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Genome-wide identification of the *NAC* family genes of adzuki bean and their roles in rust resistance through jasmonic acid signaling

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Abstract

Background Adzuki bean (*Vigna angularis*) rust, caused by the fungus *Uromyces vignae*, is an important disease affecting adzuki bean yield and quality. Previously, several NAC transcription factors (TFs) were induced by rust infection in a resistant adzuki bean variety, suggesting that NAC TF members may play important roles in rust resistance.

Results To further explore the functions of NAC TFs in rust resistance and to provide a reference for resistant varietal breeding, 101 NAC TFs were identified from the adzuki bean genome. The synteny analysis revealed 25 pairs of *VaNAC*s in the genome, which exhibited whole-genome/segmental duplication. Based on the phylogenetic relationships and conserved motif characteristics, the NAC TFs of *V. angularis* can be divided into 16 subfamilies. Previous transcriptome data showed that nine *VaNAC*s are significantly induced by rust infection. Here, a *cis*-acting element analysis of these nine genes revealed that most contain hormone responsive elements, such as abscisic acid and methyl jasmonate (MeJA). The expression levels of these nine *VaNAC*s were dynamically regulated in response to exogenous MeJA treatment, as revealed by quantitative real-time PCR analysis. Among them, seven *VaNAC*s exhibited significantly upregulated expression, peaking at 12 h post treatment (hpt) and remaining significantly higher than that of the untreated control group for 48 hpt. These results suggest that these *VaNAC*s are responsive to MeJA signaling and may play roles in the early and sustained transcriptional regulation of stress-related pathways. The exogenous MeJA decreased rust severity on adzuki bean leaves by 45.68%. Additionally, the expression levels of these nine genes in adzuki bean leaves in response to rust infection after pretreatment with MeJA were investigated. The expression of *VaNAC002* rapidly peaked at 24 h post inoculation (hpi) and remained significantly higher than the control from 120 to 192 hpi. Subsequently, transient overexpression of *VaNAC002* significantly enhanced the resistance of tobacco to *Botrytis cinerea*, indicating that *VaNAC002* positively regulates plant disease resistance.

Conclusion These findings suggest that adzuki bean NAC family members may play important roles in disease resistance through JA signaling, with *VaNAC002* having a positive regulatory role in plant immunity.

Keywords *Vigna angularis*, Adzuki bean rust, NAC, Disease-resistant, Jasmonic acid signaling pathway

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Background

Adzuki bean (*Vigna angularis*) is a vital legume crop in China, with an average annual planting area of 228,390 ha and an output of 347,900 tons in 2019, Heilongjiang Province dominates adzuki bean production, accounting for more than 44% of the national annual planting area and yield [1]. However, *Uromyces vignae* causes adzuki bean rust, which leads to premature leaf senescence and early abscission [2], substantially impacting adzuki bean yield and quality [3]. Mining resistance genes and providing candidate gene resources for rust-resistant varietal development is key for the sustainable management of adzuki bean rust.

Members of the NAM, ATAF1/2, and CUC2 (NAC) protein family, a vast group of plant-specific transcription factors (TFs), play pivotal regulatory roles in diverse physiological processes and responses to abiotic stresses. They possess a conserved sequence, the NAM domain [4], which is positioned in the N-terminal region, contains 151–159 amino acids, and has five subdomains (A to E) [5, 6]. Subdomains C and D are highly conserved among the NAC family members, whereas subdomains B and E exhibit lower conservation levels [6–8]. Among the NAM subdomains, D and E are pivotal for NAC member binding to DNA and are responsible for the functional diversity of NAC family members [9]. The transcriptionally active region (TAR) within the C-termini of NAC family proteins is a highly divergent domain [8]. The classification of NAC family members includes the use of features like branch size (minimum of three) and bootstrap values ≥ 800. Based on these criteria, the NAC families from rice and *Arabidopsis thaliana* have each been divided into 18 subfamilies [8]. Furthermore, based on the phylogenetic trees and conserved motifs, 96 NAC family members from cassava (*Manihot esculenta*) have been categorized into 16 subfamilies [10], whereas the 102 NAC members from *Capsicum annuum* have been divided into 15 subfamilies [11]. Functional studies of NAC family members show substantial differences in their activities during plant responses to biotic stresses. Notably, in pigeon pea (*Cajanus cajan*), the transient expression of the ATAF subfamily member *CcNAC55* is linked to marked plant resistance against infection by *Epicoccum sorghinum* [12]. Following the transient expression of *NAC29*, a member of the NAP subfamily, in wild tobacco (*Nicotiana attenuata*), resistance to *Alternaria alternata* is significantly enhanced through accelerated leaf senescence and regulation of defensive protein NaDLP1 expression [13]. However, excessive expression of *Gossypium hirsutum ATAF1*, a cotton ATAF subfamily member, noticeably enhances cotton's susceptibility to *Verticillium dahliae* [14]. Similarly, ATAF2 in *Arabidopsis* downregulates its disease resistance against *Fusarium oxysporum* infection by suppressing pathogenesis-related protein transcription [15]. Thus, there are intricate mechanisms behind plant NAC subfamily members' responses to phytopathogens, and the proper classification of NAC subfamilies based on sequence features and evolutionary history is critical for understanding their functions.

NAC TFs primarily regulate plant responses to pathogen infection through the jasmonic acid (JA) pathway [16]. Exogenous methyl jasmonate (MeJA) induces grapevine (*Vitis vinifera*) *NAC1* expression, and overexpressing *VvNAC1* in *Arabidopsis* leads to enhanced resistance against *Botrytis cinerea* compared with wild type [17]. In the *Arabidopsis ANAC019* and *ANAC055* double mutant, defense responsive gene *VSP1* and JA biosynthesis gene *LOX2* expression levels are notably diminished. Conversely, *ANAC019* and *ANAC055* overexpression increases *VSP1* and *LOX* expression, leading to considerable resistance against *B. cinerea*. This indicates that *ANAC055* and *ANAC019* positively contribute to *Arabidopsis* disease resistance through the JA signaling pathway [18]. These results indicated that NAC TF family members play positive roles in regulating plant immune responses. Previously, elevated expression levels of several NAC TFs were determined in a resistant adzuki bean variety during *Uromyces vignae* infection, as revealed by RNA sequencing [19], and MeJA markedly enhances rust resistance in adzuki bean [20]. However, the *VaNAC*s involved in rust resistance and their related signaling pathways remain unclear. To understand the mechanisms used by *VaNAC*s in rust resistance, *VaNAC*s were identified in the adzuki bean genome and their chromosomal locations, gene structures, and phylogenetic relationships, as well as the physicochemical properties of their encoded proteins, were studied. Subsequently, real-time quantitative PCR (qRT-PCR) was employed to assess the expression levels of *VaNAC*s in adzuki bean leaves after exposure to exogenous MeJA alone and with a *U. vignae* infection. This study increases the understanding of the molecular mechanisms used by *VaNAC*s in modulating adzuki bean rust resistance and provides a foundation for molecular-assisted rust-resistant varietal breeding.

