPR1.C01) in the plasma membrane (Table S2). This diversity suggests that NPR family proteins may participate in multiple cellular functions.

Phylogenetic analysis of NPR family genes in Brassica spp.

To explore the evolutionary relationships of *NPR* genes in Arabidopsis and Brassica, a maximum likelihood (ML) phylogenetic tree was constructed using 31 NPR proteins from *A. thaliana*, *B. napus*, *B. rapa*, and *B. oleracea* (Fig. 1). The results showed that *B. napus NPR* genes were divided into four subfamilies (Subfamily I to IV), consistent with the classification observed in Arabidopsis. Among these subfamilies, Subfamily I contained eight members, Subfamily II six members, Subfamily III five members, and Subfamily IV 13 members. Notably, all four subfamilies included genes from all four species, with no species-specific clades. Moreover, the homologs in *B. napus* clustered with their diploid progenitors — *B. rapa* (A-genome donor) and *B. oleracea* (C-genome donor) — further supporting the hybrid origin and allopolyploid history of *B. napus*.

Conserved motifs, conserved domains and gene structure analysis of NPR family genes in Brassica spp.

To investigate functional specialization and evolutionary diversity of *NPR* genes, conserved motif analysis, domain annotation, and gene structure analysis were performed. MEME analysis identified 12 conserved motifs among the 31 NPR proteins (Fig. S1). All members contained motifs 1, 3, 5, 6, 11, and 12. Motifs 8 and 10 were present in 30 members (96.77%), absent only in BnaNPR1.A08. Motifs 2 and 9

were present in 29 members, absent in BnaNPR3.C02 and BolNPR3.C02. Motif 4 was present in all except BnaNPR3.C07. Motif 7 was present in 27 proteins (87.1%), absent in BnaNPR1.A01, BraNPR1.A01, BnaNPR1.C01, and BolNPR1.C01. Motifs 10, 8, and 1 were mainly located at the N-terminus, while motifs 3 and 7 were predominantly at the C-terminus (Fig. 2B). Phylogenetic analysis showed that different subfamilies exhibited unique but conserved motif patterns, suggesting that genes within the same subfamily may share similar functions. Except for BnaNPR1.A08, all 30 other NPR proteins contained the BTB/POZ domain. Except for BnaNPR3.C02 and BolNPR3.C02, all others contained the NPR1_like_C domain (Fig. 2C). Gene structure analysis revealed that the number of exons in *BnaNPR* genes ranged from 3 to 18, with the most common structure containing four exons, observed in 16 genes (Fig. 2D). *NPR* genes exhibited considerable structural diversity, which, together with the motif distribution patterns, highlighted subfamily-specific structural similarities.

NPR family genes distribution and syntenic analyses

The chromosomal distribution of *NPR* genes in Brassica species is shown in Fig. 3. A total of 13 *BnaNPR* genes were distributed on nine chromosomes, with six located on the A subgenome and seven on the C subgenome. Chromosomes A01 and C01 harbored the highest number of *BnaNPR* genes (three each). Seven *BraNPR* genes were distributed on five chromosomes in *B. rapa*, and seven *BolNPR* genes on five chromosomes in *B. oleracea*.

To understand the evolutionary process, gene duplication events were analyzed using MCScanX. Four segmental duplication pairs were found in *B. rapa* and four in *B. oleracea*, while nine pairs were identified in *B. napus* (Fig. 4A-C; Table S3). Inter-species synteny analysis revealed that ten *BnaNPR* genes were homologous to six *BraNPR* genes, ten *BnaNPR* to five *BolNPR* genes, and 13 *BnaNPR* to four *AtNPR* genes. *BnaNPR* genes on the A subgenome of *B. napus* were homologous not only to the A genome of *B. rapa* but also to the C genome of *B. oleracea*; similarly, *BnaNPR* genes on the C subgenome showed homology to both diploid progenitors. These findings indicate extensive homology between the A and C subgenomes (Fig. 4D).

To evaluate selective pressure on Brassica *NPR* genes, the ratio of non-synonymous (Ka) to synonymous (Ks) substitutions was calculated. The Ka/Ks ratios of all 66 gene pairs were less than 1, indicating that these genes have undergone purifying selection following segmental duplication, which has been crucial for the expansion of the NPR family (Table S3).

Prediction of cis-regulatory elements in the promoter of NPR family genes in Brassica spp.

To understand the transcriptional regulation of *NPR* genes, we analyzed cis-acting elements (CREs) within the 2 kb upstream region of 31 *NPR* genes. Multiple stress-responsive, hormone-responsive, developmental, and light-responsive elements were identified (Fig. 5B). All *NPR* genes contained numerous hormone response elements, including auxin-responsive (TGA-element, AuxRR-core, TGA-box, AuxRE), gibberellin-responsive (TATC-box, GARE-motif, P-box), salicylic acid-responsive (TCA-element), abscisic acid-responsive (ABRE), and methyl jasmonate-responsive (CGTCA-motif, TGACG-motif). Except

for *BnaNPR1.C09*, all *NPR* gene promoters contained growth- and development-related elements (e.g., MRE, AACA_motif, MBSI, CCAAT-box, MSA-like, RY-element, O2-site, CAT-box, GCN4_motif, HD-Zip 1). Additionally, many *NPR* genes contained abundant light-responsive elements (e.g., MRE, ACE, G-box, 3-AF1 binding site, GT1-motif, AAAC-motif, ATCT-motif, Box 4, ATC-motif). Moreover, except for *BnaNPR1.C09*, all *NPR* gene promoters contained environmental stress-related elements, including anaerobic conditions (ARE, GC-motif), defense and stress responsiveness (TC-rich repeats), drought (MBS), low temperature (LTR), and wound-responsive (WUN-motif) elements (Fig. 5C-D). The diversity of CREs in *NPR* gene promoters suggests that these genes play important roles in regulating responses to various stresses.

GO annotation of BnaNPRs

To understand the functions of BnaNPRs, gene ontology (GO) annotation and enrichment analysis were performed on the 13 *BnaNPR* genes. As expected, most BnaNPRs were annotated as key regulators of the salicylic acid (SA)-mediated systemic acquired resistance (SAR) pathway. The enriched GO terms included regulation of jasmonic acid mediated signaling pathway, systemic acquired resistance, jasmonic acid mediated signaling pathway, cellular response to jasmonic acid stimulus, regulation of salicylic acid mediated signaling pathway, salicylic acid mediated signaling pathway, cellular response to salicylic acid stimulus, regulation of systemic acquired resistance, and induced systemic resistance. In addition, GO terms related to stress tolerance and development were also identified, including regulation of response to drug, cellular response to antibiotic, response to insect, regulation of response to biotic stimulus, and regulation of response to external stimulus (Fig. 6, Table S4). These findings suggest that BnaNPRs are mainly involved in stress resistance pathways in *B. napus*.

Spatial and temporal expression patterns of BnaNPRs

To explore the expression patterns of BnaNPRs during *B. napus* development, transcriptomic data from the BnIR database were analyzed across various tissues, including flower buds (2 mm, 4 mm), filament, petal, pollen, sepal, cotyledon, vegetative rosette leaf, root, upper/middle/lower stem epidermis, leaves (leaf 1 to leaf 23 stages), seeds (14–64 days after flowering, DAF), and siliques (2–60 DAF) (Fig. S2–S3). Data for *BnaNPR1.C09* were not available. Except for *BnaNPR3.C02*, the remaining 11 *BnaNPR* genes exhibited specific expression in most tissues. *BnaNPR1.C01*, *BnaNPR2.A01*, *BnaNPR2.C01*, and *BnaNPR4.A01* were mainly expressed in roots and stem epidermis, while *BnaNPR3.C07* was predominantly expressed in petals and sepals. The expression levels of most *BnaNPR1*, *BnaNPR2*, and *BnaNPR4* members increased with leaf development and silique maturation. Overall, the tissue- and stage-specific expression patterns of *BnaNPR* genes in *B. napus* indicate their broad functional diversity throughout the developmental process.

Expression of BnaNPRs under phytohormone treatment

The expression changes of BnaNPRs under phytohormone treatments in leaves and roots are shown in Fig. S4. Multiple *BnaNPR* genes exhibited differential transcriptional responses to various phytohormones. *BnaNPR1.C01* and BnaNPR4.A01 maintained high expression across all treatments, whereas *BnaNPR2.A01* and *BnaNPR3.A02* showed very low expression, and *BnaNPR3.C02* was not detected in any sample. Under control conditions, expression levels were relatively stable. Hormone treatments revealed that ACC (an ethylene precursor) and ABA transiently induced *BnaNPR1.C01* and *BnaNPR1*.A01 at 1 h, with BL (brassinolide) treatment at 0.5 h giving the highest value (14) for *BnaNPR1.C01*. JA treatment suppressed *BnaNPR1.A01* and *BnaNPR3.C07*. Overall, BnaNPRs displayed differentiated responses to multiple hormones with clear time-dependent patterns (Fig. S4A).

