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Genome-Wide Identification and Expression Profile Analysis of the *WUSCHEL*-Related Homeobox (*WOX*) Genes in Woodland Strawberry (*Fragaria vesca*)

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Abstract: The *WUSCHEL*-related homeobox (*WOX*) is a unique transcription factor family belonging to plants. Its members play important roles in regulation of plant stem cell division and differentiation or dynamic balance of the organ development. In this study, 16 *Fragaria vesca WOX* members (*FvWOXs*) were identified in the woodland strawberry (*Fragaria vesca*) genome. According to the phylogenetic analysis, FvWOX proteins can be classified into three clades: the ancient clade, the WUS clade and the intermediate clade. The gene structure and protein motif analysis showed that *FvWOXs* are highly conserved to each other in their respective clades. Chromosome location analysis revealed that *FvWOXs* are unevenly distributed in the *Fragaria vesca* chromosomes. Expression analysis showed that *FvWOX* genes are widely expressed in various tissues of *Fragaria vesca*. Moreover, when treated with 6-benzylaminopurine (6-BA) or gibberellin (GA_3), expression level changes of the genes highly expressed in crowns were detected at 48 h after treatment. The subcellular localization of FvWUSb was determined in tobacco leaves, indicating that the protein is located in the nucleus. This study enlarged our recognition about the *WUSCHEL*-related homeobox genes in *Fragaria vesca*, and laid a foundation for further researches about this gene family in strawberry.

Keywords: *Fragaria vesca*; *WOX* gene family; expression; phytohormone

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1. Introduction

The *WUSCHEL*-related homeobox (*WOX*) gene family, which contains a classic DNA binding domain, is one kind of unique transcription factor in plants and is essential in multiple plant growth and development processes [1]. The WOX proteins have been reported as having crucial functions in the regulation of plant stem cell division, differentiation, dynamic balance and plant organ development [2,3].

Previous studies have indicated that the *WOX* gene family members could be divided into three clades based on their evolutionary relationships, which are the ancient clade, the intermediate clade and the WUS clade [4]. WOX proteins of the lower plants only belong to the ancient clade. In the model plant *Arabidopsis thaliana*, 15 *WOX* members have been identified. Among them, *AtWOX10*, *AtWOX13* and *AtWOX14* belong to the ancient clade, *AtWOX8*, *AtWOX9*, *AtWOX11*, and *AtWOX12* are classified as the intermediate clade members, while *AtWOX1-7* and *AtWUS* are members of the WUS clade [5].

WOX genes have been identified in many plant species, e.g., there are 44 *WOX* members in wheat [3], 32 *WOX* members in cotton [6] and 33 *WOX* members in soybean [7]. Although the numbers of *WOX* genes from different species are not consistent, their functions are conserved. The most comprehensive and in-depth studies of *WOX* genes have been performed in *Arabidopsis*. In *Arabidopsis*, *AtWUS* is synthesized in the center of the tissue and then migrates to the central region of stem cells, where it directly binds to

the promoter of the meristem marker gene *AtCLV3* and thus promotes its expression [8]. *WUS-CLV3* interaction forms a feedback circuit between the tissue center and the stem cells to establish the shoot stem cell niche [1]. *AtWOX1* homologous genes have been identified as important regulators in the lateral organ and leaf development of several eudicotyledons. Overexpression of *AtWOX1* resulted in the decreased leaf size in *Arabidopsis* [9], and a similar phenomenon was also observed in cucumber overexpressing *CsWOX1* [10]. *AtWOX2* homologues play similar roles in the formation of protoderm in early embryonic development of seed plants [11]. *AtWOX3-like* genes own the highly conserved function in the lateral edge development of the vegetative and floral organs [12,13]. *AtWOX4* was reported to be required in auxin dependent cambium stimulation, and thus affects the plant lateral growth [14]. The *PttWOX4* gene, the *AtWOX4* homolog of poplar, was found to control the cell division activity in vascular cambium, thus controlling the growth of stem circumference [15]. *AtWOX5* is induced by auxin and is a negative factor that regulates the IAA homeostasis so as to maintain the stem cells in root apical meristems [16]. *AtWOX7* was reported to inhibit lateral root development in a sugar dependent way, and the overexpression of *AtWOX7* resulted in a decrease in the number of lateral root protoplasts [17]. *WOX9/STIMPY* integrates the developmental signaling and the cell cycle regulation to maintain cell division and prevent improper root differentiation [18]. *AtWOX11* homolog *OsWOX11* is found to be required for the regulation of crown root and lateral root development, and also responds to many abiotic stresses, such as drought and cold [19]. It has been reported that the overexpression of *PagWOX 11/12a* under salt stress conditions could promote the growth-related biomass of poplar [20]. *AtWOX13* in *Arabidopsis* plays a significant role in the wound triggered callus formation and organ reconnection [21].

Strawberry is a perennial herb, known as the "Queen of fruit" for its rich nutritional value, especially for its rich vitamin C content. The growth and development habit of strawberry is relatively unique. From the shortened crown, runners for vegetative reproduction and inflorescences for reproductive growth can both be extracted. Runner is the main material for the reproduction of conventional strawberry seedlings, while the inflorescence determines the production period and the yield of strawberries. As *WUS* is the key regulator of cell characteristics in the central region during development of the shoot apical meristem (SAM) [22], identification of the strawberry *WOX* genes which are highly expressed in the crown could provide us with valuable information for further research on the regulation mechanism of the induction of runner or inflorescence.

However, little is known about the *WUS-like* genes in strawberry. Recent research reported that *FvWUS1*, a homologue of *Arabidopsis WUS* in *Fragaria vesca*, is widely expressed in young flower meristem, indicating its potential function in controlling of the flower organogenesis and development [23].

In this study, we performed identification and characterization of the *Fragaria vesca WOX* members, including the gene structure analysis, conserved motif and domain identification, phylogenetic analysis, chromosomal localization, and collinearity analysis. The genes highly expressed in the crown were further screened by tissue-specific expression detection. Moreover, effects of the plant hormones treatments on *FvWOXs* expression levels were also investigated.

2. Materials and Methods

2.1. Genome-Wide Identification of WOX Genes in Fragaria vesca

A BLAST search was performed against the woodland strawberry (*Fragaria vesca*) genome data v4.0.a2 in the Genome Database for *Rosaceae* (GDR, https://www.rosaceae.org/ (accessed on 20 October 2022)) using the full-length amino acid sequences of AtWOXs. Then the sequences of retrieved woodland strawberry WOX candidates were submitted to the PFAM (http://pfam.xfam.org/ (accessed on 15 June 2021)) database [24] to annotate the HMM (Hidden Markov Model) profile of the homeobox (PF00046.32) domain [25]. Only the proteins containing the homeobox domain were considered as WOX homologs.

