



## Article

# The WRKY Transcription Factor CsWRKY70 Regulates EGCG Biosynthesis by Affecting CsLAR and CsUGT84A Expressions in Tea Leaves (*Camellia sinensis*)

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**Abstract:** Epigallocatechin gallate (EGCG) is an important contributor to bitterness and astringency in summer tea leaves; however, the transcriptional regulatory mechanisms of EGCG biosynthesis remain unclear. In this study, EGCG content was significantly decreased after foliar spraying with nano-Se fertilizers in tea leaves. A WRKY transcription factor (TF), CsWRKY70, was found to be positively related to EGCG content. The open reading frame of CsWRKY70 was 891 bp encoding 296 amino acids. CsWRKY70 is localized to the nucleus and has transcriptional activation activity. The electrophoretic mobility shift assay indicated that CsWRKY70 can directly bind to the promoters of CsLAR and CsUGT84A containing W-box (5'-C/TTGACT/C-3') sequences. Dual-luciferase reporter experiment verified that CsWRKY70 activated CsLAR and CsUGT84A expressions in tobacco leaves. In summary, these results demonstrated that CsWRKY70 may reduce EGCG biosynthesis by inhibiting the CsLAR and CsUGT84A expressions under nano-Se treatment. Our findings provide new insight into the regulatory mechanism of WRKY TFs involved in catechin biosynthesis and offer a theoretical basis for breeding low or high EGCG content tea cultivars.

**Keywords:** *Camellia sinensis*; EGCG biosynthesis; CsWRKY70; CsLAR; CsUGT84A



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## 1. Introduction

Tea (*Camellia sinensis*) is the most popular beverage in the world due to its unique flavor and multiple health benefits. Tea quality is related to several chemical compounds in tea leaves, including tea polyphenols, amino acids, and caffeine. Catechins are key phenolic compounds in tea leaves, accounting for 50–60% of the total tea polyphenols, and are crucial in the formation of tea flavor [1]. Epigallocatechin gallate (EGCG), accounting for 50–80% of total catechins, is known to process numerous health benefits, including antioxidant [2], anti-inflammatory [3], and antiviral [4] properties. In general, catechin content in tea leaves increases with the temperature increasing, resulting in a high level of catechin in summer tea leaves [5]. However, an excessive level of catechin aggravates the bitterness and astringency of tea infusion, causing a decline in tea quality [6]. Our previous study has demonstrated that moderate and high concentrations of nano-Se can improve summer tea quality by decreasing tea polyphenols, catechins, and caffeine contents and increasing theanine content in summer tea leaves (Tables S1 and S2). However, the transcriptional regulatory mechanisms of EGCG biosynthesis remain unclear.

Catechins are the final products of the flavonoid biosynthesis pathway, which is derived from phenylpropanoid. EGCG biosynthetic pathway is controlled by several crucial

genes. It has been demonstrated that non-galloylated catechins, such as catechin (C), gallocatechin (GC), epicatechin (EC), and epigallocatechin (EGC), are synthesized by several crucial enzymes, including anthocyanidin reductase (ANR), leucocyanidin reductase (LAR), and anthocyanidin synthase (ANS) [7]. Further,  $\beta$ -Glucogallin, a galloyl donor associated with the biosynthesis of EGCG, is synthesized by glucose and gallic acid in tea leaves through the UDP-glucose: galloyl-1-O- $\beta$ -D-glucosyltransferase (*CsUGT84A*) [8]. Finally, type 1A serine carboxypeptidase-like acyltransferases (*CsSCPL1A*) convert non-galloylated catechins into galloylated catechins, such as ECG and EGCG [9]. Therefore, nano-Se may regulate EGCG accumulation in tea leaves by affecting the expressions of these crucial genes.

Transcription factors (TFs) are protein molecules with special structures that can specifically bind to the *cis*-acting elements in the promoter region of functional genes. TFs can affect target gene expression by activating or inhibiting the activity of the promoter of the structural gene [10]. According to the characteristics of conserved domains, TFs can be divided into multiple families, including MYB [11], bHLH [12], WRKY [13], NAC [14], and bZIP [15]. WRKY TFs exist only in plants and feature an extremely conserved WRKYGQK sequence in N-terminal, which can specifically recognize and bind to W-box (C/T)TGAC(T/C) elements [16]. WRKY is involved in regulating different biological processes in plants [17], including plant defense regulatory networks, developmental processes, and biosynthesis of defensive metabolites [18,19]. In addition, WRKY is also engaged in mediating the biosynthesis of flavonoids. MdWRKY11 increases the accumulation of flavonoids and anthocyanins in red-fleshed apples [20]. Duan et al. find that AtWRKY41 is an inhibitor to regulate the anthocyanin biosynthesis in *Arabidopsis thaliana* [21]. Furthermore, CsWRKY57like positively impacts the biosynthesis of methylated EGCG in the tea plant [22]. However, there are few studies on WRKY TFs regulating the biosynthesis of EGCG.

In this study, different concentrations of nano-Se fertilizers were sprayed on summer tea leaves to evaluate their effect on EGCG accumulation in leaves. The CsWRKY70, which was positively associated with the EGCG content in tea leaves, was identified using transcriptome sequencing. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to further identify and validate the expression levels of differentially expressed flavonoids biosynthetic related genes. The influence of CsWRKY70 on *CsLAR* and *CsUGT84A* gene promoters was further analyzed. These results will provide a theoretical foundation for elucidating the regulatory mechanism of EGCG biosynthesis in tea plants.

## 2. Materials and Methods

### 2.1. Plant Materials and Treatments

*Camellia sinensis* var. *sinensis* cv. 'Bixiangzao' (>10-year-old) were used as test materials, which were cultivated in the Tea Germplasm Repository of Hunan Agricultural University (Changsha, China). Nano-Se fertilizers were purchased from Baiwo Huitong Technology Co., LTD., (Beijing, China); their primary component was Se nanoparticles (1500 mg/L) with an average size of 50–78 nm [23]. Five concentrations of nano-selenium fertilizer were investigated, including the control check group (CK, sterile water), 100-fold dilution (D1, 15 mg/L), 200-fold dilution (D2, 7.5 mg/L), 300-fold dilution (D3, 5 mg/L), and 400-fold dilution (D4, 3.75 mg/L). Prior to foliar spraying, all of the experimental tea plants underwent consistent pruning. Tea leaves were sprayed with nano-Se fertilizers until the leaves were moist. The spraying was performed once every 7 days for a total of 3 times in summer (July and August). The experimental field is flat with uniform fertility. Each treatment consisted of two rows of tea plants, and each row was 30 m long and 1 m wide with 1.5 m between rows. The areas of each experimental treatment were approximately 60 m<sup>2</sup>. After new leaves started sprouting, the healthy and morphologically similar tea leaves with the criterion of one bud and two leaves from three random tea plants in each treatment were collected as three independent biological replicates. The pretreatments of the tea leaves for molecular biology experiments and biochemical determinations were the

same as our published methods [24]. Briefly, fresh tea leaves were instantly partly frozen in liquid nitrogen and preserved at  $-80^{\circ}\text{C}$  for molecular biology research. In addition, some of the fresh tea leaves were also liquid-nitrogen-frozen, lyophilized, and then, mechanically crushed for subsequent biochemical determinations.

