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Genome-Wide Identification of ABCC Gene Subfamily Members and Functional Analysis of *CsABCC11* in *Camellia sinensis*

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ABSTRACT

The ATP-binding cassette (ABC) transporter is a gene superfamily in plants. ATP-binding cassette subfamily C (ABCC) protein is a multidrug resistance-associated (MRP) transporter. They play various roles in plant growth, development, and secondary metabolite transport. However, there are few studies on ABCC transporters in tea plants. In this study, genome-wide association study (GWAS) analysis of epigallocatechin gallate (EGCG) content in 108 strains of Kingbird revealed that *CsABCCs* may be involved in EGCG transport. We identified 25 *CsABCC* genes at the genomic level of the tea plant, their phylogenetic tree, gene structure, targeted miRNA and other bioinformatics were analyzed. The expression patterns of *CsABCCs* in eight different tissues and abiotic stress indicate that they have potential roles in regulating the growth, development, and defense of tea plants. The correlation analysis revealed that the expression of the *CsABCC11* gene was closely related to the EGCG content in tea buds of 108 strains of the Kingbird, and the subcellular localization experiments in tobacco showed that *CsABCC11* protein was localized on the plasma membrane. The virus-induced gene silencing (VIGS) strategy in tea plants further verified that *CsABCC11* was involved in EGCG accumulation. Our study laid a foundation for studying the biological function of *CsABCC* and provided a new candidate molecular marker gene for further EGCG-related variety breeding, which will be of great interest to breeders.

KEYWORDS

Camellia sinensis; ABCC gene subfamily; EGCG; VIGS; guizhou local variety Kingbird

1 Introduction

The tea plant (*Camellia sinensis* (L.) O. Kuntze) is a significant cash crop cultivated worldwide due to its economic importance. The plant ATP-binding cassette (ABC) protein is an important membrane protein [1]. Plant ABC transporters have multiple functions, such as participating in transporting plant secondary metabolites. It is involved in heavy metal detoxification, stress [2–4], accumulation and metabolism [5], mediating antibiotic resistance and plant hormone transport such as cytokinin and abscisic acid. Most



plant ABC transporters exist in the cell membrane and participate in organ growth, plant nutrition, plant development, and other processes [6]. It also plays an important role in plant gametogenesis, seed development, germination, organ formation, and secondary growth. It can be seen that the ABC protein can act as an input, an output, and a channel [7]. In animals, ABC transporters have been shown to have the functions of ion channels and channel regulators [8,9]. ABC transporter gene family has been characterized in many species, such as pigeon pea (*Cajanus cajan* (L.) Druce) [10], corn (*Zea mays* L.) [2], flax (*Linum usitatissimum* L.) [11], pineapple (*Ananas comosus* L.) [12,13], wheat (*Triticum aestivum* L.), soybean (*Glycine max* (L.) Merr.). In the plant genome, ABC transporters are divided into eight different subfamilies (*ABCA-ABCG* and *ABCI*). *ABCC* subfamily proteins are involved in the detoxification of metal ions and the transport of plant secondary metabolites. Studies have indicated that the maize *ABCC* subfamily proteins play a role in vesicle transport and seed phytic acid anthocyanin content [14]. However, plant ABC transporters have been well studied in other species, but it remain to be studied in tea plants.

The quality of tea depends on the secondary metabolites in tea. Catechins are a class of phenolic active substances extracted from tea plants [15]. Catechins include eight monomers, catechins, epicatechins, epigallocatechin gallate, and eight other monomers. EGCG is the most effective active ingredient in tea polyphenols. It is the monomer with the highest proportion of catechins, accounting for about 60%. EGCG is the main component that determines the color, aroma, and taste of tea, and its content is the highest in green tea. EGCG has many functions in medicine and health care, such as antioxidant [16], anti-free radical DNA damage [17], anti-inflammatory and anti-tumor effects [18,19]. It has made good progress in anti-cervical cancer research [20]. Some studies have shown that EGCG is used as a special functional preservative and skin care agent on daily chemical products to scavenge free radicals, resist the effects of pollution, sun exposure, and smoking, and prevent skin aging and wrinkles. In recent years, research has found that EGCG has a potential protective effect on COVID-19, so the author concluded that drinking green tea has a potential preventive effect on COVID-19 [21]. The anti-tumor effect of EGCG has been recognized by relevant researchers in various countries and has been listed as a potential new anti-cancer drug in many countries such as China and the United States. A large number of clinical medical experiments have proved that EGCG is no less than paclitaxel in the treatment of various cancers, and its toxicity is much lower than paclitaxel. In particular, there are many reports on the application of skin cancer [22], which has now entered the clinical stage. It can be said that EGCG is an effective and non-toxic ideal drug for the treatment of cancer, which has great development value [23,24].