To identify whole members belonging to the *WOX* family in *Fragaria vesca*, SMART database (http://smart.embl-heidelberg.de/ (accessed on 15 June 2021)) and Pfam database were then used to screen the candidates by searching for the conserved homeobox (HB) domain of the corresponding proteins. After removing the redundant protein sequences, the remaining non-repetitive *Fragaria vesca* WOX proteins were selected for further analysis.

2.2. Protein Properties and Phylogenetic Tree Construction

The physiochemical properties of *Fragaria vesca* WOX proteins, such as the molecular weight (MW), polypeptide length, isoelectric point (pI), instability index, aliphatic index and hydropathicity (GRAVY) were investigated using the online tool ExPASy (http://web.expasy.org/protparam/ (accessed on 15 November 2021)) [26]. Multiple sequence alignment of the HB domains of FvWOXs was performed with the DNAMAN software (version 5.2.2) (Lynnon Biosoft, USA). The phylogenetic tree was constructed using MEGA 7.0.26 (https://www.megasoftware.net/ (accessed on 14 July 2022)) with the Maximum Likelihood method and 1000 bootstrap values to associate the identified *Fragaria vesca* WOX proteins with known WOX family members of *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) [27]. Online website iTOL (Interactive Tree of Life, http://itol.embl.de/ (accessed on 3 November 2022)) was then used to annotate the tree [28].

2.3. Gene Structure, Domain and Protein Motif Analysis

The genomic DNA sequences and CDS sequences corresponding to *FvWOX* genes were retrieved from the strawberry genome database. The intron-exon distribution was displayed by comparing the CDS to the corresponding genomic sequence with the gene structure display server (GSDS, http://gsds.cbi.pku.edu.cn/ (accessed on 15 June 2021)) [29]. Information about locations of the FvWOX homeobox domain was obtained from the Pfam database (http://pfam.xfam.org/ (accessed on 15 June 2021)). The protein structural motif annotation was performed using the MEME program (http://meme-suite.org/tools/meme (accessed on 15 June 2021)) with the number of detected motifs as eight and the other parameters as default values [30]. TBtools software (version 1.09876) (https://github.com/twdb/tbtools (accessed on 30 September 2021)) was used to integrate and visualize these results [31].

2.4. Chromosome Location and Collinear Analysis of FvWOX Genes

Information about the physical locations of *FvWOX* genes in the chromosomes of woodland strawberry was obtained using the TBtools software (version 1.09876). The genomic sequences and annotation files of *Fragaria vesca* and other species were downloaded from GDR (https://www.rosaceae.org/ (accessed on 20 October 2022)) and PLANTS (http://plants.ensembl.org/index.html (accessed on 16 December 2021)). The collinearity was also drawn with TBtools (version 1.09876).

2.5. Cis-Elements Analysis

The 2000 bp DNA sequence upstream of the start codon (ATG) of each *FvWOX* gene was obtained from GDR. The Plant Care (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 16 December 2021)) database was used to identify the *cis*-acting elements [32]. TBtools software (version 1.09876) was used to draw the heat map of the *cis*-acting elements distribution.

2.6. Plant Material and Expression Profile Analysis

Two diploid woodland strawberry (*Fragaria vesca*) varieties 'Ruegen' (Rg; Horticulture Research International accession number FDP825), which does have runners, and 'Hawaii 4' (H4; National Clonal Germplasm Repository accession number PI551572), which has runners, were used as the plant material in this experiment.

Tissue-specific expression analyses were carried out in both varieties grown in a greenhouse under natural LD (Long Day) conditions during the spring in ShenYang (Liaoning, China; 41° N, 123° E) with a temperature of about 25 °C. 'Ruegen' plants about 8 weeks old grown in a greenhouse under natural LD conditions were used for 6-BA treatment and GA_3 treatment. A total of 20 plants were used for tissue expression detection and 60 plants were used for hormone treatment experiment.

The samples used were frozen in liquid nitrogen immediately after collection and stored at −80 °C. Total RNA was extracted using the plant tissue RNA extraction kit (TIANGEN, Beijing, China), and then the reverse transcription kit (TaKaRa, Dalian, China) was used to synthesize the cDNA for qRT-PCR experiments. qRT-PCR was performed using the SYBR Green PCR Master Mix Kit (TaKaRa, Dalian, China) on CFX96 Real-Time PCR System (Applied Bio-systems, CA, USA). Primer sequences are shown in Supplementary Table S1. Three individual samples were used for each treatment and three biological replicate analyses were also performed.

After treatment with GA_3 and 6-BA concentrations of 50 mg/L and 25 mg/L individually by spraying the whole plant, the crowns were sampled at 48 h after treatment. Transcription level changes of four *FvWOX* family members, which are highly expressed in crown were then detected by qRT-PCR.

2.7. Prediction of the Regulatory Networks Represented by FvWOX3a

The interaction network of FvWOX3a, which was highly expressed in the crown of woodland strawberry, was predicted and constructed using the online tool STRING (https://cn.string-db.org/ (accessed on 15 April 2022)). Active interaction sources included text mining, experiments, databases, co-expression, neighborhoods, gene fusions and co-occurrences. The minimum interactive score was set at 0.400 [33].

2.8. Subcellular Localization Analysis

The pRI101-GFP vector was double digested with *Sal* I enzyme and *BamH* I enzyme, and the *FvWUSb* gene with the stop codon removed was inserted into the vector. The confirmed recombinant plasmid was then transformed into *Agrobacterium tumefaciens* (GV3101). Finally, the *Agrobacterium* containing *FvWUSb* was injected into the 5 weeks old tobacco leaves, and the pRI101-GFP empty vector was used as the control. After 48 h of incubation, the localization of FvWUSb was observed under a laser confocal microscope.

3. Result

3.1. Identification of WOX Genes in Fragaria vesca

A total of 16 genes were identified as the *WOX* members in *Fragaria vesca* and named as *FvWOX1-11* according to their homologous relationship with the *Arabidopsis AtWOX* genes (Supplementary Table S2).

The *FvWOXs* are unevenly distributed on 6 chromosomes of *Fragaria vesca* (Figure 1) and no *FvWOX* member was detected on chromosome Fvb6. Five of them are found on the third chromosome, which has the most members. There are 3 *FvWOX* genes on the first chromosome, 2 on the fourth chromosome, and 4 on the fifth chromosome. For the second and seventh chromosome, only 1 *FvWOX* gene was detected individually.

Although more *WOX* members were identified in *Fragaria vesca* than in *Arabidopsis*, the homologous genes of some *Arabidopsis AtWOXs* have not been identified in strawberry, such as the homologue of *AtWOX6* or *AtWOX7*. Detailed information for each of the *FvWOX* genes is shown in Table 1. Among them, most FvWOX proteins are classified as unstable proteins because their instability indexes are predicted to be more than 40. The predicted aliphatic index of each *FvWOX* protein is between 26.04 and 33.67. All *FvWOX* proteins are predicted to be hydrophilic according to their hydropathicity (GRAVY values < 0).