## 2.2. Determination of EGCG in Tea Leaves

The major catechin in tea leaves, EGCG, was detected using high-performance liquid chromatograph (HPLC), as described in a previous study [25].

## 2.3. RNA Isolation, cDNA Synthesis, and Sequencing

Total RNA was extracted using the RNA Prep Pure Plant Kit (Tiangen, Beijing, China). The integrity and concentration of RNA were conducted by 1% agarose gel and NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The PrimeScript<sup>TM</sup> II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan) was employed to synthesize the cDNA libraries and then sequenced by the Novogene Bioinformatics Institute (Novogene, Beijing, China). The *C. sinensis* genome was used as the reference genome [26].

## 2.4. Quantitative Real-Time PCR (qRT-PCR)

For the qRT-PCR analysis, the reverse-transcribed first-strand cDNA template mentioned above was employed as the template. qRT-PCR was performed by using SYBR<sup>®</sup> Green *Pro Taq* HS premixed qPCR Kit (Accurate Biology, Changsha, China) as described previously [27]. The reference gene was  $\beta$ -actin. The reaction system was 20  $\mu\text{L}$ , and three biological replicates were performed for qRT-PCR analysis. Table S3 lists the primers that were utilized in this investigation.

## 2.5. Gene Clone and Bioinformatics Analysis

The differentially expressed transcription factors (TFs) were screened through transcriptome data (Table S4) to obtain the *CsWRKY70*, and the CDS sequence of *CsWRKY70* (TEA008808) was derived from the reference genome. The full-length sequence of *CsWRKY70* was cloned based on our published methods [28], and the gene-specific primers of *CsWRKY70* were listed in Table S3. Cloned *CsWRKY70* sequence was verified by sequencing.

The molecular weight and isoelectric point of *CsWRKY70* were analyzed by ProtParam and ProtScale tools in ExPASy (<https://web.expasy.org/protparam/>, accessed on 10 January 2022). Homology analysis of the *CsWRKY70* protein sequence were carried out in NCBI (BLAST, <http://www.ncbi.nih.gov/BLAST/>, accessed on 10 January 2022), and the sequences of neighboring species were downloaded as well. The protein sequences of *CsWRKY70* and the homologous sequences were analyzed by the NCBI Conserved Domains Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 13 January 2022) and then edited using the Genedoc software (<http://genedoc.software.informer.com/>, accessed on 15 January 2022). Amino acid sequences of WRKY family of *Arabidopsis* were downloaded from TAIR (<https://www.arabidopsis.org/>, accessed on 20 January 2022). A phylogenetic tree was constructed by using the MUSCLE program in MEGA X with 1000 bootstrap replicates (<http://www.megasoftware.net/>, accessed on 30 January 2022).

## 2.6. Promoter Isolation and Cis-Acting Elements Analysis

Total DNA was extracted using the cetyl trimethylammonium bromide method from tea leaves. The upstream 2000 bp promoter sequences of *CsLAR* and *CsUGT84A* genes were obtained from the plant-care database (<http://tpdb.shengxin.ren/>, accessed on 16 August 2022). The primers were designed using Premier 5.0 and listed in Table S3. The promoters of *CsLAR* and *CsUGT84A* were isolated using high-fidelity enzyme 2X *Pro Taq* Master Mix (New England Biolabs, Ipswich, MA, USA) by PCR [28]. Cis-acting element analysis of promoters of *CsLAR* and *CsUGT84A* was used according to the plant-care database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 18 March 2022).

### 2.7. Subcellular Location of CsWRKY70 in Tobacco (*Nicotiana benthamiana*)

The online software Cell-PLOC 2.0 (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 17 January 2022) was employed to predict the protein subcellular localization of CsWRKY70. To verify the subcellular localization of CsWRKY70, the CsWRKY70 was constructed into a transient expression vector containing the CAMV 35S promoter fused with a green fluorescent protein (GFP) to obtain CsWRKY70-GFP, and 35S-GFP empty was used as a negative control. Subsequently, both GFP-Empty and CsWRKY70-GFP vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 (Weidi Biotechnology Co., Ltd., Shanghai, China), and the constructs were then injected into tobacco leaves from the lower epidermis. After incubation for 2–3 days, the GFP signals were observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

### 2.8. Transcriptional Activation Analysis

We first used a yeast system to analyze the transcriptional activity of CsWRKY70. The full length of the CsWRKY70 gene obtained a PGBKT7-CsWRKY70 expression vector. The transformed recombinant plasmids, positive control (pGBKT7-53+ PGADT7-T), negative control (PGBKT7-Empty), and the PGBKT7-CsWRKY70 were transformed into yeast cell AH109 (Weidi Biotechnology Co., Ltd., Shanghai, China), respectively. The detailed methods for yeast two-hybrid assay were performed as described [28]. To further verify the transcriptional activity of CsWRKY70, the transcriptional activity analysis was performed in tobacco. The full length of CsWRKY70 was constructed into a 35S promoter-driven pBD vector as an effector. Reporter vector generation and dual luciferase reporter assays were carried out as previously reported [28]. The ratio of *Luciferase/Renilla luciferase* (LUC/REN) of pBD-CsWRKY70 and pBD-Empty was detected to verify the transcriptional activity of CsWRKY70.

### 2.9. Protein Expression and Purification

To obtain the recombinant protein, a sequence of CsWRKY70 containing WRKYGQK domain (130–189 aa, a total of 186 bp) was constructed into the prokaryotic expression vector pGEX 4T (Amersham Biosciences, Piscataway, NJ, USA) to generate GST-CsWRKY70 plasmids. The recombinant plasmids of GST-Empty and GST-CsWRKY70 were transformed into Rosetta DE3 (Vazyme, Nanjing, China) and cultured at 37 °C to an OD600 of 0.6, and then, induced with IPTG (Scientific Research Special, USA). After ultrasonic comminution, the GST-Empty and GST-CsWRKY70 recombinant proteins were purified with a Glutathione Resin Kit (TaKaRa, Beijing, China). The protein eluent was collected and stained with SDS-PAEG and Coomassie Bright Blue to verify the expression and purification of CsWRKY70 protein.

