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Identification, characterization, and expression analysis of *WRKY* transcription factors in *Cardamine violifolia* reveal the key genes involved in regulating selenium accumulation

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Abstract

Background *Cardamine violifolia* is a significant Brassicaceae plant known for its high selenium (Se) accumulation capacity, serving as an essential source of Se for both humans and animals. WRKY transcription factors play crucial roles in plant responses to various biotic and abiotic stresses, including cadmium stress, iron deficiency, and Se tolerance. However, the molecular mechanism of *CvWRKY* in Se accumulation is not completely clear.

Results In this study, 120 WRKYs with conserved domains were identified from *C. violifolia* and classified into three groups based on phylogenetic relationships, with Group II further subdivided into five subgroups. Gene structure analysis revealed WRKY variations and mutations within the *CvWRKYs*. Segmental duplication events were identified as the primary driving force behind the expansion of the *CvWRKY* family, with numerous stress-responsive cis-acting elements found in the promoters of *CvWRKYs*. Transcriptome analysis of plants treated with exogenous Se and determination of Se levels revealed a strong positive correlation between the expression levels of *CvWRKY034* and the Se content. Moreover, *CvWRKY021* and *CvWRKY099* exhibited high homology with *AtWRKY47*, a gene involved in regulating Se accumulation in *Arabidopsis thaliana*. The WRKY domains of CvWRKY021 and AtWRKY47 were highly conserved, and transcriptome data analysis revealed that *CvWRKY021* responded to Na_2SeO_4 induction, showing a positive correlation with the concentration of Na_2SeO_4 treatment. Under the induction of Na_2SeO_3, *CvWRKY021* and *CvWRKY034* were significantly upregulated in the roots but downregulated in the shoots, and the Se content in the roots increased significantly and was mainly concentrated in the roots. *CvWRKY021* and *CvWRKY034* may be involved in the accumulation of Se in roots.

Conclusions The results of this study elucidate the evolution of *CvWRKYs* in the *C. violifolia* genome and provide valuable resources for further understanding the functional characteristics of *WRKYs* related to Se hyperaccumulation in *C. violifolia*.

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Keywords *Cardamine violifolia*, WRKY transcription factors, Selenium, Genome-wide analysis, Gene expression

Background

Selenium (Se) is a vital trace element for humans and animals, and Se deficiency can cause various diseases in both populations. Approximately two-thirds of the soil area in China is deficient in Se, which greatly impacts the Se levels in food. Therefore, increasing the Se content in agricultural products has become essential for supplementing Se nutrition in humans [1]. In this context, *Cardamine violifolia*, which grows in Se-rich areas in Enshi, Hubei Province, China, deserves special mention. *C. violifolia* is a member of the Cruciferae family and is a plant with tender and delicious stems and leaves that is rich in nutrients such as protein, soluble sugars, amino acids, and fiber [2, 3]. Research has revealed that the average Se contents in the roots, shoots, and leaves of *C. violifolia* are 2,985, 3,329, and 2,491 mg/kg Se DW, respectively, making it a recognized Se hyperaccumulator. Due to its ability to accumulate high levels of Se, *C. violifolia* has gained significant attention as a Se-enriched plant and is highly favored by individuals [4].

WRKY transcription factors (TFs) constitute one of the largest families of transcriptional regulatory factors in plants. They play a crucial role in regulating various aspects of plant growth, development, and response to environmental stresses. Specifically, WRKY TFs bind to a specific nucleotide sequence (W-box) called the cis-acting element TTGAC (C/T) in the promoter region of target genes [5], thus regulating the process of plant life activities [6]. Most members of this gene family contain highly conserved WRKY domains, whereas a few have variations, such as WRRY, WSKY, WKRY, WVKY, or WKKY [7]. The WRKY domain is composed of approximately 60 amino acid residues and consists of a seven-peptide at the N-terminus and a zinc finger structure at the C-terminus. The zinc finger motif sequence is typically Cx4-5Cx22-23HxH (C2H2) or Cx7Cx23HxC (C2HC) [8]. Based on the number of domains and zinc finger motif sequences, WRKY TFs are categorized into three major groups: Group I, with two conserved WRKY domains, and Groups II and III, with one conserved WRKY domain [9]. Groups I and II have C2H2-type zinc finger motifs, whereas Group III has a C2HC-type zinc finger motif. Group II is further divided into five subgroups (IIa, IIb, IIc, IId, and IIe) based on differences in amino acid sequences [10].

The mechanism of Se accumulation in the Se hyperaccumulator plant *C. violifolia* is not yet fully understood, impeding its development and utilization. Transcription factors play a crucial role in plant Se metabolism [11–13]. To address this knowledge gap, we conducted a comprehensive analysis of the *C. violifolia* genome and identified a total of 118 *CvWRKY* genes. We investigated the phylogenetic association, gene structure, conserved domain composition, chromosome distribution, and homology of this gene family. Furthermore, we identified key *CvWRKY* genes involved in Se accumulation, providing a foundation for further research on their functions and the molecular mechanisms underlying their involvement in regulating Se metabolism in *C. violifolia*.

Results

Identification, physicochemical properties, and chromosomal distribution of the *CvWRKYs*

A total of 120 members of the *CvWRKY* gene family were identified from the genome of *C. violifolia* and were assigned new names from *CvWRKY1* to *CvWRKY120* (Additional file 1: Table S1). The encoded protein lengths of these genes ranged from 114 to 1,383 amino acids, with predicted molecular weights ranging from 13.52 to 155.75 kDa (Additional file 1: Table S1). The theoretical isoelectric points (pIs) of these proteins ranged from 4.76 to 9.86. CvWRKY103 had the lowest pI, whereas CvWRKY120 had the highest pI. Additionally, all CvWRKY proteins exhibited hydrophilic characteristics, as indicated by the negative range of the grand average of the hydropathicity values, which ranged from −1.169 to -0.419. Subcellular localization prediction revealed that all 120 CvWRKY proteins were located in the nucleus, which was consistent with the result that most transcription factors were located in the nucleus.

Based on the annotation information of the genome, 117 *CvWRKY*s were unevenly distributed across the 16 chromosomes of *C. violifolia*. The majority of these genes were found on Chr3 (13, 10.8%), Chr7 (10, 8.3%), Chr8 (10, 8.3%), Chr10 (14, 11.7%), and Chr13 (12, 10%). Only two *CvWRKYs* were located on Chr9, and three *CvWRKYs* were distributed in the unmounted fragments unanchor2075 and unanchor2204 (Fig. 1). These findings provide a theoretical foundation for further exploration of the purification, activity, and function of CvWRKY proteins.

Phylogenetic analysis and classification of CvWRKYs

To gain a better understanding of the phylogenetic associations among WRKY proteins from *C. violifolia*, an unrooted neighbor-joining phylogenetic tree was constructed using 1000 bootstrap replications. This analysis was based on multiple alignment of conserved regions of WRKY domain sequences from both *A. thaliana* and *C. violifolia*. Because the *WRKYs* in Group I group contains two WRKY conserved domains, it was distinguished by using near N-terminus (CvWRKYXXX C)

and C-terminus (CvWRKYXXX N). The results revealed that CvWRKY proteins were grouped together with members of the same subfamily in *A. thaliana*. Accordingly, CvWRKY proteins were classified into three distinct groups, namely, Groups I, II, and III, using a classification system established for AtWRKYs (Fig. 2) [14]. Group I consisted of 28 members, each containing two complete WRKY domains located at the N-terminus and C-terminus of the sequence. The zinc finger structure type for this group was C2H2. Group II was the largest, with 76 members. Within this group, CvWRKY proteins could be further divided into five subgroups, IIa, IIb, IIc, IId, and IIe, with 5, 16, 30, 12, and 13 members, respectively. Group II WRKY proteins generally contain a single WRKY domain, and their zinc finger structure type is C2H2. Group III had the fewest members, with a total of 16. All members of this group contained a conserved WRKY domain and two different zinc finger structure type, namely C2H/C and C2H2. Several exceptions were observed in the CvWRKY proteins, including WRKYGQK mutations. For example, CvWRKY028, CvWRKY048, CvWRKY103, and CvWRKY105 were classified as Group I, despite containing only one WRKY conserved domain. Additionally, the zinc finger structure near the C-terminus of the CvWRKY080, CvWRKY101, and CvWRKY105 proteins was lost. Furthermore, CvWRKY007 (Group IIc) and CvWRKY103 (Group I) exhibited WRKYGQK mutations that resulted in the sequence WIKYGQK, whereas CvWRKY101 (Group I) had a mutation from WRKYGQK to WQKYGQK. Moreover, WRKYGKK replaced WRKYGQK in CvWRKY027, CvWRKY085, CvWRKY063, CvWRKY107 (Group IIc), and CvWRKY048 (Group I) (Additional file 2: Fig. S1).

