Article

Genome-Wide Analyses of Tea Plant Stress-Associated Proteins (SAPs) Reveal the Role of CsSAP12 in Increased Drought Tolerance in Transgenic Tomatoes

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Abstract: Plant stress-associated proteins (SAPs) contain A20/AN1 zinc finger domains and are involved in plant response to abiotic stresses. In this study, we aimed to explore the biological function of tea plant CsSAPs. A total of 14 CsSAP genes were identified in the tea plant genome using a reference genome database (Camellia sinensis var. sinensis). The CsSAPs were divided into the following two groups: Group I, containing one AN1 domain and/or one A20 domain; and Group II, containing two AN1 domains and/or two C2H2 domains. The sequence alignments and conserved domains analysis indicated that the CsSAPs were highly structurally conserved in terms of amino acid sequence and protein structure. The CsSAPs showed different transcript levels in spatio-temporal expression and in response to cold and drought stress in tea plants. Furthermore, the expression of CsSAP12 was considerably upregulated under drought stress. The overexpression of CsSAP12 in transgenic tomatoes showed increased tolerance to drought stress compared with the wild type. Altogether, the results showed that CsSAP12 might be involved in drought stress. Thus, CsSAP12 might be a target gene in genetic engineering to improve drought tolerance in tea plants.

Keywords: SAP gene family; tea plant; CsSAP12; expression analysis; functional analysis; drought stress

1. Introduction

Environmental stresses, including biotic and abiotic stresses, severely affect plant growth, development, and even survival. However, plants have evolved complex molecular mechanisms for regulating their gene expressions in response to adverse natural environments that could minimize stress damage by altering their growth and development, and physiological and biochemical responses to stress [1]. Stress-associated proteins (SAPs) are a class of newly discovered zinc finger proteins that act as a key factor in the plant response to abiotic stresses [2].

The A20, AN1, or both A20/AN1 zinc finger domains are contained in the plant SAP family members [3]. The A20 zinc finger domain protein was first identified in human vascular endothelial cells, which is tumour necrosis factor alpha (TNFA)-inducible protein, and its C-terminus has at least one C2C2 zinc finger domain [4–6]. The protein AN1, was the first identified protein encoded by the RNA of the maternal hemisphere of Xenopus laevis, has conserved sequences that can be divided into two categories [7]. The first category is CX2CX9–12CX1–2CX4CX2HX5HXC (X stands for any amino acid, C stands for cysteine, and H stands for histidine), which is usually present without the A20 domain, and the corresponding gene contains introns in its sequence [8]. The second category is CX2CX9–12

Citation: Fan, S.-C.; Li, C.; Li, S.-H.; Tang, J.; Shi, H.-D.; Yang, T.-M.; Liang, M.-Z.; Liu, D.-D. Genome-Wide Analyses of Tea Plant Stress-Associated Proteins (SAPs) Reveal the Role of CsSAP12 in Increased Drought Tolerance in Transgenic Tomatoes. Horticulturae 2022, 8, 363. https://doi.org/10.3390/horticulturae8050363

Academic Editor: Krishna Bhattarai
Received: 7 March 2022
Accepted: 20 April 2022
Published: 21 April 2022
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CX4CX2HX5HXC [9], which contains the A20 domain and non-introns in its sequence. A total of 8, 27, 15 and 24 SAPs, belonging to the first type of AN1 zinc-finger structure, are present in rice, cotton, desert poplar (Populus euphratica), and apple, respectively [10–12].

The SAP members have been induced by several challenges and found to play a significant role in improving tolerance to abiotic stress [11,13]. The OSISAP1/OsSAP1 is the first stress-associated protein studied, which was stimulated by drought, salt, and cold [11]. In Arabidopsis, AtSAP5 could improve salt tolerance and drought resistance, and the analysis of transgenic Arabidopsis using gene chips indicated that the expression of many genes associated with stress had increased [14]. Furthermore, the SAP genes were associated with biotic and abiotic stresses in Artemisia annua, tomato, rice and banana [15–19]. In tomato, SISAP3 and SISAP4 expression increased basic resistance in Pseudomonas syringae pv. tomato DC3000 and necrotrophic fungus Botrytis cinerea, respectively [20]. Additionally, the SAP gene is associated with the regulation of signal transduction and hormone synthesis. The overexpression of OsDOG (OsiSAP11) and OsZFP185 (OsiSAP4) resulted in dwarf phenotypes, decreased gibberellic acid (GA) contents, and insufficient cell elongation [21]. Furthermore, OsZFP185 down-regulates the expression of genes associated with abscisic acid (ABA) biosynthesis and interferes with ABA-mediated tolerance to high salt, drought, and cold. The SAP genes are also associated with the regulation of plant development. Overexpression of OsZFP185 (OsiSAP4) in rice brings about a dwarfism phenotype, a decrease in endogenous GA3 and ABA content, and negative regulation of the expression of several genes bound up with ABA biosynthesis [21]. Furthermore, SAPs with E3 ubiquitin ligase activity have a hand in the regulation of redox sensors and/or regulators of gene expression under abiotic stress. In Arabidopsis, AtSAP5 acts as an E3 ubiquitin ligase to participate in drought tolerance [22].

