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Molecular Cloning, Characterization, and Expression Analysis of SIMILAR TO RCD-ONE (SRO) Family Genes Responding to Abiotic and Biotic Stress in Cucumber

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Abstract: SIMILAR TO RCD-ONE (SRO) is a plant-specific small protein family that controls many biological processes including physiological development and stress responses. The *SRO* gene family has been studied in several plant species, but no detailed characterization and expression profiles of this important gene family were performed in cucumber. In this study, we characterize the *SRO* genes in cucumber, and determined their transcript levels in various tissues and under exposure to diverse biotic and abiotic stressors. Four *SRO* genes (named as *CsSRO1–CsSRO4*) were identified and isolated, which were distributed on three different chromosomes. Gene duplication analysis showed that only one pair of segmental duplication event was identified, but no tandem duplication events were detected. All CsSROs consist of the PARP domain and a C-terminal RST domain, while the N-terminal WWE domain was only present in CsSRO2 and CsSRO4. SROs from 15 plant species are divided into two groups (I and II), and group I can be further divided into four subgroups (Ia to Id) according to the phylogenetic tree. The conserved motif and gene structure analyses showed that SROs within the same branch of the phylogenetic tree have analogous conserved motifs configuration and gene structures. However, *SRO* genes possessed variable numbers of introns in different subgroups, which may affect the evolution of new family members. RNA-Seq data and qRT-PCR results showed that the four *CsSRO* genes have distinct expression pattern in various tissues and under diverse stresses, suggesting their multiple functions in plant growth and stress responses. The findings provide a basis for further research aiming at functional characterization of the regulatory mechanism to reveal the roles of *CsSRO* genes in developmental and stress-related processes of cucumber.

Keywords: cucumber; *SRO* gene; stress response; gene expression; gene family

Citation: Xiao, L.; Zhou, Z.; Zhu, C.; Zhao, J.; Hu, Z.; Liu, S.; Zhou, Y. Molecular Cloning, Characterization, and Expression Analysis of SIMILAR TO RCD-ONE (SRO) Family Genes Responding to Abiotic and Biotic Stress in Cucumber. *Horticulturae* **2022**, *8*, 634. https://doi.org/10.3390/horticulturae8070634

Academic Editors: Giuliana Maddalena, Annamaria Mincuzzi, Francesca Garganese and Antonio Ippolito

Received: 19 June 2022
Accepted: 7 July 2022
Published: 13 July 2022

1. Introduction

Plants have produced a series of regulatory networks through the transcriptional control of numerous stress-responsive genes to protect themselves from adverse environmental effects [1,2]. Transcription factors (TFs) are a class of proteins that can bind to the *cis*-regulatory elements in promoters of target genes and thus to activate or inhibit their expression, thereby regulating plant development and the response to various stresses [3,4]. SIMILAR TO RCD-ONE (SRO) is a unique type of small TF proteins in plants, which can act as key regulators involved in regulation of many biological pathways. Reports have found that SRO proteins consist of a conserved poly (ADP-ribose) polymerase catalytic center (PARP domain, PF00644) and a C-terminal RCD-SRO-TAF4 domain (RST domain, PF12174) that interacts with other TFs. Some SROs also contain a WWE domain (PF02825)

at the N-terminus, which takes part in ADP ribosylation (ADPRylation) and ubiquitination of proteins [5–7].

The first identified *SRO* family member, *RADICAL-INDUCED CELL DEATH1* (*RCD1*) in *Arabidopsis*, was able to complement the yeast *yap1(-)* mutant which has defects in oxidative stress sensitivity, suggesting that it might participate in oxidative stress [8]. AtRCD1 also has a role in salt tolerance through physically interacting with the plasma membrane localization Na^+/H^+ antiporter SALT OVERLY SENSITIVE 1 (SOS1) [9]. The *rcd1* mutants exhibited pleiotropic phenotypes such as the aberrant leaf, root and rosette development, early flowering, and altered resistance to diverse abiotic stresses and hormones response [10–15]. Subsequently, five homologs (named as AtSRO1–5) of AtRCD1 were also identified in *Arabidopsis*. AtSRO1 is the closest homolog of AtRCD1, both of them contained WWE domain at the N-terminus and possessed similar expression patterns, but they play unequally redundant and independent roles in certain developmental processes and the response to various stresses [14,16–18]. For example, *sro1-1* mutants displayed only unconspicuous morphological phenotypes, while *rcd1-3 sro1-1* double mutants showed even more extreme phenotypes than *rcd1-3* [13,14]. Additionally, *rcd1* and *sro1* mutants also showed different responses to oxidative stress [13], but they respond similarly to $HgCl_2$ and H_2O_2 stress [19]. Another three *SRO* family members, *AtSRO2*, *AtSRO3,* and *AtSRO5*, displayed changed transcriptional levels under strong light, salt, $HgCl_2$, H_2O_2, and ozone stress conditions. In addition, overexpression of *AtSRO5* conferred salt tolerance of transgenic plants [18–20].

Besides *Arabidopsis*, *SRO* genes have also been extensively identified and functionally studied in other plant species. In rice, *OsSRO1c* functions as a core regulator to mediate abiotic stress responses via direct interaction with various TFs, such as SNAC1 (stress-responsive NAC 1) and DST (drought and salt tolerance) [21,22]. As an allele of *OsSRO1c* from the *indica* rice cultivar (*Oryza sativa* ssp. *indica*), *BROWNING OF CALLUS1* (*BOC1*) can improve genetic transformation efficiency probably through inhibiting programmed cell death caused by oxidative stress, and thus reducing callus browning [23]. Another SRO member in rice, OsSRO1a, negatively regulates the rice-*Xanthomonas oryzae* interaction via the OsMYC2-mediated jasmonic acid (JA) signaling pathway [24]. A bread wheat (*Triticum aestivum*) *SRO* gene *TaSRO1* showed promoted plant growth and multiple abiotic stress tolerance by controlling redox homoeostasis in transgenic wheat and *Arabidopsis* lines [25]. Overexpression of maize *ZmSRO1b* in *Arabidopsis* also elevated the tolerance of plants to salt, cadmium, and oxidative stress [26]. In contrast, *ZmSRO1e*-overexpressing maize and *Arabidopsis* plants exhibited increased sensitivity to oxidative and salt stress with reduced anthocyanin accumulation through disrupting the MYB–bHLH–WD40 (MBW) complex [27]. These findings demonstrated that *SRO* genes play vital roles in plant development, and response to multiple biotic and abiotic stresses.