Gene structure and conserved motif analyses of the CvWRKYs

To gain a more comprehensive understanding of the characteristics of CvWRKYs, further analysis was conducted on the sequence features of the WRKY orthology groups (Fig. 3). The results derived from the phylogenetic tree, conserved domain analysis, intron/exon numbers, and motif patterns collectively demonstrated that closely related CvWRKYs within the same orthology group shared common characteristics in terms of intron/exon numbers, conserved domains, and motifs. The presence of identical motifs indicated that the orthologous groups exhibited similar functionalities, suggesting a strong association between gene structures. Within this study, the motif composition present in CvWRKY proteins (Fig. 3B) was predicted, and a total of 20 different motifs were identified and designated Motif 1 to Motif 20. These conserved motifs ranged in length from 15 to 50 amino acids (Additional file 3: Fig. S2). Notably, highly consistent conserved motifs were observed within the same group or subgroup of CvWRKYs, with each CvWRKY protein containing motif 1 and motif 2, which were annotated as WRKY DNA binding domains (Fig. 3B). Furthermore, all CvWRKYs contained at least one WRKY conserved domain, as depicted in Fig. 3C, which was consistent with the classification results based on the phylogenetic tree (Fig. 3A). Specifically, Group I contained two WRKY conserved domains, whereas Group II and Group III contained one WRKY conserved domain. Additionally, the Group IId subfamily also contained a Plant-Zn-clust domain. The number of exons encoding *CvWRKY* genes varied from two to fifteen. Among the *CvWRKY*s, the greatest proportion of genes contained three exons (45.8%), followed by four (19.2%), two (11.7%), five (10.8%), and six (10%), and genes encoding seven, eight, and fifteen exons were present in only one copy each (Fig. 3D). It was worth noting that the classification results of *CvWRKY029* and *CvWRKY118* in Fig. 3A were inconsistent with the classification results of phylogenetic trees, mainly the phylogenetic trees constructed by using full-length gene sequences in Fig. 3A. The gene structures of CvWRKY029 and CvWRKY118 contain two WRKY conserved domains (Fig. 3C), belonging to Group I. Overall, the analysis presented above demonstrated the accuracy of the *WRKY* gene family classification and showed that closely related CvWRKYs in the phylogenetic tree exhibited similar motif compositions and might perform similar biological functions. These results provide basic and effective information for understanding the evolutionary association and function of *CvWRKYs*.

Cis-acting elements in the promoters of CvWRKYs

Cis-acting elements are essential for regulating gene expression by influencing the transcriptional activity of gene promoters. To elucidate the regulatory mechanism of CvWRKYs, this study utilized the PlantCARE online website to predict cis-acting elements within the 2,000 bp sequence upstream of the translation start site of *CvWRKYs* (Fig. 4A). The analysis revealed a total of 56 cis-acting elements in the promoter region of *CvWRKYs*, which could be broadly categorized into hormone-responsive, stress-responsive, light-responsive, and development-responsive elements (Fig. 4B). Notably, 28 types of light-responsive elements were present in nearly all the genes. Additionally, hormone-related cis-acting elements, including abscisic acid responsiveness (ABRE), auxin responsiveness (AuxRR-core, TGA-element, and TGA-box), MeJA-responsive (TGACG-motif and CGTCA-motif), gibberellin-responsive elements (GARE-motif, P-box, and TATC-box), and salicylic acid-responsive (SARE and TCA-element), were widely distributed within the promoter region. Specifically, 303 ABRE cis-acting elements were distributed in the promoter regions

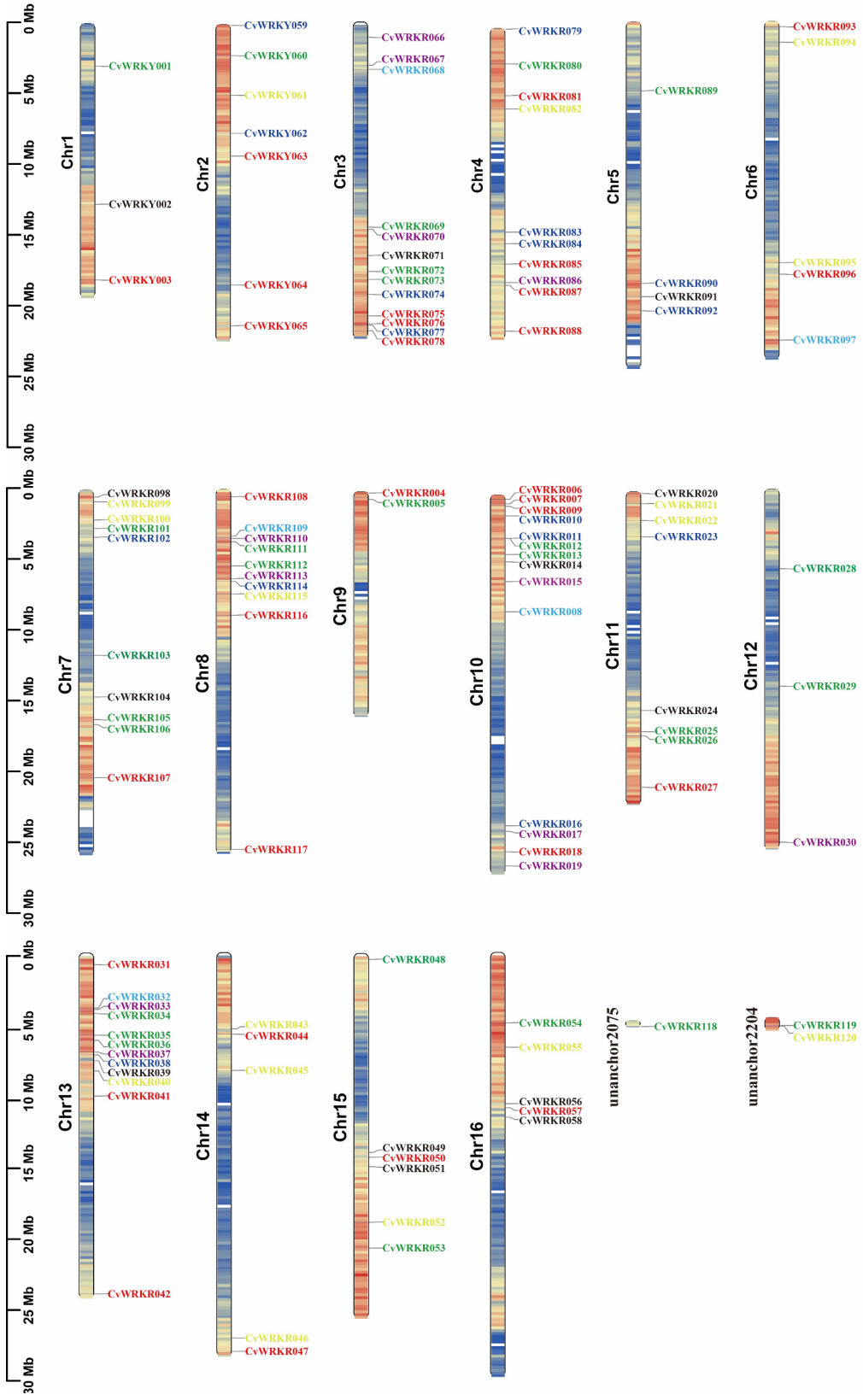

Fig. 1 Chromosomal locations of the *CvWRKY* genes on the *C. violifolia* chromosomes. Genes belonging to different subfamilies are indicated by gene names in different colors. Genes in green font belong to Group I, genes in light blue font belong to Group IIa, genes in pale yellow font belong to Group IIb, genes in red font belong to Group IIc, genes in purple font belong to Group IId, genes in black font belong to Group IIe, and genes in dark blue font belong to Group III