The genome-wide analysis of SAP genes in plants has helped understand their biological functions. In this study, the TPIA database (http://tpia.teaplant.org/, accessed on 24 March 2020) was used to excavate and identify CsSAP genes (CsSAPs). Furthermore, CsSAPs were comprehensively and systematically analysed in tea, including their conserved domains, protein and gene structures, phylogenetic relationships, cis-acting elements, and transcript levels under cold and drought stresses. Finally, CsSAP12 was isolated from tea and was transformed into tomatoes to identify its function. This study provides an analysis of the biological functions of CsSAPs under abiotic stresses in tea plant with a solid theoretical basis and reference.

2. Materials and Methods
2.1. Identification of CsSAPs in Tea Plant

Protein sequences of tea plant were downloaded from the published TPIA database to construct a local blast database. Both A20 (A20-like zinc finger, PF01754) and AN1 conservative domains (AN1 zinc finger, PF01428) were downloaded from the Pfam database (http://pfam.xfam.org/, accessed on 24 March 2020). The software HMMER 3.1 (http://hmmer.org/, accessed on 24 March 2020) was used to identify the candidate CsSAP genes from the tea plant protein sequences. The candidate genes were confirmed using NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 24 March 2020) and Pfam (http://pfam.xfam.org/, accessed on 24 March 2020) for further confirmation of the A20 and/or AN1 conserved domains.

2.2. Evolutionary Analysis of Tea Plant CsSAPs

The evolutionary tree analysis software MEGA 6 (http://www.megasoftware.net, accessed on 24 March 2020) was used to analyse the evolution of SAPs using the maximum likelihood (ML) approach (execution parameters: position correction, paired deletions, and guidance (1000 repetitions)).
2.3. Gene Structure and Genome Localization of CsSAP Genes

The data on intron, exon, and genomic mapping of CsSAPs were acquired from the TPIA database. The introns and exons in the CsSAPs of tea plant were mapped according to their location in the TPIA database.

2.4. Amino Acid Sequences and Conserved Elements of CsSAPs

Both the A20 and AN1 domains of tea plant CsSAPs were analysed using the software DNAMAN 5.0 and WebLogo 3 (http://weblogo.threeplusone.com/, accessed on 24 March 2020). The online software SWISS-MODEL was used to analyse the 3D structure of the A20 domain (AN1 topology; PDB ID: 2KZY) and the AN1 domain (AN1 topology; PDB ID: 1WFP) of CsSAPs for model matching (http://swissmodel.expasy.org/, accessed on 24 March 2020). The 3D structure was visualized using RasTop software (http://www.geneinfinity.org/rastop/, accessed on 24 March 2020).

2.5. Cis-Acting Elements in Promoters

To assess assumed cis-acting elements in the promoter of CsSAPs in tea plant, we isolated the genome sequences 1500 bp upstream of the translation start codon of CsSAPs. We then predicted putative cis-acting elements based on the Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 March 2020).

2.6. Plant Materials, Growth Conditions and Stress Treatments

Fully-expanded leaves from tea (C. sinensis var. Assamica cv. Yunkang10) that were cultured in a greenhouse were collected and included as the control group. These plants were grown in a greenhouse, and drought stress treatment was not initiated until these plants were about 50 cm high. Type YK-10 planted in a climate incubator for 15 days without water was used as samples for evaluating the effects of water deficits. The sampling schedule involved the harvesting of mature leaves from drought-treated YK-10 from the intermediate node on days of 0, 3, 5, 7 and 15 after treatment. All samples were immediately frozen in liquid nitrogen and stored in a refrigerator at −80 °C.

2.7. The qRT-PCR Analysis of CsSAPs

We extracted total RNA from the frozen leaves of tea plant using RNAprep pure Plant Kit (TIANGEN; Beijing, China). In total, 2 µg of RNA was collected for synthesizing the first strand of cDNA through the use of a PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa; Shanghai, China). For the qRT-PCR assays, reverse transcription was performed with 1 µg of total RNA from each sample, followed by amplification of 1 µL of the product. We performed qRT-PCR assays with 20 µL of reaction mixtures containing 10 µL of SYBR® Premix Ex Taq™ (TaKaRa; Beijing, China). The sequences of primers used for qRT-PCR are listed in Table S1. The ∆Ct values were calculated by using CsActin or SlActin as the endogenous control. The relative fold change in gene expression of samples was calculated using the 2−∆∆Ct method [23]. The qRT-PCR conditions were pre-denaturation at 95 °C for 30 s; followed by 35 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 15 s. The qRT-PCR was also used to calculate the copy number of foreign genes, and SlGRX1 was selected as the single copy reference gene [24,25].