Results

Identification, chromosomal localization, and collinearity analysis of *VaNAC*

Based on the sequence features of NAC TFs, 101 *VaNAC*s were identified in the adzuki bean genome. A chromosomal location analysis indicated that 89 *VaNAC*s reside on the 11 chromosomes (Fig. 1), with the highest number ($n = 15$) of members on Chromosome (Chr.) 1. Only three *VaNAC*s localized on Chr. 2, whereas two gene pairs resided on Chr. 1 and 4. An additional cluster of four members in tandem duplication was identified on Chr. 11. The remaining members were not localized to the chromosomes based on the current genome

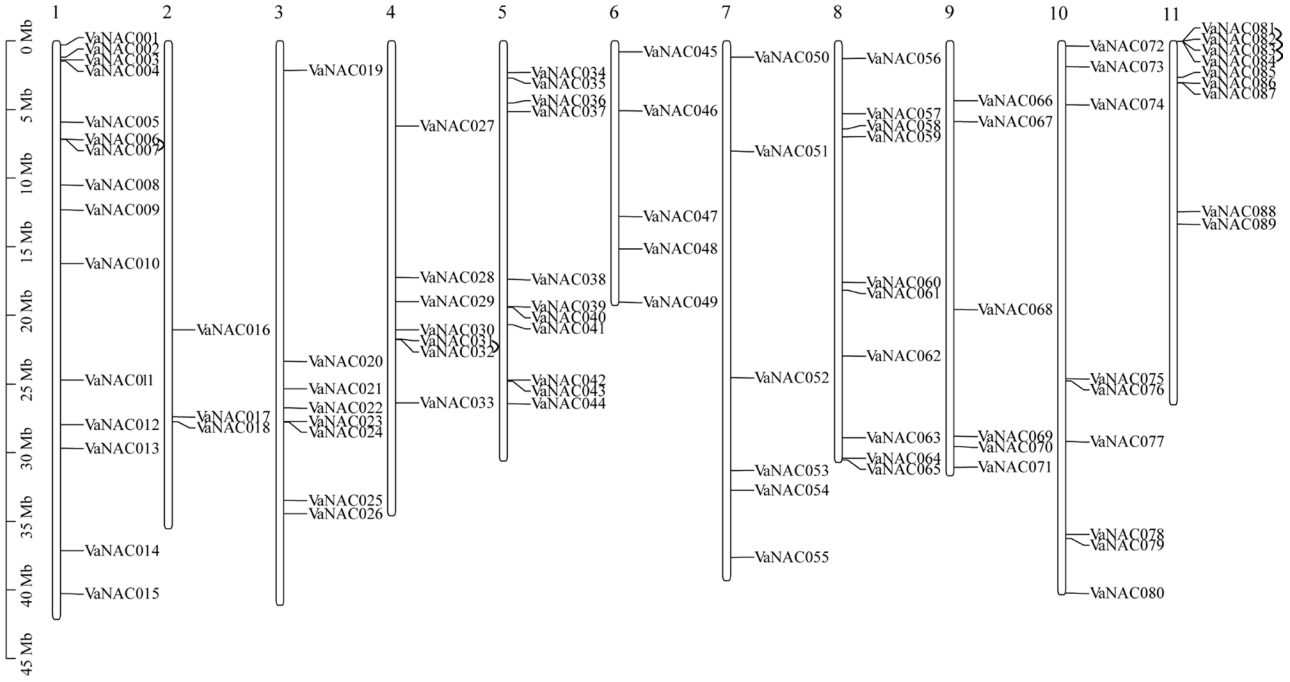

Fig. 1 Chromosomal locations of the *VaNAC*s. Numbers 1 to 11 represents the 11 chromosomes of *Vigna angularis*. Adjacent genes connected by lines indicate gene pairs or gene clusters

sequencing information and are not included in Fig. 1. In the ensuing studies, these 101 *VaNAC*s were assigned as *VaNAC001–101* based on their chromosomal locations.

An intra-species synteny analysis showed that, except for Chr. 2, the 25 pairs of *VaNAC*s on the other 10 chromosomes underwent whole-genome duplication (WGD) or segmental duplication events (Fig. 2A). The nonsynonymous (Ka)/ synonymous (Ks) values of these gene pairs ranged from 0.0811 (*VaNAC041* vs. *VaNAC069*) to 0.3657 (*VaNAC019* vs. *VaNAC078*) (Fig. 2B, Additional file 1: Table S1), indicating that the functions of these *VaNAC*s are crucial and that they may have undergone purifying selection during evolution, maintaining gene functional stability while avoiding functional redundancy or deleterious mutations. Additionally, three gene pairs (*VaNAC014* vs. *VaNAC019*, *VaNAC051* vs. *VaNAC074*, and *VaNAC061* vs. *VaNAC069*) had no Ks values (Additional file 1: Table S1), suggesting that no synonymous substitutions occurred between these gene pairs.

VaNAC gene structures

The gene structure analysis of *VaNAC*s revealed that, excluding *VaNAC099*, all the members contained 1 to 5 introns (Fig. 3). Most prevalent was the arrangement of two introns, observed in 68 genes (67.33%). Because the intron phase, which refers to the position of an intron within a gene relative to the exon, is crucial for maintaining the integrity and correctness of the coding sequence [21], the phase types of the introns in different *VaNAC*s were analyzed. The first intron phase of all the *VaNAC*s and the last intron phase of 88 *VaNAC*s was phase 0 (p0). Among the 68 *VaNAC*s with three intron phases, the intron phase order of 62 members was p0-p2-p0. This conserved arrangement suggests that these 62 *VaNAC* members have evolved from a common ancestor.

Subfamily classification and physicochemical properties of VaNACs

To determine the subclassification of the VaNACs, a phylogenetic tree containing 101 VaNACs and 30 NAC proteins from diverse plants was constructed. The analysis revealed that 97 out of 101 VaNACs segregated distinctly into 15 subfamilies (Fig. 4, shaded triangles). Four novel NAC subfamilies, denoted as VaNAC003, VaNAC009, VaNAC020, and VaNAC021, were identified in accordance with the existing NAC subfamily system [8]. The VaNAC021 subfamily consisted of VaNAC021 and VaNAC077, along with an uncharacterized subfamily member, ANAC084, from *Arabidopsis* (Fig. 4, blue branch). VaNAC003, VaNAC009, and VaNAC020 exclusively included VaNACs; however, the remaining four VaNACs (Fig. 4, red branch) were unassigned due to bootstrap values of less than 800. Consequently, conserved motifs in the TAR region were analyzed to investigate three VaNACs (VaNAC028, VaNAC042, and VaNAC052) that clustered with a TIP subfamily protein, AF281062. A conserved motif within these four proteins was identified (Fig. 4, bar chart), and these three VaNACs were assigned to the TIP subfamily. Overall, the 101 VaNACs were classified into 16 subfamilies,

