Molecular Cloning and Characterization of Galactinol Synthases in *Camellia sinensis* with Different Responses to Biotic and Abiotic Stressors

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Supporting Information

ABSTRACT: Galactinol synthase (GolS) is a key biocatalyst for the synthesis of raffinose family oligosaccharides (RFOs). RFOs accumulation plays a critical role in abiotic stress adaptation, but the relationship between expression of GolS genes and biotic stress adaptation remains unclear. In this study, two *CsGolS* genes were found to be highly up-regulated in a transcriptome library of *Ectopic oblique*-attacked *Camellia sinensis*. Three complete *CsGolS* genes were then cloned and characterized. Gene transcriptional analyses under biotic and abiotic stress conditions indicated that the *CsGolS1* gene was sensitive to water deficit, low temperature, and abscisic acid, while *CsGolS2* and *CsGolS3* genes were sensitive to pest attack and phytohormones. The gene regulation and RFOs determination results indicated that *CsGolS1* was primarily related to abiotic stress and *CsGolS2* and *CsGolS3* were related to biotic stress. GolS-mediated biotic stress adaptations have not been studied in depth, so further analysis of this new biological function is required.

KEYWORDS: *Camellia sinensis*, galactinol synthase, biofunctional investigation, gene regulation, abiotic stress, biotic stress

INTRODUCTION

Leaf wilting, root necrosis, and nutrient deficiency are common symptoms in plants subjected to biotic and abiotic stress.1 Many studies have focused on plant defense mechanisms (i.e., direct and indirect defenses) with the aim of enhancing sustainable agricultural production. For example, the lip-oxygenase pathway, which includes two biosynthetic routes mediated by allene oxide synthase or hydroperoxide lyase, was found to be an important indirect defense pathway for stress adaptation in various plants.2−4 The direct defense pathway includes raffinose family oligosaccharides (RFOs) such as raffinose, stachyose, and verbascose that are derived from sucrose and activate galactose moieties (donated by galactinol); these RFOs are extensively distributed in various plants.5 RFOs as plant storage carbohydrates (galactosyl-oligosaccharides) were found to have important roles in abiotic stress adaptation by assisting in the formation of a vitreous state that protects macromolecules.6,7 RFOs are also essential for phloem transport functions.8 When plants are subjected to abiotic stress (e.g., cold, drought, heat, or mechanical injury), RFOs accumulation occurs and osmotic pressure is regulated to maintain stable cells.9−11

Galactinol synthase (GolS, EC 2.4.1.123) belongs to glycosyltransferase family 8 and catalyzes the first step of the biosynthesis of RFOs by converting uridine diphosphate-D-galactose (UDP-D-galactose) to myo-inositol.12 GolS was first detected in a crude extract of maturing pea seeds13 and was partially purified from mature cucumber;14 the GolSs from legume seeds and cucurbit leaves were the first to be biochemically characterized.15 Recently, GolS genes have been isolated and characterized from various plant varieties, including hybrid poplar,11 *Brassica napus* L.,16 *Coffea arabica* L.,17 *Salvia miltiorrhiza*,18 grape,19 *Medicago falcate*,20 chestnut,21 *Cicer arietinum* L.,21 and many others. Most studies showed that GolS expression was regulated by various abiotic stressors (cold, drought, or heat) and that GolS activity enhanced plant tolerance to abiotic stress.1,11,16−20 While few studies have focused on the relationship between *GolS* gene expression and biotic stress adaptations, GolSs might also regulate biotic stress (pest attack or pathogen infection) responses; the mechanisms underlying GolS-mediated biotic stress responses require further investigation.11,22

Tea plant (*Camellia sinensis*) is one of the most important commercial crops in China and other Asian countries. As an evergreen woody plant, tea plants are susceptible to various biotic and abiotic stressors. Drought, freezing injury, plant diseases, and pest attack are the important biotic and abiotic stressors frequently affecting tea production.23 Although GolSs have been isolated and characterized in many plant varieties, GolS-mediated biotic and abiotic stress adaptation in *C. sinensis* has not been previously studied, despite this process being significant to tea plant cultivation and crop yields. This study, therefore, aimed to identify and characterize GolS genes in tea plant that were up-regulated under biotic and abiotic stress conditions. This information would be useful for further characterization of abiotic and biotic stress responses in tea.
plants and to breed new lines with improved resistance capabilities.

