Comprehensive analyses of the annexin (ANN) gene family in *Brassica rapa*, *Brassica oleracea* and *Brassica napus* reveals their roles in stress response

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Annexins (ANN) are a multigene, evolutionarily conserved family of calcium-dependent and phospholipid-binding proteins that play important roles in plant development and stress resistance. However, a systematic comprehensive analysis of ANN genes of Brassicaceae species (*Brassica rapa*, *Brassica oleracea*, and *Brassica napus*) has not yet been reported. In this study, we identified 13, 12, and 26 ANN genes in *B. rapa*, *B. oleracea*, and *B. napus*, respectively. About half of these genes were clustered on various chromosomes. Molecular evolutionary analysis showed that the ANN genes were highly conserved in Brassicaceae species. Transcriptome analysis showed that different group ANN members exhibited varied expression patterns in different tissues and under different (abiotic stress and hormones) treatments. Meanwhile, same group members from *Arabidopsis thaliana*, *B. rapa*, *B. oleracea*, and *B. napus* demonstrated conserved expression patterns in different tissues. The weighted gene coexpression network analysis (WGcNA) showed that BnaANN genes were induced by methyl jasmonate (MeJA) treatment and played important roles in jasmonate (JA) signaling and multiple stress response in *B. napus*.

Annexins (ANN) are a multigene, evolutionarily conserved family of calcium (Ca\(^{2+}\))-dependent and phospholipid-binding proteins present in plants, animals, and microorganisms1,2. ANN contain the characteristic annexin repeat and they regulate membrane dynamics, mediate Ca\(^{2+}\) sensing and signaling, link Ca\(^{2+}\) dynamics to cytoskeletal responses, and mediate immune or stress responses and signaling during plant growth and development1,3. A typical ANN contains four annexin repeats at the C-terminal region and a highly variable N-terminal region. Each annexin repeat usually contains a characteristic type II motif for Ca\(^{2+}\) binding1,3. The variable N-terminal region interacts with other proteins and is responsible for the functional diversity of ANN4.

Recent studies have identified the ANN gene family in *Arabidopsis thaliana* (8 genes), *Brassica rapa* (13), *Solanum lycopersicum* (9), *Solanum tuberosum* (9), *Oryza sativa* (10), *Triticum aestivum* (25), *Gossypium raimondii* (14), *Arachis hypogaea* (8), *Hordeum vulgare* (11), *Medicago truncatula* (10), *Coilhobolus sativus* (11), *Sorghum bicolor* (10), *Zea mays* (12), *Brachypodium distachyon* (11), *Selaginella mollendorffii* (5), and *Physcomitrella patens* (7) via genome-wide analysis2,5–11.

Studies have shown that ANN gene family plays a significant role in plant development and plant protection during both abiotic and biotic stresses12,13. In *Arabidopsis*, two ANN genes (*AtANN1* and *AtANN4*) were regulated by abiotic stress, negatively regulated plant tolerance to drought, salinity, and heat stress, while *AtANN8* was...
positive regulated the plant abiotic tolerance\textsuperscript{14–21}. Studies also demonstrated that AtANN1 and AtANN2 regulated root growth and development\textsuperscript{26–28}. Downregulation of AtANN5 resulted in abnormal pollen grains and severe male sterility\textsuperscript{29,30}. The rice annexin OsANN1 enhanced heat stress tolerance\textsuperscript{31}, and OsANN3 positively regulated drought tolerance to \textit{O. sativa}. ZmANN33 and ZmANN35 enhanced chilling stress tolerance during germination of maize seeds\textsuperscript{32}. \textit{Medicago truncatula} annexin 1 regulated nodulation and mycorrhization in legume plants\textsuperscript{29,33}. The tobacco annexin \textit{NtAnn12} was induced upon \textit{Rhodococcus fascians} infection\textsuperscript{34,35}. The potato annexin STANN1 promoted drought tolerance\textsuperscript{10}. The cotton annexin gene \textit{GhAnn1} was induced by various phytohormones and abiotic stress, positively regulated drought and salt tolerance\textsuperscript{36}. \textit{GhANN8b} and phosphatase \textit{GhDsPTP3a} proteins of cotton interacted with each other and regulated salt tolerance and calcium influx\textsuperscript{37}. \textit{GhAnn2} was induced by IAA and GA3, and \textit{GhAnn2} downregulation inhibited cotton fiber elongation by modulating Ca\textsuperscript{2+} influx at the cell apex\textsuperscript{38}. \textit{GhFAnnxA} regulated fiber elongation and secondary cell wall biosynthesis\textsuperscript{39}. \textit{AnxGb6} interacted with actin 1 and regulated cotton fiber elongation\textsuperscript{35}. Overexpression of cotton annexin gene \textit{AnnGh3} increased trichome density and length in \textit{Arabidopsis} leaf\textsuperscript{40}. Overexpression of \textit{Brassica juncea} annexin \textit{AnnBJ2} increased salt tolerance and abscisic acid (ABA) insensitivity in transgenic plants\textsuperscript{41,42}. Ectopic expression of \textit{B. juncea} annexin \textit{AnnBJ1} in tobacco and cotton enhanced tolerance to various abiotic stresses and fungal pathogen\textsuperscript{36–41}. \textit{AnnBJ3} promoted oxidative stress tolerance in plants\textsuperscript{42}.