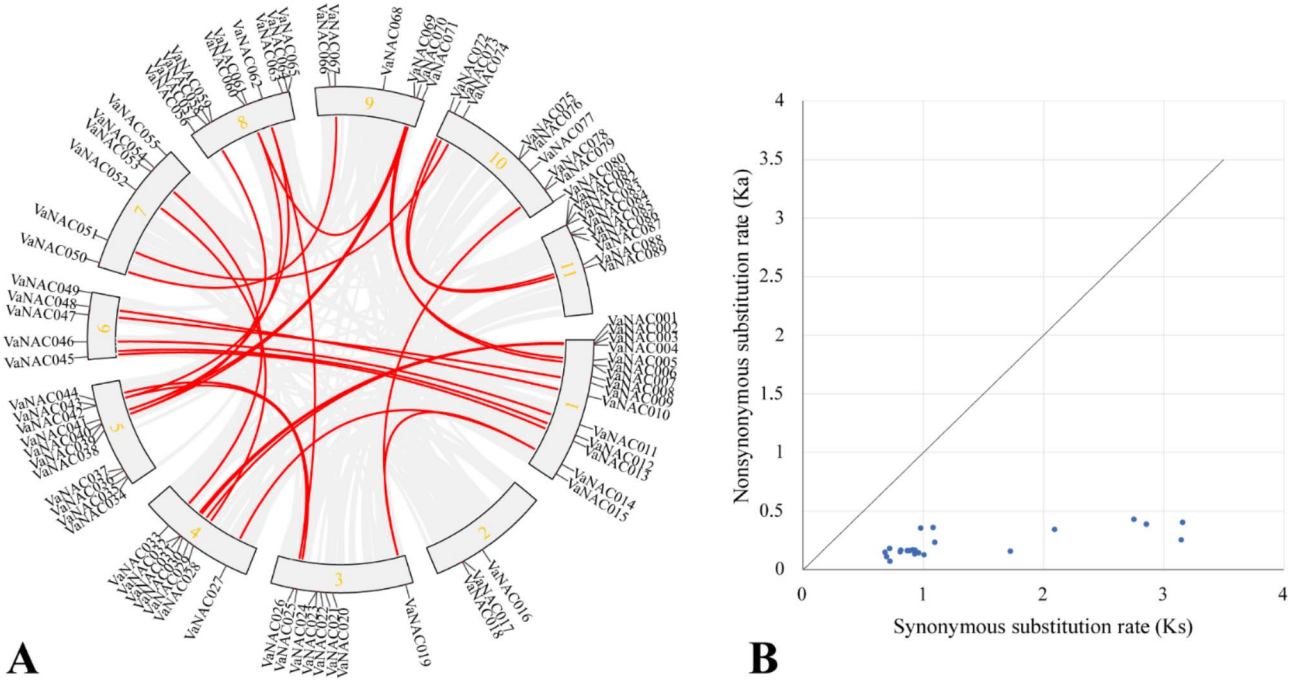

Fig. 2 Collinearity analysis and synonymous/nonsynonymous substitution frequency diagram of *VaNAC*s. (**A**) Numbers 1 to 11 represents the 11 chromosomes of *Vigna angularis*. Connecting red lines indicate homologous *VaNAC*s derived from WGD or segmental duplication events. (**B**) The blue scatter points represent the Ka/Ks values of *VaNAC*s with collinearity relationships within the adzuki bean species. The black diagonal line indicates a Ka/Ks ratio of 1

except VaNAC055, which could not be assigned to any subfamily.

The physicochemical examination of the VaNACs found notable variations among members within the same subfamily (Table 1). The 101 VaNACs possessed amino acid numbers, molecular weights, and theoretical isoelectric points spanning 66 to 694, 7.99 to 77.73 kDa, and 4.53 to 9.94, respectively. The overall average hydropathicity (GRAVY) of all the VaNACs was negative, indicating their hydrophilic nature.

Screening for *VaNAC*s related to rust resistance and their *cis*-acting element analysis

To identify the *VaNAC*s responsive to *U. vignae* infection, expression profiles of 101 *VaNAC*s were analyzed based on transcriptome data (SRP130843) obtained from a rust-resistant adzuki bean variety at 24 and 48 h post inoculation (hpi) with *U. vignae*. In total, 6 *VaNAC*s had no detectable expression, and the remaining 95 *VaNAC*s exhibited varying degrees of expression (Fig. 5). Among them, nine *VaNAC*s belonged to each of the SENU5, VaNAC021, AtNAC3, ATAF, and NAP subfamilies (Fig. 5, red font) and were induced by rust fungal infection, suggesting that they are involved in rust resistance. The *cis*-acting elements in these genes were analyzed (Table 2). Among these nine *VaNAC*s, 15 categories and 205 *cis*-acting elements related to growth, development, and abiotic stress responses were predicted. Among these *cis*-acting elements, there were 146 hormone-responsive elements (71.22% of the total), including 68 abscisic acid, 58 MeJA, and 7 indole-3-acetic acid (representing 46.58%, 39.73%, and 4.79% of the hormone-responsive elements, respectively).

Expression of rust resistance-related *VaNAC*s during MeJA-induced rust resistance

Based on transcriptomic data of rust-resistant adzuki bean inoculated with *U. vignae*, we identified nine *VaNAC*s associated with rust resistance (Fig. 5, red font). To further investigate their regulatory roles in the JA signaling pathway, further analyses were conducted. Their expression levels were examined by qRT-PCR (primers are listed in Additional file 2: Table S2) in adzuki bean leaves treated with 0.45 mg/mL MeJA. The expression levels of all the genes were affected by the exogenous MeJA (Fig. 6), with most, except for *VaNAC021* and *VaNAC091*, significantly peaking at 12 h post treatment (hpt). In addition, three of the nine genes, including *VaNAC002*, *VaNAC040*, and *VaNAC021*, were significantly higher than those of the control at 12, 24, and 48 hpt (Fig. 6).

Meanwhile, our investigation revealed that exogenous MeJA significantly enhanced rust resistance (Fig. 7A and B), as evidenced by a 45.68% reduction in the number of uredia on MeJA-pretreated leaves compared with control leaves (Fig. 7C). Subsequently, gene expression

Fig. 3 Schematic diagram of *VaNAC* gene structures. UTR: Untranslated region; CDS: Coding DNA sequence; The numbers 0, 1, and 2 indicate the intron phase types