Cucumber is an important vegetable crop with great economic value in the world, but it always encounters a variety of environmental stimulus (such as drought, salt, and pathogens) during its growth cycle [4,28]. In recent years, many stress-responsive TF gene families have been identified in cucumber, such as GARS [29,30], bHLH [31], WRKY [32], VQ [33], and TIFY [34], and some members of them were proven to function in stress responses [35,36]. However, no report has been carried out on the *SRO* family genes in cucumber until now. To elucidate whether the cucumber *SRO* family genes participate in stress response, we cloned and characterized four cucumber *SRO* genes, and the phylogenetic relationship, conserved motif, gene structure, and promoter region were analyzed by some routine bioinformatics tools. Additionally, the transcriptional levels of cucumber *SRO* genes under salt and drought stress conditions, as well as under the infection of *Pseudoperonospora cubensis* and *Meloidogyne incognita*, were also determined by qRT-PCR and RNA-seq analyses. Our findings provide a solid base for the classification and expression of the *SRO* family genes and help to facilitate further functional characterization of these genes in cucumber.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Stress Treatments

The Chinese Long cucumber inbred line 9930 plants were employed for stress treatments in this work. The seeds were soaked in water at 55 °C for 15 min, then placed on damp filter paper overnight in an artificial climate incubator at 28 °C to promote germination. After the cotyledons were fully opened, the seedlings were moved to Hoagland's nutrition for cultivation. Cucumber seedlings were soaked in Hoagland nutrient solution for hydroponics with a 16:8 h light: dark photoperiod. We transferred the two-leaf stage seedlings to a nutrition solution containing NaCl (200 mM) and PEG6000 (10%), for salt stress and drought stress, respectively, as described previously [4]. The leaf tissues were harvested for each stress condition with three biological replicates at 0, 3, 6, and 12 h after treatment. Subsequently, these samples were stored in a −80 °C freezer for RNA extraction until further experiments.

2.2. Identification and Cloning of CsSRO Genes of Cucumber

The Hidden Markov Model (HMM) profiles of the RST domain (PF12174) and PARP domain (PF00644) were downloaded from the Pfam database (http://pfam.xfam.org/, accessed on 19 June 2022) [37], and these HMM profiles were employed to search against the cucumber protein database downloaded from Cucurbit Genomics Database (CuGenDB) (http://cucurbitgenomics.org/, accessed on 19 June 2022) using HMMER software. We also searched the cucumber *SRO* family members using the BLASTP with the published *Arabidopsis* and rice SRO protein sequences as query sequences. After removing the redundant sequences obtained from the above methods and checking through Pfam and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/, accessed on 19 June 2022), we get four candidate SRO protein sequences, and their coding sequences (CDS) and corresponding genomic DNA (gDNA) sequences were also obtained from CuGenDB.

Total RNA was isolated from the leaf samples of normally growing and stress-treated seedlings using RNA-easy^{TM} Isolation Reagent Vazyme cat (Vazyme Biology Co., Ltd., Nanjing, China), and the first strand cDNAs were synthesized using the HiScript$^{®}$II Q RT SuperMix for qPCR (+gDNA wipe) (Vazyme Biology Co., Ltd., Nanjing, China) on the basis of to the manufacturer's instructions. The cDNA samples were diluted 10 times, and used as the template for PCR amplification. The PCR amplification was carried out at: denaturation at 95 °C for 3 min; 95 °C for 1 min, 60 °C for 15 s, and 72 °C for 3 min (35 cycles); followed by 72 °C for 5 min. The PCR products were detected by 1.0% agarose gel electrophoresis and subsequently sequencing. Primer sequences used for PCR amplification of each *CsSRO* gene are listed in Table S1.

2.3. In Silico Sequence Analyses of Cucumber SRO Family Members

The physicochemical characteristics of cucumber SRO proteins, including molecular weight (MW), isoelectric point (pI), and grand average of hydropathicity (GRAVY), were calculated using the ProtParam tool on the ExPASy server (http://web.expasy.org/protparam, accessed on 19 June 2022) [38]. The subcellular location of cucumber SRO proteins was predicted using CELLO v.2.5 (http://cello.life.nctu.edu.tw/, accessed on 19 June 2022) [39]. The conserved domains including PARP, RST, and WWE were examined through Pfam and InterProScan. The physical locations of *CsSRO* genes were illustrated with the MapInspect software according to the initial position information provided in CuGenDB. Moreover, these genes involved in the tandem or segmental duplications were analyzed with MCScanX on the basis of the criterion in our previous report [4]. The 2000 bp promoter sequences located upstream of the initiation codon (ATG) of the cucumber *SRO* genes were retrieved from CuGenDB, and various *cis*-regulatory elements related to stresses and hormones were determined using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 19 June 2022) [40].

2.4. Phylogenetic, Conserved Motif and Gene Structure Analyses of SRO Family Members of Cucumber and Other Plant Species

The full-length protein sequence alignments of all *SRO* family members from cucumber and different plant species were performed using the MAFFT tool under default settings, and then MEGA version 7.0 was used to construct phylogenetic trees by the Neighbor-Joining (NJ) methods and the bootstrap analysis conducted with 1000 replications [41]. MEME (http://meme-suite.org/tools/meme, accessed on 19 June 2022) was employed to examine the conserved motifs of SRO proteins of cucumber and other plant species with the default settings except the number of diverse motifs was chosen as 10 [42]. The gene structures of the *SRO* genes of cucumber, *Arabidopsis*, maize, rice, and grape were determined with the GSDS 2.0 tool (Gene Structure Display Server, http://gsds.gao-lab.org/, accessed on 19 June 2022) according to their CDS and the correspondent full-length gDNA sequences [43].

2.5. qRT-PCR Analysis of CsSRO Genes in Response to Abiotic Stress

The quantitative RT-PCR was conducted using the Roche Lightcyler 480II PCR System based on the manufacturer's instructions of the ChamQ Universal SYBR qPCR Master Mix Kit (Vazyme Biology Co., Ltd., Nanjing, China). The total qRT-PCR mixture was 20 µL including 10 µL 2 × ChamQ Universal SYBR qPCR Master Mix, 0.4 µL forward primer, 0.4 µL reverse primer, 7.2 µL ddH_2O, and 2 µL template cDNA. The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. All reactions were performed in triplicate, and *CsAct3* was used as the reference gene to determine the relative expression with the $2^{-\Delta\Delta Ct}$ method [4]. qRT-PCR-specific primers used for expression analysis of each *CsSRO* gene were listed in Table S1.