\textit{Brassica napus} (genome AnAnCnCn) is an important oil crop worldwide, which was formed by recent allopolyploidy between ancestors of \textit{B. rapa} (genome \textit{ArAr}) and \textit{B. oleracea} (genome \textit{CoCo})\textsuperscript{43}. The production and quality of \textit{B. napus} is greatly influence by adverse environmental conditions. Therefore, it is critical to improve stress tolerance in \textit{B. napus} through the identification and use of genes involved in stress response. Although there are many studies on ANN genes in various plant species, ANN genes are yet to be characterized in \textit{B. napus} and \textit{B. oleracea}. In this study, we investigate the potential role of ANN genes in environmental stress response in Brassicaceae plants. Therefore, we identified the ANN genes of \textit{B. napus}, \textit{B. rapa} and \textit{B. oleracea} and compared the gene structure, chromosomal location, evolutionary relationship, and expression pattern in different tissues and under different abiotic/biotic stresses and plant hormonal treatments. The findings of this study will provide a foundation for further studies on functional characterization of ANN genes of Brassicaceae plants under adverse environmental conditions.

Results and Discussion

Identification of ANN in \textit{B. rape}, \textit{B. oleracea} and \textit{B. napus}. A total of 13 BrANN (\textit{B. rapa} ANN), 12 BoANN (\textit{B. oleracea} ANN), and 26 BnaANN (\textit{B. napus} ANN) proteins were identified through BLASTP using \textit{Arabidopsis} ANN (AtANN) proteins (Table 1). All members were verified for the presence of annexin repeats using InterPro and Conserved Domain (CD)-search in NCBI. Brassicaceae species experienced an extra whole-genome duplication (WGD) event\textsuperscript{44–46}, based on which approximately 24 and 48 ANN genes were expected in \textit{B. rapa}/\textit{B. oleracea} and \textit{B. napus} genomes, respectively. However, only 13, 12, and 26 ANN genes were found in \textit{B. rapa}, \textit{B. oleracea}, and \textit{B. napus}, respectively (Table 1). In \textit{B. napus}, the number of genes in the An-subgenome (12) and Cn-subgenome (14) was almost the same as that in their diploid progenitors \textit{B. rapa} and \textit{B. oleracea} (Table 1). These results indicate the loss of about half of ANN genes after the Brassicaceae WGD in \textit{B. rapa} and \textit{B. oleracea}. However, most of the duplicated ANN genes were retained after the whole-genome duplication (WGD) event in \textit{B. napus}. WGD event of gene family appears to be a widespread phenomenon, such as the auxin response factor (ARF)\textsuperscript{47}, Auxin/Indoleacetic acid (Aux/IAA)\textsuperscript{48}, glutathione transferases (GST)\textsuperscript{49}, BRI1-EMS-SUPPRESSOR1 (BES1)\textsuperscript{50}, Heat stress transcription factors (Hsf)\textsuperscript{51,52}, GRAS\textsuperscript{53} family genes in diploid and allopolyploid Brassicaceae and Calcium-dependent protein kinases (CPK)\textsuperscript{54}, Jasmonate ZIM-domain (JAZ)\textsuperscript{55} and Nuclear factor YB (NF-YB)\textsuperscript{56} in diploid and allopolyploid Gossypium species (\textit{G. raimondii}; DD genome; \textit{G. arboreum}; AA genome; \textit{G. hirsutum}; AADD genome).