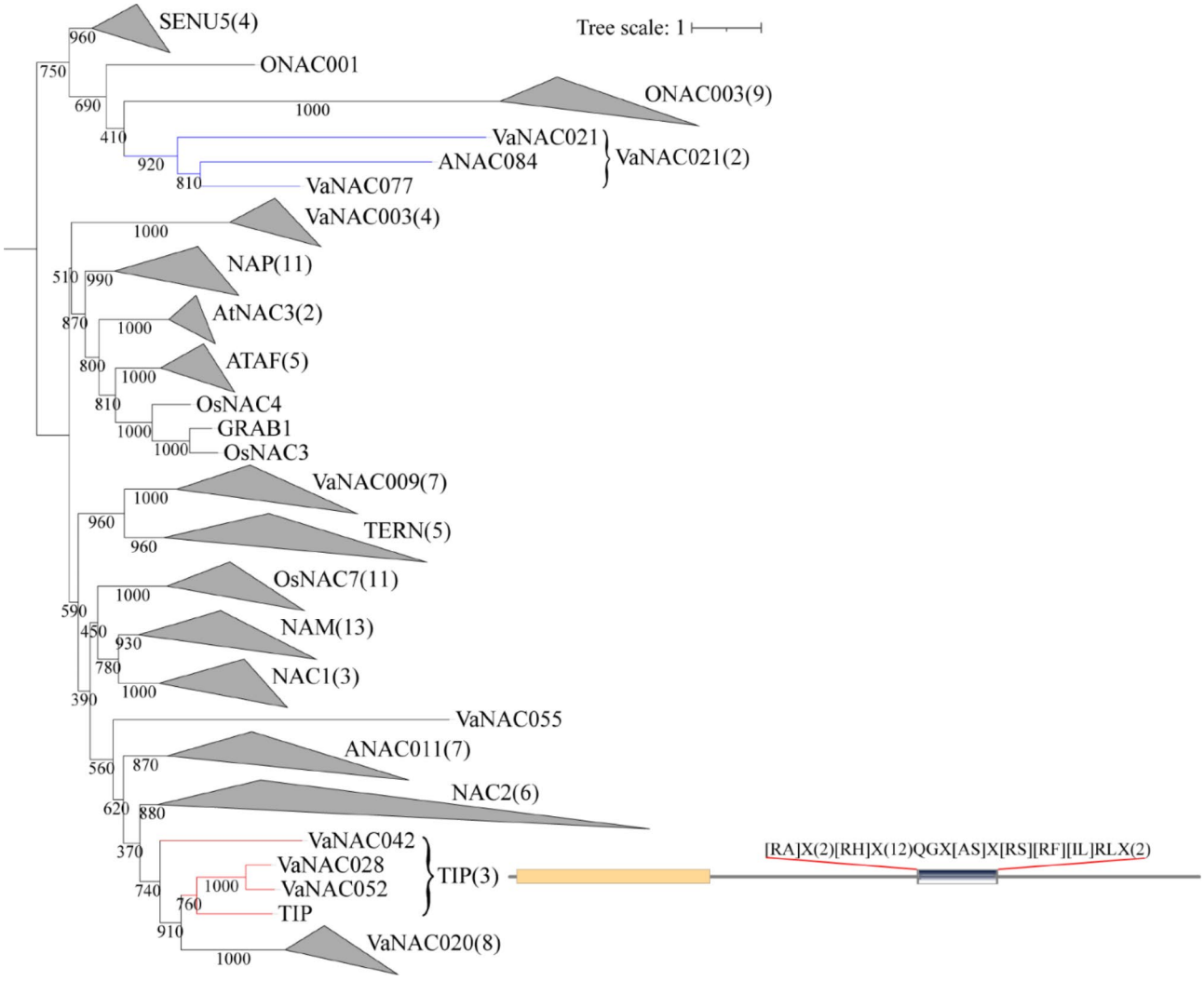

Fig. 4 Phylogenetic analysis of multi-species NAC family members. The gray triangles denote subfamily members distinctly categorized by bootstrap value (≥ 800). Red branches indicate subfamily categorization based on conserved motifs. Blue branches indicate NAC family members from *Arabidopsis* that have not yet been classified into a specific subfamily. The number in parentheses following the subfamily identifier represents the number of VaNACs within the respective subfamily. At the right, the specific conserved motifs in the TAR region of TIP subfamily members from 131 multi-species NACs, as elucidated using the MEME tool, are depicted

analysis revealed that *VaNAC040* significantly increased at 24, 48, and 120 hpi during the MeJA-induced rust resistance, but significantly decreased at 192 hpi (Fig. 8). In contrast, *VaNAC002* remained significantly higher than the control at 24, 120, and 192 hpi (Fig. 8). Further functional validation of the *VaNAC002* revealed that transient overexpression of *VaNAC002* can significantly enhance tobacco resistance to *B. cinerea* (Fig. 9A). The diameters of lesions on the leaves of plants overexpressing *VaNAC002* were 14.3% smaller than those of the control (Fig. 9B). These results indicate that *VaNAC002* positively regulates plant disease resistance.

Discussion

Plant-specific NAC TFs participate in the regulation of plant growth and stress adaptation. This study investigated 101 *VaNAC*s containing the NAM domain in adzuki bean at the whole-genome scale. Members of the NAC TF family are ubiquitous across plant genomes, with varying numbers per species. For example, 105 *NAC* genes have been identified in *A. thaliana* [8], 75 in *Oryza sativa* [8], 104 in *Solanum lycopersicum* [22], 152 in *Glycine max* [23], 121 in *Juglans regia* [24], and 91 in *Dioscorea esculenta* [25]. The large numbers of family members suggest that the NAC family in plants has undergone gene duplication events. In the current study, five tandem duplication events that included eight *VaNAC*s were found (Fig. 1), and 25 gene pairs

Table 1 The characteristics of VaNAC family members

Subfamily	No. of genes	Amino acid (aa)	Molecular weight (kDa)	pI	GRAVY
SENU5	4	94–336	11.49–39.05	8.7–9.89	-0.85–0.433
VaNAC021	2	203–213	23.40-24.15	8.58–9.47	-0.607–0.395
ONAC003	9	279–450	31.92–50.93	5.15–8.75	-0.9–0.714
VaNAC003	4	189–204	21.96–23.02	4.88–5.26	-0.798–0.511
NAP	11	233–373	26.69–41.25	6.03–9.13	-0.773–0.63
AtNAC3	2	336–341	38.07–38.28	6.55–8.11	-0.578–0.551
ATAF	5	271–295	31.01–34.06	5.87–8.74	-0.763–0.623
VaNAC009	7	148–413	17.11–46.81	6.46–9.94	-0.864–0.591
TERN	5	241–409	27.59–46.34	6.02–8.22	-0.918–0.59
ANAC011	7	66–666	7.99–75.98	4.84–9.41	-1.217–0.475
NAC2	6	168–612	19.21–66.78	4.53–7.16	-0.731–0.333
TIP	3	365–595	40.72–67.08	5.04–6.44	-0.69–0.536
VaNAC020	8	155–694	17.98–77.73	4.65–7.73	-0.745–0.35
OsNAC7	11	125–422	37.23–47.04	5.07–9.44	-0.97–0.69
NAC1	3	287–300	33.13–33.90	5.89–8.32	-0.657–0.562
NAM	13	307–401	34.46–45.51	5.6–9.08	-0.788–0.53
VaNAC055 (UC)	1	276	31.19	6.16	-0.469

pI: Theoretical isoelectric point; GRAVY: Grand average of hydropathicity; UC: Unclassified subfamily

experienced WGD or segmental duplication events (Fig. 2). This phenomenon is also observed in other species. For instance, among the 121 *NAC*s in walnut, 13 pairs are segmentally duplicated genes [24]. Similarly, in Chinese cabbage (*Brassica rapa*), 155 pairs of paralogous genes have been found among the 188 *BrNAC*s [26]. This indicates that extensive gene duplication events are an important reason for the abundance of NAC family members in various plants.

Owing to the large numbers of members, this family is often divided into different subfamilies, mainly based on the phylogenetic relationships. In *Arabidopsis* and rice, the NAC family members are classified into 18 subfamilies based on the bootstrap values (≥ 800) and the numbers of members (at least three) in each clade [8]. Applying these criteria, 97 VaNACs were partitioned into 15 subfamilies (Fig. 4), but four VaNACs remained unclassifiable. In *Arabidopsis*, the unique plant-specific WRKY TF family proteins possess two or more motifs within their TAR regions, with motif combinations being conserved across each subgroup [27]. This indicates that although certain VaNACs cannot be classified using evolutionary relationships, classification may be enhanced by examining the conserved motifs of various members within a given clade. Therefore, the conserved motifs among the members clustered in one clade with bootstrap value < 800 (Fig. 4, red branch) were investigated, and three VaNACs were assigned to the TIP subfamily. In addition, according to their evolutionary relationships, three subfamilies (denoted as VaNAC003, VaNAC009, and VaNAC020) are composed exclusively of VaNAC members that are distinct from those in other plants. This suggests that these adzuki bean NAC members may have developed along different evolutionary trajectories due to the divergence from other species, leading to gene-specific differentiation. These findings indicate that the conserved motifs in the TAR region, along with bootstrap values from phylogenetic tree branches, form the basis for the subfamily classification of NAC TFs.