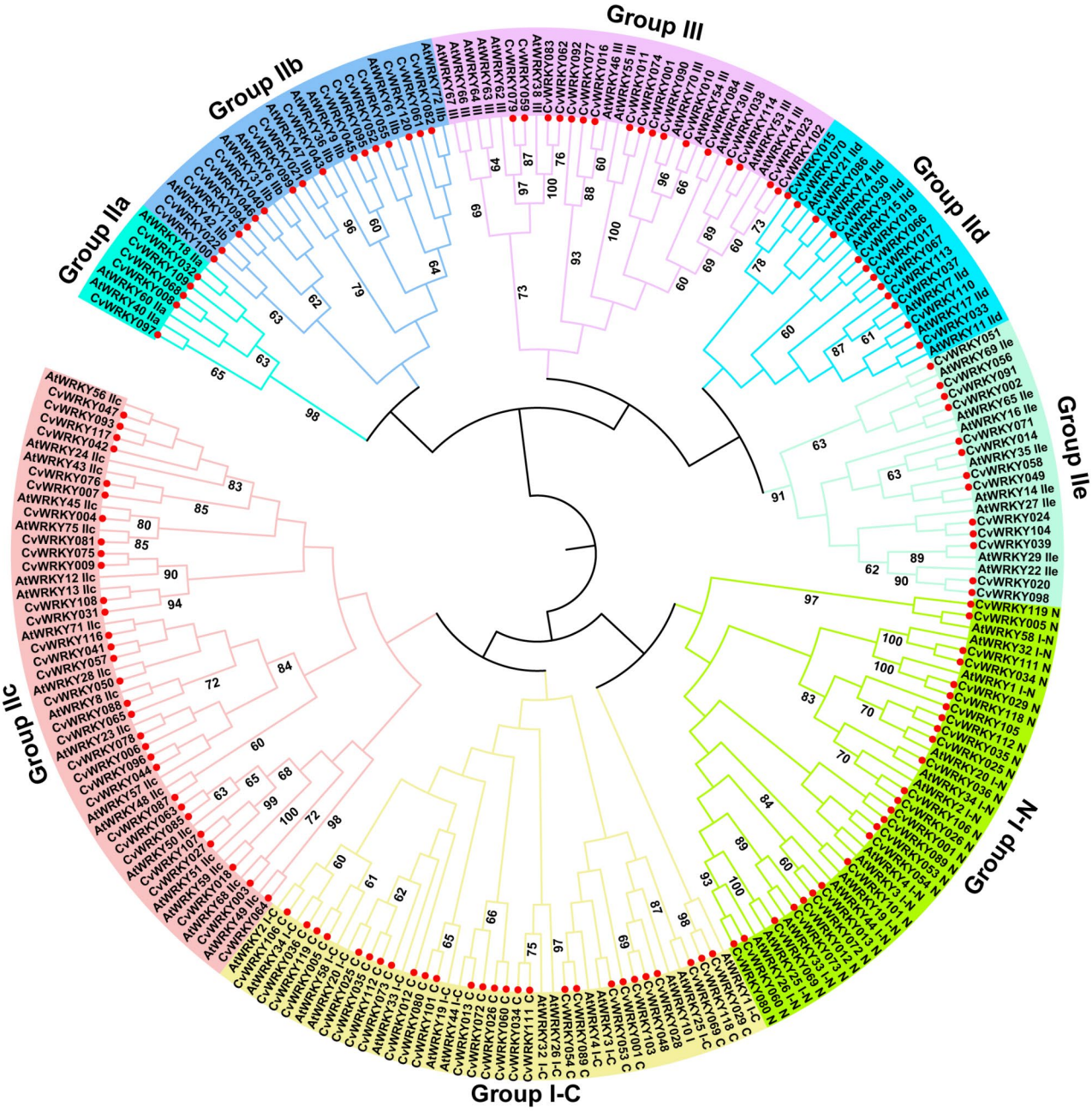

Fig. 2 Phylogenetic tree of the WRKY proteins sequences from *C. violifolia* and *Arabidopsis thaliana*. The phylogenetic tree was constructed using the MEGA 11 software, a bootstrap value of ≥ 60 was shown in the figure. Different colors indicated the various subfamilies. The red solid dot indicated the CvWRKYs

of 97 *CvWRKYs*, and 225 CGTCA motifs were distributed in the promoter regions of 95 *CvWRKYs* (Fig. 4B, Additional file 4: Table S2). Several stress-related cis-elements were also detected, including anaerobic elements (AREs), wound-responsive elements (WUN motifs), low-temperature stress-responsive elements (LTRs), drought stress-responsive elements (MBSs), defense and stress-responsive elements (TC-rich repeats), enhancer-like elements involved in anoxic-specific inducibility (GC motifs), cis-acting elements involved in dehydration, low-temperature stress, and salt stress (DRE). Furthermore, ten development-related cis-elements were identified, although the number of genes containing such cis-regulatory elements was relatively low (Fig. 4B).