2.6. Expression Analysis of CsSRO Genes Based on RNA-seq

The expression data of *CsSRO* genes in various tissues, including root, leaf, stem, female and male flowers, ovaries, tendril, and the base of tendril, were retrieved from the RNA-seq in NCBI under the accession number of PRJNA80169. The expression data of *CsSRO* genes in different biotic stresses, including the infection of *P. cubensis* (downy mildew treatment) and *M. incognita* (root-knot nematode treatment), were available in the NCBI under the accession numbers of PRJNA388584 and PRJNA419665, respectively. Based on all expression values, TBtools was used to generate expression heatmaps using the $\log_2$ transformed transcripts per million reads (TPM) values, based on the methods in our previous studies [4,44].

3. Results

3.1. Cloning and Identification of SRO Gene Sequences of Cucumber

A total of four *SRO* genes were identified in cucumber, and they were numbered from *CsSRO1* to *CsSRO4*, on the basis of their chromosomal locations (Table 1). To verify the accuracy of sequences acquired from the cucumber genome, we designed the specific primers based on the CDS sequences of *CsSRO* genes. The 1167 bp, 1398 bp, 1806 bp, and 1863 bp CDSs of the *CsSRO* genes were amplified, by using cDNA from cucumber leaf tissues as a template (Figure S1). Sequencing of PCR products confirmed the coding sequences of *CsSRO* genes, suggesting the reliability of the sequence information of the above identification (Table 1). The physicochemical characteristics including sequence length, MW, pI, and GRAVY values of CsSROs are provided in Table 1.

Table 1. Identification and characterization of *SRO* family genes in cucumber.

Gene	Locus ID	Chromosomal Position	Protein					gDNA (bp)	CDS (bp)
			Length (aa)	MW (Da)	pI	GRAVY	Subcellular Prediction		
CsSRO1	Csa1G046060	Chr1: 5287727-5290312	388	43,102.56	7.75	−0.511	Nucleus	2586	1167
CsSRO2	Csa2G372800	Chr2: 18564803-18569546	465	51,712.31	9.33	−0.324	Mitochondria/Nucleus	4744	1398
CsSRO3	Csa3G008310	Chr3: 918184-923735	601	67,660.98	5.79	−0.373	Nucleus	5552	1806
CsSRO4	Csa3G901000	Chr3: 38763905-38773671	620	70,266.98	7.55	−0.502	Nucleus	9767	1863

The basic characteristic analysis showed that the gDNA and CDS lengths of four *CsSRO* genes varied in size, with gDNA lengths varied from 2586 bp (*CsSRO1*) to 9767 bp (*CsSRO4*) and CDS lengths ranged from 1167 bp (*CsSRO1*) to 1863 bp (*CsSRO4*) (Table 1). Their gene products had molecular weight (MW) and isoelectric points (pI) with MW ranging from 43,102.56 Da (CsSRO1) to 70,266.98 Da (CsSRO4) and pI ranging from 5.79 (CsSRO3) to 9.33 (CsSRO2). The GRAVY values of CsSROs ranged from −0.511 (CsSRO1) to −0.324 (CsSRO2), which were less than zero, suggesting that the CsSRO proteins were hydrophilic proteins. The subcellular localization prediction results showed that CsSRO1, CsSRO3, and CsSRO4 were predicted to be localized in the nucleus, while CsSRO2 might be located in mitochondria and nucleus (Table 1).

3.2. Phylogenetic Analysis of SRO Family Genes from Cucumber and Different Plant Species

To investigate the phylogenetic relationship and divergence of the *SRO* family in cucumber, a phylogenetic tree was created on the basis of multiple sequence alignment of protein sequences of *SRO* gene family from cucumber and other 14 different plant species. As shown in the phylogenetic tree, these SRO proteins were irregularly divided into two groups, namely group I and group II, and group I can be further divided into four subgroups (Ia to Id) (Figure 1). Consistent with previous reports [45,46], the SRO proteins of these species were clustered in dicot- or monocot-specific patterns. For example, group II had only dicotyledonous SRO proteins, while group I contained both monocotyledonous and dicotyledonous SRO proteins. For CsSRO proteins, CsSRO3 and CsSRO4 were clustered in group Ic, while CsSRO3 and CsSRO4 were clustered in group Id and group II, respectively (Figure 1).