In roots, expression levels differed markedly. *BnaNPR4.A01*, *BnaNPR4.C01*, and *BnaNPR1.C01* maintained very high expression across all samples, whereas *BnaNPR1.A09* and *BnaNPR3.A02* showed very low expression, and *BnaNPR3.C02* remained undetectable. Unlike in leaves, the control expression levels in roots were higher and less variable. Under hormone treatments, IAA had little effect on most genes; ACC treatment induced *BnaNPR2.A01* upregulation at 3 h and 6 h; GA treatment at 6 h gave the highest root expression of *BnaNPR1.A01*; ABA treatment significantly suppressed *BnaNPR1.A01* and *BnaNPR1.C01* at 3 h and 6 h; JA treatment suppressed *BnaNPR1.C01*; BL treatment strongly induced *BnaNPR3.C07* and *BnaNPR4.A01*. *BnaNPR3.C07* reached its highest expression under IAA treatment at 1 h. Overall, root BnaNPRs responded to hormones differently from leaf BnaNPRs, with ABA showing strong inhibitory effects and BL and ACC inducing certain genes (Fig. S4B).

Expression profiles of BnaNPRs under abiotic stresses

We also investigated the responses of *BnaNPR* genes in *B. napus* ZS11 to various abiotic stresses, including salt (200 mM NaCl), drought (exposure to an air stream), freezing (–4°C), cold (4°C), heat (38°C), and osmotic stress (300 mM mannitol), using transcriptomic data from the BnIR database. The responses of *BnaNPR* genes to abiotic stresses showed tissue specificity and stress-dependent patterns (Fig. S5). Under drought and salt stress, *BnaNPR1.C01*, *BnaNPR4.A01*, and *BnaNPR4.C01* were significantly upregulated in both leaves and roots, with *BnaNPR4.A01* reaching a peak of 44.06 in roots at 12 h under drought. *BnaNPR3.C07* in roots responded most strongly to drought, salt, and osmotic stress (maximum 52.36). Under cold stress, *BnaNPR1.A08* and *BnaNPR1.C08* were upregulated in leaves, whereas freezing stress generally suppressed expression of most *BnaNPR* genes. Heat and osmotic stress induced the upregulation of some *BnaNPR2* members at later time points. *BnaNPR3.C02* was not expressed under any condition. These results indicate that different BnaNPR members may participate in the adaptation of *B. napus* to abiotic stresses through differential expression.

Expression profiles of BnaNPRs under P. brassicae stress

Clubroot disease is a devastating disease in rapeseed production. We further analyzed the expression dynamics of *BnaNPR* genes in *B. napus* line 1603 under *Plasmodiophora brassicae* infection. Using the expression levels at 0 days post-inoculation (dpi) in the resistant group (K_0) and the susceptible group (S_0) as baselines (set to 1), the relative expression levels of each gene were calculated at 7, 14, 21, and

28 dpi (Fig. 7). The results showed differentiated responses of BnaNPR members to clubroot stress. *BnaNPR2.A01* and *BnaNPR2.C01* showed markedly increased relative expression in the susceptible group at 28 dpi (S_28), reaching 7.18-fold and 4.30-fold relative to the susceptible group at 0 dpi (S_0), respectively; in the resistant group at 28 dpi (K_28), they were 3.16-fold and 2.33-fold relative to the resistant group at 0 dpi (K_0), indicating that *P. brassicae* infection further induced high expression of these two genes. *BnaNPR4.A01* decreased to 0.62-fold at S_28 relative to S_0, while K_28 remained similar to K_0, suggesting that this gene might be suppressed at the late stage of infection. *BnaNPR1.C01* showed a modest change (1.54-fold at S_28 vs. S_0, 1.50-fold at K_28 vs. K_0). *BnaNPR3.C07* slightly increased at S_7 and S_14 and then decreased. *BnaNPR1.A09* exhibited very low expression overall, and *BnaNPR3.C02* was not detected in any sample.

The focal gene of this study, *BnaNPR3.A02*, displayed a unique expression pattern: relative to S_0, its expression levels at S_7, S_14, S_21, and S_28 were 0.46, 0.92, 0.16, and 0.12, respectively, indicating a significant decrease as early as 7 dpi and extremely low levels at 21 and 28 dpi. In the resistant group (K), however, its expression at K_21 increased to 2.4–3.1-fold relative to K_0 and returned to baseline at K_28. These results indicate that *BnaNPR3.A02* is significantly suppressed during *P. brassicae* infection rather than being induced.

Based on the above expression characteristics, the specific down-regulation of *BnaNPR3.A02* under clubroot stress suggested that it might act as a negative regulator in the defense response of rapeseed to clubroot. To test this hypothesis, we performed transgenic functional analysis by heterologously overexpressing *BnaNPR3.A02* in Arabidopsis and using the Arabidopsis *npr3* mutant for functional complementation to evaluate its role in clubroot resistance.

BnaNPR3.A02 negatively regulates Arabidopsis resistance to clubroot disease

To clarify the function of BnaNPR3.A02 in clubroot resistance, we generated Arabidopsis lines overexpressing *BnaNPR3.A02* (OE-NPR3) and *npr3* knockout mutants (KO-npr3) with base deletion (Fig. S6). qRT-PCR confirmed that the target gene expression level in OE-NPR3 lines was 6- to 8-fold higher than in wild-type (WT), while *AtNPR3* expression in KO-npr3 lines was reduced to 0.2- to 0.3-fold of WT (Fig. 8A). At 28 days post-inoculation with *P. brassicae*, OE-NPR3 plants developed severe root galling, with grade-3 (most severe) plants accounting for 71.9%; the disease index (79.6–84.8) and pathogen biomass were significantly higher than those of WT. In contrast, KO-npr3 plants showed markedly reduced disease symptoms, with grade-3 plants accounting for only 15.7%; the disease index decreased to 48.4–52.9, and pathogen biomass was only 0.49-0.62 fold that of WT (Fig. 8B-E). These results demonstrate that overexpression of *BnaNPR3.A02* aggravates susceptibility to clubroot disease in Arabidopsis, whereas knockout of *NPR3* enhances resistance, confirming that *BnaNPR3.A02* negatively regulates Arabidopsis resistance to clubroot disease.

Protein-protein interaction (PPI) network and co-expression analysis of BnaNPR3.A02

To better understand the regulatory network of BnaNPR3.A02, we predicted its interacting proteins using the STRING database. Using BnaNPR3.A02 as the query, a network containing 11 interacting protein nodes and 26 edges (all with combined scores ≥ 0.478) was obtained. In this network, the node BnaCnng06860D interacted with multiple proteins (degree = 8) and might serve as a core regulatory node. Notably, BnaNPR3.A02 was predicted to interact with BnaNPR3.C02, suggesting that BnaNPRs can interact with each other (Fig. 9). Furthermore, we performed co-expression analysis of the Arabidopsis homolog of *BnaNPR3.A02*, *AtNPR3(AT5G45110)*, using the ATTED-II database (v13.0). This analysis identified 300 co-expressed genes, which were enriched in GO biological process terms such as response to stress, response to hypoxia, defense response, response to bacterium, and response to chemical stimulus, and in molecular function terms such as protein binding, nucleoside/nucleotide binding, kinase activity, and ion binding (Fig. 10A-B; Table S5). These findings indicate that BnaNPR3.A02 may play important roles in plant defense responses and stress adaptation through interactions with multiple proteins and involvement in extensive co-expression regulatory networks.