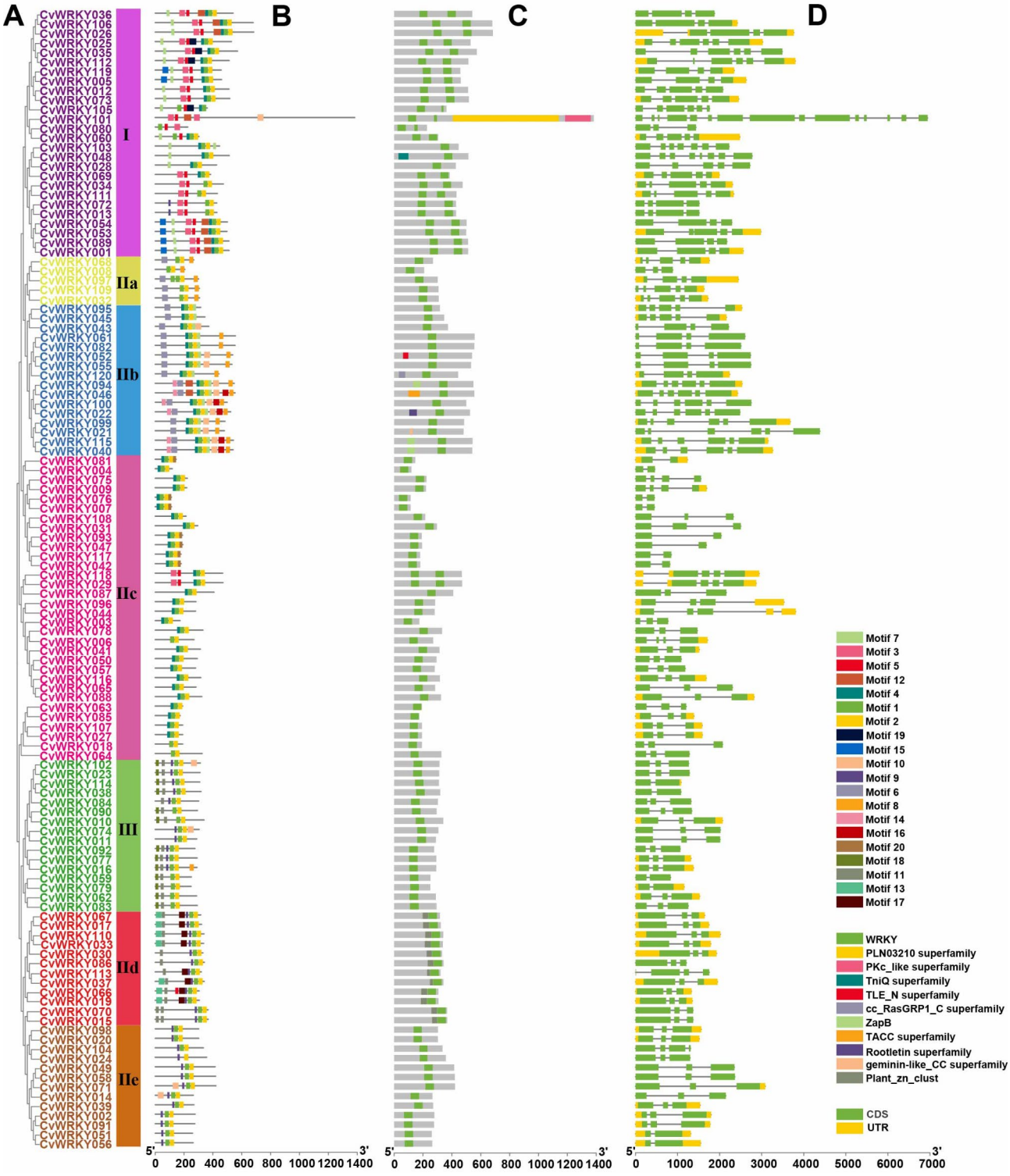

Fig. 3 The grouping, motif, conservative domains, and gene structure analyses of the CvWRKY family. **(A)** Neighbor-joining phylogenetic tree of the CvWRKY. Different colors indicate distinct subfamilies. **(B)** Conserved motifs in the CvWRKY proteins. The 20 predicted motifs were represented by different colored boxes. Sequence information for each motif was presented in Figure S2. **(C)** Conservative domains of the CvWRKY. Different colored boxes represent different conserved domains. **(D)** The Exon–intron structures of the *CvWRKY* gene family. The gray line represents an intron

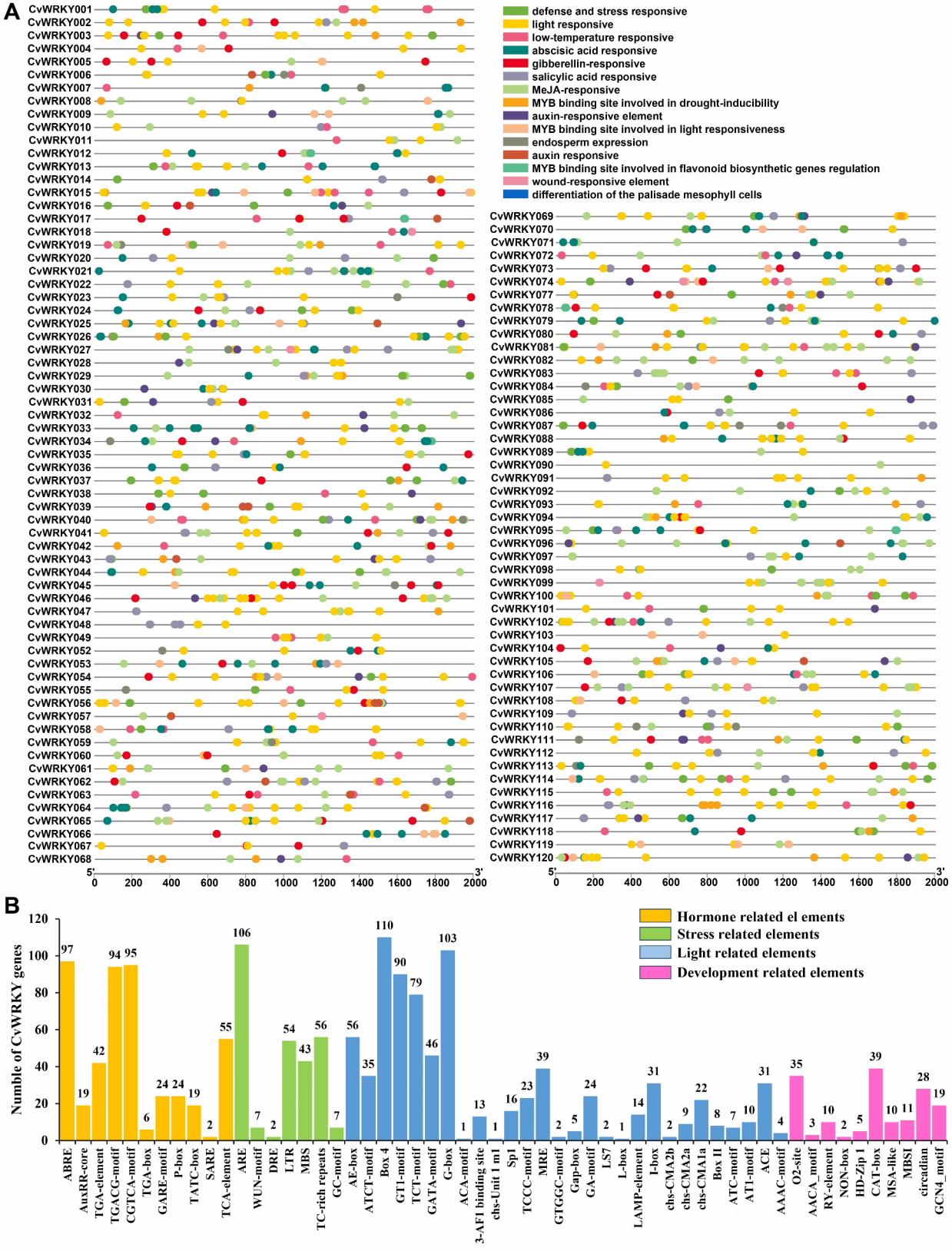

Fig. 4 Cis-acting elements analysis of the promoters of *CvWRKYs*. **(A)** Cis-element distribution on the *CvWRKY* promoters. Solid dots of different colors represent the indicated types of cis elements and their locations on the promoters of each *CvWRKY* gene. **(B)** The number of *CvWRKY* genes containing diverse cis-acting elements. X-axis and Y-axis represented the type of cis-acting elements and the number of *WRKY* gene family members, respectively

Synteny analyses of the CvWRKYs within and between species

Tandem duplication and segmental duplication events are critical in the evolutionary history of species, enabling the formation of gene families. To investigate the genomic expansion mechanism of the *CvWRKY* gene family, we analyzed the syntenic associations of the *CvWRKYs*. Conventionally, genes located on the same chromosome within a 200 kb distance are considered to originate from a gene cluster [15]. In this study, we identified 12 tandemly duplicated genes that generated six gene clusters, namely, *CvWRKY076*/*CvWRKY077* (Chr3), *CvWRKY109*/*CvWRKY110* (Chr8), *CvWRKY113*/*CvWRKY114* (Chr8), *CvWRKY035*/*CvWRKY036* (Chr13), *CvWRKY032*/*CvWRKY033* (Chr13), and *CvWRKY037*/*CvWRKY038* (Chr13) (Fig. 5). Among the 120 *CvWRKYs*, 114 (95% of all gene family members) exhibited homologous associations, and 127 segmental duplication events were observed (Additional file 5: Table S3). To investigate the molecular evolutionary association between species, MCScanX was utilized to identify orthologous *WRKY* genes among *C. violifolia*, *C. rubella*, and *A. thaliana*. A total of 177 colinear gene pairs were detected between *C. violifolia* and *C. rubella*, consisting of 115 *CvWRKYs* and 68 *CrWRKYs*. Of these, 53 *CvWRKYs* shared 2–4 orthologous *CrWRKYs*. Between *C. violifolia* and *A. thaliana*, 178 colinear gene pairs were identified, comprising 116 *CvWRKYs* and 66 *AtWRKYs*. Among these, 48 *CvWRKYs* shared 2–4 orthologous *AtWRKYs* (Fig. 5B, Additional file 6: Table S4). The study also demonstrated that *CvWRKY* genes exhibited a greater degree of homology with *WRKY* genes in *C. rubella* and *A. thaliana*. Furthermore, since *C. violifolia*, *C. rubella*, and *A. thaliana* all belong to the Brassicaceae family, their high homology can be attributed to their close genetic association.