Among the 51 \textit{Brassica} ANN genes, 35 were the typical ANN, which encoded proteins ranging from 315–325 amino acids (AA) and contained four annexin repeats. All eight ANN members (315–320 AA) homologous to AtANN4 (AT2G38750) contained 2–3 annexin repeats, as same as AtANN4. While two other ANN members (157 AA) contained only a single annexin repeat and six members (183–265 AA) contained 2–3 annexin repeats (Table 1), they may be the truncated mutated duplications.

Phylogenetic and structural analysis of ANN. A phylogenetic tree (Fig. 1A) was generated using the sequences of 59 ANN proteins from \textit{B. rapa}, \textit{B. oleracea}, \textit{B. napus}, and \textit{Arabidopsis} (Fig. S1). These ANN proteins were divided into six groups. All eight AtANN were found to have orthologous genes in \textit{B. rapa}, \textit{B. oleracea}, and \textit{B. napus} (Fig. 1A). Twelve pairs of BnaANN were found in the corresponding \textit{B. napus} An- and Cn-homoeologous chromosomes, and one pair of them had homologous genes both in \textit{B. rapa} and \textit{B. oleracea}. Meanwhile, two pairs (BnaC03g42920D/BnaA06g23966D and BnaC09g44350D/BnaA10g20320D) only had homologous genes in \textit{B. rapa}. All 12 BoANN genes were found to have homologous genes in the Cn-subgenome of \textit{B. napus}, while one BrANN (BrannB3578) had no homologous gene in An-subgenome of \textit{B. napus} (Table 1 and Fig. 1A).

Gene structure analysis revealed that majority of the homologous ANN genes paired had same gene structure (Fig. 1B). There were five introns in group IV/VI members, expect for two truncated mutant genes (Bo1g039570 and BnaC01g16910D) (Fig. 1B). This finding indicates that the ANN genes are conserved in Brassicaceae species, possibly due to their importance in plant growth and productivity.

A typical ANN protein contains four annexin repeats, each approximately 70 amino acids long\textsuperscript{13}. Annexin repeat usually contain a characteristic type II motif for binding calcium ions with the sequence GxGxT-[38 residues]-D/E\textsuperscript{14}. MEME analysis showed that 42 ANN proteins contained four annexin repeats (Fig. 1C). Motif1 was the core sequence of all the four annexin repeats, and motif4 was only found in the third annexin repeat in group I–V, while motif5 was the core sequence close to the C-terminal of Motif1 in the second and fourth annexin repeats (Fig. 1C).
Table 1. List of ANN genes identified in Arabidopsis, B. rape, B. oleracea and B. napus.

| Arabidopsis homologous gene in B. rape/B. oleracea/B. napus | Gene ID | Gene name | Chrm (bp) | AA | pI | Mw (kD) | Introns | Exons | Annexin repeats | Predicted subcellular localization | Chromosome location |
|-------------------------------------------------------------|---------|-----------|-----------|----|----|---------|---------|-------|----------------|-----------------------------------|-------------------|
| ATSG10230 (ANN7)                                            | B. rape | Bra031890 | 951       | 321| 6.3| 36.38   | 3       | 4     | 4              | Cytoplasmic                     | A10:15023513-15025336 |
|                                                             | B. oleracea | Bra017130 | 552       | 183| 8.67| 21.10   | 1      | 2     | 2              | NONE                             | C09:5098649-5098738 |
|                                                            | B. napus | Bna04j22020D | 951    | 321| 6.73| 36.43   | 3       | 4     | 4              | Cytoplasmic                     | A10:14969359-14971179 |