### 2.10. Electrophoretic Mobility Shift Assay (EMSA)

The promoters of *CsLAR* and *CsUGT84A* contain W-box (C/T)TGAC(T/C) sequences, which could be recognized by WRKY TFs. Probes were designed to contain the core element TGAC. The probes were labeled with biotin at the 3' end using DNA Labeling Kit (Thermo Scientific, Waltham, MA, USA). EMSA experiment was performed using the Light Shift™ EMSA Optimization and Control Kit (Thermo Scientific, Waltham, MA, USA), as previously described [27]. Fluorescent Chemical Imaging System (ProteinSimple, San Jose, CA, USA) was used to detect the binding of the target protein to the probes.

### 2.11. Dual-Luciferase Reporter Assay System

The CDS sequence of CsWRKY70 was constructed into the pEAQ vector to generate CsWRKY70-pEAQ as an effector, and the promoters of *CsLAR* and *CsUGT84A* were constructed into the pGreen II 0800-LUC vectors as reporter plasmids [29]. All the plasmids were then transformed into *Agrobacterium tumefaciens* strain EHA105, respectively. The effector and reporter plasmids were injected into tobacco leaves at a ratio of 9:1. After incu-

bation for 2~3 days, the LUC/REN ratios were detected by Dual-Luciferase Reporter Kit to evaluate the transcriptional effect of CsWRKY70 on the *CsLAR* and *CsUGT84A* promoters.

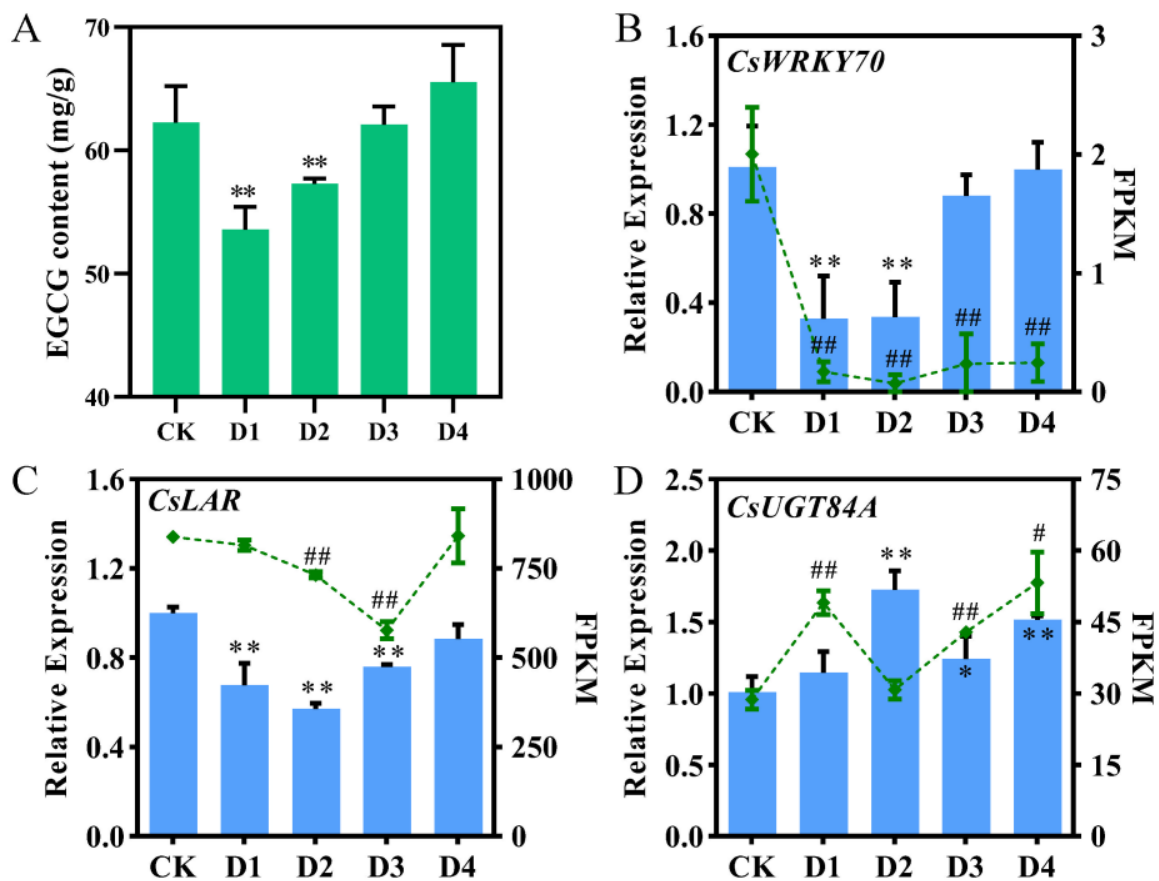
### 2.12. Statistical Analysis of Data

At least three different biological duplicates of each experiment were carried out independently. All the experimental data were recorded as mean  $\pm$  standard error. Statistical analyses were compared using one-way ANOVA or Student's *t*-tests. Significant differences were defined as  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

## 3. Results

### 3.1. Effect of Nano-Se Fertilizer on EGCG Content in Tea Leaves

The EGCG content in tea leaves after foliar application of different concentrations of nano-Se was determined using HPLC (Figure 1A). The EGCG contents in the D1 (53.57 mg/g) and D2 (57.30 mg/g) groups were significantly decreased compared to the CK group (62.25 mg/g) ( $p < 0.01$ ), while there is no significant difference in the D3 (62.08 mg/g) and D4 groups (65.55 mg/g) compared to the CK group. This result indicated that moderate and high concentrations of nano-Se could significantly decrease the EGCG content in summer tea leaves.



**Figure 1.** EGCG content and expression levels of *CsWRKY70*, *CsLAR*, and *CsUGT84A* from qRT-PCR and RNA-seq. (A) EGCG content in tea leaves after foliar application of different concentrations of nano-Se. (B–D) Expression levels of *CsWRKY70*, *CsLAR*, and *CsUGT84A* from qRT-PCR (bar graphs) and RNA-seq (line graphs). FPKM represents fragments per kilobase of transcript per million mapped reads. \* and \*\* represent significant differences ( $p < 0.05$  and  $p < 0.01$ , respectively) compared to control in qRT-PCR data. # and ## represent significant differences ( $p < 0.05$  and  $p < 0.01$ , respectively) compared to control in RNA-seq data. Data were analyzed by one-way ANOVA.