Discussion

NPR3 is a core receptor for the plant defense hormone salicylic acid (SA) and acts as a transcriptional co-repressor in systemic acquired resistance (SAR), thereby functioning as a key regulator of plant immune homeostasis [4, 5]. In this study, we performed the first genome-wide identification and systematic analysis of the *NPR* gene family in *Brassica napus* and its diploid progenitors *B. rapa* and *B. oleracea*, identifying 13 *BnaNPR*, 7 *BraNP*R, and 7 *BolNPR* genes. Unlike most gene families that typically undergo expansion in allopolyploids, the number of NPR members in *B. napus* (13) was lower than the sum of those in its two diploid parents (14), suggesting that some *NPR* genes may have been lost after allopolyploidization. This phenomenon has also been reported in other gene families. For example, Alamery et al. found that the number of NBS-LRR disease resistance genes in *B. napus* (583) was close to the sum of those in its diploid progenitors (273 in *B. rapa* and 312 in *B. oleracea*), but different subfamilies exhibited differential retention and loss patterns, with some NLR genes lost after allopolyploidization [50]. He et al. identified 287 WRKY transcription factor genes in *B. napus* (139 in the A subgenome and 148 in the C subgenome), a number also slightly lower than the sum of *WRKY* genes in the diploid parents [51]. This gene loss following allopolyploidization may be related to dosage balance and subgenome dominance. The gene balance hypothesis proposed by Bird et al. suggests that selection acts on the dosage of genes in networks, pathways, and protein complexes to maintain the balanced stoichiometry of interacting proteins; dosage-sensitive genes tend to be retained after allopolyploidization, whereas dosage-insensitive genes are more likely to be lost [52]. In addition, subgenome asymmetry in *B. napus* may also influence gene retention and loss, with the A subgenome generally exhibiting higher gene expression levels [53].

Phylogenetic analysis classified the Brassica *NPR* genes into four subfamilies (□–□), consistent with the classification in Arabidopsis [7]. All four subfamilies contained genes from all four species, with no species-specific clades. This indicates that the *NPR* gene family has maintained evolutionary conservation during the divergence of Brassica species and the allopolyploidization of *B. napus*. Notably, the homologous genes in *B. napus* clustered with their diploid progenitors — *B. rapa* (A-genome donor) and *B. oleracea* (C-genome donor) — further confirming the hybrid origin and allopolyploid history of *B. napus* and providing a direct basis for tracing the functions of different *B. napus NPR* genes back to their diploid ancestors [54]. Nine segmental duplication gene pairs were detected in *B. napus*, whereas only four such pairs were found in each of the diploid parents, suggesting that allopolyploidization may have promoted segmental duplications of *NPR* genes in *B. napus*. The Ka/Ks ratios of all homologous gene pairs were less than 1, indicating that purifying selection is the main driving force maintaining the functional stability of *NPR* genes.

Physicochemical property analysis revealed that all 31 NPR proteins are unstable (instability index > 40) and exhibit diverse subcellular localizations, including the nucleus, chloroplast, cytoplasm, cytoplasm-nucleus, and plasma membrane. This multi-compartment localization suggests that NPR proteins may perform non-canonical immune functions at different subcellular sites [55]. Previous studies have shown that Arabidopsis AtNPR1 translocates between the chloroplast and nucleus to participate in salt stress responses. Seo et al. further demonstrated that chloroplast-targeted *NPR1* overexpression enhances plant stress tolerance and photosynthetic capacity, and that chloroplastic NPR1 acts as a retrograde signal to enhance plant adaptability to stress [24]. In this study, several BnaNPR proteins were predicted to be localized in chloroplasts, suggesting that they may similarly have dual functions.

Although NPR proteins are generally unstable, they exhibit highly conserved features at the sequence and structural levels. Conserved motif analysis showed that all 31 NPR proteins contained motifs 1, 3, 5, 6, 11, and 12, and different subfamilies displayed unique but conserved motif patterns, indicating that genes within the same subfamily may share similar functions. The BTB/POZ domain and NPR1-like_C domain are present in the vast majority of NPR proteins. The BTB/POZ domain is primarily responsible for protein–protein interactions in NPR proteins and is a key structural basis for the formation of NPR1–NPR3/NPR4 complexes and for the interaction of NPR proteins with TGA transcription factors [56]. In this study, all except BnaNPR1.A08 contained the BTB/POZ domain, and all except BnaNPR3.C02 and BolNPR3.C02 contained the NPR1-like_C domain, indicating that *B. napus* NPR proteins have largely retained molecular interaction capabilities similar to those of Arabidopsis NPR proteins during evolution.

Gene structure analysis revealed that the number of exons in *NPR* genes ranges from 3 to 18, with the four-exon structure being the most common. This diversity in exon number suggests that NPR genes may produce different transcript variants through alternative splicing, thereby expanding their functional diversity. Previous studies have shown that alternative splicing of OsNPR3 in rice produces a truncated protein variant that compromises defense gene activation by sequestering OsNPR1, thereby enhancing susceptibility to pathogens [18]. Therefore, the observed differences in exon numbers among *NPR* genes

suggest that similar post-transcriptional regulatory mechanisms may exist in *B. napus NPR* genes, a possibility that awaits further investigation.

Promoter cis-element analysis revealed that the promoters of all *BnaNPR* genes are enriched with various hormone (SA, JA, ABA, GA, IAA) and stress (drought, low temperature, hypoxia, wounding) response elements, which is highly consistent with the differential expression patterns of *BnaNPRs* under phytohormone and abiotic stress treatments. Under hormone stress, ACC and ABA treatments transiently induced upregulation of *BnaNPR1.C01* and *BnaNPR1.A01* in leaves, while ABA treatment suppressed *BnaNPR1.A01* and *BnaNPR1.C01* expression in roots, suggesting that BnaNPRs may act as integration nodes in hormone signaling networks and play roles in tissue-specific stress adaptation. Under abiotic stress, drought and salt stress strongly induced the expression of *BnaNPR1.C01*, *BnaNPR4.A01*, and *BnaNPR4.C01* in both leaves and roots, consistent with reports that *AtNPR1* is induced under drought and salt stress in Arabidopsis [57]. Notably, *BnaNPR3.C07* in roots reached a peak expression of 52-fold under drought and osmotic stress, much higher than other members, suggesting a special role in root water stress responses. Freezing stress generally suppressed the expression of most *BnaNPR* genes, whereas cold stress (4°C) selectively induced the upregulation of *BnaNPR1.A08* and *BnaNPR1.C08*, indicating that different low-temperature regimes trigger distinct NPR-dependent signaling pathways.

In response to biotic stress (*P. brassicae*), the expression patterns of *BnaNPR* genes were more complex. *BnaNPR2.A01* and *BnaNPR2.C01* were strongly induced at 28 days post-inoculation, whereas *BnaNPR4.A01* was suppressed. Notably, the focal gene of this study, *BnaNPR3.A02*, was significantly down-regulated during *P. brassicae* infection but was up-regulated by 2.4–3.1 fold at 21 days in the resistant group. This pattern — increased expression at later stages in the resistant group but drastic reduction upon infection — suggests that, on one hand, the expression of this gene may be regulated by developmental stage, and on the other hand, *P. brassicae* may actively suppress *BnaNPR3.A02* transcription through secreted effector proteins, thereby dampening host defense responses.

Transgenic functional validation directly confirmed the negative regulatory role of *BnaNPR3.A02*. Overexpression of *BnaNPR3.A02* in Arabidopsis led to increased disease severity, higher disease index, and elevated pathogen biomass, whereas knockout of *AtNPR3* significantly enhanced resistance. These findings are consistent with previous reports that *AtNPR3* functions as an SA receptor and negatively regulates immunity [11, 12, 13]. Importantly, heterologous overexpression of the *B. napus* gene *BnaNPR3.A02* also enhanced susceptibility, indicating that this negative regulatory function is conserved in cruciferous plants. Protein-protein interaction network prediction showed that BnaNPR3.A02 may interact with BnaNPR3.C02. While STRING database predictions provide initial interaction clues, these need further validation by yeast two-hybrid or co-immunoprecipitation (Co-IP) experiments. Co-expression analysis enriched for GO terms such as defense response and response to bacterium, further supporting its involvement in immune regulation. These findings are largely consistent with the previously established functional framework of Arabidopsis NPR1, NPR3, and NPR4 in the SA signaling pathway: NPR1 acts as a co-activator positively regulating defense gene expression [9], whereas NPR3

and NPR4 function as co-repressors that attenuate immune responses by promoting NPR1 degradation, thereby maintaining plant immune homeostasis [11, 12, 13].

Taken together, we propose a working model in which, under normal conditions, BnaNPR3.A02 may function as a maintainer of immune homeostasis, moderately suppressing excessive activation of defense genes. Upon *P. brassicae* infection, the pathogen down-regulates this gene through unknown effector proteins, thereby relieving the suppression of defense responses and allowing the host to mount an effective resistance reaction. However, in overexpressing plants, the high level of this gene blocks defense activation, resulting in increased susceptibility. This hypothesis is consistent with the concept of a "defense suppressor" in plant immunity, but the specific molecular mechanisms — such as whether BnaNPR3.A02 directly binds SA and its interaction mode with TGA transcription factors — require further investigation.