Using the transcriptomic data SRP130843 [19], the expression levels of *VaNAC*s in response to *U. vignae* infection were examined, and nine *VaNAC*s from each of the SENU5, AtNAC3, ATAF, VaNAC021, and NAP subfamilies were induced during infection (Fig. 5). A further analysis revealed that the promoter regions of these nine genes contained large numbers of hormone-responsive elements, such as abscisic acid- and JA-responsive elements. In other plants, NAC family members are closely involved with the JA signaling pathway. The promoter region of wheat *NAC069* contains a MeJA-responsive element. The expression of *NAC069* in wheat is significantly upregulated after 24 h of MeJA treatment, and wheat lines with *NAC069* knocked out exhibit reduced resistance to *Puccinia triticina* infection [28]. Similarly, the promoter region of grapevine *VvNAC1* contains MeJA-responsive elements. The expression of *VvNAC1* is significantly upregulated in grapevine following MeJA treatment, and *VvNAC1* transgenic *Arabidopsis* lines show enhanced resistance to *B. cinerea* [17]. These studies indicate that exogenous MeJA regulates the expression of NAC members through JA-responsive elements, thereby functioning when plants are under biotic stress. The expression levels of these nine rust-resistant VaNAC members were all affected by exogenous MeJA (Fig. 6). Further gene expression analyses indicated that *VaNAC002* was significantly upregulated at key stages of *U. vignae* infection following MeJA treatment (Fig. 8). In addition, the transient overexpression of *VaNAC002* in tobacco confirmed that *VaNAC002* indeed plays a positive regulatory role in plant immunity (Fig. 9). These findings suggest that adzuki bean NAC family members play important roles in disease resistance through JA signaling, especially *VaNAC002*. However, the molecular mechanisms by which *VaNAC002* regulates plant immunity still require in-depth investigation.

Conclusions

This study identified 101 NAC TF family proteins in the adzuki bean genome and assigned them to 16 subfamilies. Gene expression profiling revealed that nine *VaNAC*s were closely related to the rust resistance of adzuki bean. A subsequent expression analysis in infected leaves pretreated with MeJA and transient overexpression experiment concluded that *VaNAC002* may

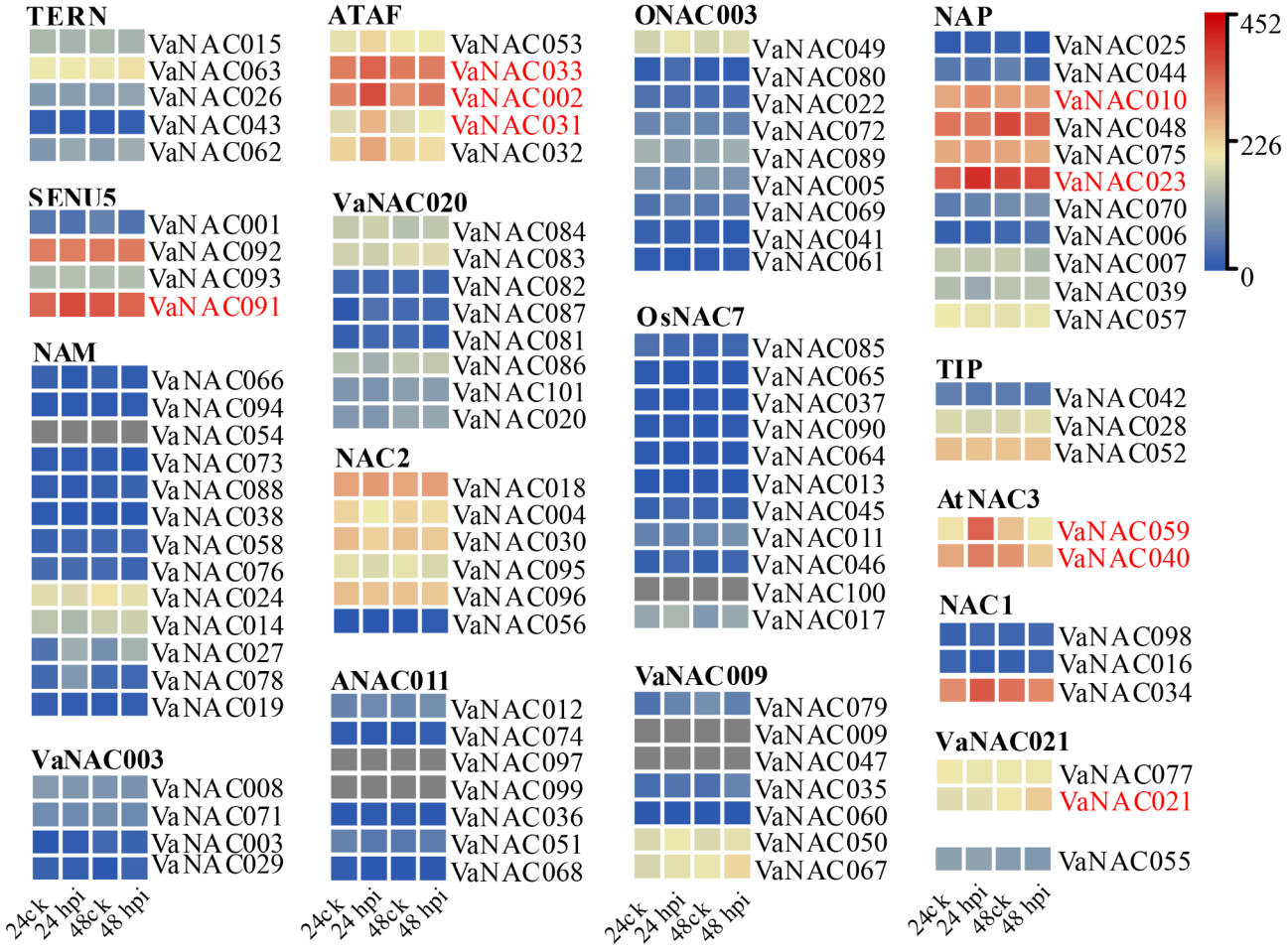

Fig. 5 Expression analysis of *VaNAC* subfamily members with rust infections. The transcriptome data-based Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values of *VaNAC*s from the rust-resistant variety 'QH1' 24 and 48 h after inoculation with rust fungus; 24 CK: control treatment after 24 h; 24 hpi: Rust infection for 24 h; 48 CK: Control treatment after 48 h; 48 hpi: Rust infection for 48 h. The red highlighted members were candidates for subsequent gene expression measurements. An FPKM value-based color scale appears in the upper right side

positively regulate plant disease resistance through the JA signaling pathway.

Materials and methods

Identification, chromosomal localization, and collinearity analysis of VaNACs

Genomic data for the adzuki bean 'Jingnong 6' were obtained from the NCBI database [29]. The hidden Markov model of the NAM domain (PF02365) [30] was utilized to identify the *NAC* family genes with HMMER3.0 software [31]. Candidate protein sequences were submitted to the Pfam and NCBI CDD databases for further refinement and to identify the *NAC* family members in adzuki bean [32]. Using TBtools, the chromosomal locations of each gene were retrieved and mapped onto the different adzuki bean chromosomes in ascending order according to their physical position (Mbp) [33]. In addition, the gene duplication relationships among the *VaNAC*s were analyzed using the MCscanX comparative genomics program in TBtools, which determined the collinearity relationships within species. Additionally, Ka and Ks substitution rates between *VaNAC*s were characterized through a synonymous analysis [33].

***VaNAC*gene structural characteristics analysis.**

Using the adzuki bean genome annotation file (gff3) as a template, the exon, CDS, 3' UTR, and 5' UTR positional information for the *VaNAC*s on chromosomes were extracted using the gene structure visualization function of TBtools software [33].