### 3.2. Expression Patterns of *CsWRKY70*, *CsLAR*, and *CsUGT84A*

Differentially expressed genes were obtained from our RNA-seq data (Figure S1). A WRKY TF with the same expression pattern as the EGCG content was identified and named *CsWRKY70* (Figure 1B). Simultaneously, two flavonoid biosynthetic-related genes, *CsLAR* and *CsUGT84A*, were identified and cloned; they were positively and negatively correlated with the expression pattern of *CsWRKY70*, respectively (Figure 1C,D). To further verify the expression patterns of *CsWRKY70*, *CsLAR*, and *CsUGT84A* in tea leaves under foliar spraying of different concentrations of nano-Se, qRT-PCR was used. Results showed that nano-Se significantly decreased the *CsWRKY70* expression in the D1 and D2 groups and showed no significant difference in the D3 and D4 groups compared to the CK (Figure 1B;  $p < 0.01$ ), which was consistent with our RNA-seq data. Further, expression levels of *CsLAR* in nano-Se treated groups were all reduced compared to the CK group (Figure 1C; D1, D2, and D3,  $p < 0.01$ ). In contrast, expression levels of *CsUGT84A* in nano-Se treated groups were increased compared to the CK group (Figure 1D; D2, D3, and D4,  $p < 0.01$ ). The qRT-PCR results were basically consistent with our RNA-seq data, supporting the accuracy of the transcriptomic data.

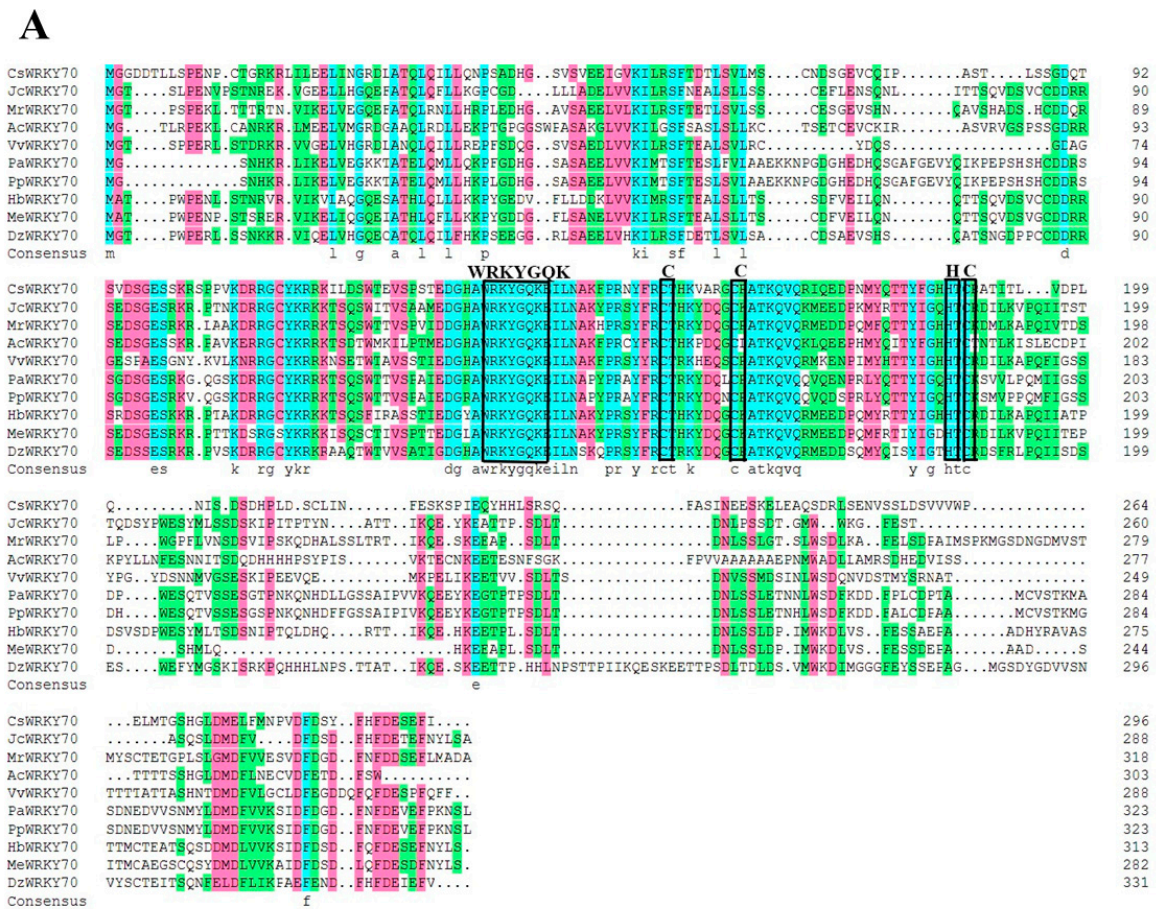
### 3.3. Identification and Bioinformatics Analysis of *CsWRKY70*

The CDS sequence of the WRKY TF was cloned by PCR and named *CsWRKY70*. The open reading frame of *CsWRKY70* was 891 bp and encoded a protein containing 296 amino acids. The predicted molecular weight and isoelectric point of *CsWRKY70* were 33.39 kDa and 5.23, respectively.

To further characterize the molecular features of *CsWRKY70*, multiple sequence alignments and phylogenetic analyses were carried out. Sequence alignment showed that *CsWRKY70* has a typical WRKYGQK structure, which is an important characteristic of WRKY TFs. There is a WRKYGQK HeptaPeptide sequence at the N-terminal of *CsWRKY70* and followed by a C2HC zinc finger structure (Figure 2A), indicating that *CsWRKY70* belongs to the WRKY family. Further, a phylogenetic tree of amino acid sequences between *CsWRKY70* and WRKY TFs from *Arabidopsis thaliana* was constructed to determine the phylogenetic characteristics of *CsWRKY70*. As shown in Figure 2B, WRKY TFs were divided into three groups: WRKY I, WRKY II, and WRKY III. There were five subgroups in the WRKY II group, including WRKY IIa, WRKY IIb, WRKY IIc, WRKY IId, and WRKY IIE. *CsWRKY70* belonged to WRKY III group and was homologous to AtWRKY70 and AtWRKY54. It has been revealed that AtWRKY70 and AtWRKY54 are crucial in plant responses to biotic and abiotic stresses and in regulating plant tolerance to osmotic stresses [30]. According to these findings, *CsWRKY70* could be critical for physiological and biochemical reactions, such as the biosynthesis of secondary metabolism in tea plants.