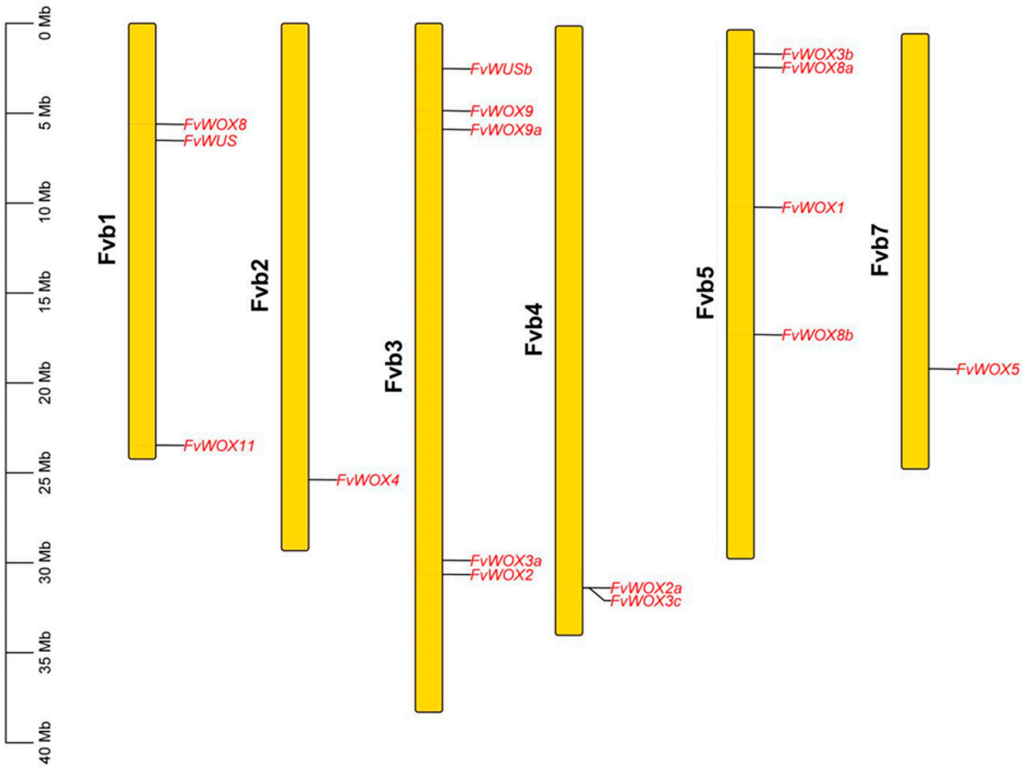

Figure 1. Chromosome localization map of *FvWOXs*. Yellow vertical bars with various lengths represent the woodland strawberry chromosomes. Short black horizontal lines indicate the position of each *FvWOX*.

Table 1. Summary information of *FvWOX* genes.

Name	Gene Locus ID	Number of Amino Acids	Molecular Weight (MW)	Isoelectric Point (pI)	Instability Index	Aliphatic Index	Hydropathicity (GRAVY)
FvWUS	FvH4_1g11910.1	891	73,066.77	5.09	60.27	26.04	−0.764
FvWUSb	FvH4_3g04400.1	885	72,820.74	5.09	59.21	27.68	−0.808
FvWOX1	FvH4_5g17270.1	1158	95,638.53	5.01	49.94	29.88	−0.936
FvWOX2	FvH4_3g35730.1	807	65,343.68	5.12	41.45	26.39	−0.728
FvWOX2a	FvH4_4g32650.1	795	65,214.18	5.12	46.02	31.32	−0.856
FvWOX3a	FvH4_3g34410.1	768	65,226.52	5.08	54.45	28.13	−0.947
FvWOX3b	FvH4_5g02150.1	972	79,499.19	5.08	54.78	32.2	−0.869
FvWOX3c	FvH4_4g32660.1	972	79,784.03	5.07	55.51	33.13	−0.931
FvWOX4	FvH4_2g34030.1	696	57,459.4	5.11	57.53	29.31	−0.958
FvWOX5	FvH4_7g24100.1	528	42,815.14	5.23	38.05	28.79	−0.663
FvWOX8	FvH4_1g10280.1	1173	94,627.87	5.07	36.63	30.86	−0.756
FvWOX8a	FvH4_5g03570.1	540	43,069.95	5.21	35.86	33.15	−0.843
FvWOX8b	FvH4_5g25470.1	600	47,987.51	5.19	39.52	33.67	−0.86
FvWOX9	FvH4_3g08310.1	1215	102,295.82	4.98	59.31	26.17	−0.929
FvWOX9a	FvH4_3g10130.1	939	78,480.07	5.04	58.91	24.49	−0.855
FvWOX11	FvH4_1g29830.1	840	69,986.65	5.09	39.08	25.48	−0.775

3.2. Phylogenetic Analysis and Multiple Sequence Alignment

A completely conserved HB domain is the typical feature of WOX proteins. Domain sequence alignment results revealed that the HB domain was detected in all the FvWOX proteins and the domains have 56 or 57 amino acids (Supplementary Figure S1). It was also found that 15 conserved amino acids were detected in the FvWOX HB domain. In *Arabidopsis thaliana*, there are 16 conserved amino acids in the HB domain, indicating that the HB domains of WOX are variable in different species.

In order to better understand the evolutionary and phylogenetic relationships of the WOX members among different species, a phylogenetic tree was constructed using the

known WOX members of *Arabidopsis*, tobacco, tomato, potato and the identified *Fragaria vesca* WOX proteins (Figure 2). According to the phylogenetic relationship listed in the tree, the FvWOXs could also be classified into three clades: the WUS clade, the ancient clade and the intermediate clade. As shown in the tree, FvWOX1-FvWOX5 belong to the WUS clade; FvWOX8, FvWOX8a and FvWOX8b are the ancient clade members; while FvWOX9, FvWOX9a and FvWOX11 are classified into the intermediate clade. It was also found that WUS clade is the largest one among the three clades.