The role of epigallocatechin gallate (EGCG) in a variety of abiotic stresses has become a topic of concern in recent literature. Studies have shown that exogenous application of EGCG can alleviate a variety of abiotic stresses, such as high temperature, salt, and drought, thereby enhancing stress tolerance [25]. The study also reported that EGCG levels increased during drought stress, suggesting that this compound may play a role in mitigating the effects of water deficit on plants [26]. In addition, catechin synthesis (EGCG is its derivative) has been found to be sensitive to environmental disturbances such as drought and salinity, which highlights the importance of these compounds in plant stress responses [27]. In addition, the signaling pathways of plants in response to cold, salt, and drought stress have also been extensively studied, providing clues for revealing the molecular mechanism of stress tolerance. EGCG may play a role in regulating these pathways, potentially helping plants treated with this compound to enhance stress tolerance [28], and the compound EGCG found in green tea may have a protective effect on plants against drought and salt stress. More research is needed to elucidate how EGCG enhances stress resistance and to explore its potential applications in agriculture and crop improvement.

In our previous research, the EGCG content of the 108 strains of Kingbird was analyzed, and it was found that there were differences in EGCG content among different strains, and then GWAS was mapped to a gene *CsABCC11*. In this study, we performed a genome-wide identification of *CsABCCs* in tea

plants, and the chromosome mapping, phylogeny classification, conserved motifs, promoter components, miRNA, and expression pattern were analyzed. Subcellular localization experiments confirmed the subcellular localization of CsABCC11 protein in tobacco. Subsequently, correlation analysis revealed that the expression of the *CsABCC11* gene was closely related to the EGCG content of tea plant buds, and the VIGS strategy carried out in tea plants further verified that *CsABCC11* was an indispensable part in the formation of EGCG. Therefore, it is highly significant to investigate the ABCC transporter in tea plants to enhance the EGCG content, providing relevant insights for further research.

2 Materials and Methods

2.1 Experimental Materials

The tea cultivar ‘FudingDabai’ was planted in the experimental field of Guizhou Academy of Agricultural Sciences (Guiyang, Guizhou, China). The buds of 108 strains (‘Kingbird’(Niaowangzhong)) were taken from Guiding Yunwu (Guiding, Guizhou, China). Tobacco for subcellular localization was planted in our laboratory.

2.2 Identification of CsABCC Subfamily Genes in Tea Plant

Firstly, the Arabidopsis *AtABCC* gene is derived from the Arabidopsis genome database (Table S1). Taking the *Arabidopsis thaliana* gene sequence as a reference, the tea tree genome annotation obtained in the tea tree genome database was used. The BLASTP alignment was performed using the *ABCC* gene-specific conserved domain model [29], and the threshold was set to (E-value < 10^{-5}) to obtain the hypothetical *CsABCCs*. The protein characteristics of the *CsABCCs* were analyzed by the NCBI-CDD database to ensure that the *CsABCCs* only had its domainI (Accession: cl33195) [30]. The obtained sequence was sorted and the repetitive items were removed, and finally, the *CsABCC* gene family members of the tea plant were obtained. The amino acid length of ABCC protein in tea plants was analyzed according to the Perl script of the downloaded tea plant genome database. The relative molecular mass, isoelectric point and subcellular localization were identified by ExPASy and Plant-mPloc online [31].

2.3 Phylogenetic Analysis of ABCCs in Different Plant Species

MEGA X was used to analyze the evolutionary relationship of proteins from tea plants and *Arabidopsis thaliana* based on the adjacency method. The Bootstrap test is repeated 1000 times [32]. Use the FigTree software (version 1.4.2) to display the phylogenetic tree. The protein sequence of *Arabidopsis thaliana* was obtained from *Arabidopsis thaliana* Information Resource (TAIR).

2.4 Analysis of Chromosome Position, Gene Structure and Conserved Motif of CsABCC Genes in Tea Plant

The chromosome location information of the *CsABCCs* (including chromosome distribution, length, start, and end position) was downloaded from TPIA and visualized by TBtools. TBtools software was used to visualize the exon-intron structure of the *CsABCCs* based on genome annotation. The conserved protein motifs of CsABCC protein were analyzed by the MEME program.