Identification of key *CvWRKYs* involved in regulating Se accumulation

To further analyze the *CvWRKYs* involved in the regulation of Se metabolism in *C. violifolia*, 120 *CvWRKY* genes were aligned to the complete transcriptome database with an identity threshold of 87.5%. Duplicate gene IDs were eradicated, resulting in the identification of 67 unique *WRKYs* in the transcriptome data (Additional file 7: Table S5). Analysis of the FPKM values revealed that all 67 *WRKYs* exhibited differential expression patterns under treatment with varying concentrations of Na2SeO4. Five distinct types of expression patterns were primarily observed (Additional file 8: Fig. S3), suggesting that these genes respond to Na_2SeO_4 induction. To further explore the potential key genes involved in Se metabolism in *C. violifolia*, the correlations between the FPKM values of the 67 *WRKYs* and the contents of total Se, organic Se, and inorganic Se in *C. violifolia* leaves treated with different concentrations of Na_2SeO_4 were analyzed (Additional file 9: Table S6 and Additional file 10: Table S7). There was a significant positive correlation between the expression level of *CvWRKY034* (F01_transcript/25855) and the Se content (Fig. 6A). The correlation coefficients between *WRKY034* expression and total Se, inorganic Se, and organic Se content were greater than 0.88 ($P<0.001$) (Fig. 6B, C, D), suggesting that *CvWRKY034* may play a role in the biological processes of Se metabolism and accumulation in *C. violifolia*.

Previous research has demonstrated the crucial role of AtWRKY47 in maintaining Se homeostasis and tolerance in *A. thaliana*, as well as its involvement in Se accumulation [16]. In this study, the *AtWRKY47* sequence was used as the seed sequence to identify CvWRKY021 and CvWRKY099 in *C. violifolia*, which exhibited high sequence similarity to AtWRKY47 according to BLAST analysis. *AtWRKY47*, *CvWRKY021*, and *CvWRKY099* were classified as Group IIb. The WRKY conserved domains of AtWRKY47 and CvWRKY021 showed identical sequences (Fig. 7A), and they displayed close phylogenetic associations with *WRKYs* from other plant species, confirming their placement within the *WRKY* gene family (Fig. 7B).

Aligning *CvWRKY021* and *CvWRKY099* with transcriptome data revealed that they corresponded to *F01_transcript/22,479*. Furthermore, the expression level of this gene significantly increased as the concentration of Na_2SeO_4 increased, with the highest expression observed under the 4 mg/L Na_2SeO_4 treatment. Although the expression level of this gene decreased in the 16 mg/L Na_2SeO_4 treatment group compared to the 4 mg/L Na_2SeO_4 treatment group, the difference was not statistically significant (Fig. 7C). These findings suggest that *CvWRKY021* may play a role in Se accumulation in *C. violifolia*. Interestingly, both *CvWRKY021* and *CvWRKY034* were induced to be expressed in response to Na_2SeO_4 treatment. However, in the shoots of *C. violifolia*, the expression of these two genes was downregulated compared to that in the control group, whereas in the roots, the expression of these genes was upregulated (Fig. 7D). Additionally, the Se content in both the shoots and roots of *C. violifolia* significantly increased following Na_2SeO_4 treatment, with the roots showing a much greater Se content than the shoots (Fig. 7E). This suggests that *CvWRKY021* and *CvWRKY034* may primarily contribute to the Se accumulation process in *C. violifolia*.

Discussion

C. violifolia is a unique plant in China that is highly enriched in selenium, earning the title of "king of Se-enriched plants". This plant not only has a delicious taste

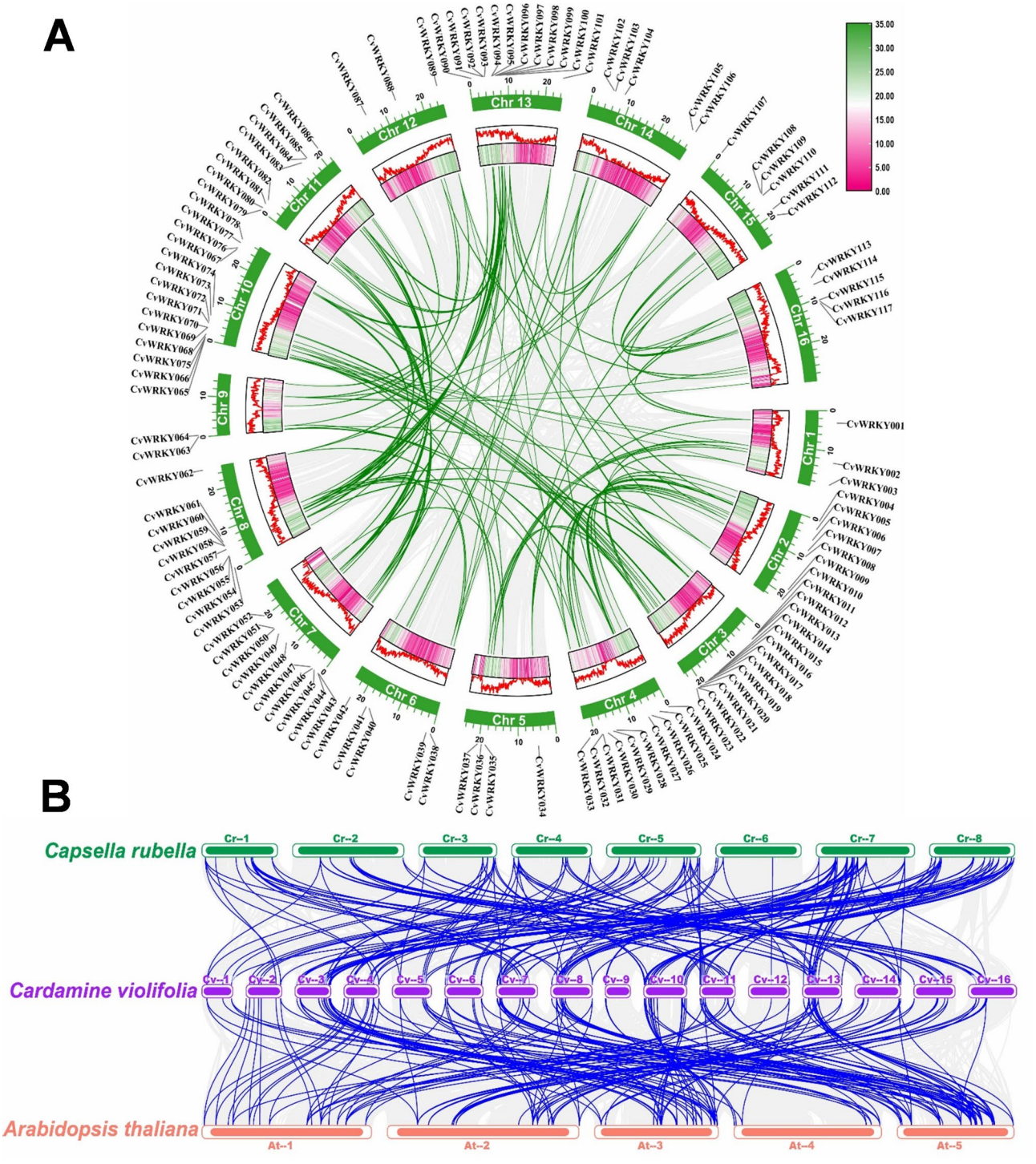

Fig. 5 Syntenic analyses of *CvWRKY* genes. **(A)** Gene collinearity of the *CvWRKY* gene family. Green lines connecting two chromosomal regions indicated synteny blocks in the *C. violifolia* genome. **(B)** The eight chromosomes of *Capsella rubella* (Cr1–Cr8), 16 chromosomes of *Cardamine violifolia* (Cv1–Cv16), and five chromosomes of *Arabidopsis thaliana* (At1–At5) were mapped in different colors. Blue lines represent syntenic *CvWRKY* gene pairs

and is packed with nutrients but is also extensively cultivated and processed. Its cultivation not only enhances people's diet but also generates significant economic income for growers, promoting industrial restructuring in suitable regions and providing social and economic benefits. Additionally, the exceptional ability of *C. violifolia* to accumulate Se has led to its utilization as a novel source of Se in various fields, including the livestock industry [17].