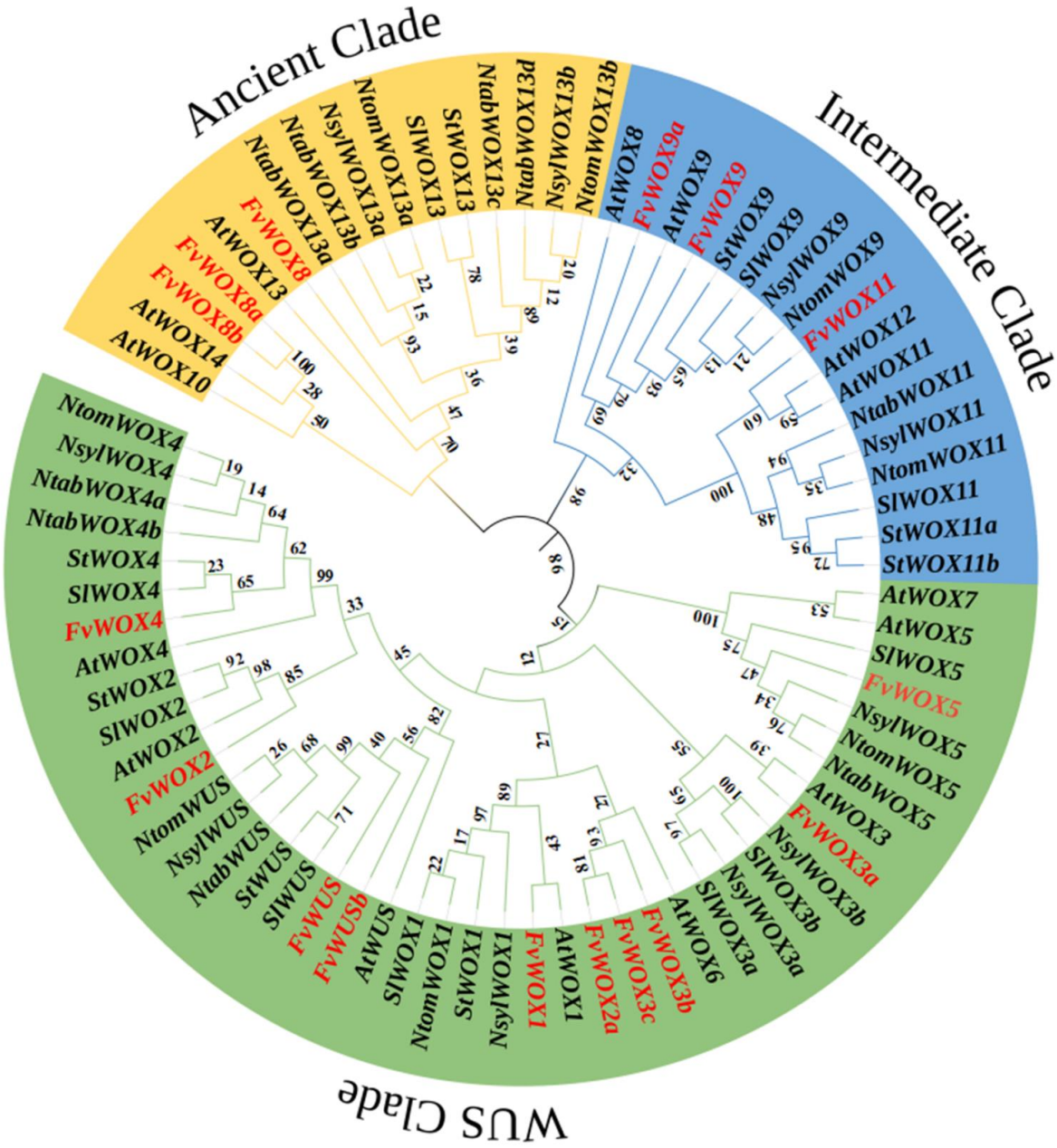

Figure 2. Phylogenetic analysis of WOX homologs in different species. All WOX proteins were divided into 3 clades (ancient, WUS, and intermediate), represented by clusters in different colors. The evolutionary history was inferred by the Maximum Likelihood method using MEGA7 software with 1000 bootstrap replicates. Fv, *Fragaria vesca*; At, *Arabidopsis*; St, *Solanum tuberosum*; Sl, *Solanum lycopersicum*; Ntab, *Nicotiana tabacum*; Ntom, *Nicotiana tomentosiformis*; Nsyl, *Nicotiana sylvestris*. WOX proteins of *Fragaria vesca* are marked in red font.

3.3. Conserved Motif and Gene Structure Analysis

To better understand the diversity of *FvWOX* genes, the conserved motifs in FvWOX proteins were analyzed with MEME. Result showed that there are totally 8 predicted conserved motifs (Figure 3A). The amino acid sequences of the identified 8 conserved motifs are shown in Supplementary Table S3. It was also found that motif1 and motif 2 could be detected in all FvWOX proteins, while motif 3 is unique to the members of the ancient clade, motif 4 is unique to the members of the WUS clade, and motif 5 is unique to the intermediate clade. Such characterization indicates that the same clade may have similar biological functions for their similar motif distributions. It is noteworthy that motif 6 is detected not only in the WUS clade members, such as *FvWOX2a*, *FvWOX3b* and *FvWOX3c*, but also in *FvWOX9* belonging to the intermediate clade. Further analysis showed that the HB domains (motif1+2) of most FvWOX proteins are located at the N-terminus (Figure 3B).

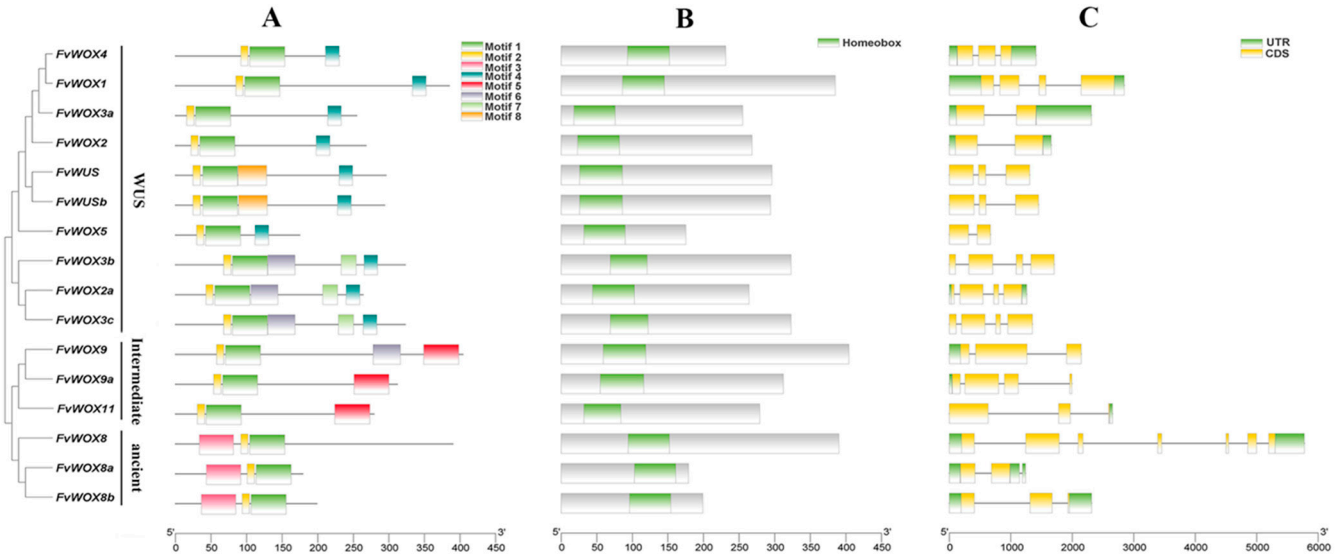

Figure 3. Protein and gene structures of FvWOXs. (**A**) Schematic diagram of the conserved motifs of FvWOX proteins. Different colored boxes represent different conserved protein motifs. (**B**) Positions of the HB domain in each FvWOX protein. (**C**) The exon-intron structures of *FvWOX* genes: the yellow box indicates the exon, the black line indicates the intron, and the green box indicates the untranslated 5′- or 3′-region.