2.5 Analysis of Cis-Regulatory Elements of CsABCC Genes Promoter

The promoter sequences of all *CsABCC* genes were obtained from TPIA and analyzed using PlantCARE and visualized by TBtools.

2.6 Prediction of Putative miRNA Targeting CsABCC Genes

Based on the miRNA of *Arabidopsis thaliana*, the hypothetical miRNA targeting *CsABCC* was predicted by online software (<https://www.zhaolab.org/psRNATarget/analysis>, accessed on 21 June 2024),

and the miRNA-target gene network was constructed by online software (<https://www.bioinformatics.com.cn>, accessed on 18 June 2024).

2.7 Expression Pattern Analysis of *CsABCC* Genes

The *CsABCC*s Transcriptome data of eight tissues (top bud, young leaf, mature leaf, old leaf, stem, root, flower and fruit) and salt, drought and cold stress of tea variety “Shuchazao” were downloaded from open database (TPIA) and heat maps were created using TBtools.

2.8 EGCG Correlation Analysis

The primers of *CsABCC11* gene were designed by IDT. The cetyltrimethylammonium bromide (CTAB) method was used to extract the total RNA from the bud heads of 7 groups of 108 strains of VIGS and Kingbird, the retrotranscription system is as follows: 50 ng–5 µg Total RNA, 1 µL Oligo (dT) 18 (0.5 µg/µL), 3.6–4 µL 5 × TRUE Reaction Mix, RNase free H₂O to final volume 20 µL. The cDNA was then used as a template for qRT-PCR analysis. Each reaction system contained 10 µL SYBR Green qPCR Mix, 1 µL primers, 3 µL cDNA template, and 5 µL H₂O. The reaction process is as follows: 1 cycle at 95°C for 30 s; 95°C 10 s, 60°C 30 s, 40 cycles; after 40 cycles of 72°C 60 s, The relative expression level was calculated with *CsGAPDH* gene as reference.

2.9 Subcellular Localization

The PCAMBIA1300-35S-*CsABCC11*-GFP fusion expression vector was constructed (Fig. S1). he constructed plant fusion expression vector PCAMBIA1300-35S-*CsABCC11*-GFP and the empty vector PCAMBIA1300-35S-GFP were transformed into GV3101 competent cells, and co-expressed and injected into the lower epidermis of 4–6-week-old *N. benthamiana* [33]. After 2 days of low light culture, the distribution of green fluorescence signal was observed under laser confocal microscope [34].

2.10 Virus-Mediated VIGS and HPLC Determination of EGCG Content

The pTRV2-*CsABCC11* vector was constructed, and pTRV1, pTRV2 and pTRV2-*CsABCC11* were introduced into competent cells GV3101. Agrobacterium strains were inoculated into solid YEP medium (yeast powder 10 g/L, peptone 10 g/L, NaCl 5g/L, Rif ampicin 50 mg/L, Kanamycin 100 mg/L), and the single colony was cultured in the corresponding liquid YEP medium. The bacterial liquid was collected and resuspended. The pTRV1 and pTRV2, pTRV2-*CsABCC11* bacterial liquid were mixed at 1:1, and then the tea tree cuttings were vacuum infiltrated [35]. Stored in dark for 3 days, cultured at 25 degrees Celsius, the light duration and dark duration were 16 and 8 h, respectively. The content of EGCG in VIGS buds in silenced cuttings was determined by high performance liquid chromatography (HPLC). The sample preparation method is as follows. Weigh 0.2 g tea powder (accurate to 0.0001 g) through 40 mesh sieve, place it in a 15 mL centrifuge tube, add 10 mL of 70% methanol. The extraction was carried out in 70°C water bath for 10 min (shake once every 2 min), centrifuge it in a high-speed centrifuge (low temperature 4°C, 6000 r/min) for 5 min, and transfer the supernatant to a 25 mL measuring bottle. The residue was repeatedly extracted with 5 mL 70% methanol, and the supernatant was combined. The supernatant was diluted with stable liquid to 25 mL, shaken, and filtered through 0.22 µm microporous membrane. The detection method of EGCG content was based on the national standard GB/T8313.

2.11 Data Analysis and Mapping

All results were expressed as mean ± standard deviation of at least 3 biological replicates. SPSS and Excel software were used for data analysis, drawing with Graphpad Prism and TBtools.