**MATERIALS AND METHODS**

**Preparation of Plant Materials and Treatments.** Two-year-old tea plant clone cuttings (C. sinensis ‘Shuchazao’) were obtained from the Dechang tea plantation (Shucheng, China), which were grown under controlled environment (12 h day/night ratio with 3000 lx light intensity at 25 °C). The low-temperature groups were treated at 0 and 4 °C, and the control group was kept at 25 °C. A total of 15 branches for each temperature group were inserted into floral foam and incubated at the specified temperatures for 3, 6, 9, 12, and 24 h. The drought group was watered for 5 days and the control every 1–2 days; samples were collected and analyzed on day 5, 8, 11, 14, and 17.

In the post-attacked group, tea geometrids (*Ectropis oblique*) at the third larval stage were placed on tea plants (10 geometrids per individual tea plant). After one-third of each leaf was consumed, the pests were removed and the surviving leaves were collected within 24 h. Tea plant leaves from the nonattacked group were used as the control. The phytohormone groups were evenly sprayed with 1.0 mM abscisic acid (ABA), 4.0 mM methyl jasmonate (MeJA), or 1 mM methyl jasmonate (MeJA), containing 0.05% Tween-20 until the leaves were wet; cuttings sprayed with 0.05% Tween-20 or distilled water were used as the control. Leaves of the same age and position were collected 3, 6, 12, 24, and 48 h after chemical treatments.

To determine the constitutive expression levels of GolS genes in different organs, apical buds, developing leaves, mature leaves, young stems, roots, petals, flower buds, and fruits were collected and evaluated. Unless specified otherwise, all treatments in this study were performed in triplicate, and the collected samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

**Nucleotide and Amino Acid Sequence Analyses.** Phylogenetic analysis of the CsGolS gene sequences was performed using BLAST searches in the National Center for Biotechnology Information database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The possible bootstrap values determined using 1000 replications.26

**RNA Extraction and cDNA Synthesis.** Total RNA was extracted from plant leaves (200 mg) using an RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The total RNA quality was examined by agarose electrophoresis and spectrophotometric measurement at an absorbance ratio of A260/A280. First-strand cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit (Takara) following the manufacturer’s instructions.

**Molecular Cloning of the Full-Length cDNAs of CsGolS Genes.** Full-length cDNAs of CsGolS genes were cloned using a SMART RACE Kit (Clontech, Dalian, China) according to the manufacturer’s instructions.30

**Protein Expression and Purification.** The ORFs of CsGolS1, CsGolS2, and CsGolS3 were amplified by PCR with pfu polymerase (Takara) using the cDNA generated from the total RNA of C. sinensis leaves, and the gene-specific primers P30–P35 were used (NotI and XhoI sites underlined, Supplementary Table 1, SI). After PCR amplification, the recombinant plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells (Novagen, Shanghai China). The transformed cells were incubated in LB broth containing ampicillin (50 μg/mL) at 16 °C, 25 °C, and 37 °C overnight for optimal temperature evaluation. Until the cell density reached 0.6 (OD600 absorbance), the broth was induced by adding a range of isopropyl β-D-1-thiogalactopyranoside concentrations (IPTG, 0.25–2.0 mM) for different periods of time (1–5 h). The optimal overexpression conditions were evaluated by examining the recombinant protein yield and quality using SDS–PAGE. His-tagged recombinant proteins in disrupted cells were purified by affinity chromatography with nickel-nitrilotriacetic acid agarose resin (Ni-NTA, Qiagen, CA), and the purified recombinant proteins were dissolved in phosphate buffer (pH 7.0).