2.8. Vector Construction, Plant Transformation and Drought Stress Treatment

The open reading frame (ORF) of CsSAP12 was amplified by PCR, which consisted of denaturation at 94 °C for 5 min; followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The cDNA of CsSAP12 was cloned into pBWA(V)KS plant transformation vectors containing the kanamycin resistance gene. Tomato transformations were performed as previously described [26]. The transgenic plants were screened and confirmed by kanamycin-resistance and PCR using a primer pair specific to the CsSAP12 primers (Table S1). Additionally, tomato seeds were screened on MS culture medium containing
kanamycin to identify transgenic plants. Therefore, MDA content, oxygen free radical content, and antioxidant enzyme activities were determined using T3 generation plants.

The transgenic tomato and wild-type plants were planted for 3 months before being used for drought stress in a greenhouse. Drought stress treatment was performed for 20 days without water. The plants were analysed using the determination of physiological indices, such as leaf relative water content, MDA content, oxygen free radical content, and antioxidant enzyme activities.

2.9. Measurement of Physiological Indices

For the measurement of leaf relative water content, leaves were weighed using a balance (Suzhou Science Instrument Co., Ltd., Suzhou, China) and recorded as fresh weight (FW). The leaves were dried to a constant weight at 100 °C in an oven (Shanghai Badh Machinery Equipment Co., Ltd., Shanghai, China). Next, the leaves were weighed as dry weight (DW). The calculation formula of leaf water content was as follows:

\[
\text{Leaf water content} = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100\%.
\]

The accumulation assay of two Reactive Oxygen Species (ROS), hydrogen peroxide (H₂O₂) and oxygen free radicals (O₂⁻), MDA content, and activities of antioxidant enzyme, including SOD (EC: 1.15.1.1), CAT (EC: 1.11.1.6), and POD (EC: 1.11.1.7) were performed using reagent kits of A064-1-1, A052-1-1, A003-1, A001-1, A007-1-1 and A084-3-1 (Jiancheng Bioengineering Institute, Nanjing, China), respectively.

The osmotic potential (\(\Psi_w\)) was calculated according to the formula: \(\Psi_w = -iCRT\); \(i\), dissociation coefficient (CaCl₂ = 2.60), the concentration of CaCl₂ solution comparable to the water potential of the plant material was found by varying the weight of the plant material. C, isotonic concentration (mol/L). R, Gas constant 0.008314 LMPa/(mol·K), T, Kelvin temperature.

3. Results

3.1. Identification of CsSAPs in Tea Plant

In total, 14 CsSAPs were obtained from the proteins of tea plant. The gene name, ID, conserved domains, protein length, molecular weight, theoretical pI, and chromosome location of the CsSAPs are listed in Table 1. The genomic DNA sequences of the CsSAPs ranged from 435 bp to 9197 bp, whereas the length of the coding DNA sequence of the CsSAPs ranged from 435 bp to 1737 bp. Furthermore, the CsSAPs contained different types of zinc-finger domain, i.e., both CsSAP6 and CsSAP7 contained the AN1-AN1 domain, whereas CsSAP11 contained only an AN1 domain, 11 other CsSAPs contained the A20-AN1 domain. Multiple sequence alignment analysis showed that the A20 and AN1 domains and multiple amino acid sites were highly conserved (Figure 1).

Sequence logos were then produced, further indicating that these domains were highly conservative at every residue position (Figure 2a,b). Furthermore, by modelling and analysing the A20 and AN1 domains of the CsSAPs using the SWISS-MODEL online software, the homologous models of the A20 and AN1 domains are shown in Figure 2c,d, respectively. The results showed clearly that the A20 and AN1 domains of the CsSAPs work together with the A20 domain of ubiquitin receptor ZNF216 and the zf-AN1 domain of Arabidopsis F5O11.17 protein (PDB ID: 2KZY.A, 44.7% sequence identity for residues 10–48; 1WFP.1.A, 53.2% sequence identity for residues 107–153), suggesting that the A20 and AN1 domains of the CsSAPs were highly conserved.