3 Results

3.1 Identification and Phylogenetic Tree Analysis of CsABCC Subfamily Members in Tea Plant

A total of 25 *CsABCC* genes were identified, all containing a common domain (MRP). All *CsABCC* protein sequences are provided in Fig. S1. According to its position on the chromosome, it was named *CsABCC1* to *CsABCC25*. The basic information of *CsABCC* genes was analyzed (Table 1). The protein range was 778 to 1556 bp, the molecular weight was 86.15 to 173.39 kDa, and the isoelectric point was 5.45~8.61. The 25 *CsABCC* proteins are located in the plasma membrane, which shows that the ABCC transporter is located on the plasma membrane and plays a role in transport.

Table 1: Basic information of *CsABCC* genes

Gene name	Sequence ID	Chr no.	Length	MW	pI	Subcellular localization
CSS0004166.1	CsABCC1	Chr1	1475	164459.6	6.91	Plasma membrane
CSS0010150.1	CsABCC2	Chr3	1508	168136.2	7.88	Plasma membrane
CSS0042039.1	CsABCC3	Chr3	1373	153347.5	8.61	Plasma membrane
CSS0043095.1	CsABCC4	Chr4	1190	132505.6	7.45	Plasma membrane
CSS0028519.1	CsABCC5	Chr7	1469	163211.7	6.49	Plasma membrane
CSS0049253.1	CsABCC6	Chr7	1298	144629.9	5.63	Plasma membrane
CSS0022155.1	CsABCC7	Chr7	1341	149878.6	5.97	Plasma membrane
CSS0016358.1	CsABCC8	Chr7	1447	161990.4	6.55	Plasma membrane
CSS0029571.1	CsABCC9	Chr7	1405	157420.6	7.51	Plasma membrane
CSS0011010.1	CsABCC10	Chr9	1489	166125.3	7.1	Plasma membrane
CSS0027340.1	CsABCC11	Chr11	1505	169582.4	7.33	Plasma membrane
CSS0024181.1	CsABCC12	Chr11	1505	169445.2	7.54	Plasma membrane
CSS0005866.1	CsABCC13	Chr12	1413	157634.7	6.17	Plasma membrane
CSS0003692.1	CsABCC14	Chr12	1399	156362.1	6.15	Plasma membrane
CSS0028245.1	CsABCC15	Chr12	1483	166000.1	6.64	Plasma membrane
CSS0019380.1	CsABCC16	Chr12	1385	154567.1	6.09	Plasma membrane
CSS0029302.1	CsABCC17	Chr13	1556	173395.1	7.46	Plasma membrane
CSS0040468.1	CsABCC18	Chr13	1545	172382.8	7.27	Plasma membrane
CSS0010084.1	CsABCC19	Chr13	1509	169153.9	7.29	Plasma membrane
CSS0012613.1	CsABCC20	Chr14	1475	164508.4	6.62	Plasma membrane
CSS0044500.1	CsABCC21	Chr15	778	86148.27	5.45	Plasma membrane
CSS0012465.1	CsABCC22	Chr15	865	95609	6.53	Plasma membrane
CSS0004622.1	CsABCC23	Contig1125	1502	168030.5	7.08	Plasma membrane
CSS0016470.1	CsABCC24	Contig1175	1371	152060.5	6.27	Plasma membrane
CSS0045946.1	CsABCC25	Contig1212	1454	162574.5	7	Plasma membrane

In order to explore the evolutionary relationship between the *ABCC* gene of tea plant and other plant homologues, the phylogenetic tree of tea plant and *Arabidopsis thaliana* was constructed by neighbor-joining method. According to the conserved motifs between them, all ABCC proteins were divided into four groups. As shown in Fig. 1, there were 13 members in the first and second groups, and 7 members in the third and fourth groups, respectively.