Gene structure is an important factor that determines the relationship between genome evolution and the functional differentiation of multi-gene family members. Results showed that most *FvWOX* genes contain 1–3 introns (Figure 3C). *FvWOX3a*, *FvWOX5* and *FvWOX2* have only one intron, while six introns were detected in *FvWOX8*. Results also indicated that members with high homology have the similar exon-intron distribution structures, and the gene structures of the members belonging to the WUS clade were significantly more conserved than those of the other two clades.

3.4. Collinear Analysis of FvWOX Genes

Collinearity analysis is usually performed to identify the conservation of gene sequences and gene types among different species. The collinear analysis results of *FvWOXs* and their homologues in other species are shown in Figure 4; two genes connected by a line are syntenic genes.

In total, there are 33 syntenic *WOX* genes between *Fragaria vesca* and the other three species. Among them, 14 syntenic *WOX* genes were identified in *Arabidopsis*; 9 genes were identified in tomato, and 10 genes were identified in potato. Collinear analysis demonstrated that not all *FvWOX* members are orthologous to *WOX* family members of other species. This finding suggested that the duplicated genes may have been lost

during evolution, and also indicated that the divergence time of *WOX* family members might be after the divergence of *Fragaria vesca* and these species. There is only one pair of putative tandem duplication genes, on the Fvb4 chromosome of *Fragaria vesca*, suggesting that tandem duplications may contribute less to the expansion of the *WOX* gene family.

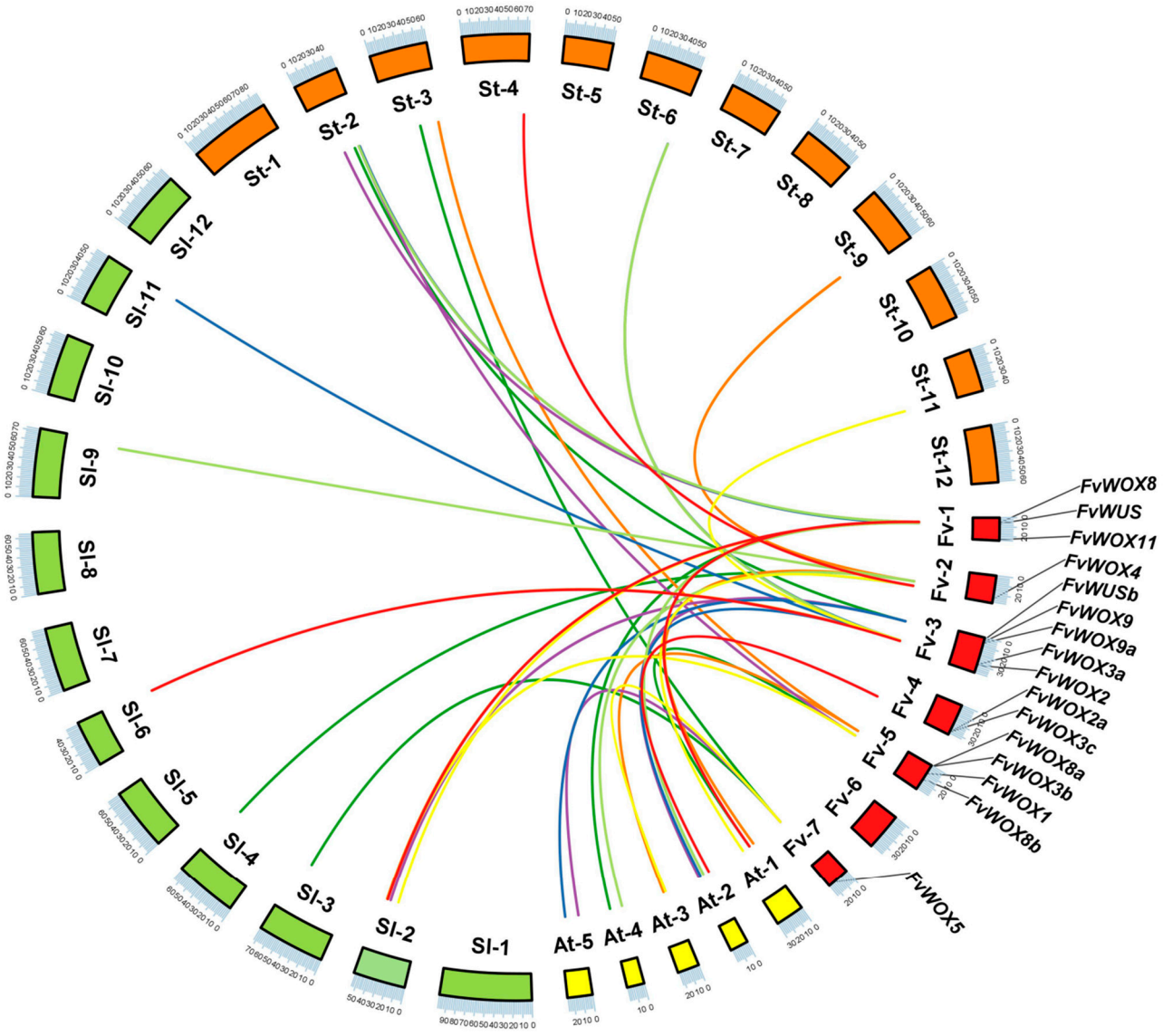

Figure 4. Synteny analysis of *WOX* genes between *Fragaria vesca* and three representative plant species. Genes connected by lines are homologous genes. Fv-1 to Fv-7: the chromosomes of *Fragaria vesca*. At-1 to At-5: the 5 chromosomes of *Arabidopsis*, Sl-1 to Sl-12: the 12 chromosomes of *Solanum lycopersicum*, St-1 to St-12: the 12 chromosomes of *Solanum tuberosum*.

3.5. Cis-Elements Analysis of FvWOX GENES

In total, 58 major *cis*-acting elements were identified in the 2000 bp upstream promoter regions of the *FvWOX* genes (Supplementary Figure S2), including 20 light-responsive elements, 13 hormone-responsive elements, 11 stress-responsive elements and 14 developmental-responsive elements. The light-responsive category has the maximum number of components. The main light-responsive elements are G-box and GT1-motif. The most common hormone-responsive element is the Ja-responsive element (MYC), while MYB is the most common stress-responsive element. Such results indicated that the transcription of *FvWOX* genes may be mainly regulated by light, hormones and stress signals.