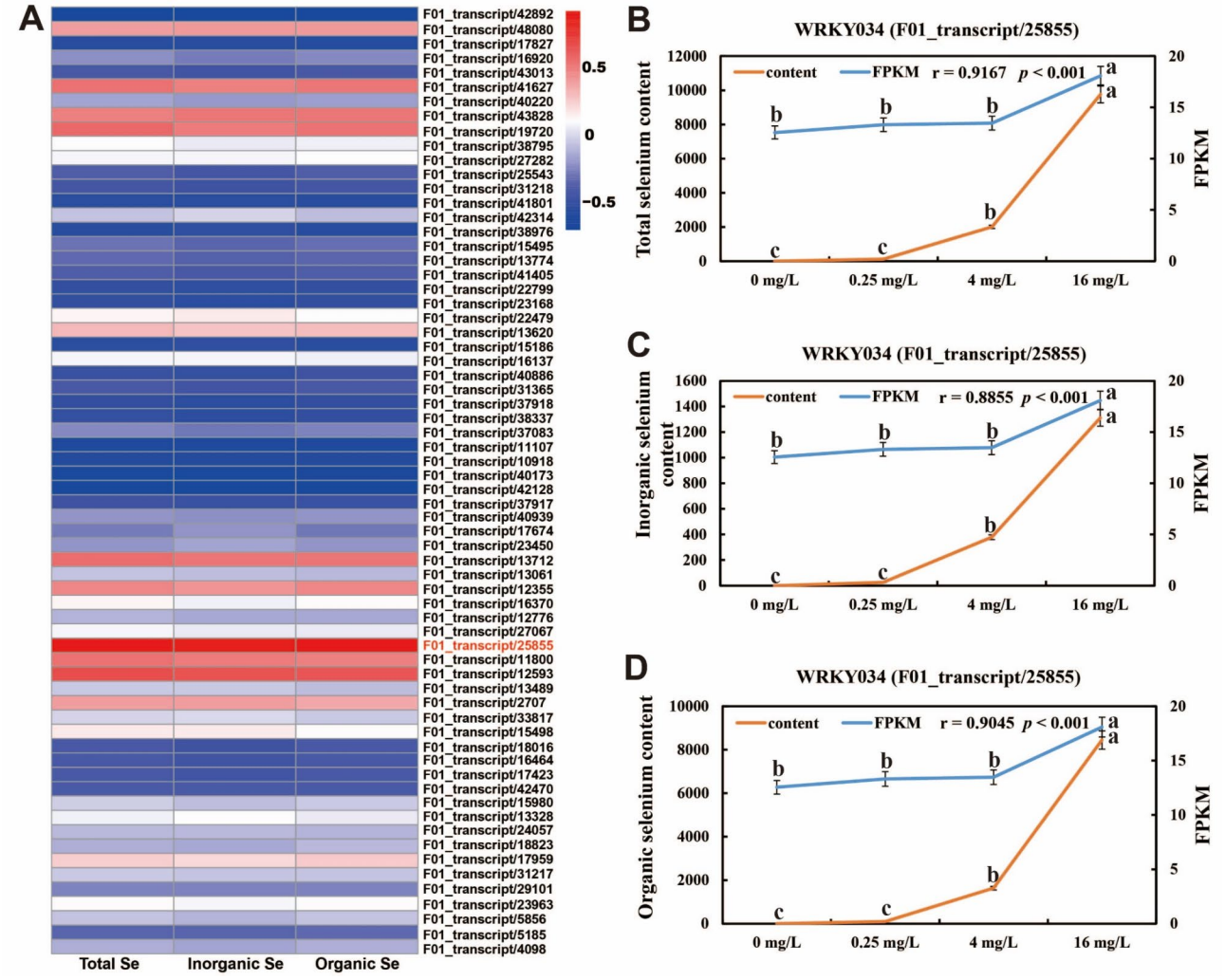

Fig. 6 Correlation analysis. **(A)** Correlation analysis between *WRKY034* expression level and selenium content. **(B)** Correlation analysis between the expression level of *WRKY034* and the content of total selenium. **(C)** Correlation analysis between the expression level of *WRKY034* and the content of inorganic selenium. **(D)** Correlation analysis between the expression level of *WRKY034* and the content of organic selenium

The *WRKY* gene family plays a crucial role in regulating stress reactions in plants [18]. Although genome-wide analysis of the WRKY family has been conducted in several species [19, 20], the *WRKY* family of *C. violifolia* remains unidentified. This knowledge gap hampers the study of *CvWRKY* gene function. Therefore, our research focused on identifying the *WRKY* family of *C. violifolia* through systematic bioinformatics analysis and expression analysis. This approach aimed to identify the key *WRKY* genes involved in regulating Se metabolism in *C. violifolia*, which will provide a theoretical foundation for understanding the molecular mechanism underlying Se enrichment in this plant and enhancing its development and utilization.

At the genome level, we identified a total of 120 *CvWRKYs* (CvWRKY001 ~ CvWRKY120) in *C. violifolia*, surpassing the number of *WRKYs* found in other plants, such as *Cucumis sativus* (61) [21], *Hippophae rhamnoides* (48) [22], *Lycopersicon esculentum* (81) [23], and *Oryza sativa* (109) [24]. However, *C. violifolia* had fewer *WRKY* genes than did herbaceous plants such as *Triticum aestivum* (171) [25] and *Raphanus sativus* (126) [26]. Among them, the number of *WRKY* family members in *R. sativus*, which also belongs to the Brassicaceae family, was the greatest. The expansion of the *WRKY* gene family in *C. violifolia* was mainly attributed to tandem and segmental duplication events [10]. Chromosome distribution and collinearity analysis revealed that 6 pairs, totaling 12 *CvWRKYs*, were tandem repeat genes, whereas 114 *CvWRKY*s were segmental repeat genes originating from chromosome regions or entire chromosomes. The presence of duplicated *WRKY* genes suggests that these genes may have similar biological functions and can form complex regulatory networks, particularly in gene expression regulation under exogenous Se treatment. Collinearity analysis with other plants