3.2. Analysis of CsSAPs Structure and Conservative Elements

The evolution, exon-intron structure, and conserved domains were analysed to explore the structural variability of the SAP family genes. An evolutionary analysis of 14 CsSAPs showed that they can be separated into two groups: Group I, including one AN1 domain and/or one A20 domain; and Group II, including two AN1 domains and/or other domains (Figure 3a). Gene structure analysis results showed that CsSAP1-CsSAP5, CsSAP8, CsSAP9,
CsSAP11, and CsSAP14 had no introns, CsSAP7 and CsSAP10 had only one intron, and CsSAP6 contained two introns (Figure 3a). Conservative domain analysis showed that all the CsSAPs contained A20 and/or AN1 conserved domains (Figure 3b). In Group I, CsSAP5, CsSAP8, CsSAP9, CsSAP11 and CsSAP14 had no introns, and CsSAP7 and CsSAP10 contained A20 and AN1 conserved domains. CsSAP6 and CsSAP7 in Group II contained two AN1 conserved domains.

**Table 1. Properties of CsSAPs identified from tea plant genome.**

| Gene Name | Gene ID | Zinc-Finger Domain | CDS Length (bp) | Protein Length (aa) | Molecular Weight (kDa) | No. of Introns | Theoretical pI | Scaffold Location |
|-----------|---------|---------------------|-----------------|---------------------|-------------------|-----------------|----------------|------------------|
| CsSAP1    | TEA003271.1 | A20-AN1             | 519             | 173                 | 18.31             | 0               | 7.72           | Scaffold622:157553:157987:+ |
| CsSAP2    | TEA007661.1 | A20-AN1             | 522             | 174                 | 17.45             | 0               | 7.51           | Scaffold3002:1448206:1457402:− |
| CsSAP3    | TEA007758.1 | A20-AN1             | 483             | 161                 | 17.06             | 0               | 8.14           | Scaffold1761:663031:663537:− |
| CsSAP4    | TEA008252.1 | A20-AN1             | 516             | 172                 | 18.14             | 0               | 7.44           | Scaffold435:1131711:1132217:+ |
| CsSAP5    | TEA009516.1 | AN1-AN1             | 492             | 164                 | 17.53             | 0               | 7.76           | Scaffold4677:9737:10201:+ |
| CsSAP6    | TEA013656.1 | AN1-AN1             | 1737            | 579                 | 63.76             | 4               | 9.66           | Scaffold942:754898:755419:+ |
| CsSAP7    | TEA013661.1 | AN1-AN1             | 573             | 191                 | 20.92             | 1               | 8.52           | Scaffold3002:1448206:1457402:− |
| CsSAP8    | TEA014231.1 | A20-AN1             | 465             | 155                 | 17.24             | 0               | 8.91           | Scaffold4677:9737:10201:+ |
| CsSAP9    | TEA016540.1 | A20-AN1             | 507             | 169                 | 17.96             | 0               | 8.46           | Scaffold435:1131711:1132217:+ |
| CsSAP10   | TEA016572.1 | AN1-AN1             | 507             | 169                 | 18.75             | 0               | 9.11           | Scaffold1761:663031:663537:− |
| CsSAP11   | TEA021384.1 | A20-AN1             | 498             | 166                 | 17.85             | 0               | 8.08           | Scaffold4125:30076:307293:+ |
| CsSAP12   | TEA023579.1 | A20-AN1             | 492             | 164                 | 17.64             | 0               | 8.7            | Scaffold5358:143051:143542:+ |
| CsSAP13   | TEA025409.1 | A20-AN1             | 435             | 145                 | 15.69             | 0               | 8.6            | Scaffold2300:157553:157987:+ |

*'+' and ‘−’ indicate that the gene is located in the forward and reverse strand, respectively.

**Figure 1.** Multiple alignments of the A20/AN1 domain in tea plant CsSAPs. The conserved domains are shown in boxes, identical amino acids are shown with a dark blue background (similarity: dark blue = 100%; pink > 75%; cyan > 50%).
Sequence logos were then produced, further indicating that CsSAP10 contained A20 and AN1 conserved domains. CsSAP6 and CsSAP7 in Group II contained two AN1 conserved domains.

**Figure 2.** (a) Sequence logos of A20 domain in the CsSAPs. (b) Sequence logos of the AN1 domain in the CsSAPs. (c) Three-dimensional tertiary structural model of the A20 domain (PDB ID: 2KZY.1.A); and (d) three-dimensional tertiary structural model of the AN1 domain (PDB ID: 1WFP.1.A). The logos of the A20 and AN1 domains were obtained through multiple alignments of the CsSAPs. In each stack, the symbol height represents the relative frequency of each amino acid at that position; numbers 1–5 represent β-sheets in A20 and AN1 domains; α-helices are red, β-sheets (numbers 1–5) are yellow, and strands are blue/green.

**Figure 3.** Sequence analysis of CsSAPs. (a) Phylogenetic relationships and Gene structure analysis; (b) analysis of the conserved domains for CsSAPs in tea plant. The phylogenetic tree of full-length amino acid sequences was constructed using MEGA software.