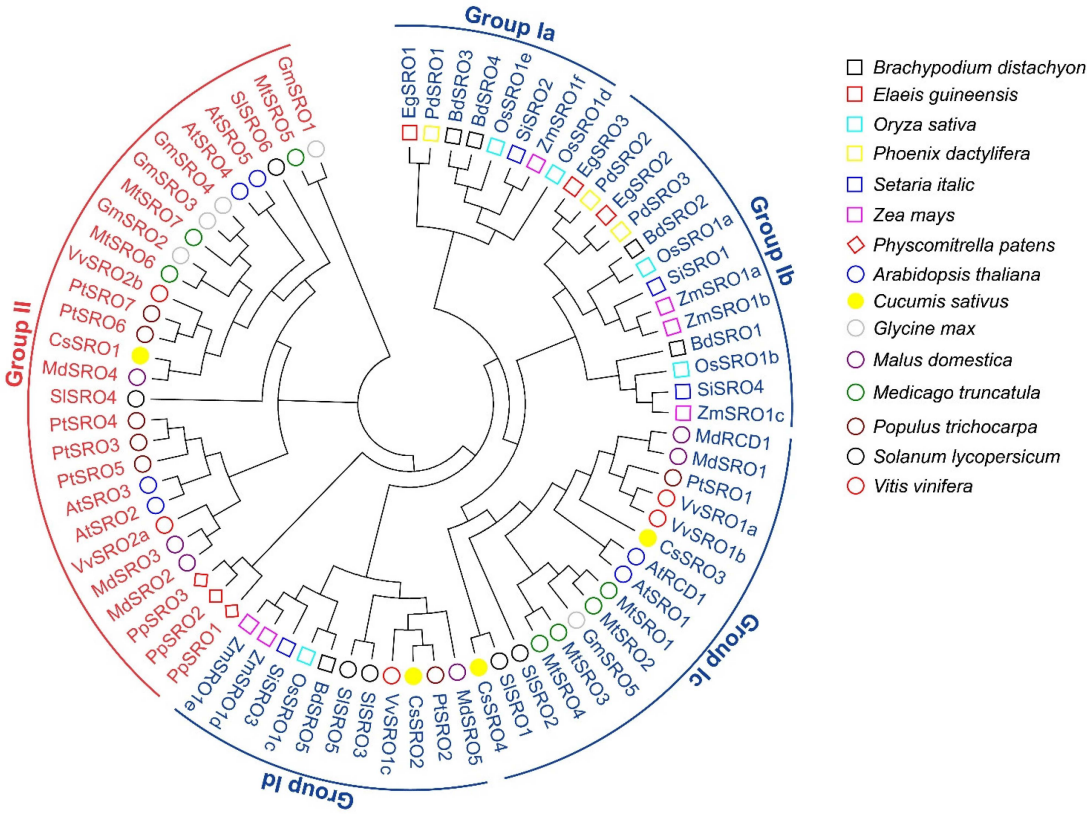

Figure 1. Phylogenetic relationships of *SRO* family genes from cucumber and 14 other plant species. The phylogenetic tree was created with MEGA 7.0 using the Neighbor-Joining (NJ) method with bootstrap analysis performed using 1000 replicates. Two groups, namely group I and group II, are colored with blue and red, respectively, and the colored circles, rhombuses and squares represent different plant species.

3.3. Conserved Motifs of SRO Proteins from Cucumber and Other Plant Species

Domain analysis by Pfam and InterProScan tools showed that all CsSROs consist of the PARP domain and a C-terminal RST domain, while the N-terminal WWE domain was only present in CsSRO2 and CsSRO4 (Figure S2). To further investigate the diversification of SRO proteins, the conserved motif analysis of SRO proteins from cucumber and other plant species was performed based on the results of MEME, and a phylogenetic tree was also created. The results showed that 10 conversed motifs were predicted and their details are shown, and the SROs in the same group had similar motif composition (Figure 2). Motifs 9, 4, 8, 2, and 1 made up the PARP domain, which were widely distributed in a large proportion of SRO proteins, with the exception of several SROs, such as OsSRO1d and VvSRO1c (lack of motif 2), ZmSRO1d, ZmSRO1e, and AtSRO3 (lack of motif 9). Motifs 7 and 5 were annotated as the RST domain, which were present in most SRO proteins, except for CsSRO2, OsSRO1d, VvSRO1c, ZmSRO1d, and ZmSRO1e. In addition, motif 3 and motif 6 were present in the N-terminal region of all SRO proteins of group I, while they were absent in group II SRO proteins. Motif 10 was specific to SRO proteins of group Ia and Ib, as well as present in most members of group II, with the exception of AtSRO4 and AtSRO5 (Figure 2).

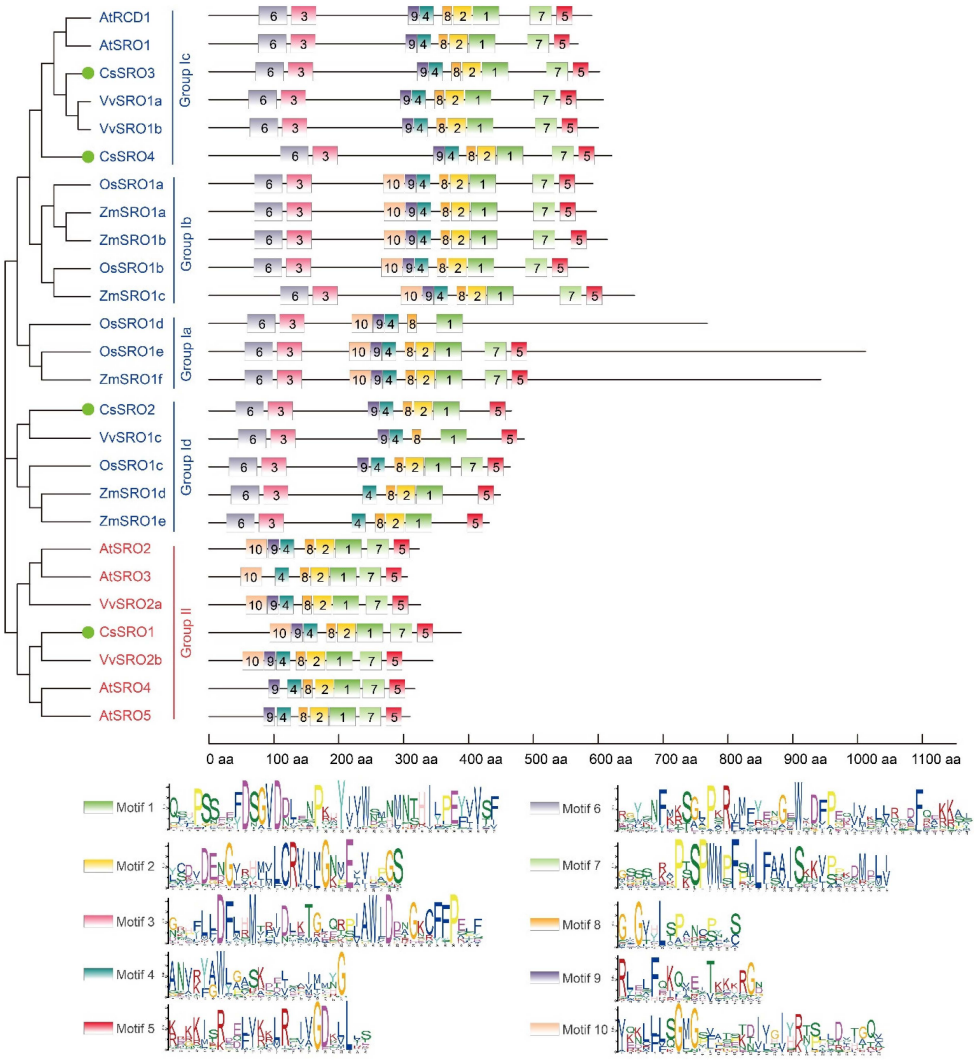

Figure 2. Conserved motif configurations of the *SRO* family members from cucumber, *Arabidopsis*, maize, rice, and grape on the basis of phylogenetic relationship. The clustering was shown according to the results of phylogenetic analysis. The colorful boxes delineate different motifs (numbered 1–10) and their details were shown at the bottom of the figure.