**Galactosyl Synthase Activity Assay.** The purified recombinant proteins (CsGolSs) were used for the galactosyl synthase activity assay. A total reaction volume of 50 μL comprising 20.0 mM myoinositol, 4.0 mM UDP-Gal, 50 mM HEPES (pH 7.0), 2.0 mM dithiothreitol, 4.0 mM MnCl2, 4.0 μg BSA, and 10.0 μL recombinant protein (0.1 mg/mL) was obtained; the supernatant of the pET-32a (+) transformant and phosphate buffer were used as negative controls.27 The enzyme reaction was performed at 30 °C for 5 h and stopped by the addition of 50 μL of 100% ethanol. After 25.0 μg of phenyl α-D-glucoside was added as an internal standard, the reaction mixture was incubated at 80 °C for 30 min, passed through a 10 000 Da cutoff filter (NANOSEP microconcentrators, Pall Filtron), and evaporated to dryness under a nitrogen stream. Trace water in the resulting residue was removed by phosphorus pentoxide in a desiccator overnight. The thoroughly dried residue was derivatized with 200 μL of trimethylsilyl imidazole-pyridine (1:1, v/v) at 80 °C for 45 min and analyzed for phenyl α-D-glucoside and galactosyl using a gas chromatography–mass spectrometry (GC–MS, Shimadzu) analysis.32 The ORFs of CsGolS1, CsGolS2, and CsGolS3 were amplified using the 2 μL of 100% ethanol. After 25.0 μg of phenyl α-D-glucoside was added as an internal standard, the reaction mixture was incubated at 80 °C for 30 min, passed through a 10 000 Da cutoff filter (NANOSEP microconcentrators, Pall Filtron), and evaporated to dryness under a nitrogen stream. Trace water in the resulting residue was removed by phosphorus pentoxide in a desiccator overnight. The thoroughly dried residue was derivatized with 200 μL of trimethylsilyl imidazole-pyridine (1:1, v/v) at 80 °C for 45 min and analyzed for phenyl α-D-glucoside and galactosyl using a gas chromatography–mass spectrometry (GC–MS, Shimadzu) analysis.32 The recombinant plasmids were enriched and sequenced using the ABI PRISM 3730XL Sequencing System (Applied Biosystems, Foster City, CA).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis.** SYBR Green qRT-PCR amplification was performed on a CFX96 Touch real-time PCR detection system (Bio-Rad) with a total volume of 25.0 μL containing 12.5 μL of 2X SYBR Premix Ex Taq (Takara), 2.0 μL of cDNA template (100 ng/μL), 0.5 μL (10 μM) of forward primer, 0.5 μL (10 μM) of reverse primer, and 9.5 μL of Milli-Q water. Three technical replicates were run for each sample using the following cycling parameters: 95 °C for 3 min followed by 40 cycles of 94 °C for 45 s, 60/55 °C for 30 s, and 72 °C for 20 s. The gene β-Actin was selected as an internal control for qRT-PCR amplification, and the specific primers P20–P29 listed in Supplementary Table 1 were used for qRT-PCR amplification.39 The amplification efficiencies of all genes tested in this study ranged from 90% to 110%. Data were analyzed according to the threshold cycle (Ct). The relative changes in gene expression were quantified using the 2−ΔΔCt method.30 Significant differences were determined using Duncan’s multiple range tests, calculated using DPS software (www.chinads.net).

**Northern Blot Analysis.** To validate the qRT-PCR results, Northern blot analyses using RNA obtained from different tissues.
apical buds, developing leaves, mature leaves, young stems, roots, petals, flower buds, and fruits) of *C. sinensis* ‘Shuchazao’ without biotic or abiotic stress and RNA obtained from leaves of drought-stressed plants were conducted. A total of 3 g of frozen sample was ground in liquid nitrogen and RNA was isolated using an RNAprep Pure Plant Kit (Qiagen) according to the manufacturer's instructions. Total RNA quantification was determined using a Qubit (Invitrogen, Shanghai, China) and Northern blotting was performed as previously described.1

**RFOs Extraction and Determination.** Samples treated at 4 °C for 3, 6, 12, and 24 h were used for the abiotic stress group analyses; samples collected 3, 6, 12, and 24 h after *E. oblique* attack were used for the biotic stress group analyses. For each sample, 0.3 g of fresh leaves were used for the extraction of RFOs (i.e., raflnose, stachyose, and verbascose). The extraction method and chromatography analyses were conducted as previously described.31

**Statistical Analysis.** Three biological replicates were analyzed for each treatment, and GraphPad Prism software was used for data analysis. Data are presented as means ± SD. Significant differences were determined using the Student's t-test with p values < 0.05 considered to be statistically significant.