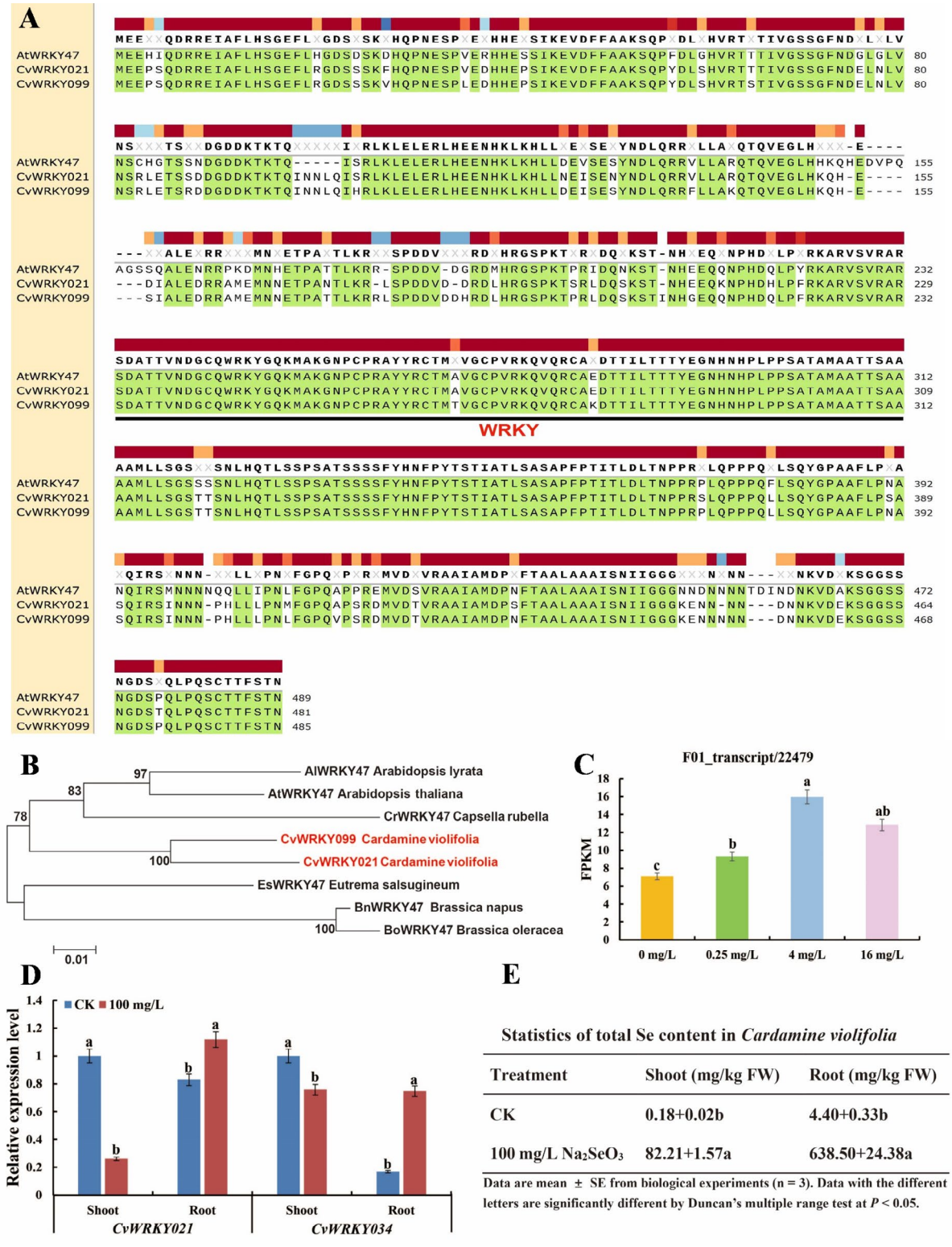

Fig. 7 Analysis of candidate genes involved in Se accumulation in *C. violifolia*. **(A)** Multiple sequence alignment of WRKYs. *AtWRKY* was a gene from *Arabidopsis* that participated in Se accumulation. **(B)** Phylogenetic tree of WRKY proteins from different plants. AlWRKY47 (ID: XP_020879345, *Arabidopsis lyrata*), CrWRKY47 (ID: XP_023636950, *Capsella rubella*), EsWRKY47 (ID: XP_006396367, *Eutrema salsugineum*), BnWRKY47 (ID: XP_013726314, *Brassica napus*), BoWRKY47 (ID: XP_013609023, *Brassica oleracea*). **(C)** Expression pattern analysis of *CvWRKY* (*F01_transcript/22479*) treated with different concentrations Na_2SeO_4. **(D)** Analysis of gene expression levels of *CvWRKY021and CvWRKY034* under Na_2SeO_3 treatment. **(E)** Determination of total Se content in different parts of *C. violifolia* treated with Na_2SeO_3

demonstrated the existence of conserved *WRKY* genes in *C. violifolia* that are evolutionarily related to those in other plants, such as *A. thaliana*, known as orthologous genes. Therefore, the functional analysis and validation of *CvWRKYs* can be guided by the functions of *WRKYs* in other plants.

WRKY transcription factors are characterized by a conserved structural domain consisting of approximately 60 amino acids. This conserved domain is composed of a highly conserved WRKYGQK motif at the N-terminus and a variable zinc finger structure at the C-terminus. WRKY transcription factors play a critical role in regulating gene expression by binding specifically to the W-box motif within the promoter region of target genes [27]. In the model plant species *A. thaliana*, the *WRKY* family has been classified into three groups. Within this classification, 120 identified *CvWRKYs* were assigned, with Group II further subdivided into five subgroups. These findings indicate that CvWRKY proteins show a high degree of conservation and similarity in their grouping compared to *WRKY* families found in other plant species [28].

However, some variations have been identified in the CvWRKY protein sequences. For instance, CvWRKY028, CvWRKY048, CvWRKY103, and CvWRKY105, which cluster into Group I, possess only one conserved WRKY domain. Furthermore, the zinc finger structures near the C-terminus of the conserved WRKY domain of the CvWRKY080, CvWRKY101, and CvWRKY105 proteins were lost. Previous research has demonstrated that the conserved residues within the WRKY domain are crucial for the proper folding of the DNA-binding zinc finger. Moreover, the loss of the zinc finger structure sequence may result in reduced or eliminated DNA binding activity [29].

In addition to these variations, exceptions to the WRKYGQK motif have been observed in CvWRKY proteins. For example, the protein sequences of CvWRKY007 (Group IIc) and CvWRKY103 (Group I) exhibit WIKYGQK variation, whereas CvWRKY101 (Group I) possesses WQKYGQK variation. Furthermore, the protein sequences of CvWRKY027, CvWRKY085, CvWRKY063, CvWRKY107 (Group IIc), and CvWRKY048 (Group I) exhibited WRKYGKK variation. In soybean, the W-box binding ability of GmWRKY6 and GmWRKY21 was affected by a mutation in the WRKYGKK motif [30]. Additionally, NtWRKY12 in tobacco, which features a conserved structural domain mutated to WRKYGKK, exhibits specific binding to the WK box sequence (TTTTCCAC) [31]. Notably, mutations within Group IIc may alter DNA binding affinity, resulting in a range of binding specificities [32]. The genetic variations detected in *CvWRKYs* may give rise to the emergence of novel functions, subfunctions, and pseudogenes within the *CvWRKY* gene family [33]. The cis-acting elements within gene promoters have a significant impact on gene expression patterns by binding transcription factor sites [34]. The promoters of *CvWRKYs* contain a diverse range of cis-acting elements, including elements related to development, plant hormone responsiveness, stress response, and light response. This indicates their involvement in various biotic and abiotic stress responses. It is speculated that the cis-acting elements of *CvWRKY* genes interact with various stress-related trans-acting factors to regulate the expression and response of stress resistance genes in *C. violifolia*. WRKY proteins have been shown to function under various abiotic stress conditions, such as cadmium stress, salt tolerance, and iron deficiency [35–38]. However, it remains unclear whether *CvWRKYs* play a role in regulating Se tolerance in *C. violifolia*. The close association between gene expression and function is well recognized, emphasizing the importance of studying gene expression patterns in different tissues or under stress conditions to understand gene functions [39].

Given the interest in understanding the molecular mechanisms underlying Se enrichment in *C. violifolia*, this study tentatively suggested that *CvWRKY034* may be involved in the biological processes of Se metabolism and accumulation in *C. violifolia*. This was based on the analysis of the correlation between gene expression levels and the contents of total selenium, inorganic selenium, and organic Se in tissue samples under exogenous Se treatment ($r>0.88$, $P<0.001$). Previous studies have reported the involvement of *AtWRKY47* in regulating Se accumulation processes [16]. Genes with similar sequences often exhibit similar biological functions [40]. In *C. violifolia*, CvWRKY021 and CvWRKY099 showed high sequence similarity to AtWRKY47 and were classified into Group IIb, with the WRKY conserved domain highly conserved between AtWRKY47 and CvWRKY021. Transcriptome data analysis revealed that *CvWRKY021* and *CvWRKY099* corresponded to *F01_transcript/22,479*, and this *transcript* was inducible by Na_2SeO_4, suggesting that *CvWRKY021* may participate in the Se accumulation process. Further analysis revealed that both *CvWRKY021* and *CvWRKY034* responded to Na_2SeO_3 treatment, and their expression was significantly upregulated in the roots. Moreover, the Se content in roots significantly increased, indicating that *CvWRKY021* and *CvWRKY034* might participate in the regulation of Se accumulation in roots. However, further validation of the specific functions of *CvWRKY034* and *CvWRKY021* in Se metabolism in *C. violifolia* is needed to comprehensively understand their mechanisms of action.

Conclusion

This study comprehensively investigated the WRKY family in *C. violifolia*, identifying 120 *CvWRKYs* organized into three main groups with five subgroups in Group II. Detailed analyses of their physicochemical properties, chromosomal positions, phylogeny, gene structures, motifs, cis-acting elements, and collinearity provided insights into their evolutionary and functional roles. Integrating gene expression and Se content data from prior studies, we found a significant positive correlation between the expression level of *CvWRKY034* and Se content. CvWRKY021, showing high homology to the Se-accumulating AtWRKY47 in *A. thaliana*, responded positively to the induction of Na_2SeO_4. Both *CvWRKY034* and *CvWRKY021* were also responsive to the induction of Na_2SeO_3, with their expression downregulated in shoots and upregulated in roots, and the Na_2SeO_3 treatment leading to increased Se accumulation in roots. These findings suggest *CvWRKY034* and *CvWRKY021* may as key regulators of Se accumulation in *C. violifolia* roots, contributing to its hyperaccumulation capacity. This study on the WRKY family in *C. violifolia* provides a theoretical basis for revealing the role of *WRKYs* in the Se hyperaccumulation capacity of *C. violifolia*. Moreover, this study provides important insight for future functional validation endeavors.