3.4. Gene Structure of SRO Family Genes

A detailed *SRO* structure analysis was performed by comparing the CDS sequences with the corresponding gDNA sequences of *SRO* family genes from cucumber, *Arabidopsis*, maize, rice, and grape. As a result, the number of introns in these *SRO* genes ranged from 3 to 7, and the structural characteristics of *SRO* genes clustered together were similar but varied among different groups (Figure 3). Notably, *SRO* genes in group Ia were found to have 4 or 7 introns, group Ib *SRO* genes had 5 or 6 introns, group Ic *SRO* genes harbored 4–7 introns, group Id *SRO* genes contained 3 introns each, while group II *SRO* genes were found to have 3 or 4 introns (Figure 3). The numbers of introns in four *CsSRO* genes were 4, 3, 5, 7, respectively.

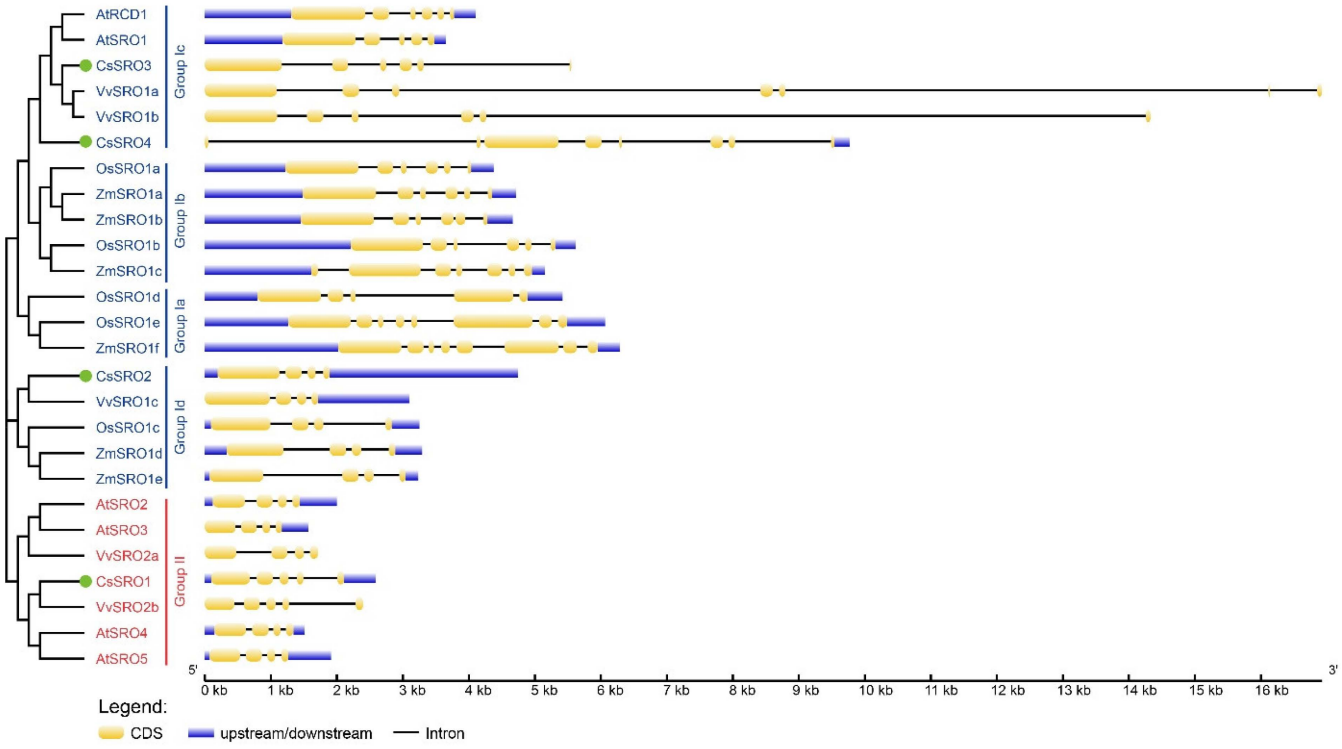

Figure 3. The gene structures of *SRO* family genes from cucumber, *Arabidopsis*, maize, rice, and grape on the basis of phylogenetic relationship. The clustering was shown based on the results of phylogenetic analysis. The boxes colored blue and yellow indicate upstream/downstream and CDS, respectively, the black line indicates intron.

3.5. The Distribution and Gene Duplication of CsSRO Genes

Chromosomal mapping of *CsSRO* genes showed that these genes were located on 3 out of 7 chromosomes of the cucumber genome. Chromosome 3 had the most *CsSRO* genes (two), whereas chromosomes 1 and 2 each possessed only one gene (Figure 4). The analysis of the duplication event indicated that a segmental duplication event was identified (*CsSRO3* and *CsSRO4*), while no tandem duplication event was detected (Figure 4).

3.6. Bioinformatics Analysis of Putative Promoters of CsSRO Genes

The *cis*-regulatory elements within the promoter regions of genes would provide valuable information for their expression and possible regulatory patterns. Therefore, the PlantCARE tool was employed to analyze the stress- and hormone-related *cis*-regulatory elements within the promoter regions of the *CsSRO* genes, and 16 kinds of *cis*-regulatory elements were identified (Figure 5). Eight kinds of *cis*-regulatory elements were found to be responsive to various hormones, such as ABRE for abscisic acid (ABA), TGACG-motif and CGTCA-motif for methyl jasmonate (MeJA), TGA-element for auxin, ERE for ethylene, TCA-element for salicylic acid (SA), P-box and TATC-box for gibberellin. It is worth noting

that ABRE was most abundant in the promoter sequences of *CsSRO2*, implying that it may play a critical role in responsive to ABA. In addition, eight kinds of *cis*-regulatory elements are stress-responsive, including ARE (anaerobic induction), GC-motif (anoxia), LTR (low-temperature responsive), STRE (stress-responsive), W-box (fungal elicitors), TC-rich repeats (defense and stress response), WRE3 and WUN-motif (wounding responses) (Figure 5).