Phylogenetic and physicochemical property analyses of VaNACs

From the literature, NAC family proteins of clearly defined subfamily were collected [8], and their protein sequences were downloaded from the NCBI database for phylogenetic analysis of NAC TF family members across multiple species. The gene names and their GenBank

Table 2 Analysis of *cis*-acting elements in *VaNAC*s promoters

Annotation of cis-acting elements	Name	Gene Name								
		VaNAC002	VaNAC010	VaNAC021	VaNAC023	VaNAC031	VaNAC033	VaNAC040	VaNAC059	VaNAC091
Auxin responsive	TGA-element	-	1	-	-	1	1	1	-	-
	AuxRR-core	-	1	2	-	-	-	-	-	-
MeJA responsive	CGTCA-motif	4	3	1	4	1	3	5	7	1
	TGACG-motif	4	3	1	4	1	3	5	7	1
Gibberellin responsive	GARE-motif	-	-	-	1	-	-	-	-	-
	P-box	-	1	1	3	-	-	1	-	-
	TATC-box	-	1	-	-	-	-	-	-	1
Salicylic acid responsive	TCA-element	2	-	-	-	2	-	-	-	-
Abscisic acid response reaction	ABRE	9	1	4	5	6	11	20	10	2
Elements involved in defense and stress responses	TC-rich repeats	1	1	-	-	-	1	2	2	2
Components involved in low-temperature response	LTR	1	-	-	1	2	-	-	-	-
Components necessary for anaerobic induction	ARE	3	3	-	-	2	1	3	1	9
Components involved in circadian rhythm control	Circadian	-	-	1	-	-	-	1	-	-
Elements involved in seed-specific regulation	RY-element	-	1	-	-	-	-	-	-	-
Corn-soluble protein metabolism regulatory element	O2-site	3	-	3	-	1	1	-	1	-
Elements related to meristematic tissue expression	CAT-box	-	-	-	-	-	-	1	-	1
MYB-binding sites participating in drought induction	MBS	1	-	-	-	1	2	-	-	-

Table 2 (continued)

Annotation of cis-acting elements	Name	Gene Name								
		VaNAC002	VaNAC010	VaNAC021	VaNAC023	VaNAC031	VaNAC033	VaNAC040	VaNAC059	VaNAC091
MYB-binding sites participating in photoreaction	MRE	-	-	1	1	1	1	-	-	-
MYBHv1-binding site	CCAAT-box	-	-	-	1	-	-	-	-	1

The numbers indicate the *cis*-acting elements in each gene

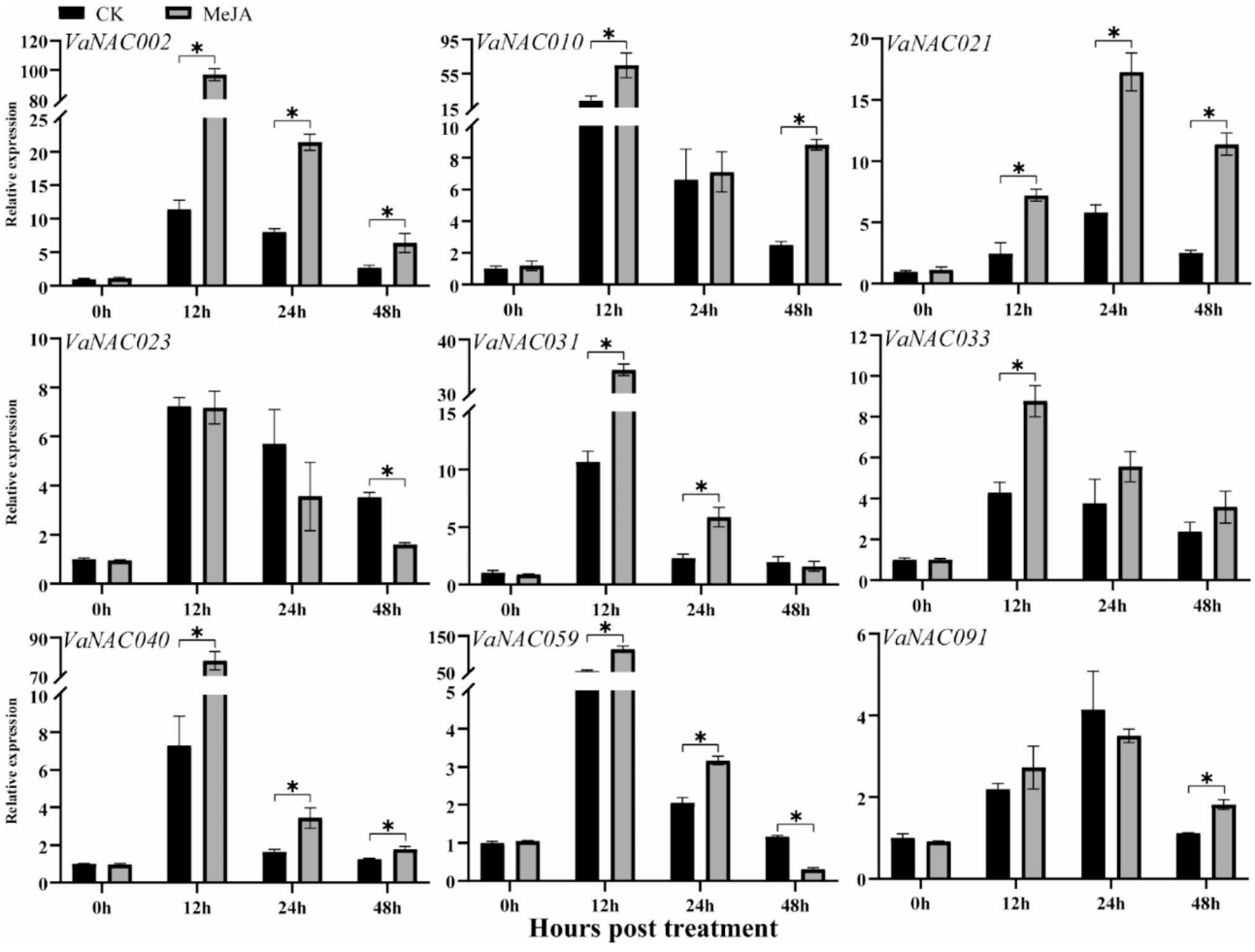

Fig. 6 *VaNAC* expression levels during MeJA-induced rust resistance in adzuki beans. CK and MeJA indicate infected leaves of adzuki bean pretreated with sterile water and MeJA, respectively. * Indicates a significant difference between the two treatments at the same time point ($P < 0.05$)

accession numbers are listed in Additional file 3: Table S3.

The NAC family protein sequences from adzuki bean and other plants underwent multiple sequence alignments using the MAFFT tool [34]. A phylogenetic tree was constructed utilizing the Maximum Likelihood method in IQ-TREE [35], with a bootstrap value of 1,000 for validation. Using the description of Ooka et al. [8], branches containing three or more members and bootstrap value ≥ 800 were used as the primary criteria to classify VaNAC members into subfamilies. For members that could not be classified using these criteria, the conserved motif in the TAR region, as described by Eulgem et al. [27], was utilized. The conserved motifs were analyzed using the MEME online tool (https://meme-suite.org/meme/doc/meme.html) [36]. To further elucidate