In summary, this study provides the first genome-wide identification and functional analysis of the *NPR* gene family in *B. napus*, revealing the evolutionary characteristics and expression divergence of this family after allopolyploidization. Transgenic functional validation demonstrates for the first time that *BnaNPR3.A02* negatively regulates Arabidopsis resistance to clubroot disease, providing new evidence for understanding the functions of *NPR* genes in cruciferous plant immunity and identifying a potential gene target for breeding clubroot-resistant *B. napus* varieties.

Conclusion

In this study, we identified 13, 7, and 7 NPR family genes in *Brassica napus*, *B. oleracea*, and *B. rapa*, respectively, and classified them into four subfamilies (□–□). Phylogenetic tree, conserved domain, and gene structure analyses revealed the conservation and diversity of this gene family. Spatiotemporal expression analysis demonstrated that BnaNPRs exhibit tissue-specific expression patterns. Promoter analysis, along with expression profiling under phytohormone and abiotic stress treatments, indicated that BnaNPRs are involved in various stress responses. Furthermore, expression analysis under *Plasmodiophora brassicae* stress highlighted the potential role of *BnaNPR3.A02* in clubroot resistance. Overexpression of this gene in Arabidopsis significantly enhanced susceptibility, whereas knockout of *AtNPR3* increased resistance, confirming that *BnaNPR3.A02* negatively regulates clubroot resistance. Protein-protein interaction (PPI) network and co-expression analyses further suggested that BnaNPR3.A02 may participate in plant immune responses through interaction with other NPR members and involvement in defense-related biological processes.

Declarations

Abbreviations

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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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Author Contribution

L.Z. and F.Z. conceived and designed the experiments. Y.L., K.X., Z.G., R.S., X.L. performed the experiments. J.N., H.S., Z.C. performed the data analyses. L.Z., Y.M., and W.S. wrote the manuscript. K.L. and Z.H. revised the manuscript. All authors reviewed the manuscript.

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Data Availability

The RNA-seq data from B. napus ZS11 under various hormone and stress treatments are available through the BnIR database (https://yanglab.hzau.edu.cn/BnIR) developed by the Yang Lab at Huazhong Agricultural University.

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Figures

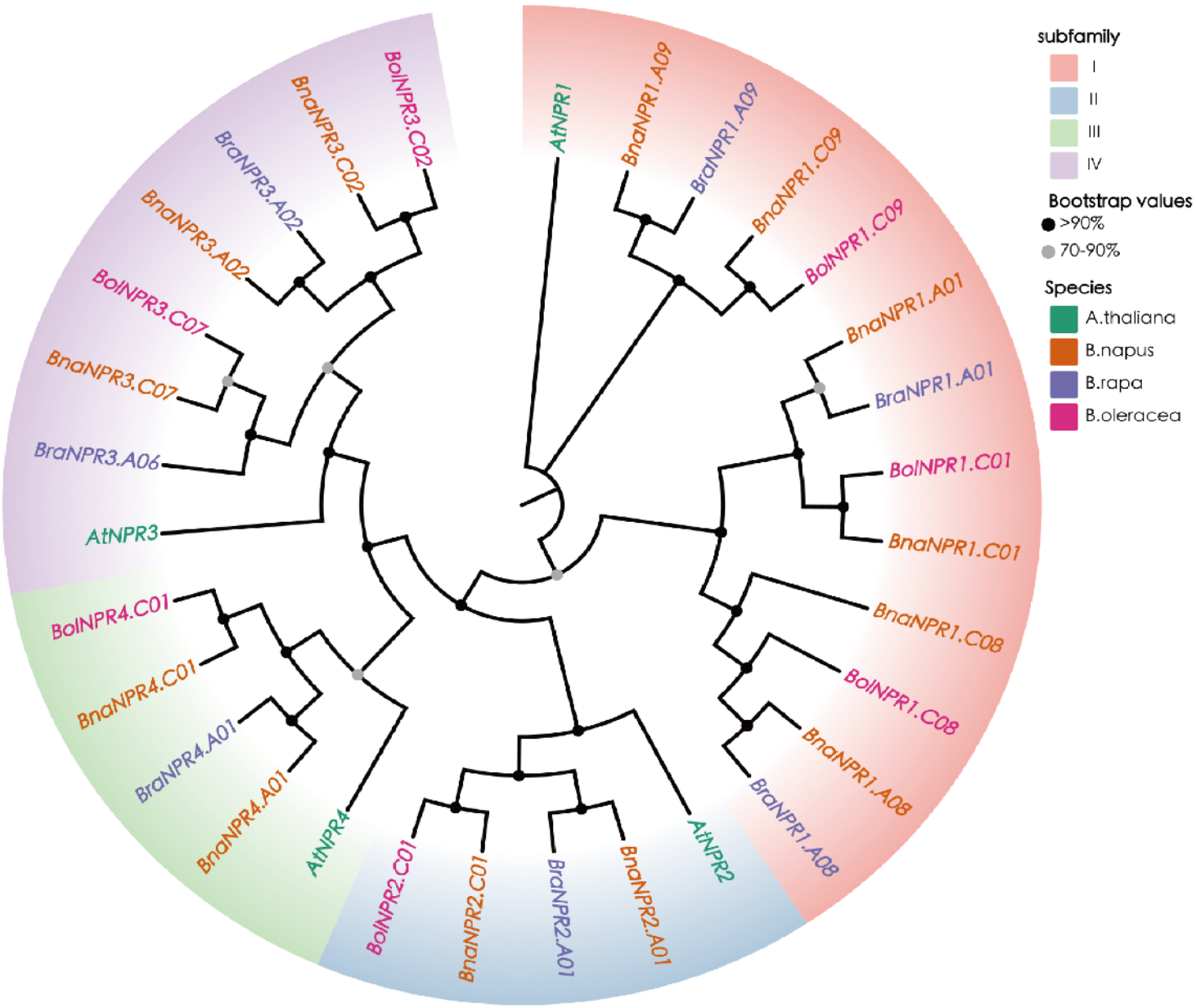

Figure 1

Phylogenetic relationships of NPR proteins from *Arabidopsis thaliana*, *Brassica napus*, *B. rapa* and *B. oleracea*. The maximum likelihood (ML) tree was constructed using the amino acid sequences of 31 NPR proteins. The four subfamilies (I–IV) are highlighted in different colors. Bootstrap values (≥ 70%) from 1,000 replicates are shown at nodes. At: *Arabidopsis thaliana*; Bna: *Brassica napus*; Bra: *Brassica rapa*; Bol: *Brassica oleracea*.