### 3.3. Phylogenetic Analysis of CsSAPs

To examine the evolutionary relationships among plant SAPs in tea plant and other plants, such as *Actinidia chinensis* and *Vitis vinifera*, full-length protein sequences encoded
by 118 SAP genes from 20 species were used to construct unrooted phylogenetic trees. The evolutionary analysis of CsSAPs showed that they were classified into following two groups: CsSAP1-CsSAP5 and CsSAP8-CsSAP14 belonged to Group I, containing an AN1 domain and/or an A20 domain; CsSAP6 and CsSAP7 belonged to Group II, containing two AN1 domains and/or other domains (Figure 4). Group I was classified into two subgroups (Ia–Ib): Ia, containing one AN1 domain and one A20 domain, including CsSAP1-5, CsSAP8-10 and CsSAP12-14; and Ib, containing only one AN1 domain, CsSAP11. Group II can be divided into three subgroups (IIa–IIb): IIa, containing two AN1 domains and two C2H2 domains, including CsSAP6; IIb, two AN1 domains, including CsSAP7.

Figure 4. Phylogenetic analysis of 118 SAPs from 20 species of plants. The unrooted NJ tree was constructed using the full-length amino acid sequences. Ia, Ib, IIA, IIB indicates four subgroups. 14 CsSAPs in tea were showed in red and plus stars.

3.4. Functional Prediction of CsSAPs

To identify the likely cis-regulatory elements (CREs) of CsSAPs, we isolated genomic sequences 1500 bp upstream of the start codon from CsSAPs. A total of 100 CREs belonging to 10 typical types of abiotic and biotic response elements or transcription factors binding sites were obtained in 14 promoters. These were related to responses to hypoxia, cold, drought, pathogens, trauma, and hormones (Table 2). Most CsSAPs promoters contained the anaerobic response element, ethylene-responsive element, and W-box element. Additionally, some hormone-related CREs, such as ABA, salicylic acid, methyl jasmonate, and ethylene, were also found in CsSAPs gene promoters. The results showed that the CREs of CsSAPs played a significant role in the stress responses of plant and might be associated with the circadian clock, cell differentiation, and morphogenesis regulation.
Table 2. The cis-acting elements analysis of CsSAPs.

| Cis-Acting Elements | ABRE | ARE | CGTCA | ERE | LTR | MBS | TCA | TC-Rich Repeat | W-Box |
|---------------------|------|-----|-------|-----|-----|-----|-----|----------------|-------|
| Stress to Response  | ABA  | Hypoxia | MeJA | Ethylene | Chilling | Drought | SA | Defence | Pathogen |
| CsSAP1              | 0/5  | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP2              | 0/2  | 1/1 | 0/1 | 0/1 | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP3              | 1/1  | 3/0 | 1/0 | 0/1 | 1/0 | 1/0 | 1/0 | 1/0 | 2/0 |
| CsSAP4              | 2/0  | 2/2 | 1/0 | 1/0 | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP5              | 1/0  | 1/1 | 0/1 | 1/0 | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP6              | 0/1  | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP7              | 1/1  | 1/0 | 1/0 | 0/1 | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP8              | 1/1  | 0/1 | 0/1 | 1/0 | 0/1 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP9              | 2/2  | 1/2 | 1/0 | 1/0 | 1/0 | 1/0 | 1/0 | 0/1 | 1/0 |
| CsSAP10             | 1/1  | 2/1 | 0/1 | 2/0 | 0/1 | 1/0 | 1/0 | 0/1 | 2/1 |
| CsSAP11             | 0/3  | 0/1 | 0/1 | 1/0 | 1/0 | 0/1 | 1/0 | 0/1 | 1/0 |
| CsSAP12             | 0/1  | 1/0 | 0/1 | 3/2 | 1/0 | 0/1 | 1/0 | 1/0 | 1/0 |
| CsSAP13             | 0/1  | 1/0 | 1/0 | 0/1 | 1/0 | 0/1 | 1/0 | 1/0 | 1/2 |
| CsSAP14             | 0/1  | 1/0 | 1/0 | 0/1 | 1/0 | 0/1 | 1/0 | 0/1 | 1/0 |

ABRE (ABA response element), ARE (anaerobic response element), CGTCA (MeJA-responsiveness), ERE (ethylene-responsive element), LTR (low-temperature response element), MBS (MYB binding site involved in drought response), TCA (salicylic acid response element), TC-rich repeat (defence and stress responsiveness), and W-box (elicitation; wounding and pathogen responsiveness; binding site of WRKY type transcription factors); digits represent the number of regulatory elements on the positive and negative chain. The blank area indicates no corresponding cis-acting element in any chain of the promoter.