| ATSG10220 (ANN6)                                            | B. rape | Bra031890 | 951       | 321| 6.3| 36.38   | 3       | 4     | 4              | Cytoplasmic                     | A10:15026227-15027937 |
|                                                            | B. oleracea | Bra017130 | 552       | 183| 8.67| 21.10   | 1      | 2     | 2              | NONE                             | C09:5098649-5098738 |
|                                                            | B. napus | Bna04j22020D | 951    | 321| 6.73| 36.43   | 3       | 4     | 4              | Cytoplasmic                     | A10:14969359-14971179 |
| ATSG65020 (ANN2)                                            | B. rape | Bra04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
|                                                            | B. oleracea | Bra0110220 | 951       | 321| 6.3| 36.38   | 3       | 4     | 4              | Cytoplasmic                     | A10:14969359-14971179 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
| ATIG355720 (ANN1)                                           | B. rape | Bra039578 | 789       | 265| 6.2| 30.43   | 4       | 5     | 3              | NONE                             | Scaffold001641814-143464 |
|                                                            | B. oleracea | Bra039578 | 960       | 319| 5.27| 36.39   | 5       | 6     | 4              | Cytoplasmic                     | C06:11348510-11350276 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
| ATIG12380 (ANN8)                                            | B. rape | Bra039578 | 789       | 265| 6.2| 30.43   | 4       | 5     | 3              | NONE                             | Scaffold001641814-143464 |
|                                                            | B. oleracea | Bra039578 | 960       | 319| 5.27| 36.39   | 5       | 6     | 4              | Cytoplasmic                     | C06:11348510-11350276 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
| AT2G387600 (ANN3)                                           | B. rape | Bra039578 | 789       | 265| 6.2| 30.43   | 4       | 5     | 3              | NONE                             | Scaffold001641814-143464 |
|                                                            | B. oleracea | Bra039578 | 960       | 319| 5.27| 36.39   | 5       | 6     | 4              | Cytoplasmic                     | C06:11348510-11350276 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
| ATIG680909 (ANN5)                                           | B. rape | Bra039578 | 789       | 265| 6.2| 30.43   | 4       | 5     | 3              | NONE                             | Scaffold001641814-143464 |
|                                                            | B. oleracea | Bra039578 | 960       | 319| 5.27| 36.39   | 5       | 6     | 4              | Cytoplasmic                     | C06:11348510-11350276 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |
| AT2G387500 (ANN4)                                           | B. rape | Bra039578 | 789       | 265| 6.2| 30.43   | 4       | 5     | 3              | NONE                             | Scaffold001641814-143464 |
|                                                            | B. oleracea | Bra039578 | 960       | 319| 5.27| 36.39   | 5       | 6     | 4              | Cytoplasmic                     | C06:11348510-11350276 |
|                                                            | B. napus | Bna04j23464 | 951    | 321| 6.57| 36.00   | 3       | 4     | 4              | Cytoplasmic                     | A06:15094250-15096876 |