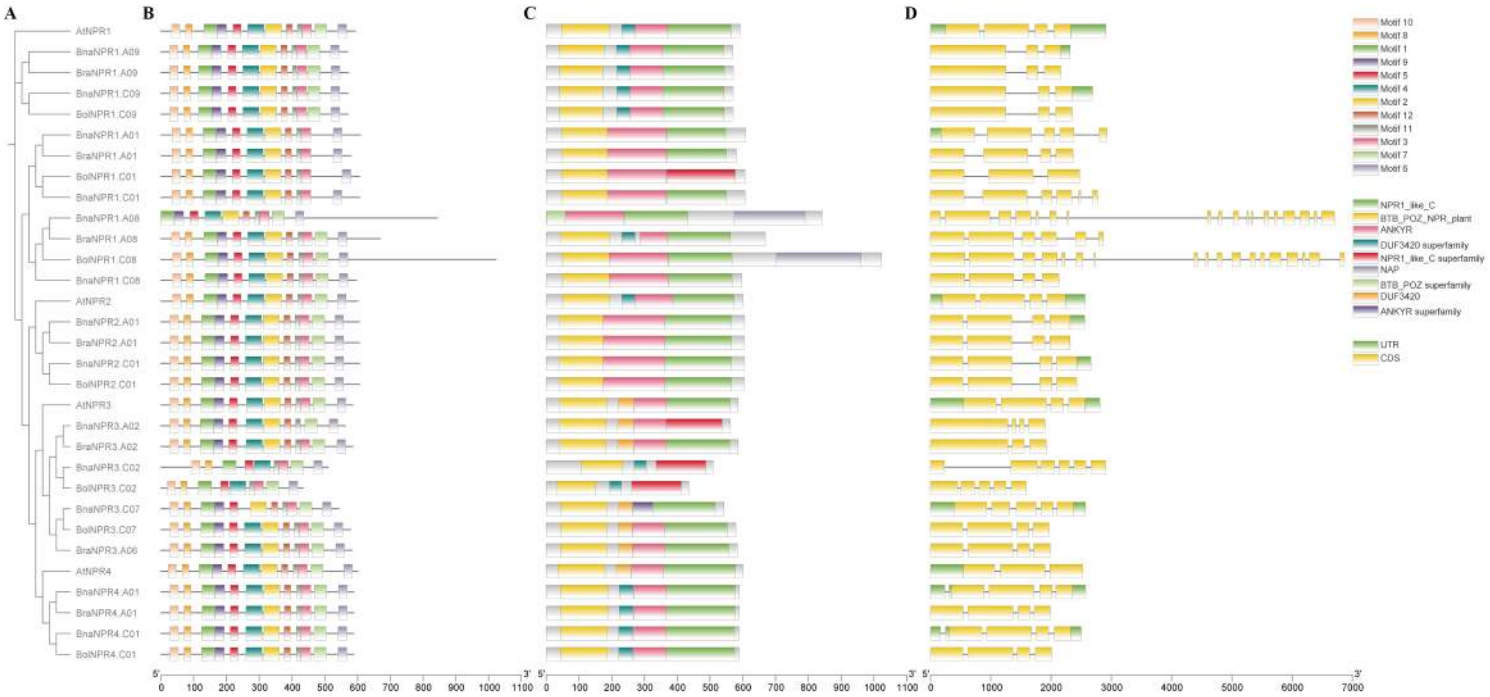

Figure 2

Conserved motifs, domains and gene structures of NPR genes in *A. thaliana* and Brassica species. **(A)** Phylogenetic tree of 31 NPR proteins**. (B)** Distribution of conserved motifs (1–12) identified by MEME. Motif patterns are indicated by colored boxes. **(C)** Conserved domain architectures annotated by InterPro. **(D)** Exon-intron structures. Exons, introns and untranslated regions (UTRs) are represented by yellow boxes, black lines and green boxes, respectively. The horizontal axis indicates the length of each gene (bp).

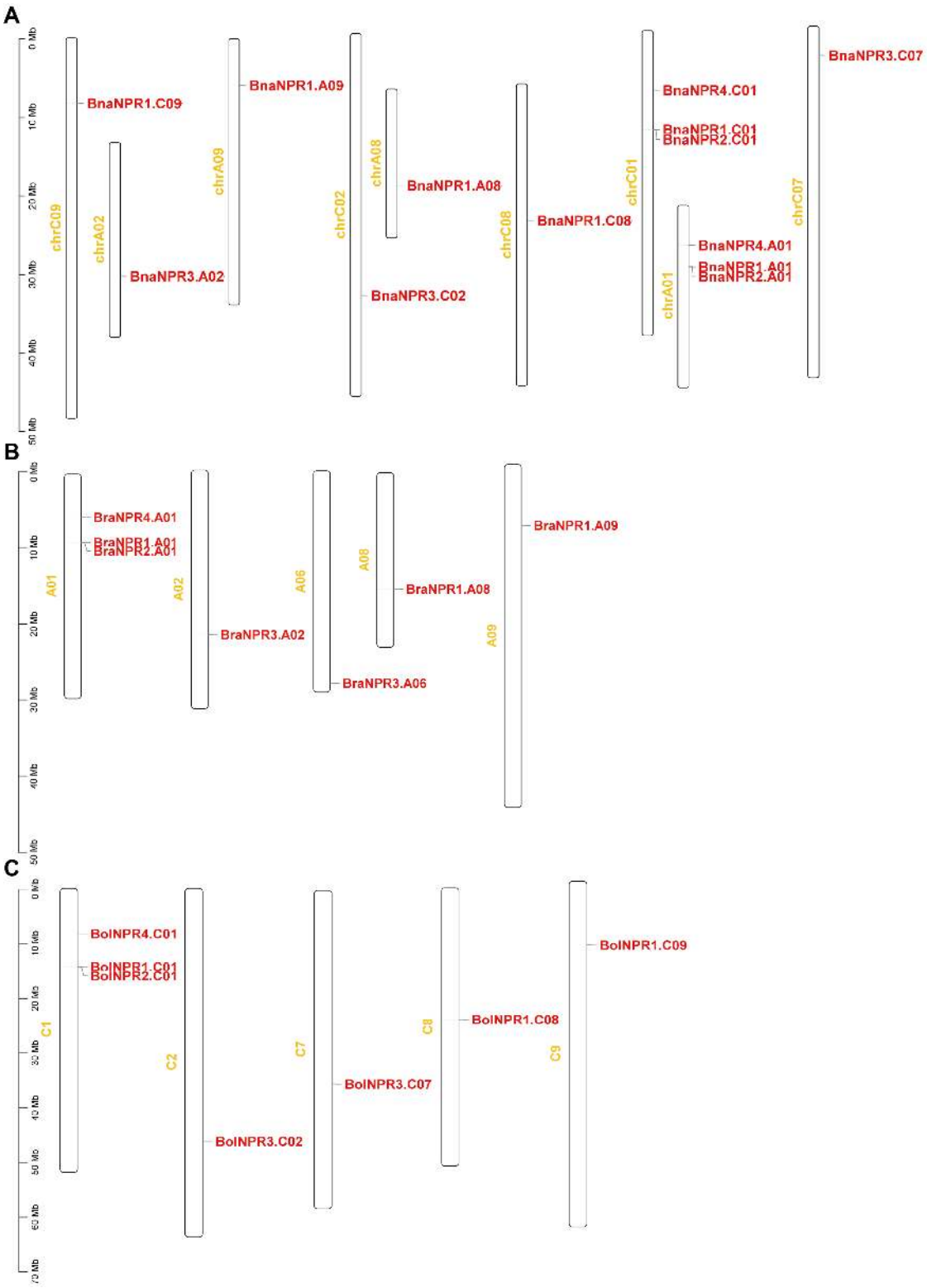

Figure 3

Chromosomal distribution of *NPR* genes in *Brassica napus* (A), *Brassica rapa* (B) and *Brassica oleracea* (C). Chromosomes are shown as gray bars, with chromosome numbers indicated on the left. The scale bar represents megabases (Mb). Gene names are placed to the right of each chromosome.