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**RESULTS**

**Isolation and Sequence Analysis of CsGolS Genes.** In our preliminary studies, three EST sequences were selected from an *E. oblique*-attacked tea plant transcription library; these genes were found to be similar to GolS genes using BLAST searching against the GenBank database. The full-length sequences of the three CsGolS genes, designated CsGolS1, CsGolS2, and CsGolS3 (1386 bp, 1574 bp, and 1350 bp, respectively) were obtained by 3' and 5' RACE-PCR. The full-length sequences of CsGolS1, CsGolS2, and CsGolS3 were submitted to GenBank database with the accession numbers JX624168, KP757767 and KP757768, respectively. The CsGolS1 putative protein was 339 amino acids (aa) long with a calculated *M*<sub>w</sub> of 38.97 kDa and a theoretical pI of 5.20. The CsGolS2 putative protein was 338 aa long with a calculated *M*<sub>w</sub> of 38.69 kDa and a theoretical pI of 4.96. The CsGolS3 putative protein was 326 aa long with a calculated *M*<sub>w</sub> of 37.80 kDa and a theoretical pI of 4.80. No signal peptide was observed in the three CsGolSs by analyzing the protein sequence with SignalP 4.1 and no internal transmembrane segment was found by the transmembrane topology predictions. Scratch protein predictor analysis showed that no disulfide bonds were detected for these proteins, and the ratio of helix, strand, and loop in the secondary structure was 23.89:10.91:65.19 for CsGolS1, 23.67:10.06:66.27 for CsGolS2, and 29.14:11.96:58.90 for CsGolS3.

**Multiple Sequence Alignment and Phylogenetic Analysis.** The BLASTp analysis showed that the aa sequence of CsGolS1 and CsGolS2 displayed the highest homology (85.0% and 84.7% identity) with a GolS protein from *C. arabica* (GenBank no. ADM92588), while CsGolS3 displayed the highest homology (77.9% identity) with a GolS protein from *Manihot esculenta* (GenBank no. AGC51778). Moreover, 73.1% identity was observed between CsGolS1 and CsGolS3, and 70.5% identity was observed between CsGolS2 and CsGolS3. The protein sequences of CsGolS1 and CsGolS2 displayed the highest homology (85.0% and 84.7% identity) with a GolS protein from *C. arabica* (GenBank no. ADM92588), while CsGolS3 displayed the highest homology (77.9% identity) with a GolS protein from *Manihot esculenta* (GenBank no. AGC51778). Moreover, 73.1% identity was observed between CsGolS1 and CsGolS3, and 70.5% identity was observed between CsGolS2 and CsGolS3. The protein sequences of CsGolS1 and CsGolS2 showed the highest similarity (92%) to each other. Multiple sequence alignment of the three CsGolSs with highly homologous GolS proteins from *C. arabica* and *M. esculenta* indicated that the three functional domains of the CsGolSs were conserved in GolS proteins (Figure 1). The active residues in the three functional domains consisted of a manganese-ligation motif (DXD), a serine phosphorylation site (S), and a typical hydrophobic pentapeptide (APSSA) in the C-terminal are contained in the red boxes.

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![Figure 1. Multiple sequence alignment of CsGolS1 (AGQ44777), CsGolS2 (AKS29170), and CsGolS3 (AKS29171) with the galactinol synthases from other plant species.](image-url)
typical hydrophobic pentapeptide (APSAA) at the C-terminal.