### 3.4. Subcellular Localization of *CsWRKY70*

WRKY TFs are typical nuclear proteins with transcriptional activity [31]. *CsWRKY70* was predicted to be a nuclear protein using the online software WoLF POSRT. We further verified this result by subcellular localization assays using tobacco. Results showed that *CsWRKY70*-GFP was localized in the nucleus (Figure 3), while the positive control of GFP-Empty was distributed in the whole cell, indicating that *CsWRKY70* is a nuclear localization protein.



**B**

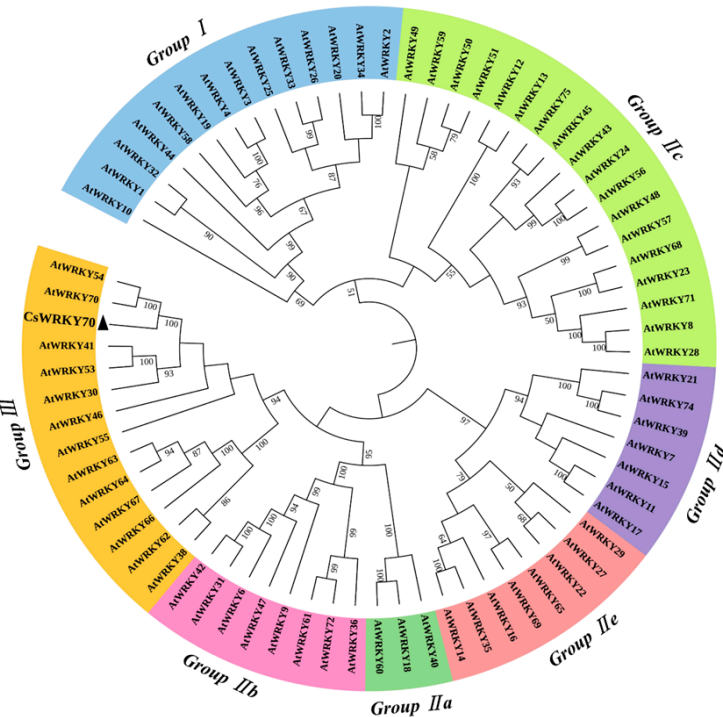
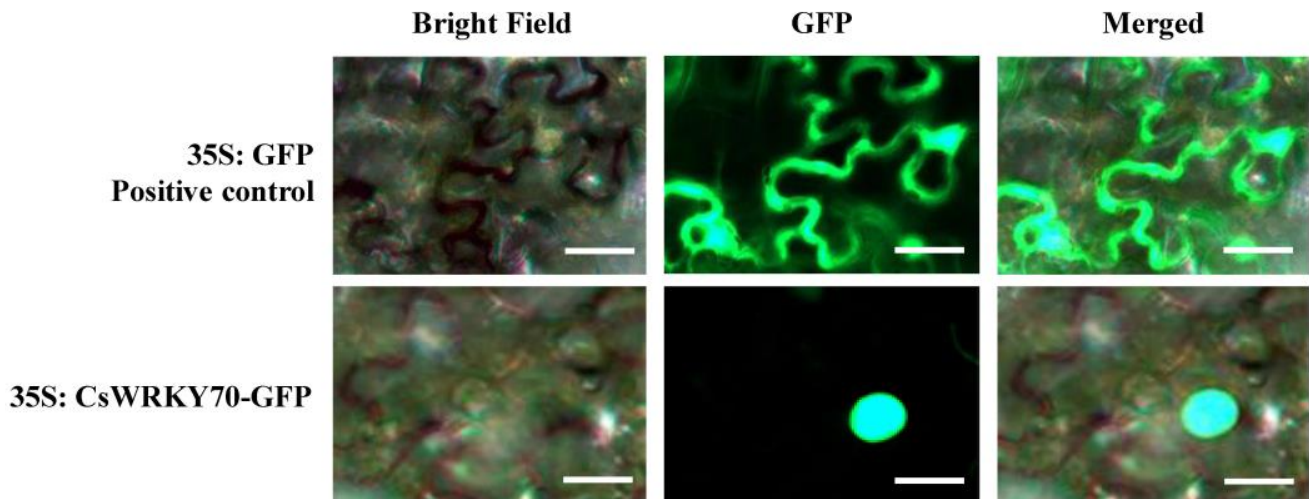


Figure 2. Multiple sequences alignment and phylogenetic analyses of CsWRKY70. (A) Homology

protein sequence alignment between CsWRKY70 and WRKY70 protein sequences of other species (*Jatropha curcas*, *Myrica rubra*, *Actinidia chinensis*, *Vitis vinifera*, *Cerasus avium*, *Amygdalus persica*, *Hevea brasiliensis*, *Manihot esculenta*, and *Durio zibethinus*). Identical or similar amino acids were indicated by blue, red, and green shading. The WRKY domain and C2HC zinc finger were indicated by black boxes. (B) Phylogenetic analysis of CsWRKY70 and *Arabidopsis*-derived WRKY TFs family. CsWRKY70 was marked with a black triangle.



**Figure 3.** Subcellular localization of CsWRKY70 via transient expression in tobacco leaves (*Nicotiana benthamiana*). The white bars denote 45  $\mu$ m. GFP, green fluorescent protein.