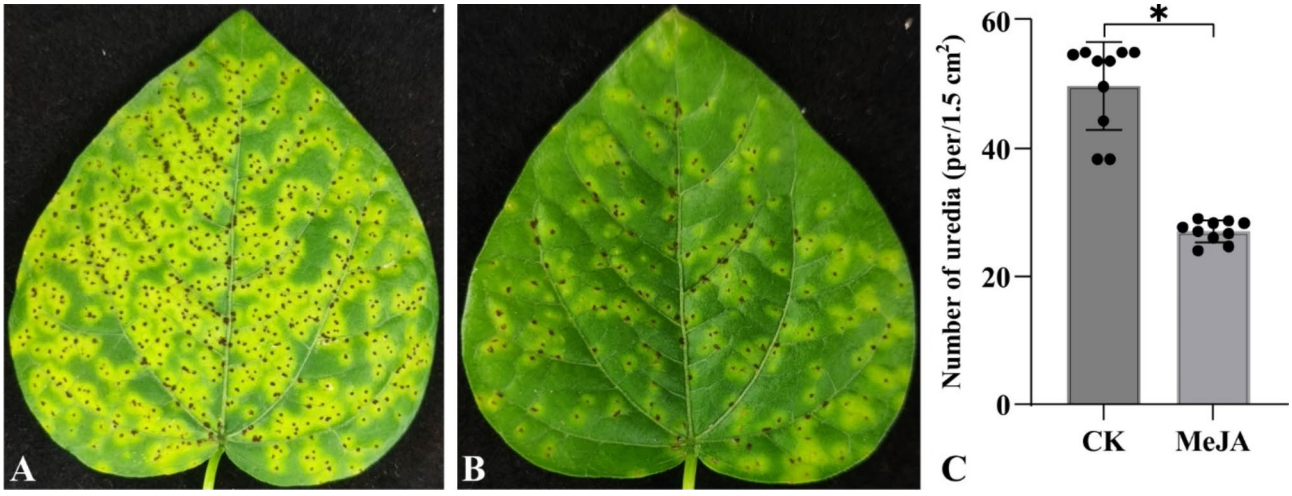

Fig. 7 Adzuki bean leaf phenotypes and *U. vignae* spore masses after infection with or without MeJA pre-treatment. A, B: Adzuki bean leaves at 10 d post inoculation (dpi) with *U. vignae*. (**A**) Control. (**B**) Pre-incubated for 2 with 0.45 mg/mL MeJA prior to infection. (**C**) Number of urediniospore masses on adzuki bean leaves at 10 dpi with *U. vignae* with or without (CK) the MeJA pre-treatment. Values represent means ± SEs of 10 biological replicates. Differences were assessed using *t*-tests. *, $P < 0.05$

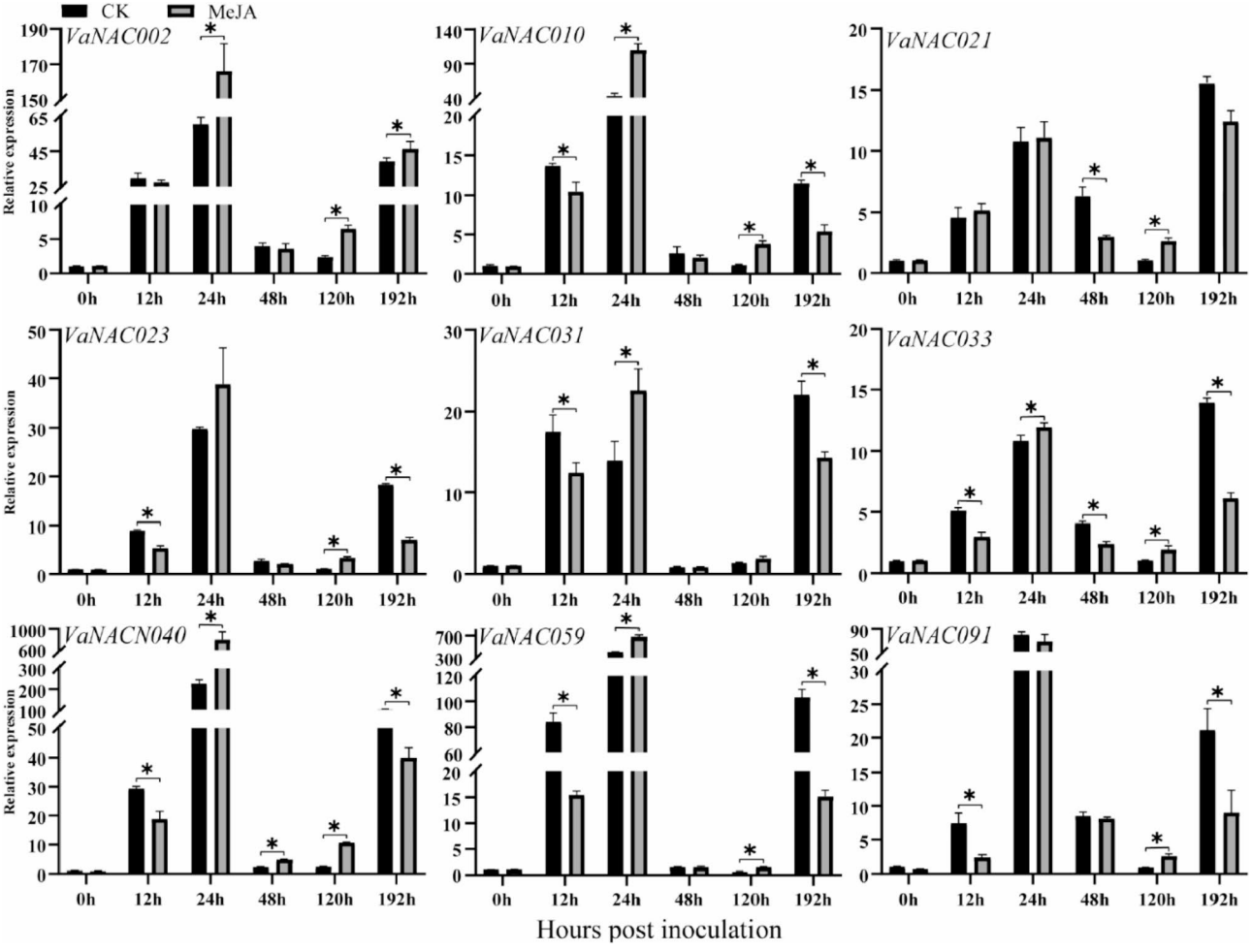

Fig. 8 MeJA-induced *VaNAC*s expression after *U. vignae* inoculation. CK and MeJA indicate the leaves of adzuki bean inoculated with rust fungi after pre-treatment with sterile water and MeJA aqueous solution, respectively. * Indicates a significant difference in relative expression between the two treatments ($P < 0.05$)

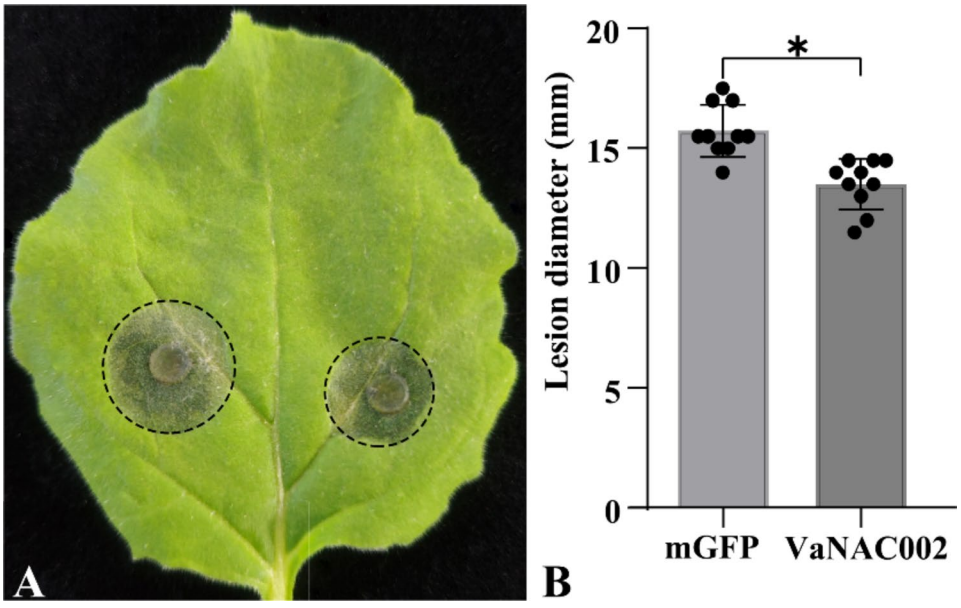

Fig. 9 Effects of *VaNAC002* transient expression in tobacco against *B. cinerea* infection. (**A**) Symptoms of tobacco leaves at 36 h after inoculation with *B. cinerea*. 'mGFP' indicates the transient expression of mGFP (pBin-mGFP), and 'VaNAC002' indicates the transient expression of *VaNAC002* (pBin-VaNAC002-mGFP). (**B**) Lesion diameters on the infected tobacco leaves. Values represent means ± SEs of 10 biological replicates. Differences were assessed using *t*-tests. *, $P < 0.05$