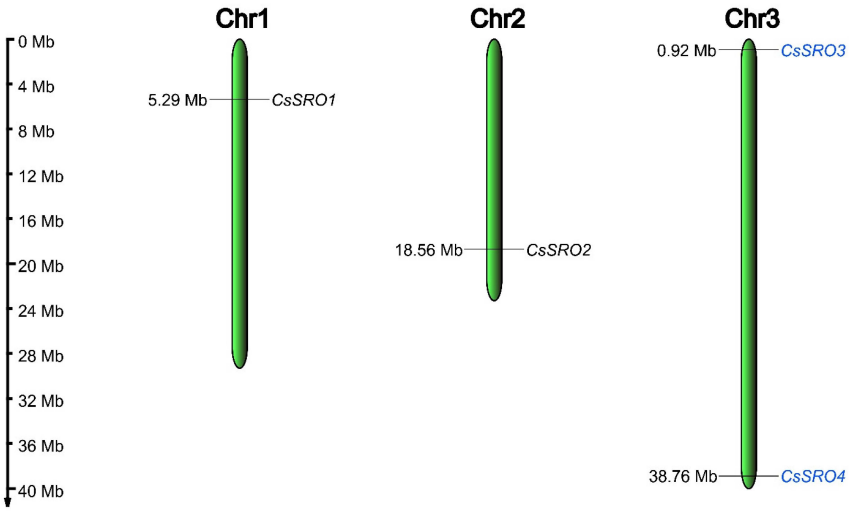

Figure 4. Distribution of *CsSRO* genes on cucumber chromosomes. The segmental duplication genes are colored with blue. The chromosome numbers are labeled on the top of the bars, and the chromosome length represents the size of chromosomes. Positions are in Mb.

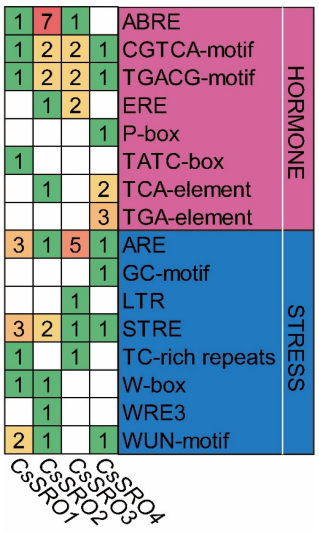

Figure 5. Stress- and hormone-responsive *cis*-regulatory elements in the promoter regions of *CsSRO* genes. The number in box represents the copy of the cis-element of individual *CsSRO* genes. ABRE, *cis*-element involved in the abscisic acid (ABA) responsiveness; CGTCA-motif and TGACG-motif, *cis*-element involved in the MeJA responsiveness; ERE, ethylene-responsive element; P-box and TATC-box, *cis*-elements involved in the gibberellin responsiveness; TCA-element, salicylic acid (SA)-responsive element; TGA-element, auxin-responsive element; ARE, *cis*-acting regulatory element essential for the anaerobic induction; GC-motif, anoxia-specific inducibility element; LTR, low-temperature responsive element; STRE, stress responsive element; W-box, WRKY binding site involved in fungal elicitors; TC-rich repeats, *cis*-acting element involved in defense and stress responsiveness, WRE3 and WUN-motif, wound-responsive elements.

3.7. Tissue Expression Profiles Analysis of CsSRO Genes

RNA-Seq data from different tissues (root, stem, leaf, flowers, and tendrils) and three stages of developing ovaries were used to examine the tissue-specific expression profiles of *CsSRO* genes, and an expression heat map was generated. As a result, *CsSRO* genes were universally expressed in all tested tissues, and *CsSRO3* and *CsSRO4* exhibited relatively higher expression levels than the other two *CsSRO* genes (Figure 6). *CsSRO1* displayed a much higher expression in root than in other tissues, and *CsSRO2* showed the most abundant gene expression levels in root, unfertilized ovary, male and female flowers (Figure 6).

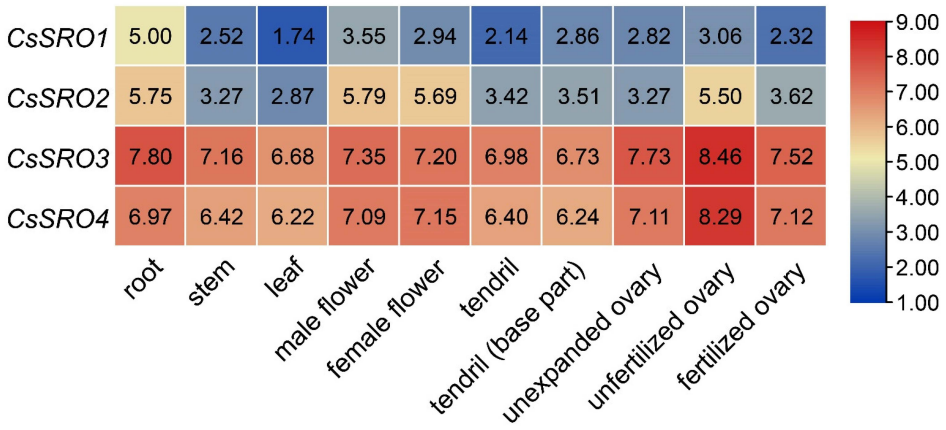

Figure 6. Expression profiles of the *CsSRO* genes in different tissues of cucumber based on RNA-Seq data. Data were normalized based on the $\log_2$-transformed TPM values of each gene in all tissues analyzed, and genes highly or weakly expressed are colored red or blue, respectively.

3.8. Expression Patterns of CsSRO Genes under Salt and Drought Stress

Since the promoter analysis revealed several stress-related *cis*-elements, we examined the expression patterns of *CsSRO* genes under salt and drought stress. Under salt stress, the expression of *CsSRO1*, *CsSRO3*, and *CsSRO4* was induced and peaked at 3 h, followed by an obvious decrease at 6 h and 12 h (Figure 7A). The expression level of *CsSRO2* was increased rapidly and then maintained at 6 h, and then dropped at 12 h. Under drought treatment, the *CsSRO* genes exhibited similar expression profiles as under salt treatment (Figure 7B). These results indicated that *CsSRO* genes may play a role in response to various abiotic stresses.