**Table 1.** List of ANN genes identified in Arabidopsis, B. rape, B. oleracea and B. napus.
According to the gene structure and motif analysis, the missing parts of the truncated mutant members were readily apparent. Both the first and fourth annexin repeats were absent in Bo9g172330 and BnaC09g46400D, and the first annexin repeat was absent in BnaAnng37420D. Bo1g039570 and BnaC01g16910D had only the second annexin repeat at the C-terminal (80–159 AA), and the core sequence of annexin repeat was not detected at the N-terminal (1–79 AA). It is similar in the N-terminal of Bo6g043900, BnaC06g08410D, and AtANN4 homologues (Fig. 1B,C).

Chromosomal location and synteny analysis of ANN of B. rapa, B. oleracea, and B. napus. As showed in Fig. 2, the distribution of BnaANN in An- and Cn-subgenome was nearly even with 12 ANN genes from the An-subgenome and 14 from the Cn-subgenome. However, the ANN genes’ distribution was uneven on each chromosome. Three pair (2 genes/pair) of ANN genes from the An-subgenome were repeated in tandem on chromosome Bn_A03, Bn_A04, and Bn_A10 (Fig. 2); and three pair (2 genes/pair) of ANN genes from the Cn-subgenome were repeated in tandem on chromosome Bn_C03, Bn_C04, and Bn_C09 (Fig. 2C). B. napus gene analysis showed that the An- and Cn-subgenome were largely collinear to the corresponding diploid Ar and Co genomes43,57. Most of the An-Ar and Cn-Co orthologous gene pairs demonstrated similar chromosomal locations. The distribution of ANN genes in B. rapa and B. oleracea were similar to the distribution of the orthologous BnaANN genes in the B. napus An-subgenome and Cn-subgenome, respectively (Fig. 2). Two BnaANN (BnaAnng94520D and BnaAnng37420D) and one BrANN (Brn039578) genes were located on the unanchored scaffolds that were not mapped to a specific chromosome (Fig. 2). The sequence and phylogenetic analyses revealed BnaAnng94520D-Brn034402 and BnaAnng37420D-Brn031890 as two An-Ar orthologous gene pairs. Based on this, we predicate Bn_A02 and Bn_A05 as the true chromosomal locations of BnaAnng04520D and BnaAnng37420D, respectively. BnaANN (BnaC03g49290D and BnaC09g44350D) had no orthologous genes in B. oleracea (Fig. 2), though they had homologous genes in An-subgenome. These findings indicate that duplication of BnaA06g23960D and BnaA10g20320D led to the formation of BnaC03g49290D and BnaC09g44350D, respectively. Analysis of the synteny among An-subgenome and Cn-subgenome showed high collinearity between Bn_A01-Bn_C01, A02-C02, A03-C03, A04-C04, A05-C05, A06-C06, A07-C07, A08-C08, A09-C09, and A10-C09, and 83.7% orthologous gene pairs between B. rapa and B. oleracea were retained as homologous gene pairs in B. napus43,57. 90.9% ANN gene pairs (10/11 pairs) between B. rapa and B. oleracea were retained as homologous gene pairs between B. napus An-chromosomes and Cn-chromosomes (Fig. 2).
There were two tandem pairs (AtANN3/4 and AtANN6/7) on chromosome 2 and chromosome 5 in Arabidopsis, respectively. Bra009048/Bra009049, Bo9g172330/Bo9g172340, BnaA10g22010D/BnaA10g22020D, and BnaC09g46400D/BnaC09g46410D were homologous to AtANN6/7 tandem pair in B. rapa, B. oleracea, B. napus An-subgenome and Cn-subgenome, respectively. We identified two tandem pairs each homologous to AtANN3/4 in B. rapa (Br_A03 and Br_A04), B. oleracea (Bo_C03 and Bo_C04), B. napus An-subgenome (Bn_A03 and Bn_A04), and Cn-subgenome (Bn_C03 and Bn_C04) (Fig. 2). AtANN8 (AT5G12380) was located near the AtANN6/7 tandem pair on chromosome 5 in Arabidopsis. Correspondingly, there was a gene homologous to AtANN8 located near the tandem pair homologous to AtANN6/7 in B. rapa (Br_A10), B. napus An-subgenome (Bn_A10) and Cn-subgenome (Bn_C09) (Fig. 2). There was no gene homologous to AtANN8 in B. oleracea (Bo_C09). Instead, we found a truncated mutated gene (Bo1g039570) homologous to AtANN8 in B. oleracea (Bo_C01). Meanwhile, a truncated mutated gene (BnaC01g016910D) was homologous to Bo1g039570 in B. napus Cn-subgenome (Bn_C01) (Fig. 2). Bra039578 had no homologous gene in An-subgenome of B. napus. These findings suggest that majority of the ANN genes are conserved in Brassicaceae species, only a few ANN genes are missing or duplicating in B. napus.

To better understand the evolutionary constraints acting on the ANN gene family, we estimated the number of nonsynonymous substitutions per nonsynonymous site (Ka), the number of synonymous substitutions per synonymous site (Ks), and the Ka/Ks ratio. Ka/Ks value < 1 indicates that a gene pair has experienced purifying selection; Ka/Ks > 1 indicates positive selection; and Ka/Ks = 1 indicates neutral selection. The Ka/Ks ratio was...
Figure 3. Heat map showing expression of ANN genes in different tissues at different developmental stages of Arabidopsis (A), B. rapa (B), B. olearacea (B), and B. napus (C). Coloured rectangles indicate the gene FPKM values.

<1 for majority of the ANN collinear gene pairs (209/210), except for the gene pair Bra024346/BnaA06g23960D (Ka/Ks > 1) (Table S1). These results indicate that majority of genes experienced purifying selection, whereas Bra024346 and BnaA06g23960D experienced positive selection.

Expression profile of ANN genes in different tissues. ANN genes exhibit tissue-specific expression, which is usually consistent with their substantially differentiated functions.4,16–18,22,23,58. We investigated the expression of all ANN genes in different tissues of Arabidopsis, B. rapa, B. olearacea, and B. napus based on the Arabidopsis eFP Browser data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and RNA-Seq data (B. rapa: GSE43245; B. olearacea: GSE42891; B. napus: PRJNA394926) (Table S2). The ANN genes were expressed across different vegetative and reproductive organs during different developmental stages of the four species (Fig. 3). In general, the ANN expression pattern was different between groups; however, expression pattern was very similar within a group in the four plant species.