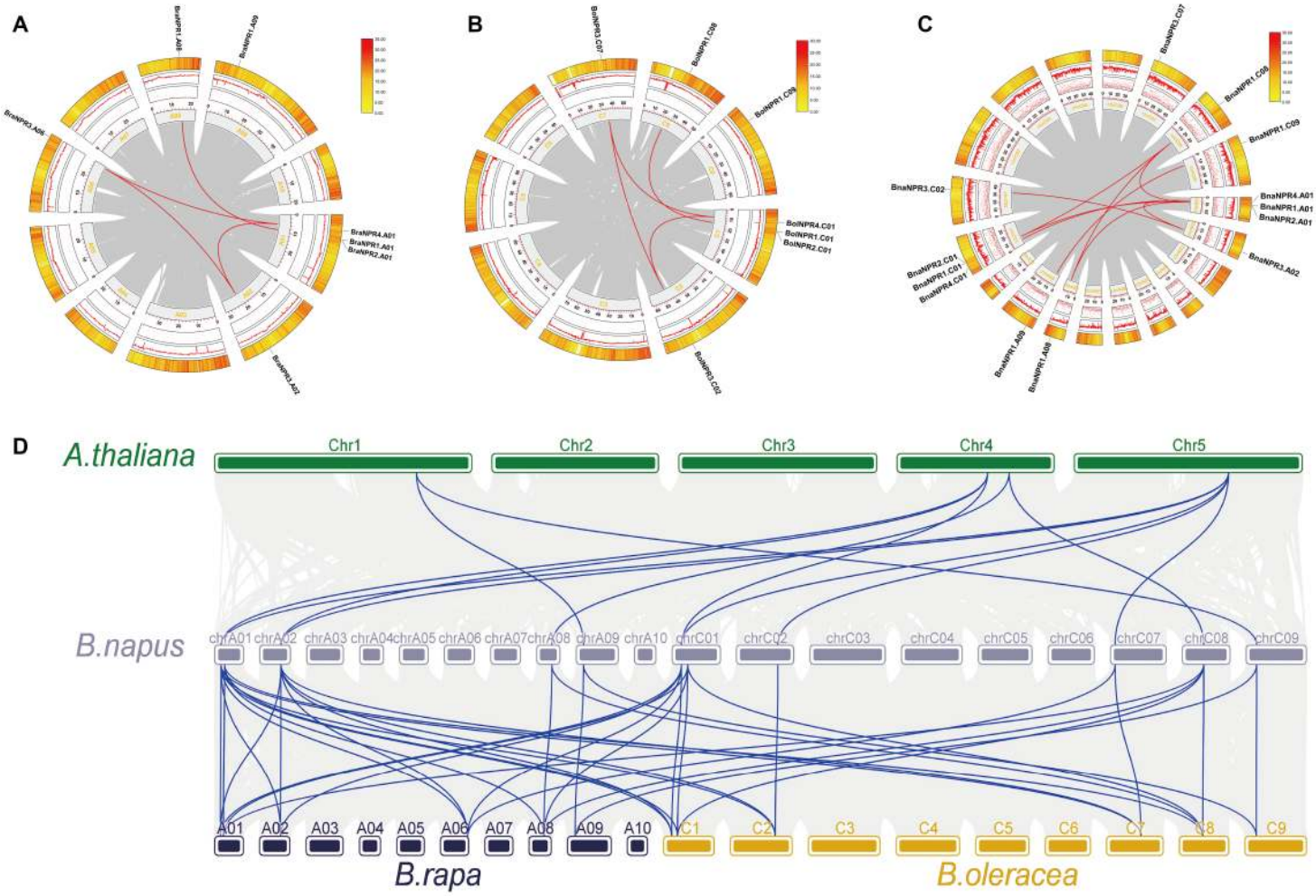

Figure 4

Synteny analysis of *NPR* genes among Brassica species. **(A)** Intra-species synteny in *B. rapa*. **(B)** Intra-species synteny in *B. oleracea*. **(C)** Intra-species synteny in *B. napus*. Gray lines in the background represent all syntenic blocks, and red lines highlight NPR gene pairs. **(D)** Inter-species synteny among *A. thaliana, B. napus*, *B. rapa* and *B. oleracea*. Syntenic connections of *NPR* genes are indicated by blue lines.

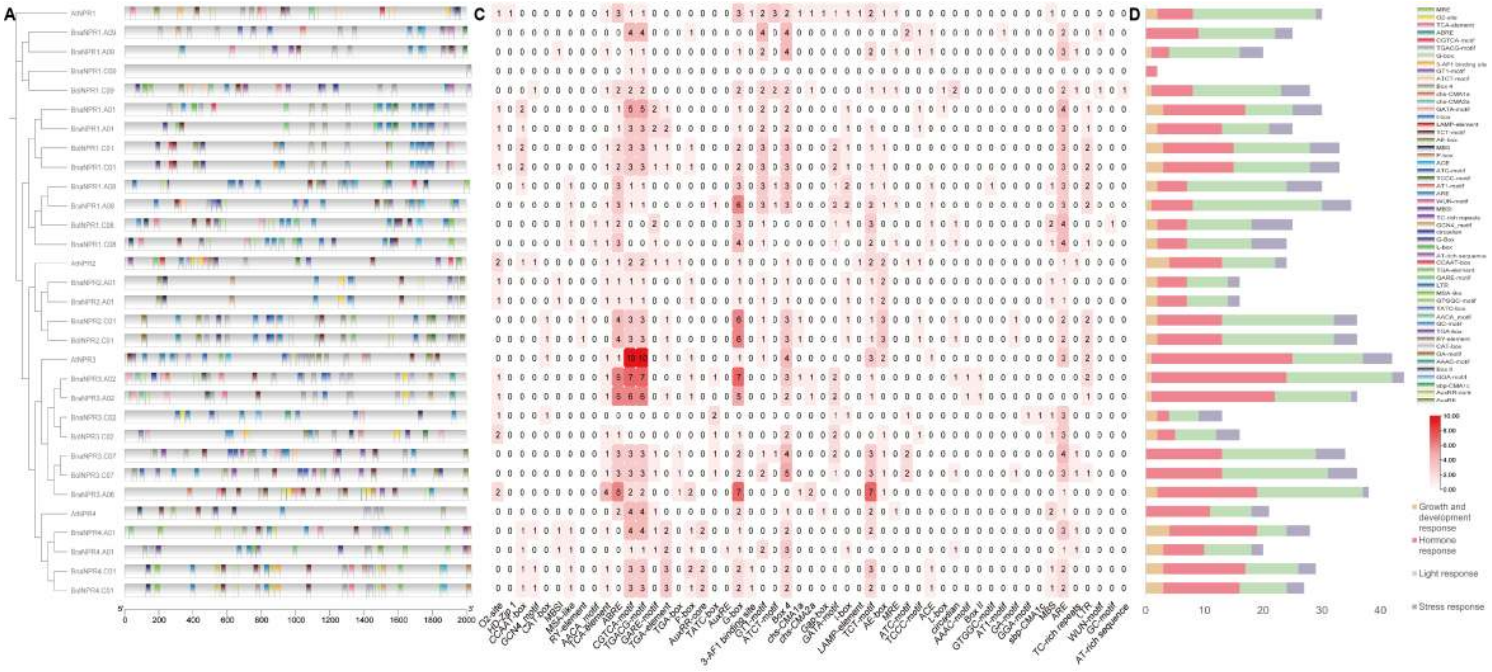

Figure 5

Cis-regulatory elements (CREs) in the promoters of NPR genes. **(A)** Phylogenetic tree of 31 NPR proteins. (B) The distribution of various CREs in the 2 kb upstream regions. **(C)** Heatmap showing the number of individual CREs per gene. **(D)**Bar plot showing the total number of CREs classified into four major categories: hormone-responsive, stress-responsive, developmental, and light-responsive elements.

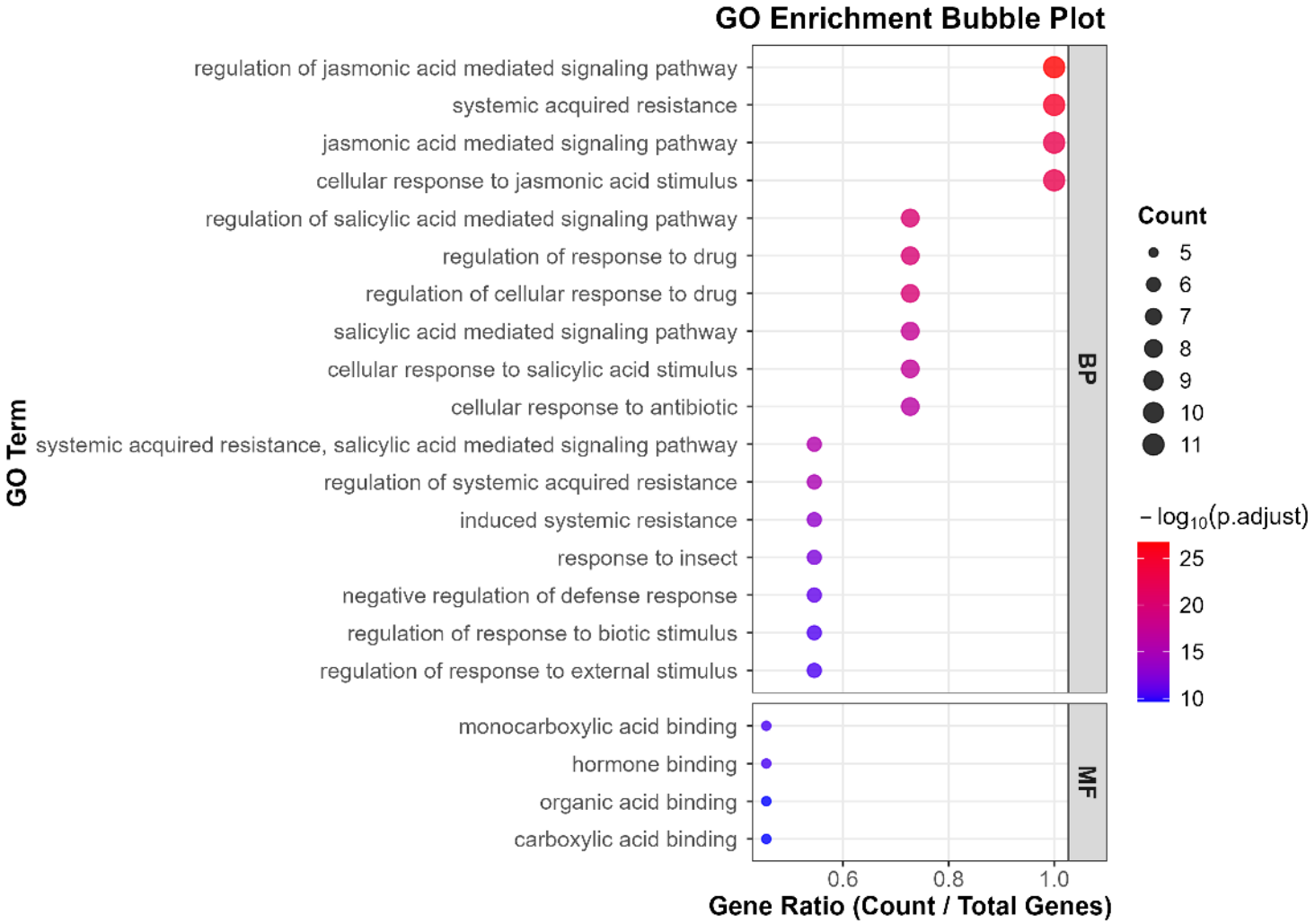

Figure 6

Gene Ontology (GO) enrichment analysis of BnaNPR genes. The x-axis represents the gene ratio, and the y-axis lists the GO terms. The size of the dots represents the number of genes, and the shade of the color indicates the -log10(P-value).