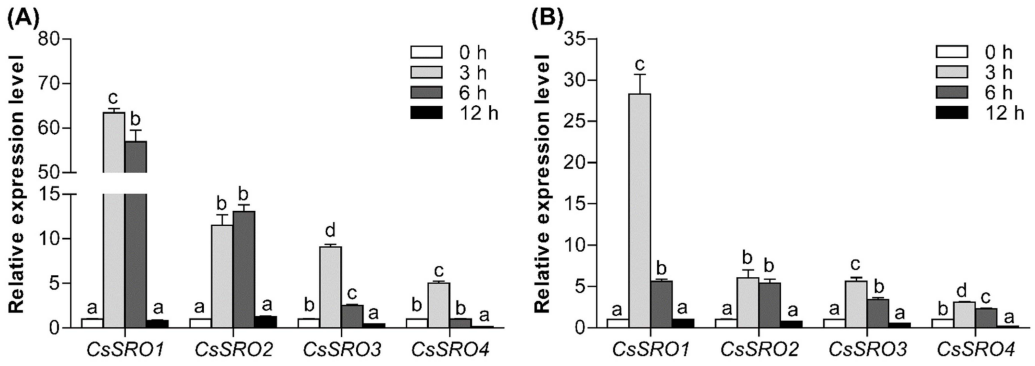

Figure 7. qRT-PCR analysis of *CsSRO* genes expression patterns under salt (**A**) and drought (**B**) stress. Cucumber seedlings were treated with salt (200 mM NaCl) and drought (10% PEG6000) conditions at different time points (0, 3, 6, and 12 h), and leaf samples were collected from three biological replicates of each treatment. Relative gene expression levels were normalized to *actin* expression levels, and the expression levels of the control (0 h) were set to 1.0. Error bars represent the standard deviations. The small letters above the bars indicate the significant differences between means ($p < 0.05$).

3.9. Expression Patterns of CsSRO Genes in Responses to Various Pathogen Infections

To analyze whether the *CsSRO* genes responded to biotic stress, their expression patterns under the infection of *P. cubensis* (DM treatment) and *M. incognita* (RKN treatment) were determined on the basis of the published RNA-Seq data [4,47]. In the Vlaspik (DM-susceptible) cucumber plants, *CsSRO1* displayed increased expression after *P. cubensis* infection at 2, 4, and 6 dpi compared to the control, while *CsSRO3* and *CsSRO4* were down-regulated at certain time points. In the PI 197088 (DM-resistant cucumber plant), *CsSRO1* displayed increased expression after *P. cubensis* infection at the earlier time point (1 dpi), while the other three *CsSRO* genes displayed decreased expression at certain time points, especially at 2 dpi (Figure 8A). Under RKN inoculation, the transcription levels of *CsSRO1* and *CsSRO2* were remarkably increased in susceptible CC3 lines, but no changed regulation of *CsSRO* genes was found during resistant IL10-1 lines response to RKN inoculation (Figure 8B).

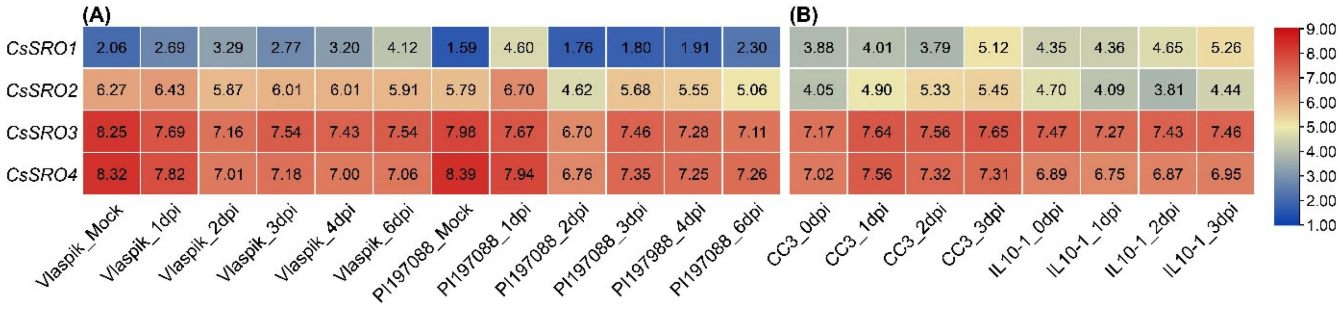

Figure 8. Expression profiles of *CsSRO* genes in response to DM (**A**) and RKN (**B**) based on RNA-Seq data. (**A**) Expression profiles of *CsSRO* genes in Vlaspik (DM-susceptible cucumber plant) and PI 197088 (DM-resistant cucumber plant) leaves that were mock inoculated or collected 1, 2, 3, 4, and 6 dpi with *P. cubensis* infection. (**B**) Expression profiles of *CsSRO* genes in root tips from CC3 (RKN susceptible cucumber plant) and IL10–1 (RKN resistant cucumber plant) at 0, 1, 2 and 3 dpi with *M. incognita* inoculation. Genes highly or weakly expressed are colored red or blue, respectively, based on the $\log_2$ transformed TPM values.

4. Discussion

In recent decades, *SRO* gene family has been identified in diverse plant species whose genomes were sequenced [7,21,48]. However, no comprehensive identification and characterization has been performed for *SRO* genes in cucumber. In this study, the cucumber *SRO* family genes were identified and cloned, and these genes were named as *CsSRO1–CsSRO4*, making it a small gene family. The number of *SRO* family genes was compared to those of previously identified plant species, such as sesame, rice, *Arabidopsis*, maize, *Brachypodium distachyon*, tomato, banana, and apple, which generally contained 4–6 *SRO* genes in their genomes [46,48–50], but was much less than that in Chinese cabbage (12 genes) [51], and wheat (30 genes) [45]. These findings indicated that the expansion of the *SRO* gene family was relatively conservative, and the number of *SRO* family genes may not possess an absolute correlation with genome size during plant evolution. In addition, a segmental duplication event was found in this study (Figure 4), and similar results were also found in other plants, including wheat [45], and tomato [50].

Phylogenetic tree analysis showed that CsSRO proteins along with SRO proteins from 14 other different plant species were grouped into two groups (group I and group II) based on their sequence relationships, and group I can be further divided into four subgroups (Ia to Id) (Figure 1). The SRO proteins of these tested species were clustered in dicot- or monocot-specific patterns, and monocot species contained only group I SROs. In group I, monocotyledonous and dicotyledonous SRO proteins were also had individual distribution in special subgroups, of which group Ia and group Ib SROs were monocotyledonous, while SROs in group Ic and group Id were coming from mono- and dicot plants (Figure 1), suggesting that the evolution of SROs in these groups followed the monocot and dicot

divergence. Notably, SROs including the WWE domain were distributed in group I, while all SROs in group II lack the WWE domain, which is consistent with the previous studies [7,48]. It can be speculated that the variation of the WWE domain is a divergent force for the expansion of the *SRO* gene family. We also identified 10 diverse conserved motifs, and there is an obvious difference in the arrangements of these conserved motifs in the two groups, while the SROs in the same group showed conservative conserved motif arrangements (Figure 2). Gene structure analysis provides extra clues into the procedure of evolution in different gene families [3]. In the current study, most *SRO* genes in the same subgroup displayed conservative gene structure, but they have variable numbers of introns in different subgroups (Figure 3), indicating that gain or loss of introns may present in *SRO* genes during the evolution, and the functions of phylogenetically related *SRO* genes may be conserved.