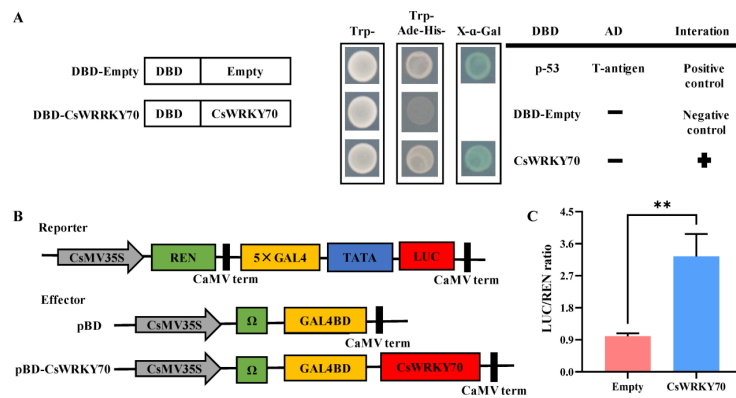
### 3.5. Transcriptional Activity Analysis of CsWRKY70

To evaluate the transcriptional activity of CsWRKY70, a GAL4-responsive reporter system was conducted in yeast. The yeast strains transformed with the positive control pGBKT7-53+pGADT7-T, negative control pGBKT7-Empty, and pGBKT7-CsWRKY70 plasmids were grown on the SD/-Trp medium plates, indicating that the yeast transformation was successful (Figure 4A). The transformed strains were transferred into the medium plates (SD/-Trp/-His/-Ade) for further cultivation. The positive control and pGBKT7-CsWRKY70 yeasts grew on the selected plates and exhibited  $\alpha$ -galactosidase activity, suggesting that CsWRKY70 functions as a transcription activator with self-activating activity. We further verified the transcriptional regulatory activity of CsWRKY70 using tobacco. The pBD-Empty and pBD-CsWRKY70 were transformed into tobacco and detected by dual luciferase system. Results showed that the *LUC/REN* ratio of the pBD-CsWRKY70 was substantially higher than that of pBD-Empty (Figure 4B,C), suggesting that CsWRKY70 has transcriptional self-activation activity. The transcriptional activity assay in yeast and tobacco revealed that CsWRKY70 may act as a transcriptional activator.

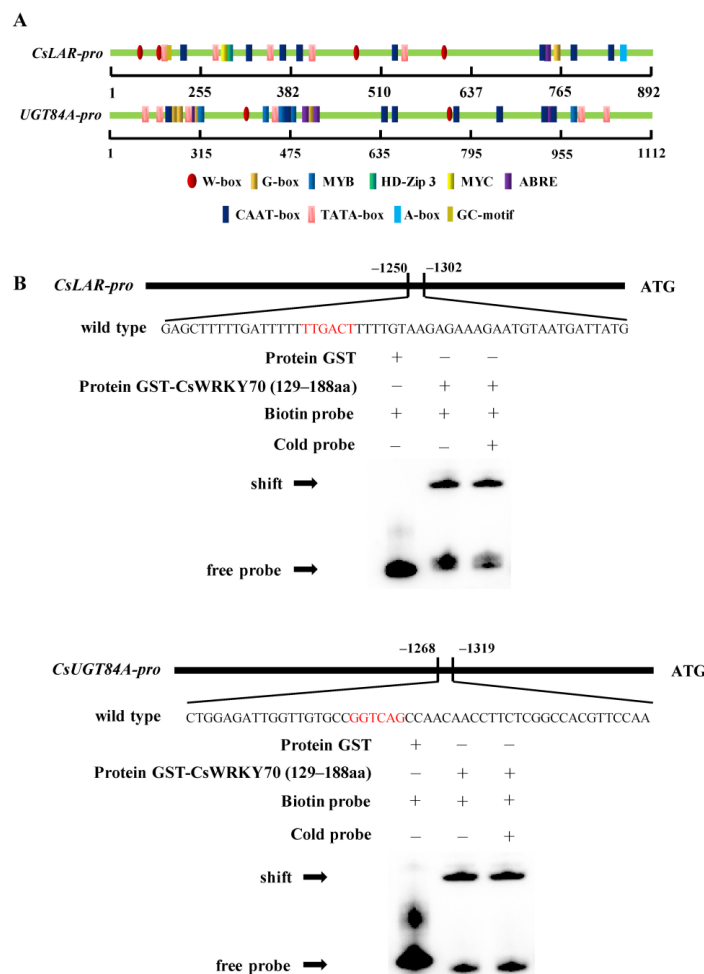
### 3.6. CsWRKY70 Binds to the Promoters of CsLAR and CsUGT84A

WRKY TFs specifically bind to the W-box elements on the promoter region of the target gene to affect gene expression [32]. Multiple W-box (5'-C/TTGACT/C-3') sequences were found in *CsLAR* and *CsUGT84A* promoters (Figure 5A). The purified recombinant GST-CsWRKY70 protein (130–189 aa) was used to confirm the DNA–protein interactions between CsWRKY70 and the *CsLAR* and *CsUGT84A* promoters using EMSA. Results showed that the protein electrophoretic mobility shifts were observed in the presence of recombinant GST-CsWRKY70 protein and biotin-labeled probes of *CsLAR* and *CsUGT84A* promoters containing the W-box elements, while the GST-empty protein only the band for the free probe was observed (Figure 5B). These results indicated that the CsWRKY70 fusion protein could directly bind to the W-box motif on the promoters of *CsLAR* and *CsUGT84A*.





**Figure 4.** Transcriptional activity characteristics of CsWRKY70 protein. **(A)** Transcriptional activity of CsWRKY70 in yeasts. Co-transformed yeasts were grown on selective media (SD medium without tryptophan, histidine, and adenine, SD/−Trp/−His/−Ade). The X-α-galactosidase test was performed to evaluate the transcription activation ability of CsWRKY70. **(B)** Transcriptional activity of CsWRKY70 in tobacco system (*Nicotiana benthamiana*). **(C)** The LUC/REN fluorescence intensity ratio was determined to evaluate the transcription activation ability of CsWRKY70. SD, synthetic defined medium; −Trp/−His/−Ade, medium lacking tryptophan, histidine, and adenine; LUC, Luciferase; REN, Renilla luciferase. \*\* represents significant differences ( $p < 0.01$ ) compared to control. Data were analyzed by Student’s *t*-tests.