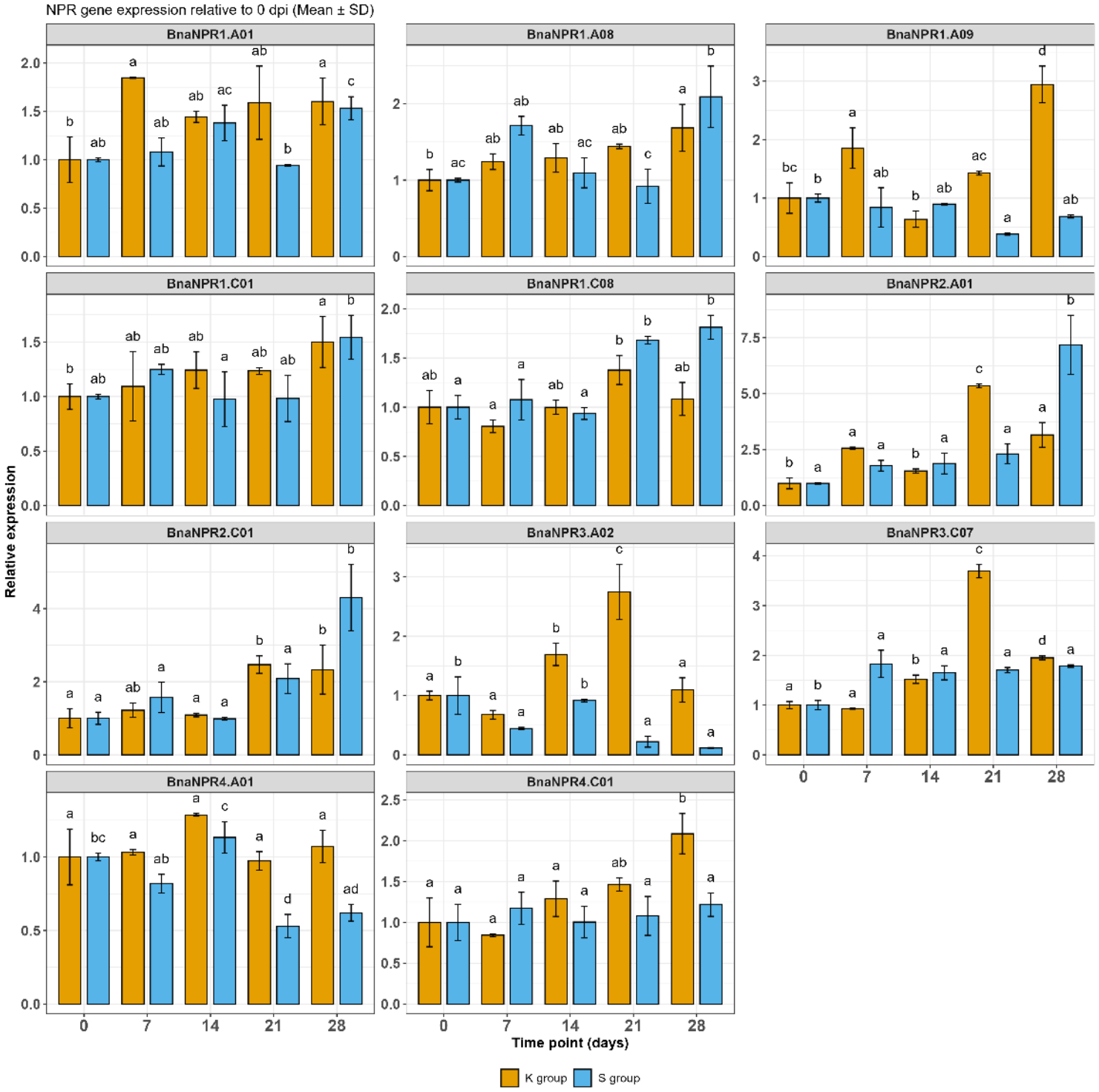

Figure 7

Expression profiles of BnaNPR genes under *Plasmodiophora brassicae* infection. Relative expression levels of 12 BnaNPR genes (BnaNPR3.C02 was not detected) in resistant (K) and susceptible (S) *B. napus* line '1603' at 0, 7, 14, 21, and 28 days post-inoculation (dpi). Data are presented as mean ± SD (n = 3).

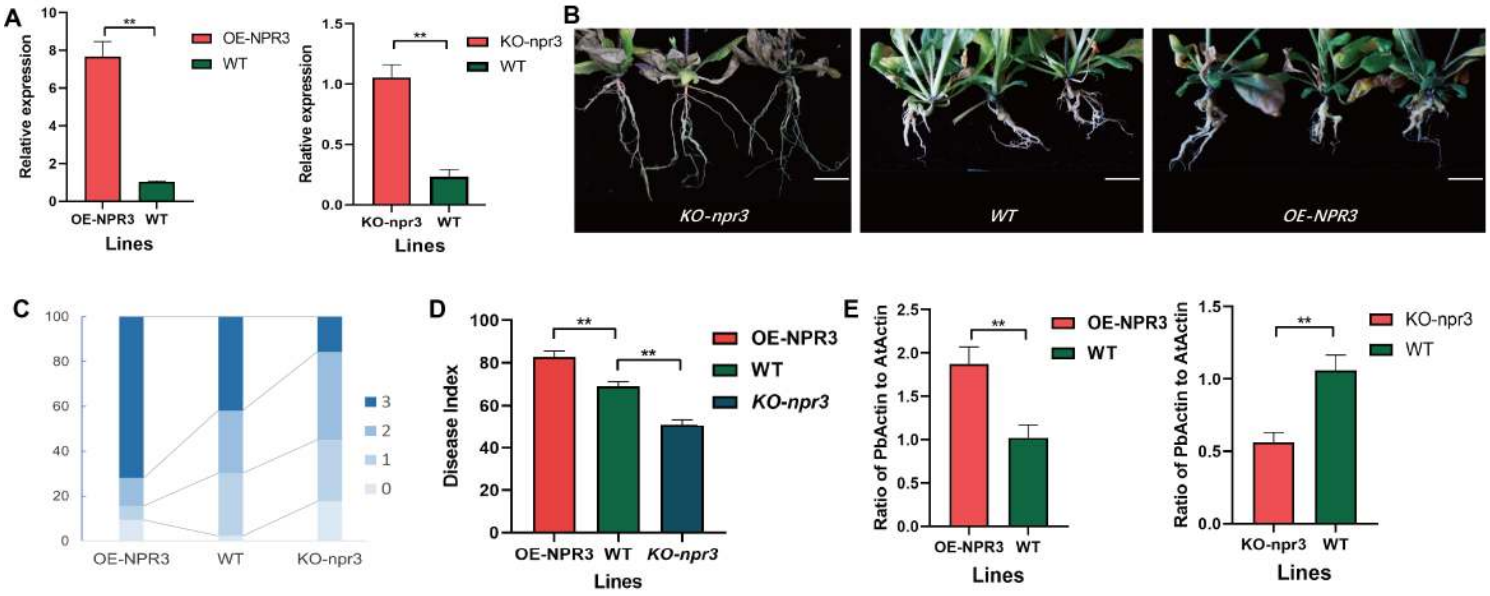

Figure 8

BnaNPR3.A02 negatively regulates resistance to clubroot disease in Arabidopsis.