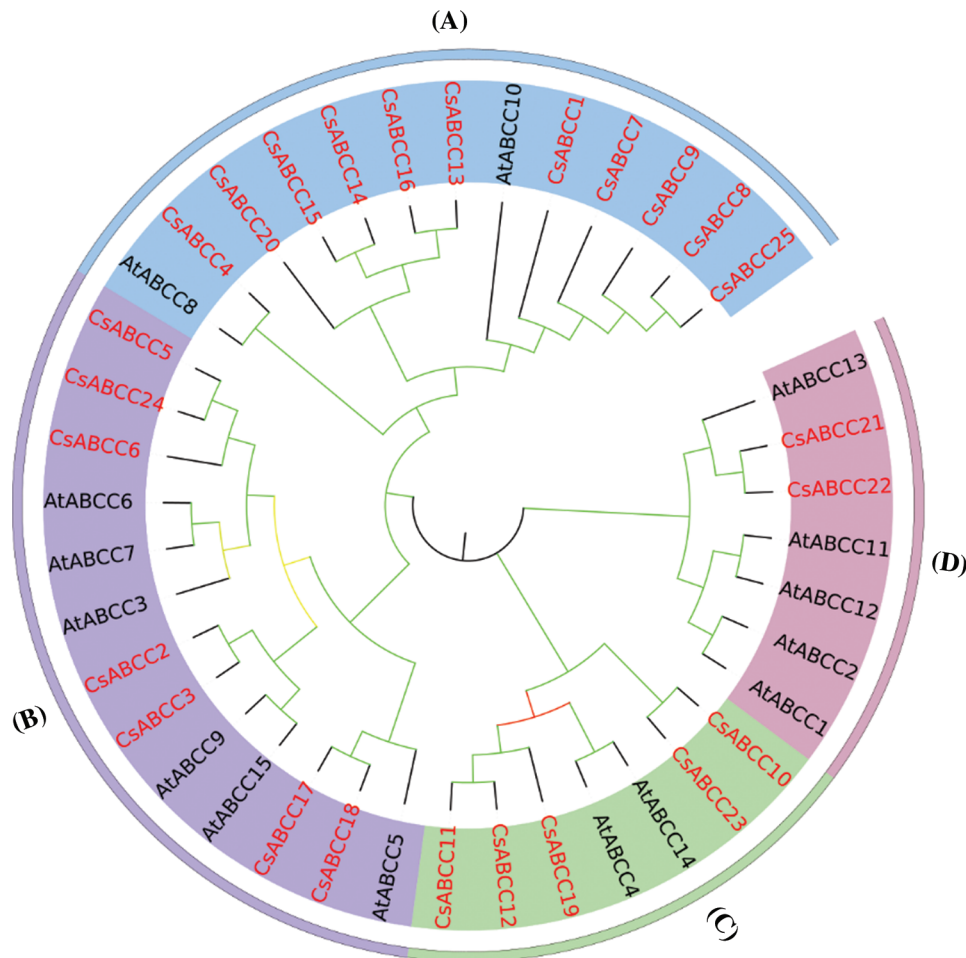


Figure 1: The phylogenetic relationship and classification of *Camellia sinensis*, *Arabidopsis thaliana*, and ABCC proteins. All ABCC proteins were divided into three groups. Gene ID is represented by different colors, red represents the tea plant, and black represents *Arabidopsis thaliana*

3.2 Analysis of Conserved Motifs of Genes

To obtain the conserved domain of CsABCC proteins, we used the MEME program to analyze it. A total of 10 conserved motifs are predicted, containing about 34–50 conserved amino acids (Fig. 2), and *CsABCC* showed highly similar motif composition in the same subfamily. It shows that these motifs are conserved during the evolution of tea plants. Majority proteins have the same conserved motifs, but the number of motifs is different. All *CsABCC* genes have more than 9 conserved motifs, up to 15. There are motif 1, motif 2, motif 3, motif 4, motif 5 and motif 6, motif 9 and motif 10 in each *CsABCC* sequence. There is no motif 7 and motif 8 in *CsABCC2* and *CsABCC19*. *CsABCC1*, *CsABCC3*, *CsABCC5*, *CsABCC7*, *CsABCC16*, *CsABCC17*, *CsABCC20*, *CsABCC24* and *CsABCC25* have the same motifs, including 12 conserved motifs, which also indicates that the same *CsABCC* gene subfamily members have similar functions.