3.5. Expression Pattern of CsSAPs in Tea Plant

Identifying the expression patterns of CsSAPs in different tissues can help us understand their functions. Real-time quantitative PCR results showed that CsSAP1, CsSAP2, CsSAP6, CsSAP13 and CsSAP14 were highly expressed in roots and leaves, CsSAP3 and CsSAP8 were expressed strongly in stems, and CsSAP4, CsSAP5, CsSAP7, CsSAP9, CsSAP10, CsSAP11 and CsSAP12 were highly expressed in stems and flowers (Figure 5a). In addition, the relative expressions of CsSAPs were determined in tea plants under drought stress. Under 5 days of drought treatment, the expression of CsSAP9 and CsSAP12 increased significantly, whereas the transcripts of CsSAP4 was significantly reduced. The expression of the other CsSAPs showed no obvious changes (Figure 5b).

3.6. CsSAP12 Overexpression Enhances Drought Tolerance in Transgenic Tomato

Transcript analysis showed that CsSAP12 was significantly accumulated under drought stress. The full-length cDNA of the CsSAP12 obtained from tea plant was 498 bp, which encoded a predicted protein containing 165 amino acid residues with a calculated molecular mass of 17.86 kDa and an isoelectric point of 8.44. It contained both A20 and AN1 zinc finger domains and its amino acid sequence showed a similarity of up to 53.27% with that of SlSAP4 (Figure S1), which corresponded to drought stress in tomato plants (Solanum lycopersicum) [27].

To further identify its function, CsSAP12 was transformed into tomatoes. After detection, 12 transformants were identified and confirmed with obviously increased levels of CsSAP12 transcripts; it could not be observed in wild type (WT) (Figure 6a). In transgenic tomato seedlings, the copy number of CsSAP12 was 1.39 times that of the reference gene, SIGRX1, indicating that CsSAP12 was a single copy gene in transgenic tomatoes (Figure S2). We then selected transgenic tomatoes with high CsSAP12 expression to assess their potential function when responding to drought stress.
Identification of transgenic tomato lines under drought stress. (a) Phenotypes of WT and transgenic lines under drought stress. The values are represented as the mean ± standard deviation, and different letters of the alphabet represent significant differences calculated by Duncan’s multiple range test ($p < 0.05$).

**Figure 5.** Tissue-specific expression and drought response of 14 CsSAPs. (a) Expression patterns of CsSAPs in different tissues of tea plant. (b) Expression patterns of CsSAPs in response to drought stress.

**Figure 6.** Identification of transgenic tomato lines under drought stress. (a) Semi-quantitative PCR analysis of the CsSAP12 expression level. (b) Relative expression of CsSAP12 in leaves of WT and transgenic plants. (c) Phenotypes of WT and transgenic lines under drought stress. The values are expressed as the mean ± standard deviation, and different letters of the alphabet represent significant differences calculated by Duncan’s multiple range test ($p < 0.05$).
The WT tomato and CsSAP12-overexpressed seedlings showed similar growth characteristics without any treatment, and there were no obvious changes in seeds germination, leaf development, flower fruit, and leaf development between transgenic and wild tomatoes (Figure S3). After 20 days of drought stress, the green leaves of WT plants were wilted and subsequently turned yellow and drooped significantly; CsSAP12-overexpressed transgenic tomatoes displayed slightly wilted leaves but without drooping (Figure 6b). Furthermore, the WT plants did not recover normal development when rehydration began (2 days of refreshment), the transgenic tomato leaves recovered to upright growth (Figure 6c), and the expression of CsSAP12 in the transgenic lines treated with drought stress was significantly higher ($p$-value < 0.05) than that under 2 days of refreshment (Figure 6b), suggesting that the resistance of transgenic tomatoes to drought was increased due to the increased transcript level of CsSAP12.

3.7. Measurement of Physiological Indices of CsSAP12 Transgenic Plants

In a further effort to appraise the growth conditions of CsSAP12 transgenic lines under drought stress, leaf relative water content, malondialdehyde (MDA) content, and oxygen free radical content (O$_2^-$) were evaluated in WT and transgenic tomatoes. The leaf relative water content of transgenic lines was remarkably higher than that of WT under drought stress and after 2 days of refreshment (Figure 7a). The MDA content and oxygen free radical content of WT were significantly higher than those of the transgenic lines. After 2 days of refreshment, the MDA and oxygen free radical content of both WT and transgenic lines decreased rapidly (Figure 7b,c).

Moreover, antioxidant enzyme activities, including peroxidase (POD), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) were also measured. Compared with WT after 20 days of drought, CAT, POD, and SOD activities in the transgenic lines were 1.37-, 1.29- and 1.47-times higher than those of WT, respectively. The antioxidant enzyme activities of WT and transgenic lines after 2 days of refreshment displayed a sharp downward trend compared with that of WT and transgenic lines after 20 days of the drought-contrasting trend; the CAT, POD, and SOD activities of the transgenic lines were 1.74-, 1.16- and 1.82-times greater than those of the WT (Figure 7d).