Group I (ANN6/7) members showed expression in young siliques (ovules) and seeds, which indicate their importance in ovule and seed development in Brassicaceae plants. However, two truncated mutated members (Bo9g172330 and BnaC09g24600D) homologous to ANN7 were at low abundance expression levels (Fig. 3). Unlike Bo9g172330 and BnaC09g24600D, other five truncated mutated members (BnaAm9g37420D, BnaA06g23960, BnaC06g08410D, Bo1g039570 and BnaC01g16910D) have a similar expression level to their homologous genes which have complete gene structure (Figs. 1 and 3). So, truncated mutated gene structures may decrease their own genes’ expression level, but not always. The expression levels of group 2 (ANN2) members were highest in roots and young siliques (ovules), while that of group 3 (ANN1) members were higher in roots, stems, and young siliques (pericarps) (Fig. 3). These expression levels are consistent with the role of ATANN1 and ATANN2 in root growth and development.50–22. It was indicated that ANN1/2 regulates the development of young siliques and seeds. We detected low level of expression for group 4 (ANN8) members. ATANN5, which regulates pollen development, showed specific expression in mature pollen. The B. napus genes homologous to AtANN5 were mainly expressed in buds and new pistils. The genes homologous to ATANN3 and ATANN4 demonstrated similar expression pattern. Both genes were expressed in flowers and young siliques (ovules), though they belong to group IV and VI, respectively (Fig. 3). All these indicated that ANN genes may be involved in various developmental processes with different functions. In Arabidopsis, ATANN3 and ATANN4 had similar expression pattern because they share a 5′ promoter region (2654 bp). In B. rapa, Bra000090 and Bra000091 share a 5′ promoter region (2079 bp), while in B. olearacea, Bo3g032760 and Bo3g032770 share a 5′ promoter region (2412 bp); In B. napus, BnaA03g18070D and BnaA03g18080D share a 5′ promoter region (2455 bp) and BnaC03g21600D and BnaC03g21600D share a 5′ promoter region (6199 bp). They were homologous to ATANN3/ATANN4 pair, and had similar expression pattern. But another gene pairs (Bna017102/Bna017103, Bo4g187790/Bo4g187780, BnaA04g22190D/BnaA04g22180D, and BnaC04g45920D/BnaC04g45910D) homologous to ATANN3/ATANN4 pair were at low abundance expression levels (Fig. 3B,C). All the results suggested that there were gene duplications, gene expression pattern differentiations and subsequent functional diversifications in ANN family genes in Brassicaceae species, and the functions of homologs of a given group ANN genes might be redundant as they share similar expression patterns.
Expression pattern of ANN genes in response to abiotic stress and hormonal treatment.

Accumulating evidence from various plant species has shown the regulation of ANN genes in response to abiotic stress and hormonal treatment \(^5\)–\(^7\),\(^9\),\(^58\). To examine the expression pattern of BnaANN genes under various abiotic stress conditions and hormonal treatments, we utilized the data on transcriptional profiling (Table S3). As shown in Fig. 4, most of the expressed BnaANN genes in group II/III/V/VI were up-regulated under salinity and PEG stress in roots and MeJA treatment in leaves. BnaA06g23960, BnaA03g18070D and BnaC03g49290D were down-regulated under cold stress, whereas BnaAnng04520D and BnaC05g27530D were up-regulated under cold stress at 12 hours point (Fig. 4).

B. napus is a winter biennial crop with excellent tolerance to low-temperature stress during vegetative stage. The response mechanisms are different under chilling and freezing temperatures, as well as cold shock and cold acclimation in plants\(^5\),\(^2\),\(^6\). Based on the transcriptional profiling of early-maturing, cultivated B. napus varieties under different low-temperature treatments with or without cold acclimation (GSE129220: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129220) (Table S4)\(^6\),\(^4\), transcriptome analysis revealed that group III BnaANN were induced slightly by chilling stress, and were up-regulated by freezing stress strongly, regardless of cold acclimation (Fig. 5A). This finding indicates that group III BnaANN genes play important roles in freezing stress in B. napus.

Sclerotinia sclerotiorum is a hemibiotroph pathogen with a wide host range. It is the causative agent of stem rot, one of the most devastating diseases of B. napus\(^6\),\(^6\). Previous studies have shown the role of JA signaling in plant resistance to hemibiotroph pathogens\(^6\),\(^7\),\(^9\). The transcriptional profiling of B. napus susceptible (Westar) and tolerant (ZY821) genotypes infected with S. sclerotiorum (GSE81545: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81545) (Table S5) showed that the group II BnaANN were induced by S. sclerotiorum infection, and the expression level in the susceptible genotype (Westar) was more than that in the tolerant (ZY821) genotype; some members from group III and group V BnaANN were induced, while some members were repressed by S. sclerotiorum infection (Fig. 5B). These findings indicate a complex response mechanism and the role of some BnaANN in B. napus response to S. sclerotiorum.