Previous reports revealed that the transcription of *SRO* genes exhibited constitutive expression or tissue-specific expression patterns. For example, *Arabidopsis AtSRO1* and *AtRCD1* [16], rice *OsSRO1d* and *OsSRO1e* [21], tomato *SolySRO5* and *SolySRO6* [50] were found to have a broad expression in various tissues. In sesame, *SiSRO1a* and *SiSRO1b* were highly transcribed in various tissues, while *SiSRO2a* and *SiSRO2b* exhibited the highest transcript levels in root [46]. We thus investigated the spatio-temporal expression patterns of *CsSRO* genes during the development of various tissues based on the published RNA-Seq data. The results showed that *CsSRO1* was expressed at a much higher level in root than other tested tissues, *CsSRO2* was highly expressed in root, unfertilized ovary, female and male flowers, while *CsSRO3* and *CsSRO4* were ubiquitously expressed in the tested tissues (Figure 6). In apple, the expression of *MdSRO1* and *MdSRO4* was highest in roots, whereas *MdSRO5* displayed the highest expression in flowers [52]. In tomato, the highest transcript levels of *SolySRO2*, *SolySRO3,* and *SolySRO5* were observed in flowers, roots, and 3 cm fruits, respectively [50]. In addition, *CsSRO2* and *CsSRO4* displayed obviously higher transcription levels in unfertilized ovary than unexpanded and fertilized ovaries (Figure 6), thus fueling our speculation that they may participate in ovary development of cucumber. Similarly, *SolySRO4* was highly expressed in mature tomato fruits (breaker + 10 fruits), and its values were much higher than that in mature green fruits and breaker fruits, suggesting that this gene may participate in fruit development and ripening [50]. Moreover, the identified segmental duplication gene pairs, *CsSRO3* and *CsSRO4*, showed very similar spatio-temporal expression patterns (Figure 6), suggesting their possible similar functions.

It was reported that genes in *SRO* family play vital roles in the resistance to various abiotic stresses. In this work, many *cis*-elements responding to stresses (especially ARE and STRE) were distributed in the promoters of *CsSRO* genes (Figure 5), and similar results were reported in the promoter regions of *SolySRO* and *SiSRO* genes [46,50]. The qRT-PCR analysis also revealed that the expression of four *CsSRO* genes were significantly induced under salt and drought stress (Figure 7), suggesting their involvement in stress tolerance. In previous reports, rice *SRO* genes (*OsSRO1a–OsSRO1e*) were induced by a series of abiotic stresses, including heat, cold, drought, UV, salt, wound, and submergence, and *OsSRO1c* was found to play positive roles in cold, drought, and oxidative stress [21,22]. Transgenic *Arabidopsis* plants overexpressing *IcSRO1* from *Ipomoea cairica* also exhibited enhanced tolerance to salt and drought stress [53]. Overexpression of *MdRCD1* in *Arabidopsis* promotes plant adaptation to drought, salt, and H_2O_2 stresses [52]. Overexpression of *SiSRO2b* also conferred the tolerance of transgenic yeast cells to osmotic, salt, and oxidative stress [46]. *SRO* family genes are also known as regulators function in plant defense response against pathogens, and the regulatory mechanisms depend on the hormone signaling pathways, such as JA, SA, and so on. For example, OsSRO1a was found to regulate JA-dependent disease susceptibility to the *Xanthomonas oryzae* pv *oryzae* (*Xoo*) [24]. The presence of *cis*-elements associated with hormones and defense responses in the promoters of *CsSRO* genes implied the potential roles of *CsSRO* genes in response to biotic stress through the hormone signal transduction pathway (Figure 5). Additionally, all *CsSRO* genes showed changed expression levels in both sensitive and resistant cucumber plants inoculated with DM,

except for *CsSRO2*, whose expression was unchanged in DM-susceptible Vlaspik plants (Figure 8). *CsSRO1* and *CsSRO2* displayed significantly increased expression at certain time points in susceptible CC3 plants inoculated with RKN (Figure 8). In banana, a total of four out of six *MaSRO* genes showed increased expression levels during *Fusarium oxysporum* f. sp. *Cubense* tropical race 4 (*Foc* TR4) infection [48]. Therefore, *CsSRO* genes may also play important roles in the response of plants to adverse environmental conditions.

5. Conclusions

In this work, four *SRO* family genes were isolated from cucumber, and their evolutionary relationship, conserved motif composition, gene structure, chromosomal location, *cis*-regulatory elements within the promoter region, followed by expression patterns in various tissues and differential expression in response to abiotic and biotic stresses were systematically analyzed. Expression analysis showed that the four *CsSRO* genes were differentially expressed in various tissues and under diverse stresses, suggesting their roles in plant physiological processes and defense response. Our findings facilitate further study to the functional characterization of *CsSRO* genes in response to abiotic and biotic stresses in cucumber, and these *CsSRO* genes can be considered as potential candidates for breeding of cucumber stress-tolerant varieties in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8070634/s1, Figure S1: PCR amplification of *CsSRO* genes. M, DL ladder 2000 DNA Marker; 1, *CsSRO1*; 2, *CsSRO2*; 3, *CsSRO3*; 4, *CsSRO4*, Figure S2: Schematic diagram of domain distributions in CsSRO proteins; Table S1: The gene-specific primers used in this study.

Author Contributions: Data curation, L.X., Z.Z., C.Z. and Y.Z.; formal analysis, C.Z. and J.Z.; funding acquisition, S.L. and Y.Z.; investigation, L.X., Z.Z. and J.Z.; methodology, L.X., Z.Z., C.Z., J.Z., Z.H. and Y.Z.; resources, L.X., Z.Z., Z.H. and S.L.; software, C.Z. and Y.Z.; writing—original draft, L.X. and Y.Z.; writing—review and editing, S.L. and Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Academic and Technical Leader Plan of Jiangxi Provincial Main Disciplines (20204BCJ22023), the Natural Science Foundation of Jiangxi Province (20202BABL205002), and the National Natural Science Foundation of China (31860566).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article and Supplementary Materials.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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