3.6. Expression Patterns of FvWOXs in Different Woodland Strawberry Tissues

As shown in Figure 5, *FvWOX* genes are found to be widely expressed in various tissues, indicating their potential varied biological functions in different tissues. The *FvWOXs* members belonging to the ancient clade, such as *FvWOX8*, *FvWOX8a* and *FvWOX8b*, exhibited their expression in various tissues with lower expression levels. Members belonging to the intermediate clade, *FvWOX9* and *FvWOX9a*, showed their higher expression levels in flower, indicating their potential roles in the development of flowers.

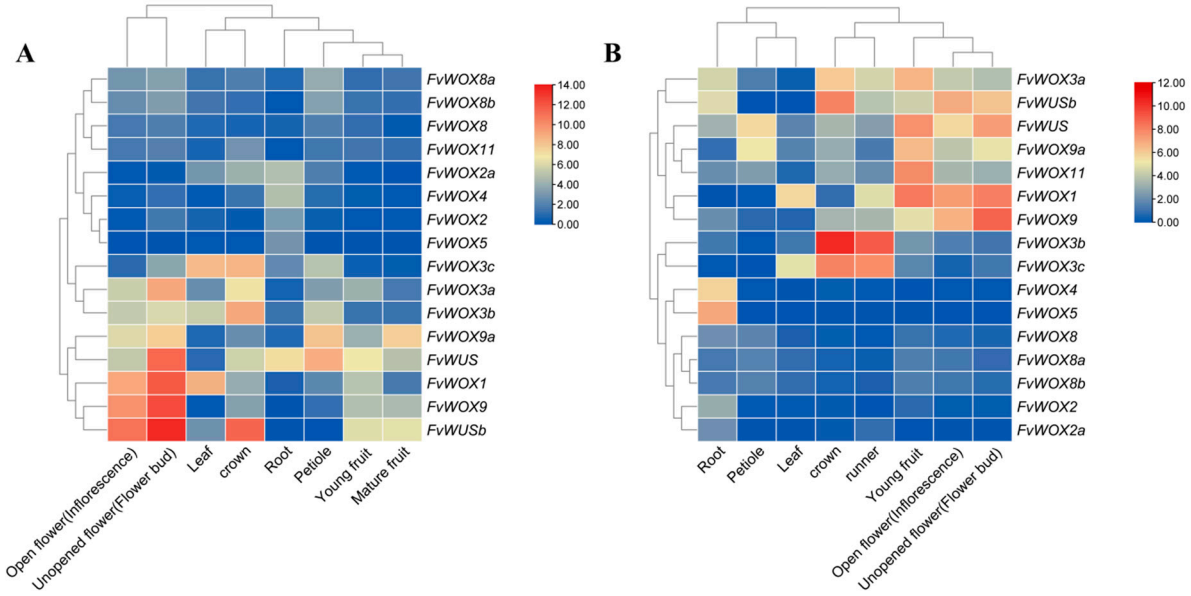

Figure 5. Tissue-specific expression of *FvWOXs* in two woodland strawberry varieties, Ruegen (**A**) and Hawaii 4 (**B**). The color scale represents gene expression levels. Blue indicates low expression level, red indicates high expression level. Clustering based on log2-transformed FPKM values of 16 *FvWOX* genes that are detected in at least one tissue based on the FPKM 95% confidence interval lower boundary greater than zero.

FvWOX3a, *FvWOX3b* and *FvWOX3c* are mainly expressed in crown and runner. *FvWOX4* and *FvWOX5* are highly expressed in roots and hardly expressed in other tissues. The expression level of *FvWOX1* is relatively high in young leaf, flower and fruit. Both *FvWOX2* and *FvWOX2a* are expressed at low levels in all tissues. *FvWUS* and *FvWUSb* are highly expressed in several tissues, especially in the crown and flower bud.

The *FvWOXs* showed a similar expression pattern in the tissues of the two woodland strawberry varieties. At the same time, the *FvWOXs* which were highly expressed in crowns, such as *FvWOX3b* and *FvWOX3c*, also had high expression levels in runners, indicating that such genes may play similar roles in these two tissues which are important for the agricultural traits.

3.7. FvWOXs Highly Expressed in Crown Are Regulated by 6-BA and GA_3

The results showed that after 6-BA treatment, expression of *FvWOX3a* and *FvWUSb* were significantly inhibited compared those in the control lines (Figure 6A), while the expression levels of *FvWOX3b* and *FvWOX3c* were significantly up-regulated compared to the control lines at 48 h (Figure 6A). With the GA_3 treatment, transcription levels of the four candidate genes were all up-regulated at different levels (Figure 6B). These results suggest that expression of those four *FvWOXs* could be regulated by such phytohormones.