The phylogenetic analysis showed that GolSs from different plants examined in this study were evenly distributed in four major classes (Figure 2). CsGolS3 clustered in class I and was closely related to CaGolS2, while CsGolS1 and CsGolS2 clustered in class III and were closely related to CaGolS1. We, therefore, hypothesized that CsGolS3 was likely to possess different functions from CsGolS1 and CsGolS2. The result of this phylogenetic analysis was consistent with the multiple sequence alignment of the CsGolS aa sequences that showed that, for the manganese-ligation motif, CsGolS3 had the aa residues DGD while CsGolS1 and CsGolS2 had DAD. CaGolS1 (class III) has previously been shown to be constitutively expressed under natural field conditions and was sensitive to various environmental stresses, while expression of CaGolS2 (class I) was not detected unless the plants were cultivated under extreme drought conditions or in a high-salt environment.

Production of CsGolS Recombinant Protein and Its Hydrolytic Activities. Three CsGolS genes were efficiently expressed as a soluble protein fraction in E. coli BL21 (DE3) pLysS cells. The recombinant protein yield and quality results indicated that the optimum expression condition was 25 °C with 0.25 mg/mL IPTG induction for 3 h. The apparent Mw of recombinant CsGolS proteins was about 50 kDa, and the Mw values of fusion proteins (including Trx A, His-Tag, and S-Tag recombinant proteins) determined by SDS–PAGE were slightly bigger than the theoretical Mw described above (Figure 3).

Enzymatic activity assays showed that when the substrates of myoinositol and UDP-Gal existed in the reaction systems, galactinol was synthesized by fusion proteins of CsGolS1, CsGolS2, and CsGolS3 (Figure 4), indicating that the three CsGolS fusion proteins showed GolS activity. Further quantitative determination showed that the specific activity of the CsGolS1, CsGolS2, and CsGolS3 fusion proteins was 0.19, 0.08, and 0.30 μmol/min·mg, respectively.
CsGolSs Constitutive Expression Levels in Different Tea Plant Organs. The constitutive expression results of CsGolSs showed that the three genes had quite different transcription patterns (Supplemental Figure 1, SI). The highest level of CsGolS1 transcription was detected in mature leaves and the next highest in roots; the highest transcription level of CsGolS2 was detected in flower buds, followed by mature leaves; and the highest transcription level of CsGolS3 was detected in fruits, followed by mature leaves. For all three genes, the transcription levels in other organs were very low. As the three CsGolS genes were constitutively expressed in the mature leaves, this tissue was selected for expression analyses in the subsequent abiotic and biotic stress studies.

Northern Blot Validation for CsGolS1 Transcription. For the validation of CsGolS expression patterns determined using qRT-PCR, CsGolS1 transcription levels in different organs under natural field conditions and CsGolS1 transcription levels in mature leaves under drought stress were further examined by Northern blotting. These results showed that CsGolS1 expression in mature leaves was much higher than in the other organs, with the next highest expression observed in roots; the level of CsGolS1 expression in other organs was low. These results were completely consistent with those obtained using qRT-PCR (Supplemental Figures 1 and 2A).

Abiotic and Biotic Stress Regulations of CsGolS Expression in Tea Plant. Under drought stress, the expression of CsGolS1 was down-regulated for the first 5 days (0.3-fold), returned to the level of the control on day 11, was rapidly up-regulated 2.6-fold (compared with the control) on day 14, and returned to control levels on day 17 (Figure 5A). Under low-temperature stress, the expression of CsGolS1 was up-regulated 26.6-fold (compared with the control) after 6 h at 0 °C and 30-fold after 3 h at 4 °C; these time points had the highest CsGolS1 expression for each respective temperature treatment. After the maximum expression points, CsGolS1 expression decreased rapidly at both temperatures and reached the control level at 9 h (Figure 5B). These results indicated that low-temperature stress of 4 °C resulted in a more rapid change in CsGolS1 expression than 0 °C. The expression levels of CsGolS2 and CsGolS3 did not change significantly with low temperatures or drought stress (data not shown).