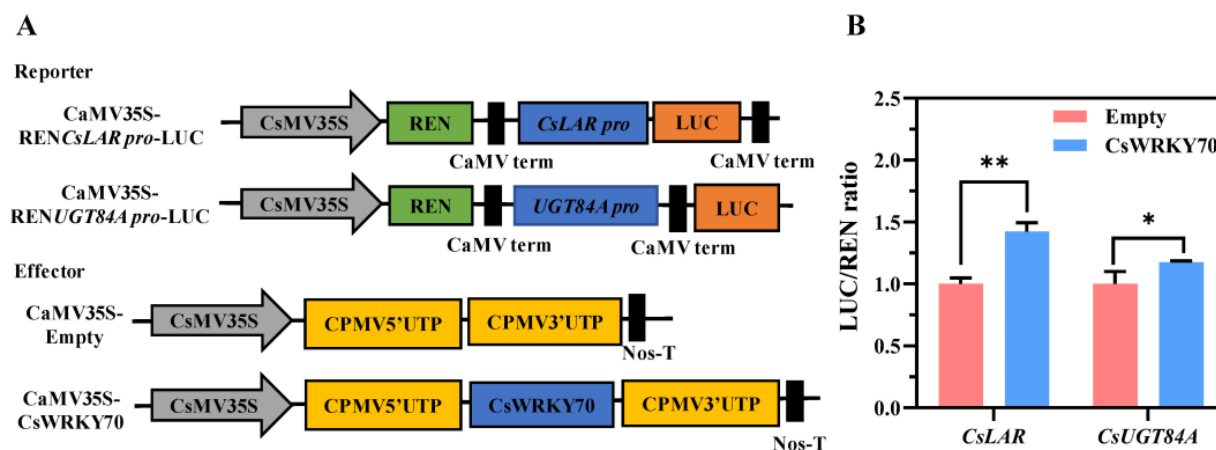


**Figure 5.** Cis-elements analysis of *CsLAR* and *CsUGT84A* promoters and electrophoretic mobility

assays (EMSA) of CsWRKY70 binding to *CsLAR* and *CsUGT84A* promoters. (A) Prediction of *cis*-elements on the *CsLAR* and *CsUGT84A* promoters. Ellipses represent W-box elements that can bind to WRKY TFs. (B) Recombinant GST-CsWRKY70 fusion protein binds directly to the promoters of *CsLAR* and *CsUGT84A* containing the W-box (T/CTGACT/C). The DNA–protein complexes were separated on 6% native polyacrylamide gels. The ‘–’ and ‘+’ indicate the absence and the presence of specific proteins, respectively.

### 3.7. Transcriptional Regulatory Effects of CsWRKY70 on *CsLAR* and *CsUGT84A* Promoters

To further determine whether CsWRKY70 can promote the promoter activities of *CsLAR* and *CsUGT84A*, a dual luciferase reporter gene assay was performed in tobacco. As shown in Figure 6A, the pEAQ-Empty and pEAQ-CsWRKY70 vectors were used as effectors, and the *CsLAR* and *CsUGT84A* promoters were transformed into the pGreen II 0800-LUC vectors as reporters. Compared to the empty control, the LUC/REN ratios of co-transformations of CsWRKY70 and *CsLAR* pro-LUC and CsWRKY70 and *CsUGT84A* pro-LUC were remarkably increased (Figure 6B). These results indicated that CsWRKY70 may influence EGCG biosynthesis in tea leaves by regulating *CsLAR* and *CsUGT84A* expressions.



**Figure 6.** The ability of CsWRKY70 to activate transcription of *CsLAR* and *CsUGT84A* promoters in tobacco leaves (*Nicotiana benthamiana*). (A) Schematic diagram of vector plasmids. (B) CsWRKY70 activates the promoter activities of *CsLAR* and *CsUGT84A*. \* and \*\* represent significant differences ( $p < 0.05$  and  $p < 0.01$ , respectively) compared to the empty control. Data were analyzed by Student’s *t*-tests.

## 4. Discussion

### 4.1. *CsLAR* and *CsUGT84A* Encode Key Enzymes for EGCG Biosynthesis

EGCG biosynthesis is regulated by several crucial enzymes. *LAR* has been demonstrated in association with flavonoid biosynthesis in multiple plants, including *Vitis vinifera* [33], *Populus* [34], and *Lycium ruthenicum* Murr [35]. Further, numerous studies have found that *LAR* is closely related to catechin biosynthesis in tea plants. Kumar et al. found that *LAR* is one of the key regulators involved in catechin biosynthesis during the development and seasonal variation among different tea plant cultivars [36]. *LARs* can also promote the biosynthesis of catechin monomers and inhibit their polymerization [37]. Furthermore, *LAR* gene expression is up-regulated in the pruned tea plants, which is highly correlated with changes in EGCG content in pruned tea plants. Additionally, glycosylation by UGT enzymes is commonly the last process for natural product biosynthesis in plants [38]. Two glycosyltransferases, *UGT79B1* and *UGT84A2*, were found to be involved in anthocyanin biosynthesis in *Arabidopsis* [39]. In tea plants, it was found that the important precursor of EGCG biosynthesis,  $\beta$ -glucogallin is catalyzed by UGT enzymes from gallic acid and glucose urate diphosphate [40]. Therefore, *CsLAR* and *CsUGT84A* may be crucial genes for regulating catechin biosynthesis in tea plants, especially for the most abundant EGCG. In this study, we found that EGCG content is significantly decreased after foliar application

of moderate and high concentrations of nano-Se, which is accompanied by the same expression patterns of *CsLAR* and *CsUGT84A* genes, indicating that *CsLAR* and *CsUGT84A* may be the target genes in response to the effect of nano-Se on EGCG content in tea leaves.

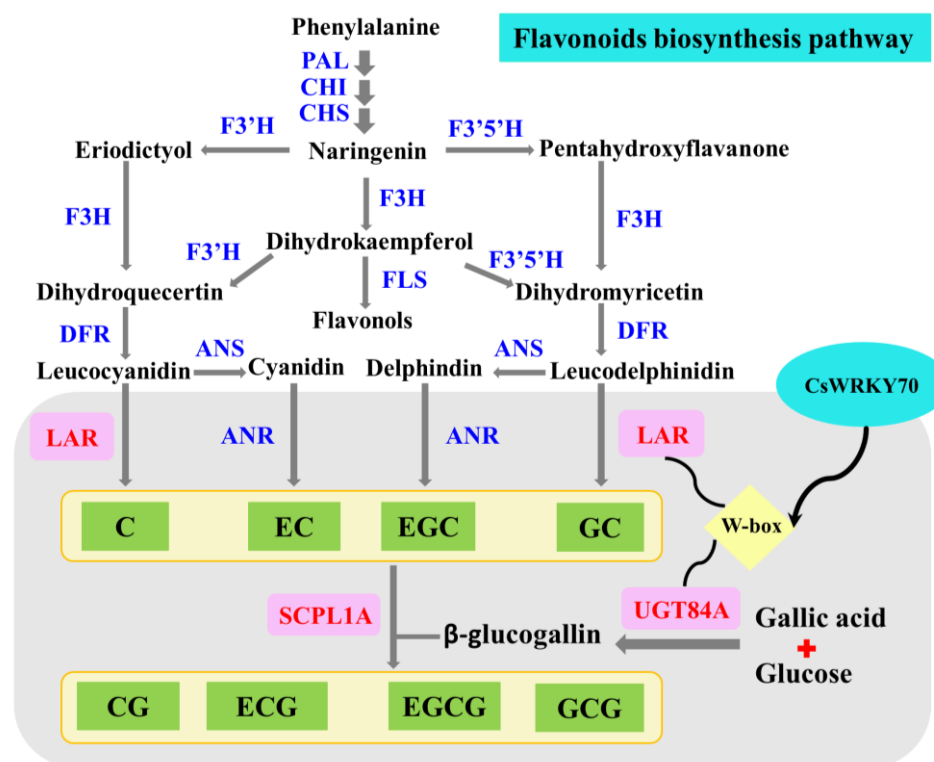
#### 4.2. *CsWRKY70* Belongs to the Group III of the WRKY Family and Is Positively Correlated with EGCG Content