Furthermore, to identify the physiological differences between the WT and transgenic tomatoes at the early stage of drought stress, osmotic potential and two Reactive Oxygen Species (ROS), hydrogen peroxide (H$_2$O$_2$) and oxygen free radicals (O$_2^-$) were also detected using WT and transgenic tomatoes under 7 days drought stress. The results showed that the osmotic potential of CsSAP12 transgenic plants was much lower than that of wild plants after 7 days of drought and 2 days of refreshment (Figure 7e). Both hydrogen peroxide and oxygen free radicals accumulated mostly in leaves, and they were decreased obviously in the WT and transgenic tomatoes after 7 days of drought (Figure 7f,g). Interestingly, no obvious changes were found in the hydrogen peroxide contents of stems between the WT and CsSAP12 transgenic tomatoes, while the content of oxygen free radicals in CsSAP12 transgenic plants was lower than that of the WT. After 2 days of refreshment, the hydrogen peroxide and oxygen free radical content were decreased in both the WT and transgenic lines. Moreover, the hydrogen peroxide content showed no obvious changes in roots and stems of WT and transgenic tomatoes, but oxygen free radical contents of roots were lower in CsSAP12 transgenic lines than in the WT (Figure 7f,g).
Figure 7. Stress physiological indices and antioxidant enzyme activities of wild types (WT) and CsSAP12−overexpressed transgenic lines under drought stress. (a) Relative water content of leaves. (b) Oxygen free radical content. (c) MDA relative content. (d) Activities of antioxidant enzyme (CAT, POD, SOD) in WT and transgenic lines under drought stress and refreshment assay. (e) Osmotic potential. (f) Hydrogen peroxide and oxygen free radical content (g) of different tissues in WT and transgenic lines. The values are expressed as the mean ± standard deviation, and different letters of the alphabet a–d suggest significant differences calculated by Duncan’s multiple range test (p < 0.05), error bars represent standard errors.

4. Discussion

Recently, along with the discovery and sustained development of gene sequencing and sequence assembly technology, the preparation of high-quality profiles of plant genomes provides great convenience in the study of plant-specific agronomic traits, the acceleration of plant breeding, an increase in production, and the ability to resist biotic and abiotic stresses [28]. Tea, one of the world’s most important beverage crops, has a variety of secondary metabolites that are good for the human body, and has economic, medical, and cultural significance [29,30]. As the quality of tea genome profiles has improved, it provides great opportunities and platforms for identifying tea family genes [27,28]. Recently, several tea plant gene families have been identified, such as GRAS, LEA, HSP, WOX, WRKY, bZIP, and LBD [31–39]; however, reports about the tea plant SAP gene family are not available. The SAP gene families have been identified and analysed in rapeseed, cotton maize, tomato and Arabidopsis (Table 3). The numbers of SAP gene family members in rapeseed and cotton
are 57 and 37, respectively, whereas the numbers of SAP gene family members in maize, tomato, and Arabidopsis are 11, 13 and 14 [3,40,41]. In this study, 14 CsSAPs were identified by searching the tea plant genome. Compared with the number of SAP gene families in other species, the tea plant CsSAP gene family has fewer members. As reported in previous studies, gene replication, which includes a segment, tandem and genome-wide replication, is essential for the diversification of gene functions and the rearrangement and expansion of the genome [42,43]. The tea genome emerged in two rounds of whole-genome duplication (WGD) events 30–40 million years ago and 90–100 million years ago, respectively [32]. These WGD events and subsequent paralogous repeats impacted considerably on the copy number of secondary metabolite-related genes of tea plant. However, because of the lack of chromosome localization information, we could not specifically analyse whether members of the SAP gene family participated in gene replication events. With developments in the accuracy and assembly of tea genome sequencing, the promotion and replacement of search and analysis software, and the discovery of variable shear, the aforementioned problems will be overcome, and other SAP gene family members will be determined. Furthermore, the zinc finger structures of the SAP gene family are not the same in different plants. The number of A20 zinc finger structures among members of the SAP gene family of rapeseed, apple, and rice were 7, 3, and 1, respectively. The A20-A20-AN1 zinc-finger structure was also found in the Arabidopsis and rapeseed SAP gene families [40,44]. Altogether, the loss or increase in zinc-finger type genes in these genomes is essential for the complex enzyme activity of these plants.