In the pest-attacked group, CsGolS1 showed only limited up-regulation in the first 3 h and then rapidly returned to control levels. Comparatively, CsGolS2 expression was very sensitive to pest attack, with its expression up-regulated 5.8-fold at 6 h after pest attack; CsGolS2 expression quickly returned to control levels between 6 and 12 h. Similar to CsGolS2, the expression of CsGolS3 was up-regulated 4.2-fold after 6 h and then rapidly returned to the control level after 9 h (Figure 5C). These results suggested that the CsGolS2 and CsGolS3 isoforms were much more sensitive to pest attack than the CsGolS1 isoform.

The expression patterns of CsGolS1 and CsGolS2 in response to ABA were similar, with both slightly down-regulated in the first 3 h before being steadily up-regulated until 24 h, while the expression of CsGolS3 was consistently up-regulated from 0 to 9 h before rapidly returning to control levels (Figure 5E). Of the three genes, CsGolS2 expression was most sensitive to SA (Figure 5D), suggesting that it may play a role in the disease response. Compared to SA and ABA, the phytohormone MeJA had limited effects on CsGolS1 and CsGolS2 expression, while CsGolS3 expression was sensitive to MeJA and increased 6.3-fold after 3 h before rapidly returning to control levels (Figure 5F). The phytohormone treatments showed that CsGolS1 and CsGolS2 had similar responses to ABA and MeJA while CsGolS2 and CsGolS3 had similar responses to SA.

Tea Plant RFOs Change under Abiotic and Biotic Stresses. Under low-temperature stress, the raffinose concentration began to increase at 6 h, reached the maximum concentration of 0.367 mg/g fresh leaves at 12 h (p < 0.05), and then returned to the level of the control by 24 h (Figure 6A). On the other hand, CsGolS1 expression in mature leaves was much higher than in the other organs, with the next highest expression observed in roots; the level of CsGolS1 expression in other organs was low. These results were completely consistent with those obtained using qRT-PCR (Supplemental Figures 1 and 2A). Under drought stress, CsGolS1 transcription levels determined by Northern blotting were also consistent with those determined using qRT-PCR, with expression decreasing for the first 5 days, highly up-regulated on the day 14, and then recovered to the same level as the control day 17 (Supplemental Figure 2B and Figure 5A). The Northern blot results for different organs and environmental conditions indicated that the tea plant CsGolSs transcription levels determined by qRT-PCR in this study were reliable.

Figure 4. Synthesis product of recombinant CsGolS proteins determined by gas chromatography–tandem mass spectrometry: (A) standard mixture (i.e., phenyl-α-D-glucoside and galactinol, 8.0 mg/L for each analyte), (B) synthesis product of PET32α (+) expressed proteins (negative control), (C) synthesis product of recombinant CsGolS1, (D) synthesis product of recombinant CsGolS2, and (E) synthesis products of recombinant CsGolS3.

Figure 5A. Constiutive expression levels for CsGolSs. The expression levels of CsGolSs were similar in response to each respective temperature treatment. After the maximum expression points, CsGolS1 expression decreased rapidly at both temperatures and reached the control level at 9 h (Figure 5B). These results indicated that low-temperature stress of 4 °C resulted in a more rapid change in CsGolS1 expression than 0 °C. The expression levels of CsGolS2 and CsGolS3 did not change significantly with low temperatures or drought stress (data not shown).

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was observed before levels reduced slightly at 24 h (Figure 6C). With low-temperature treatment, raffinose levels increased significantly only at 12 h, while verbascose levels were significantly higher only at 12 and 24 h. Under pest attack stress, the raffinose concentration began to increase after 3 h and quickly increased to the maximum concentration of 0.922 mg/g fresh leaves at 6 h. After 6 h, the raffinose concentration reduced to approximately the same level as at 3 h and was still significantly higher than in the control (Figure 6B). Verbascose was significantly higher at 6 h after pest attack and increased continuously to 24 h (0.078 mg/g, Figure 6D). Stachyose was not detected in any samples examined (Figure 6). Comparing the RFO results of the low-temperature experiment to the pest attack experiment indicated that RFO synthesis was regulated by both abiotic and biotic stress, but that biotic stress had a greater effect than abiotic stress.