(A) qRT-PCR validation of *BnaNPR3.A02* expression in wild-type (WT), overexpression (OE-NPR3) lines, and *AtNPR3* expression in npr3 knockout (KO-npr3) lines. **(B)** Disease phenotypes of WT, OE-NPR3 and KO-npr3 plants at 28 dpi with *P. brassicae*. Scale bar: 1 cm**. (C)** Percentage distribution of plants among disease severity grades (grade 0 to grade 3) for each genotype.**(D)** Disease index values. **(E)** Relative pathogen biomass (*P. brassicae* PbActin / plant Actin7). Data are mean ± SD (n = 3). Asterisks indicate significant differences compared with WT (**P < 0.05, **P < 0.01*)**.**

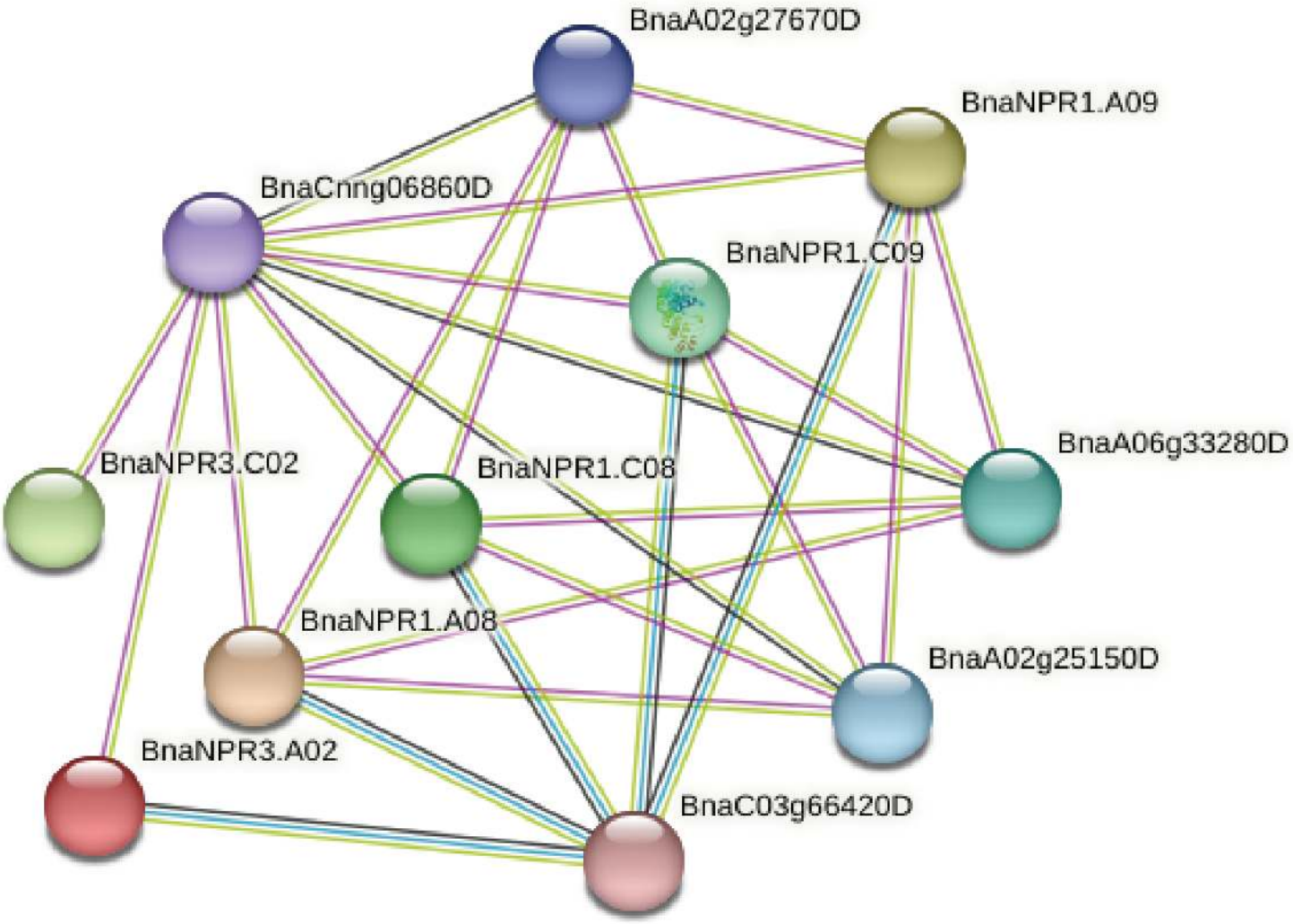

Figure 9

Protein-protein interaction (PPI) network of BnaNPR3.A02. The network was predicted using the STRING database. Nodes represent proteins, and edges indicate predicted functional associations.

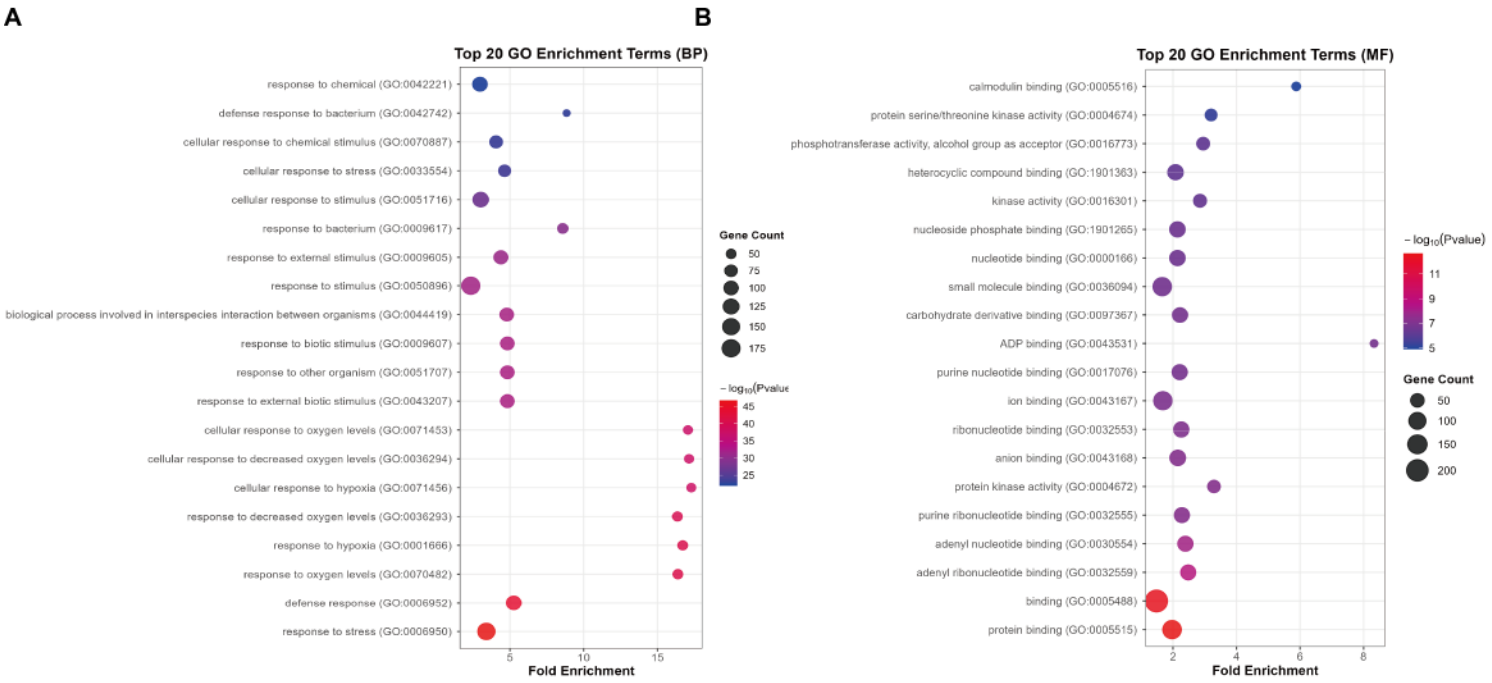

Figure 10

Co-expression analysis of *AtNPR3* (the Arabidopsis homolog of *BnaNPR3.A02*). **(A)** GO enrichment analysis of co-expressed genes in biological process (BP) category. **(B)** GO enrichment analysis of co-expressed genes in molecular function (MF) category. P-values are indicated by color scale, and the number of genes is shown by dot size.

Supplementary Files

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