To validate the results on transcriptional profiling, we performed a qRT-PCR to detect the transcript levels of three genes (BnaA03g49290D, BnaC05g27530D, and BnaC03g21590D) from group II/III/VI in the roots challenged with salt and PEG and in the leaves treated with cold and MeJA. The expression pattern (of these BnaANN genes) was consistent with the RNA-Seq data (Figs. 4–6). All three BnaANN genes were induced under salinity and PEG stress in roots and induced by MeJA in leaves (Fig. 6A). BnaC03g49290D and BnaC03g21590D were repressed by cold treatment (Fig. 6A). BnaC05g27530D was significantly upregulated under freezing stress, with or without cold acclimation (Fig. 6B). In B. rapa, Bna034402 (gene to homologous BnaC05g27530D) was strongly induced by hormone and stress treatments\(^1\). All these results indicated the role of these three genes in multiple abiotic stress response and JA signaling response in B. napus.
Weighted gene co-expression network analysis (WGCNA) of BnaANN in response to environmental stress.

Weighted gene co-expression network analysis (WGCNA) is an effective way to identify clusters of highly correlated genes. To reveal the divergent functions of BnaANN genes in development, abiotic stress response, and hormone signaling, coexpression networks were constructed on the basis of pairwise correlations of all B. napus gene expression across 12 tissues samples and 8 treatment (abiotic stress and hormone) samples using WGCNA. The analysis identified 56 distinct modules (labeled with different colors) as shown in the dendrogram (Fig. S2). In total, 16 of 26 BnaANN genes were identified in six different modules: light green module (5), blue module (3), green module (3), turquoise module (3), salmon module (1), and magenta module (1) (Table S6).

The light green module (845 genes) was positively correlated with the MeJA treatment in leaves (Fig. S2). Five BnaANN genes (BnaA03g18070D, BnaA03g18080D, BnaC03g21590D, BnaC03g21600D, and BnaC05g27530D) were induced by MeJA treatment in light green module (Fig. 4 and Table S6). The top two hub genes with the highest module membership kME (k-means clustering algorithm) values were BnaA03g18070D (BnaANN 4A-1) and BnaC06g31830D (BnaTIFY7) in the light green module (Fig. 7A and Table S6). The jasmonate acid (JA) signaling repressor, TIFY, was induced by JA and regulates plant development and stress response. Additionally, there were some B. napus JA biosynthesis genes and JA responsive genes in the light green module, such as the Lipoxygenase (LOX), Allene oxide cyclase (AOC), Allene oxide synthase (AOS), 12-oxophytodienoate reductase (OPR), Jasmonate O-methyltransferase (JMT), and Ethylene-responsive factor (ERF) (Fig. 7A and Table S6). Transcriptional profiling and qRT-PCR analysis results showed that BnaA03g18070D/BnaANN 4A-1, BnaC06g31830D/BnaTIFY7, BnaC04g38070D/BnaERF42, and BnaC02g43450D/BnaAOS were all induced by MeJA (Fig. 7B and Table S7). However, there was little research at the functions of annexins in JA signaling. ZmAnx6.1 and ZmAnx7 were induced at 12 h by JA, and the JA-responsive cis-elements exist in their promoters. We analyzed the promoter sequences (2000 bp upstream of transcription start sites) of BnaANN, and found that there were so many cis-elements involved in stress (drought, low-temperature, heat, anaerobic, wounding, defense and stress) response and plant hormones (MeJA, ABA and SA) response in their promoters. BnaANN members contain MeJA-responsive cis-elements (1 to 9) in promoters (Fig. S3). It suggested that the BnaANN genes in light green module involved in JA signaling response in B. napus.

Three BnaANN genes (BnaA04g22190D, BnaC03g49290D, and BnaC02g43450D) in the blue module were expressed with NaCl and PEG treatments in roots, while genes (BnaC08g96690D, BnaA10g20320D...
and BnaC09g44350D) in the green module were expressed in roots. BnaC01g16910D, BnaA06g23960D, and BnaC06g08410D in the turquoise module were positively correlated with bud, stamen, ovule, and silique (Fig. S2 and Table S6). All the results indicate the different functions of B. napus ANN genes during plant development and stress response.