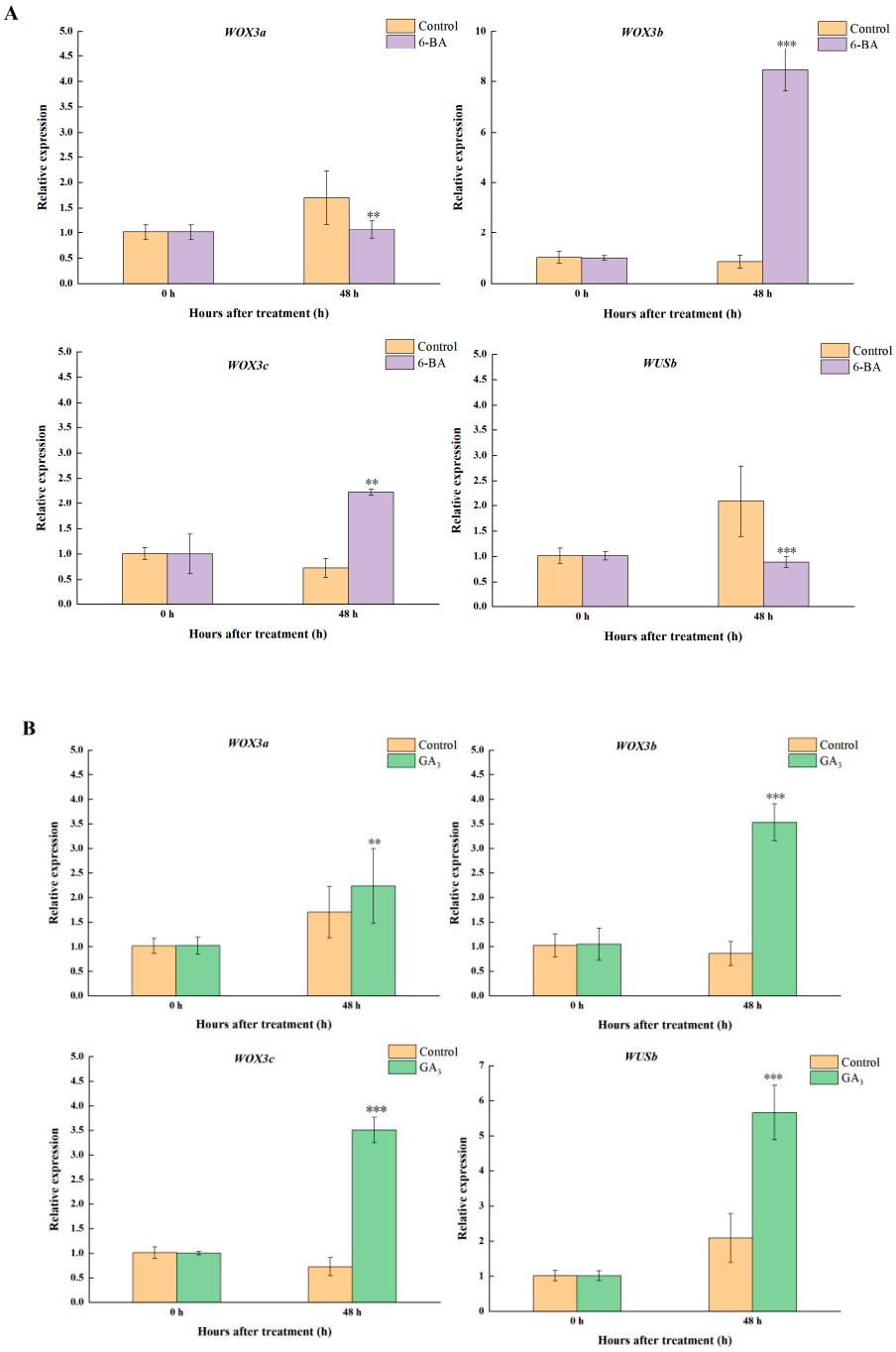

Figure 6. Expression changes of four *FvWOX* genes under 6-benzylaminopurine (6-BA) (**A**) and gibberellin (GA_3) (**B**) treatment. The crowns were taken as samples at 0 h and 48 h to detect the transcriptional level changes of the genes. Each value is the mean ± SD of three biological replicates. **, $p < 0.01$; ***, $p < 0.001$.

3.8. Subcellular Localization of FvWUSb

Considering the high expression level of *FvWUSb* in crowns of both varieties, the subcellular localization of its coded protein was further investigated. When injected with 35S:GFP, the GFP signal was detected throughout the entire cell. However, when injected with 35S:FvWUSb-GFP, the signal was restricted to the nucleus. Such results suggested that FvWUSb specifically accumulates in the nucleus (Figure 7).

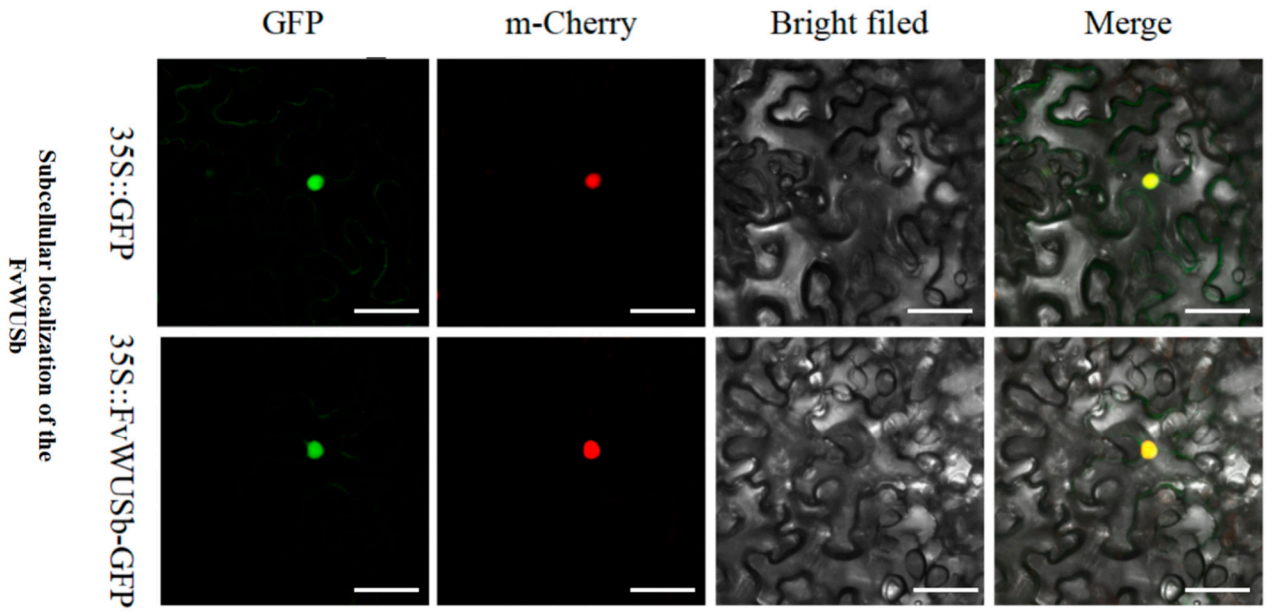

Figure 7. Subcellular localization of the *FvWUSb* protein. From left to right: GFP fluorescence, nuclear localization marker protein m-Cherry, bright field and merged microscope images. Scale bars = 25 μm.

3.9. Protein Interaction Network Prediction of FvWOX3a

In order to better understand the possible roles of the four *FvWOXs* highly expressed in the crown, STRING v11.0 (https://string-db.org/ (accessed on 15 April 2022)) was used to screen their possible interacting proteins.

The prediction (Supplementary Figure S3) indicated that *FvWOX3a* may interact with the proteins involved in the plant growth and development, such as the homologue of LBD protein (XP_004302187.1), the proteins involved in the light response, such as the homologue of SRR1 protein (XP_004300678.1), and the proteins involved in the secondary metabolism, such as the homologue of SWEET3 protein (XP_004308007.1). However, for other candidate proteins (*FvWUS3b*, *FvWUS3c* and *FvWUSb*), no possible interacting proteins were detected.

4. Discussion

The *WOX* gene family members are crucial for the normal growth and development of plants, which has been reported in many species [3,6,7]. In this study, we identified 16 different *FvWOX* genes from the woodland strawberry genome database. According to their phylogeny relationship, the 16 *FvWOXs* could be divided into three clades, which is consistent with findings of other researches such as in *Cucumis sativus* [34]. Like most species, the largest members were detected in the WUS clade [3,6]. Such findings indicate that *WOX* gene members of different species are somewhat conserved in the evolutionary process.

All FvWOX proteins are found to be hydrophilic unstable protein, which implies that they may have some similar functions based on their similar physicochemical properties. Specific motifs are conserved among the proteins belonging to certain branches and subclasses. The differences in the conserved motifs of each branch may account for the diversification of their functions; similar results were also reported in different species [3,6,7].