WRKY TF family members are critical to plant growth and development, morphogenesis [41], hormone signal transduction [42,43], biological and abiotic stress [44], and secondary metabolite biosynthesis [45]. Our results showed that *CsWRKY70* belongs to Group III of the WRKY TF family. Further, *CsWRKY70* is homologous to *AtWRKY70* and *AtWRKY45* in *Arabidopsis thaliana* (Figure 2B). *AtWRKY70* and *AtWRKY54* are found to be involved in plant responses to biotic and abiotic stresses and regulate plant tolerance to osmotic stresses [46,47]. The WRKY TF family is also widely investigated in tea plants. Most of the identified WRKY genes in tea plants may be involved in response to drought, cold, ABA, or salt stresses [48]. Recently, WRKY TFs are involved in regulating phenylalanine metabolic pathways in various systems and play crucial roles in the flavonoid biosynthesis in plants. Several WRKY protein members were discovered to be involved in the biosynthesis of anthocyanins and proanthocyanidins in plants [49–51]. WRKY TFs are also found to associate with catechin and methylated catechin biosyntheses in tea plants [27,52]. These findings suggest that *CsWRKY70* may exhibit the same biological functions in tea plants. In this study, we found that moderate and high concentrations of nano-Se dramatically down-regulated the *CsWRKY70* expression in tea leaves. This finding is compatible with the variations in EGCG content after nano-Se treatments. Therefore, we speculated that *CsWRKY70* may regulate EGCG biosynthesis affected by nano-Se.

#### 4.3. *CsWRKY70* Binds to Cis-Elements of *CsLAR* and *CsUGT84A* Promoters and Acts as a Transcriptional Activator

WRKY TFs usually bind to the W-box elements on the promoter region of the target gene [45]. Cis-elements analysis revealed that *CsLAR* and *CsUGT84A* promoters contain several W-box elements, indicating that there exist potential interactions between *CsWRKY70* and *CsLAR* and *CsUGT84A*. Previous research indicated that *CsWRKY57*like activated the transcription of methylated EGCG biosynthesis-related genes (*CcCoAOMT*, *CsLAR*, and *CsDFR*) by binding to the W-box elements [22]. This finding is similar to our result. In our results, *CsWRKY70* is localized in the nucleus and processes transcriptional activation ability. However, no information is currently available concerning the regulatory effect of WRKY TFs on *CsUGT84A*. Further, we demonstrated that *CsWRKY70* directly binds to the *CsLAR* and *CsUGT84A* promoters and activates the expressions of *CsLAR* and *CsUGT84A*. Therefore, *CsWRKY70* may regulate EGCG biosynthesis under moderate and high levels of nano-Se treatments by affecting *CsLAR* and *CsUGT84A* gene expressions.

The transcriptional regulatory mechanism of *CsWRKY70* on *CsLAR* and *CsUGT84A* in the flavonoids biosynthesis pathway is schematized in Figure 7. *CsLAR* catalyzes the formation of non-galloylated catechins from phenylalanine. *CsUGT84A* is involved in the formation of  $\beta$ -glucogallin. Further, non-galloylated catechins and  $\beta$ -glucogallin are catalyzed to galloylated catechins by the key enzyme *SCPL1A* [9]. Recently, *CsMYB1* is found to regulate galloylated catechins biosyntheses, such as EGCG and ECG, by regulating *CsANR* and *CsSCPL1A* gene expressions in tea leaves [53]. Our results enriched the understanding with regard to how *CsWRKY70* might regulate EGCG biosynthesis. In the future, we will explore the regulatory mechanisms of the two crucial gene families, *UGT84A* and *SCPL1A*, on the biosynthesis of galloylated catechins in tea plants more deeply.



**Figure 7.** Diagram depicting the mechanism of CsWRKY70 affecting EGCG biosynthesis by regulating *CsANR* and *CsSCPL1A* expressions in tea leaves after nano-Se foliar application. PAL, phenylalanine ammonia lyase; CHI, chalcone isomerase; CHS, chalcone synthase; F3'H, flavanone 3-hydroxylase; F3'5'H, flavonoid 3', 5'-hydroxylase; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase; SCPL1A, serine carboxypeptidase-like acyltransferase; UGT84A, uridine diphosphate glucuronosyltransferase; C, catechin; EC, epicatechin; EGC, epigallocatechin; GC, galocatechin; CG, catechin gallate; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; GCG, galocatechin gallate.

## 5. Conclusions

This work demonstrated that nano-Se fertilizers can significantly decrease the EGCG content in summer tea leaves. Transcriptome sequencing data suggested that expressions of a WRKY TF (*CsWRKY70*) and catechin biosynthetic-related genes (*CsANR* and *CsSCPL1A*) were positively correlated with EGCG content. *CsWRKY70* was further isolated from tea leaves and found to be a transcriptional activator. *CsWRKY70* is a nuclear localization protein, which can specifically bind to *cis*-acting elements (*W*-box) on the promoters of *CsLAR* and *CsUGT84A*. These results indicated that *CsWRKY70* affects EGCG biosynthesis by regulating *CsANR* and *CsSCPL1A* expressions in tea leaves after nano-Se foliar application. Our results enrich the knowledge of the molecular regulatory mechanism of WRKY TFs involved in catechin biosynthesis and provide a theoretical basis for molecular breeding and innovation of tea cultivars.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9010120/s1>, Table S1: contents of major chemical components in tea leaves after foliar spraying of nano-Se (%). Table S2: contents of catechins in tea leaves after foliar spraying of nano-Se (mg/g). Table S3: list of primers used in this study. Table S4: differentially expressed transcription factors after foliar application of different concentrations of nano-Se. Figure S1: gene expression levels of differentially expressed catechin biosynthesis-related genes from transcriptome.



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