their functions, the molecular weights, isoelectric points, and hydrophilicities of VaNAC proteins were analyzed using the ExPasy ProtParam online tool (https://web.expasy.org/protparam/) [37].

Gene expression and*cis*-acting element analyses of*VaNACs*.

Using the transcriptomic data (SRP130843) of a rust-resistant adzuki bean cultivar acquired at 24 and 48 hpi [19], the relative expression levels of all the *VaNAC* members during rust infection were analyzed based on Fragments Per Kilobase of transcript per Million mapped read values to preliminarily understand the roles of *VaNAC*s in response to rust infection. On this basis, *VaNAC* members with upregulated expression in the resistant variety after rust infection were selected. The promoter regions 2,000 bp upstream of the transcription start sites of the candidate genes were analyzed using the online tool PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Identifying the *cis*-acting elements contained in the candidate genes provides a necessary reference for understanding their expression-related regulatory mechanisms [38].

Plant cultivation, MeJA treatment, and urediniospore inoculation

Using the rust-susceptible adzuki bean variety 'Baoqinghong' as the experimental material, a qRT-PCR analysis was conducted. The plants were grown in a glass greenhouse under 16-h light (25 ℃ ± 2 ℃) and 8-h dark (20 ℃ ± 2 ℃) conditions. A 0.45 mg/mL solution of MeJA was prepared using sterile water, and the MeJA solution was sprayed onto the true leaves of 8-day-old seedlings using a manual sprayer, with sterile water sprayed as a control. The plants were then placed in a humid chamber at 20 ℃ ± 2 ℃ in the dark for 12 h, after which they were transferred back to the greenhouse for continued cultivation at 25 ℃ ± 1 ℃ during the day and 20 ℃ during the night (16- h light/8-h dark photocycle) [20]. True leaf samples were collected at 0, 12, 24 and 48 h after the MeJA treatment.

The urediniospores of *U. vignae* isolate ZXL01 were prepared as a suspension at a 1×10^5 urediniospore/mL concentration in sterile water. At 48 h after MeJA treatment, the true leaves of the adzuki beans were inoculated with *U. vignae* urediniospores. Following inoculation, the plants were kept in a dark, humid chamber at 20 ℃ ± 2 ℃ for 24 h before being transferred back to the glasshouse for continued cultivation under normal conditions [2]. True leaf samples were collected at 0, 12, 24, 48, 120, and 192 h after inoculation with the urediniospore suspension.

To eliminate individual differences, leaf samples used in this study were collected from four plants that underwent the same treatment. These samples were pooled and thoroughly ground in liquid nitrogen.

At 10 dpi with *U. vignae*, the number of urediniospore masses on a 1.5-cm^2 leaf area was used to determine disease severity. The numbers of urediniospore masses in the MeJA pretreated and control groups were then recorded and compared [39].

Nicotiana benthamiana plants were grown in a climate chamber (16 h photoperiod at 22 °C). They were used for the transient expression of rust resistance related *VaNAC*s to validate their roles in plant immunity.

Total RNA isolation and quantitative RT-PCR

Total RNAs of the leaf samples were isolated using TRIzol methodology [40]. After quantification and quality assurance using a NanoDrop 1000 and agarose gel electrophoresis, cDNA synthesis was executed utilizing the HiScript III 1st Strand cDNA Synthesis Kit (+ gDNA wiper). The adzuki bean actin gene (*VaACT*) served as a control, and qRT-PCR was used to analyze the expression levels of target genes during MeJA treatment and the MeJA-elicited rust resistance process in adzuki beans. The qRT-PCR reaction system had a total volume of 10 μL, including 5.0 μL of 2× SYBR Premix ExTaq, 0.5 μL of Primer-F, 0.5 μL of Primer-R, 3.0 μL of ddH_2O, and 1 μL of cDNA. The PCR cycling conditions were as follows: 95 ℃ for 3 min; 40 cycles of 95 ℃ for 30 s and 60 ℃ for 30 s. Treatments were replicated three times, and relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method [41]. Statistical evaluations were performed using GraphPad Prism 9 software [42].

Transient gene expression in tobacco and *B. cinerea* inoculation

The *VaNAC002* gene was cloned from cDNA of *V. angularis* using primers VaNAC002-orf-F and VaNAC002-orf-R (primers are listed in Additional file 2: Table S2). The PCR product was inserted into *Kpn*I- and *BamH*I-digested pBin-mGFP, creating a fusion vector with mGFP (pBin-VaNAC002-mGFP). The vector was transformed into *Agrobacterium tumefaciens* GV3101. The constructed pBin-VaNAC002-mGFP and pBin-mGFP vectors were independently transiently expressed in *N. benthamiana* leaves after infiltration of the suspended *Agrobacterium* (OD600 = 0.6) using a needleless syringe [43]. Each treatment was repeated 10 times. The treated seedlings were incubated for 24 h in a greenhouse and then inoculated with *B. cinerea*. For fungal inoculation, the inoculum of *B. cinerea* was maintained on potato dextrose agar at 25 ℃ in the dark for 3 days. Leaves of each treatment were detached and inoculated with fresh mycelial plugs (diameter 0.5 cm) at the injection points, and then, they were placed into a transparent box to maintain humidity in the dark. At 36 hpi, the lesion diameters were measured for each treatment.

Abbreviations

TFs	Transcription factors
VaNACs	NAC TFs of V. angularis
MeJA	Methyl jasmonate
hpt	Hours post treatment
hpi	Hours post inoculation
dpi	Days post inoculation
NAC	NAM, ATAF1/2, and CUC2
TAR	Transcription active region
qRT-PCR	Quantitative real-time PCR
Chr	Chromosome
WGD	Whole-genome duplication
p0	Phase 0
p1	Phase 1
p2	phase 2
pI	Theoretical isoelectric point
gff3	Genome annotation file
GRAVY	Grand average of hydropathicity
FPKM	Fragments Per Kilobase of transcript per Million mapped reads

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11478-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Not applicable.

Author contributions

LSM, KXW, and YLH planned and designed the research. LSM, DX, and LY performed the experiments and analyzed the data. LSM and DX wrote the manuscript. KXW and ZYH reviewed and edited the manuscript. All the authors read and approved the final manuscript.

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Data availability

The RNA-seq data utilized in this article are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive Database at [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA431103] and can be accessed with [BioProject PRJNA431103].

Declarations

Ethics approval and consent to participate

The use of plant materials in the paper fully complied with our institutional guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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