Table 3. Numbers of SAP gene family members in various species.

| Plant Species          | A20-AN1 | A20-AN1 | A20-AN1 | A20-AN1 | A20-AN1 | A20-AN1 | A20-AN1 | Total Number |
|------------------------|---------|---------|---------|---------|---------|---------|---------|-------------|
| Camellia sinensis      | 11      | 0       | 0       | 1       | 2       | 0       | 0       | 14          |
| Arabidopsis thaliana   | 10      | 0       | 0       | 1       | 1       | 1       | 1       | 14          |
| Brassica napus         | 32      | 0       | 7       | 9       | 5       | 4       | 0       | 57          |
| Gossypium hirsutum     | 28      | 0       | 0       | 3       | 4       | 0       | 2       | 37          |
| Malus domestica        | 23      | 0       | 3       | 0       | 3       | 0       | 1       | 30          |
| Medicago truncatula    | 11      | 0       | 0       | 2       | 1       | 0       | 2       | 16          |
| Oryza sativa           | 11      | 1       | 1       | 3       | 1       | 0       | 1       | 18          |
| Populus euphratica     | 15      | 0       | 0       | 0       | 2       | 0       | 1       | 18          |
| Solanum lycopersicum   | 9       | 0       | 0       | 1       | 2       | 0       | 1       | 13          |
| Zea mays               | 8       | 0       | 0       | 1       | 1       | 0       | 1       | 11          |

The SAP gene family members of Arabidopsis, rice, tomato, and cotton are classified into five groups [32,41,43], while in apple (Malus domestica Borkh.), they are divided into the following two groups: Group I, with an A20 domain and an AN1 domain; and Group II, including two AN1 domains [5]. In the present study, we performed an evolutionary analysis of tea plant SAPs and compared them with 118 SAP genes from 20 species. The evolutionary analysis of tea SAPs showed that tea SAPs can be classified into the two groups: Group I (A20-AN1 type) and Group II (AN1 type). A typical feature of SAP in each plant is the absence of introns. Among the 12 CsSAP members of Group I, 11 CsSAPs were found to contain no introns, whereas all Group II-type CsSAPs contained introns. The 11 CsSAPs with no popular intron genes indicated the ancient origins of the SAP genes and their close relationship with the rapid accumulation of transcripts because of a reduction in post-transcriptional processes.

Plant SAP genes can be quickly induced by several abiotic stresses. The OsiSAP1/OcSAP1 gene can be upregulated by high salt, aridity, chill, ABA, submergence, and mechanical
wounding induction [44]. The OsSAP9 gene is upregulated responding to cold, heat, PEG6000, and other stresses [45,46]. The OsSAP8 gene is upregulated by salt, heat, cold, ABA, desiccation, heavy metals, submergence, and wounding [47]. Similar results induced by abiotic stress have also been found in Aeluropus littoralis, Arabidopsis, maize, cotton, Populus euphratica, Brassica napus, and apple [8,47–53].

The expression patterns of 14 SAP genes in tea plants were comprehensively analysed in this study. Among the 14 genes, CsSAP12 was, not only highly expressed in various tissues of plants, but its expression increased in drought conditions. Drought stress allowed the formation of hydrogen peroxide and oxygen free radical in plants, and could cause MDA accumulation because of membrane lipid peroxidation, indicating that antioxidant activity in plants can improve plant tolerance to different stress factors [53]. The overexpression of CsSAP12 can effectively reduce osmotic potential in leaves and the content of hydrogen peroxide, oxygen free radical in different tissues, promoting lower MDA content and higher leaf water content. And after drought treatment, the anti-oxidase activity of CsSAP12-overexpressed transgenic lines was higher than wild species and rapidly reduced after rehydration; MDA content and oxygen free radical content in CsSAP12-overexpressed transgenic lines were lower than in WT lines, while the relative water content of leaves was even higher, suggesting that transgenic tomatoes had better drought tolerance than that of wild-type tomatoes.

5. Conclusions

In this study, 14 CsSAP genes were identified in tea plant. We studied the function of CsSAP genes in apple growth and development using bioinformatics, gene expression, and functional analysis. Functional characterization showed that CsSAP12 might play an important role in salt stress. It will provide a basis for future studies to comprehensively and comparatively analyse the functional characteristics of CsSAP to deeply investigate aspects of drought stress in tea.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050363/s1. Figure S1: Protein sequence alignment of SISAP4 (GenBank accession number: XP 019066408) and CsSAP12 by Clustalx; A20 and AN1 domains are indicated at the top. Figure S2. The qRT-PCR amplification curve of CsSAP12 and SIGRX1. Figure S3. Phenotypes of CsSAP12 over-expressed transgenic and wildtype tomatoes. Table S1: Application of primers used for PCR and qRT-PCR.

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

Funding: This research was funded by the Natural Science Foundation of China (Grant No. 31660566), and The Open Fund of the State Key Laboratory of Yunnan Provincial Key Laboratory of Tea Science (Grant No. 2021YNCX002).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

Acknowledgments: We thank TopEdit (www.topeditsci.com, accessed on 25 March 2020) for its linguistic assistance during the preparation of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.
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