Materials and methods

Identiffcation and analysis of *CvWRKY* gene family in *C. violifolia*

The *CvWRKY* gene family was analyzed using the *C. violifolia* genome obtained from the Genome Warehouse BIG Data Center (https://ngdc.cncb.ac.cn/) [41]. The hidden Markov model (HMM) file of the WRKY domain (PF03106) from the Pfam database (http://pfam.xfam.org) was used as the seed sequence to identify candidate *CvWRKYs* using HMMer software (version 3.3.2) with an E value < 1e-5. The conserved domains of the candidate CvWRKYs were detected using the NCBI CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (https://smart.embl.de/) online tools. Multiple sequence alignment was performed to manually remove sequences with incomplete conserved domains and redundant sequences, resulting in the identification of members of the *CvWRKY* gene family. The physicochemical properties of the proteins were determined using the ExPASy online website (http://web.ExPASy.org/protparam/), whereas their subcellular localization information was predicted using the Cell-PLoc 2.0 online tool (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/).

Construction of phylogenetic tree and chromosomal distribution

The WRKY family gene sequences of *Arabidopsis thaliana* were downloaded from the *A. thaliana* database (www.Arabidopsis.org). Multiple sequence alignments of the WRKY conserved domains of WRKY proteins in *A. thaliana* and *C. violifolia* were carried out using ClustalX, and the neighbor-joining (NJ) method was used to construct a phylogenetic tree with pairwise deletions and 1000 bp replications via MEGA 11. The phylogenetic tree was visualized using iTOL online software (https://itol.embl.de/). Gene density and the distribution of CvWRKY on chromosomes were obtained from the annotation file (gff3 file) of the *C. violifolia* genome data and visualized using TBtools software to display the distribution of CvWRKY proteins on chromosomes.

Gene structure identification, motif composition, and cis-acting elements

The conserved domains of the CvWRKY protein were predicted using the Batch CD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) provided by NCBI. Analysis of conserved CvWRKY motifs was performed using MEME online software (https://meme-suite.org/meme/tools/meme), with 20 motifs selected and default parameter settings. TBtools software was used to extract the upstream 2000-bp sequences of the *C. violifolia* genome as promoter sequences, which were then submitted to the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for the prediction of cis-regulatory elements. Visualization of gene structure, motif distribution, and cis-acting elements was carried out using TBtools software [42].

Collinearity analysis

The genome annotation files of *C. violifolia*, including the chromosome scaffolds, gene density files, and files indicating the positions of *CvWRKY* genes on the chromosomes, were obtained using TBtools software. Intraspecific collinearity analysis of *C. violifolia* was performed using TBtools, and the results were visualized. The genome data and annotation files for *Capsella rubella* and *A. thaliana* were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/). Collinearity analysis was conducted to evaluate the covariance between the *C. violifolia*, *C. rubella*, and *A. thaliana* genomes. The collinearity associations between *C. rubella* and *C. violifolia*, as well as between *A. thaliana* and *C. violifolia*, were analyzed using the one-step MCScanX button in TBtools software. The collinearity analysis results were further simplified using the File Merge for the MCScanX button. The annotation file information in the collinearity analysis result was extracted after running the Quick

Run MCScanX Wrapper button, and the results were visualized.

Plant materials and treatment

C. violifolia seeds, donated by Enshi Se-Run Material Engineering Technology Co., Ltd., were germinated in substrate soil. Once they reached 4–5 true leaves, the plants were transplanted into hydroponic tanks (36 cm × 24 cm × 12.5 cm) filled with 1/4 strength Hoagland's nutrient solution. The plants were grown under controlled conditions: the temperature was maintained at 20±1 °C, the relative humidity was approximately 75%, and the plants were subjected to a light cycle of 14 h (200 μmol/m^2/s) followed by 8 h of darkness. After 10 days, hydroponic tanks with 9 *C. violifolia* plants exhibiting consistent growth were selected for further treatment. The nutrient solution in the tanks was then replaced with 1/2 Hoagland's nutrient solution containing different concentrations of Na_2SeO_4 (0, 0.25, 4.0, or 16.0 mg/L). The nutrient solution with varying Na_2SeO_4 concentrations was refreshed every 10 days. Each treatment group comprised three independent biological replicates, with each replicate containing 9 *C. violifolia* plants. After three consecutive treatments, all plants from each replicate were harvested, washed three times with deionized water, wiped to remove surface moisture, frozen in liquid nitrogen, thoroughly mixed, and stored at -80 °C for future use.

Using the same procedure as described above, *C. violifolia* plants with consistent growth were obtained and subjected to continuous culture for 45 days. The nutrient solution in the tanks was then replaced with 1/2 Hoagland's nutrient solution containing different concentrations of Na_2SeO_3 (0 or 100 mg/L). After 15 days of treatment, the plants were divided into shoots and roots for collection. The samples were frozen in liquid nitrogen and stored at -80 °C for later use.

Screening and expression analysis of *CvWRKY* gene involved in Se metabolism and accumulation in *C. violifolia*

The research group obtained the full-length transcriptome, transcriptome sequencing, and Se content data of *C. violifolia* from the aforementioned plant materials [3]. These data were utilized for subsequent analysis in this study. Protein sequences with a similarity greater than 85% were selected for further analysis by aligning them to the full-length transcriptome database using TBtools software (E-value≤1e-5), using the CvWRKY protein identified from the genome as a seed sequence. Additionally, a correlation analysis was conducted between the screened gene expression levels and the total Se content, organic Se content, and inorganic Se content. The resulting data were visually represented using a heatmap tool within TBtools.

Determination of Se content and qRT-PCR analysis

The fresh samples, weighing 0.2 g, were placed into a digestion vessel and mixed with 8 mL of HNO_3. They were then predigested for 15 min at 180 °C using an automatic graphite digester (G-400, Shanghai Yiyao Instrument Technology Development Co., Ltd., China). After the predigestion process was completed and the mixture had cooled, the vessel was sealed and transferred to a microwave digestion system (YMW40, Changsha Yong Le Kang Instrument, China) for further digestion. The digestion program was as follows: heating to 120 °C within 5 min and maintaining for 3 min; subsequently heating to 160 °C within 5 min and maintaining for 3 min; and finally heating to 180 °C within 5 min and maintaining for 20 min. The acid was then removed at 180 °C on an automatic graphite digester until the liquid volume decreased to approximately 1 mL. The vessel was then removed and cooled to room temperature. The volume was adjusted to 10 mL using 2% HNO_3 to obtain the liquid to be detected. The total Se content in the shoots and roots was determined via hydride generation atomic fluorescence spectrometry (HG-AFS, AFS8510, Beijing Haiguang Instrument Co., China), as described by Rao et al. [3].

A ChamQ Universal SYBR qPCR Master Mix kit (Vazyme Biotech) was used for qRT−PCR analysis of total RNA from the shoots and roots of *C. violifolia* treated with Na_2SeO_3. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with *β-actin 3* serving as the reference gene. The specific primers for all genes can be found in Additional file 11: Table S8.

Abbreviations

Se	Selenium
DW	Dry weight
TFs	Transcription factors
FPKM	Fragments per kilobase of transcript per million mapped reads
NJ	Neighbor-joining
NCBI	National center for biotechnology information
qRT-PCR	Quantitative real-time PCR

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Author contributions
F.X. and X.L. conceived the idea and designed the experiment. Z.Y., S.R., W.Z., and J.Y. carried out various experiments. S.C. did the investigation. X.L. and F.X. wrote the manuscript. All authors have read and approved the manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article Additional file. The datasets used and/or analyzed during the current study are available from the authors on reasonable request (Feng Xu, xufeng198@126.com; Xiao-Meng Liu, LiuXM925@163.com).

Declarations

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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