**DISCUSSION**

Many studies have shown that the abiotic stressors can regulate the expression of *GolS* genes, and therefore, result in RFOs accumulation. RFOs are important for carbohydrate storage and assist plants to overcome adverse environments. Expression of *GolS* genes was regulated by pest attack in hybrid poplar, suggesting that RFOs may also be involved in combatting biotic stress. However, only a few studies have focused on the relationships between *GolS* expression and plant biotic stress adaptations, and the mechanism of *GolS*-mediated biotic stress adaptation has not been characterized in depth. In this study, two significantly up-regulated *GolS* genes (*CsGolS2* and *CsGolS3*) were identified in the transcriptome library of *E. oblique*-attacked tea plant. Therefore, the CsGolS regulation in the tea plant was considered to be an important biological agent for adaptation to biotic stress. On the basis of this assumption, three *CsGolS* genes were cloned and characterized from tea plant, and their expression in response to abiotic and biotic stressors was investigated.

The transcript regulation analyses under abiotic and biotic stress showed that the expression level of *CsGolS1* was significantly affected by abiotic stresses (water deficiency, low temperature, and ABA treatment); conversely, other than moderate up-regulation following SA treatment, *CsGolS1* expression did not change significantly with the biotic stressors of pest attack and MeJA. This suggests that the *CsGolS1* isoform is primary related to abiotic stress responses, as well as being somewhat responsive to specific biotic stress (i.e., tea plant disease). The result of *CsGolS1* regulation was consistent with the majority of *GolS* studies in other plants. In contrast to *CsGolS1*, the expression of *CsGolS2* and *CsGolS3* showed only minor changes with the abiotic stressors of water deficiency and low temperature. The expression of *CsGolS2* and *CsGolS3* was significantly regulated by biotic stressors including *E. oblique* attack, SA treatment (related to plant disease), and MeJA treatment (related to pest attack). This suggests that the *CsGolS2* and *CsGolS3* genes should primarily relate to biotic stress adaptations, while *CsGolS1* may play a role in abiotic stress responses.

Figure 5. Expression of *CsGolS* genes in mature leaves under abiotic and biotic stress conditions determined using qRT-PCR. Gene expression in nontreated controls was set to 1.0. Data represent the means ± SD (n = 3) of three biological replicates. Different letters above the bars represent significant differences at p < 0.05.
stress adaptation, which is consistent with the new biological role hypothesized in hybrid poplar.22 RFOs concentrations in samples subjected to abiotic and biotic stresses also supported the CsGolSs regulation results, the RFO synthesis was significantly up-regulated by pest attack, and the influence of biotic stress was more important than low temperature as an abiotic stress. Taken together, these results suggested that the CsGolS1 gene was primarily related to abiotic stress and CsGolS2 and CsGolS3 genes were closely related to biotic stress. Interestingly, ABA treatment yielded significantly up-regulated CsGolS2 and CsGolS3 expression, suggesting that these two isoforms might also be related to some unknown abiotic stress, although the two isoforms did not respond to the abiotic stressors of water deficiency and low temperature. The results of gene regulation and RFOs determination were not consistent with the phylogenetic analysis, which suggested that CsGolS3 may have a biological role different from those of CsGolS1 and CsGolS2, but the actual reasons need to be further investigated. The role of the three genes in biotic stress responses in C. sinensis represents a new biofunctional role for the GolS family in tea plant.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b00377.

Primers used in this study (Supplemental Table 1), expression of CsGolS genes in different tea plant organs grown under field conditions determined using qRT-PCR [Supplemental Figure 1, where data represent the means ± SD (n = 3) of three biological replicates and different letters above bars represent significant differences at p < 0.05], and CsGolS1 transcription level validation by Northern blotting showing (A) CsGolS1 transcripts in different tea plant tissues and (B) CsGolS1 transcripts in mature leaves following drought stress (Supplemental Figure 2) (PDF)

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Figure 6. HPLC analysis of RFOs content in tea plants under low temperature and pest attack stress. Data represent the means ± SD (n = 3) of three biological replicates. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with the control.
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