Materials and Methods
Identification of ANN of B. rapa, B. oleracea, and B. napus. B. rapa, B. oleracea and B. napus ANN proteins have been identified using BLASTP (E-value < 1e-5) to look for homologs of Arabidopsis ANN among B. rapa, B. oleracea and B. napus genome sequences database in Ensembl genomes (http://ensemblgenomes.org)77. The annexin motifs in ANN proteins were characterized using InterPro (http://www.ebi.ac.uk/interpro/)78 and the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The molecular weight (Mw), isoelectric point (pI), and subcellular localization of ANN proteins were predicted using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/)79 and ProtComp 9.0 (http://linux1.softberry.com/). The exon and intron organization of the ANN genes were analyzed using the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/)80. The conserved motifs of ANN were analyzed with MEME (http://meme.nbcr.net/meme/cgi-bin/meme.cgi)81.

Phylogenetic analysis. Multiple sequence alignment of all identified ANN proteins (Arabidopsis, B. rapa, B. oleracea, and B. napus) were performed using ClustalW and a phylogenetic tree was constructed using the neighbour-joining (NJ) phylogenetic method in MEGA782 with 1000 bootstrap replicates.

Chromosomal localization of ANN genes. The position of ANN genes on the chromosomes of B. rapa, B. oleracea, and B. napus were obtained using TBtools v0.6683183. DnaSP (DNA Sequence Polymorphism) v684 was used to calculate the ratio of the nonsynonymous substitution rate (Ka) to the synonymous substitution rate (Ks) and the Ka/Ks value between paralogous gene pairs.

Plant materials and treatments. ZS11 (B. napus L. cv. Zhongshuang 11)57 seeds were allowed to germinate and then the seedlings were transplanted to pots containing soil or vermiculite. The growth conditions, hormone treatments, and abiotic stress conditions were as described previously85. Hormone treatments were performed by spraying leaves of 6-week-old seedlings with ABA (100 μM), MeJA (100 μM), SA (1 mM), and ETH (10 μg/ml); To simulate hot and cold stresses, seedlings were grown in chamber with 40 °C or 4 °C. To simulate salt and PEG stresses, seedlings were irrigated with NaCl (200 mM) or PEG-6000 (20%) solutions.

For chilling and freezing treatments with or without cold acclimation, the seedlings of two early-maturing semi-winter rapeseed varieties (HX17 and HX58) were used. They were treated as described previously 64. Seedlings were cultured in incubators under 20 °C (14 h light: am6:00–pm8:00)/16 °C (10 h dark: pm8:00–am6:00) 4 weeks, then treated with 4 °C (14 days) → 4 °C (12 h) (CA) or −4 °C (12 h) (FA), 20 °C/16 °C (light/dark) 6 weeks → 4 °C (12 h) (CB), 20 °C (14 h light: am6:00–pm8:00)/16 °C (10 h dark: pm8:00–am6:00) 6 weeks → −4 °C (12 h) (FB). For the acclimation condition, after the 14 days at 4 °C, 4 °C/−4 °C (12 h) mean a treatment with 4 °C or −4 °C at pm8:00–am8:00 (10 h dark and 2 h light).

RNA isolation and sequencing and gene expression analysis. The collected samples were sent to the sequencing cooperations of Sangon Biotech (Shanghai) Co., Ltd. and Novogene Co., Ltd. for RNA isolation, examination, and sequencing44,85. qRT-PCR analysis was performed as described previously85. The primers used in this study were listed in Table S8.

Figure 6. qRT-PCR analysis of three BnaANN genes under abiotic stress and hormone treatments. The relative qRT-PCR expression level (blue bar) is shown on the left y-axis. The RNA-Seq TPM/FPKM values (red line) are shown on the right y-axis. BnaActin (BnaC05g34300D) was used as the endogenous reference gene. The relative transcript levels were averaged over the three technical replicates.
Heat map analysis. The RPKM (Reads Per kb Per Million reads) and TPM (Transcripts Per Million) values were used to represent the expression levels of the ANN genes. A heat map of the expression profile of the ANN genes was plotted using Heatmap Illustrator, version 1.0.

Weighted gene coexpression network analysis (WGCNA). Weighted gene coexpression network analysis was performed using WGCNA package in R. The networks were visualized using Cytoscape v3.

Data availability
The authors declare that all the data and plant materials will be available without restrictions.

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Author contributions

Xin He designed the overall study, performed and analyzed most of the experiments, and wrote the manuscript. Li Liao, Sai Xie, Min Yao, Pan Xie, Wei Liu, Yu Kang, Luyao Huang, Mei Wang, Lunwen Qian assisted with experimental data analysis and graph draw. Chunyun Guan and Zhongsong Liu made a significant contribution to the manuscript. Wei Hua and Mei Guan revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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