The HB domain contains a helix-loop-helix-turn-helix structure, which can recognize the sequence-specific targets in a precise spatiotemporal manner [35]. Furthermore, this domain is conserved across species, thus maintaining its functional integrity [36]. In this study, it was found that the HB domains of FvWOXs are composed of two conserved motifs, namely as Motif1 and Motif2. The two motifs were detected in all the FvWOX proteins, similar to the findings for cotton [6]. At the same time, results of the comparison

of their genetic structures and their domain amino acid sequences also support the above phylogenetic relationship and classification.

As a transcription factor, the AtWUS homolog FvWUSb was predicted to be localized in the nucleus by the website. Our results further demonstrated that FvWUSb is indeed nuclear localized, which is consistent with the localization of the TaWUS protein, the AtWUS homolog in wheat [3].

As a diploid species, few duplication events occurred in the genome of woodland strawberry. Our results did not find yet the existence of fragment duplication in *FvWOXs*. In the chromosomal mapping, *FvWOX2a* and *FvWOX3c* are adjacent genes on Fvb4. The adjacent homologous genes on a single chromosome are the most representative tandem duplication genes [37]; *FvWOX2a* and *FvWOX3c* may be the result of tandem duplication.

The expression pattern of one gene is usually related to its function. The *WUS* gene was originally identified as a master regulator required for *Arabidopsis* shoot and floral meristem integrity [38]. Meanwhile, *WUS* gene expression is restricted to a small fraction of cells in the central region of the shoot apical meristem (SAM), which is required to maintain the stem cell fate in *Arabidopsis* [8,39]. As the shoot apical meristem of strawberry, the crown can produce both flower buds and runners, which is of great significance to the production of strawberries [40]. It is important to identify whether any *FvWOX* members are involved in this process. As the homolog of *AtWUS*, *FvWUSb* was detected to be highly expressed in the crown. At the same time, *FvWOX3a*, *FvWOX3b* and *FvWOX3c*, the homologs of *AtWOX3*, were also detected as having high expression levels in the crown. *AtWOX3-like* genes have been reported as having highly conserved functions in the development of lateral margins of vegetative and floral organs [12,13]. Whether the homologues showing high expression in the crown of *Fragaria vesca* have the same function remains to be elucidated.

Interestingly, most of the four genes highly expressed in the crown were also found to have high expression levels in the runner tips. It can be speculated that these genes may play similar roles in the SAM differentiation of the woodland strawberry.

Expression profiles of some *FvWOXs* in the flower organs of woodland strawberry have been reported by Cao et al. using transcriptional data [41]. Similar results are also found in our qRT-PCR expression measurement in the flower organs. In this study, two woodland strawberry varieties with or without runner, "Hawaii 4" and "Ruegen", were used further to characterize the expression profiles of *FvWOXs*. The results (Figure 5) revealed that there was little difference in the expression profiles of most family members between the two varieties, except that *FvWOX11* was expressed to a certain extent in the young fruit of "Hawaii 4"and hardly expressed in the young fruit of "Ruegen". This may be related to the specificity between the varieties, and the mechanism of such difference remains to be elucidated.

Previous reports indicated that *WOX5* of *Arabidopsis* interacts with *HAM2*, which is expressed in the root apical meristem (RAM) and involved in shoot control and maintenance, together with the root stem cell niche [42,43]. Our results also suggest that the *WOX5* homolog of *Fragaria vesca*, *FvWOX5*, is specifically expressed in the root. The homology of the two proteins was 57.37%, indicating that the function of *WOX* gene was conserved among different species, but the specific function and regulatory mechanism of *FvWOX5* in the strawberry remain to be elucidated.

At the same time, it was also found that, compared with members of the other two branches, members of the ancient clade are not expressed or are expressed at very low levels in all tissues of both varieties. Such findings were also reported in other species such as soybean [7]. Such results may suggest that these members may be expressed in some undetected tiny tissues or their expression may be induced by specific conditions, such as stresses.

Plant hormones are small molecular signals that control many aspects of plant development and physiology. Recent studies have shown that gibberellin and cytokinin signaling pathways can regulate shoot apical meristem and influence the expression of *WUS-like*

genes [44]. Special roles are reported for these two kinds of hormones. In the strawberry, gibberellin can promote the multiplication of runners and the elongation of stems, which are essential to the yield and propagation [45]. It has also been reported that application of 6-BA could promote flower bud differentiation [46]. In this study, the expression detection result (Figure 6) indicated that the application of 6-BA induced the expression of *FvWOX3b* and *FvWOX3c* at 48 h after spraying. However, the expression of *FvWOX3a* and *FvWUSb* was inhibited. Previous studies have shown that the type B proteins of *Arabidopsis* response regulator (ARR) can activate the expression of genes encoding type A, such as ARRS, ARR7 and ARR15, which are blockers of the CK signaling pathway and then downregulate the expression of *WUS* [44]. This is consistent with our finding that expression of *FvWUSb* was inhibited by 6-BA treatment.

Although expression levels of these members were found to be regulated by cytokinin treatment, no cytokinin-responsive elements were detected among the predicted putative regulatory elements (Supplementary Figure S2). This result indicates that the cytokinin response elements may be outside the analyzed 2000 bp promoter region, or the cytokinin may first regulate other upstream target genes and then affect the expression of these *WOX* genes.

Studies have shown that *WOX* transcription factors play a role in the signaling pathways of plant hormones such as gibberellins [47]. At the same time, previous studies suggested that gibberellins activity may control the induction of cell division in the apical tissue of the crown and act as one of the signals that determine the fate of the bud [48]. Expression of the detected four genes which are highly expressed in the crown were also somewhat up-regulated after GA_3 treatment. Our study revealed that expression of these *WOX* members in woodland strawberry crown could be regulated by such plant hormone signals; the expression changes may subsequently affect the differentiation fate of the SAM in the crown.

Taken together, our research has laid the foundation for subsequent researches and provided a useful resource for further study about the function of *WOX* genes in strawberries or other *Rosaceae* plants.

5. Conclusions

In this study, 16 *Fragaroa vesca WOX* members were identified and characterized by systematic bioinformatics analysis. Among these *FvWOX* members, the candidates that highly expressed in crown were identified, and further work showed that the transcription of such genes could be affected by GA_3 or 6-BA. Subcellular localization showed that FvWUSb protein is localized in the nucleus. This research provides the basis for further studies of the strawberry *WOX* family, and established a method for the identification and characterization of the *WOX* genes in other species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8111043/s1, Figure S1: Sequence alignments of the HB domains of FvWOXs; Figure S2: Prediction of *cis*-acting element components of *Fragaria vesca WOX* genes promoter; Figure S3: The interaction network prediction of FvWOX3a with other proteins; Table S1: Primer sequences usd in qRT-PCR; Table S2: List of *FvWOX* genes; Table S3: The amino acid sequences of 8 conserved motifs.

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