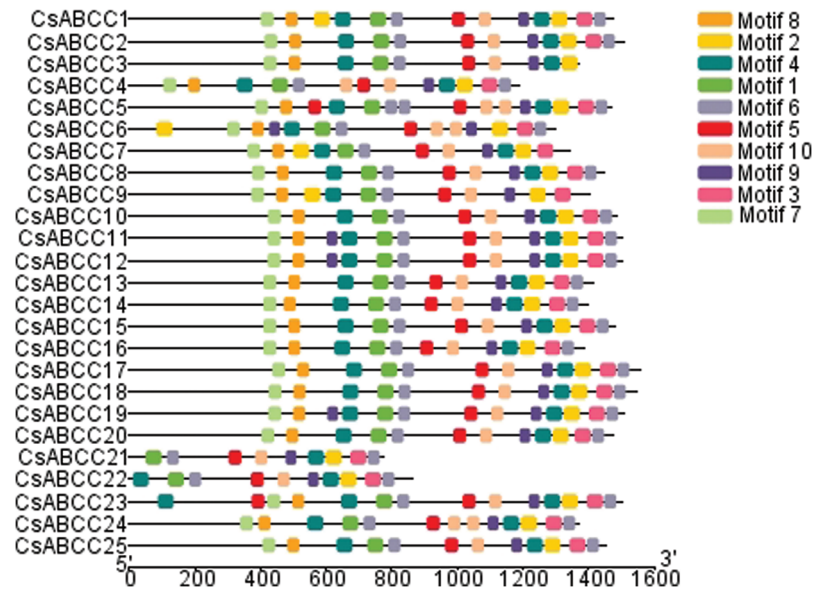


Figure 2: Conserved motifs of *CsABCCs* in tea plant. The left side represents the position of the motif on the gene, the right side represents the motif name, and different color blocks represent different motifs

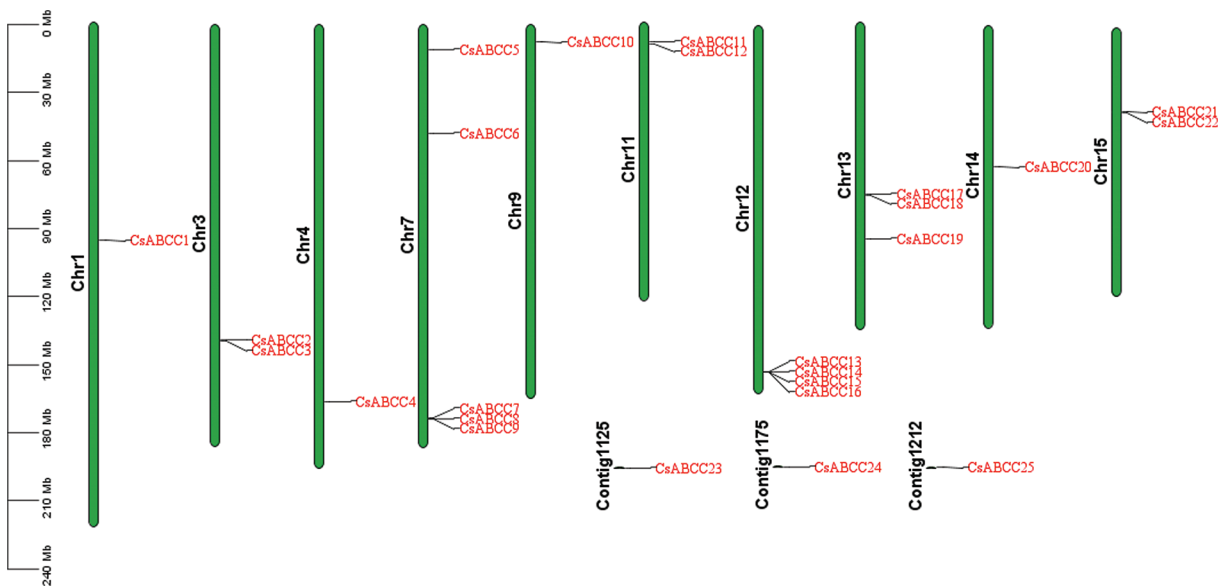


Figure 3: Chromosome distribution of *CsABCC* genes. The numbering of the *CsABCC* genes is shown on the right side of the chromosome. A ruler describes the relative length of a chromosome, denoted by megabases (Mb)

3.3 Gene Structure and Chromosome Location of *CsABCC*

In order to further study the effect of gene evolution on the *CsABCC* genes, the chromosome localization of the *CsABCC* gene in tea plants was analyzed by TBtools software. As shown in Fig. 3, the results show that 22 of the 25 members of *CsABCC* are unevenly distributed on 10 chromosomes (Fig. 3), and 3 *CsABCC* genes were located on the unanchored scaffold. The largest number of *CsABCC* genes is on chromosome 7,

containing 5 genes, which is the largest of all chromosomes. Chromosomes 3, 11 and 15 contained the same number of *CsABCC* genes, namely 2, and chromosomes 1, 4, 9 and 14 also contained the same number of *CsABCC* gene, namely 1.

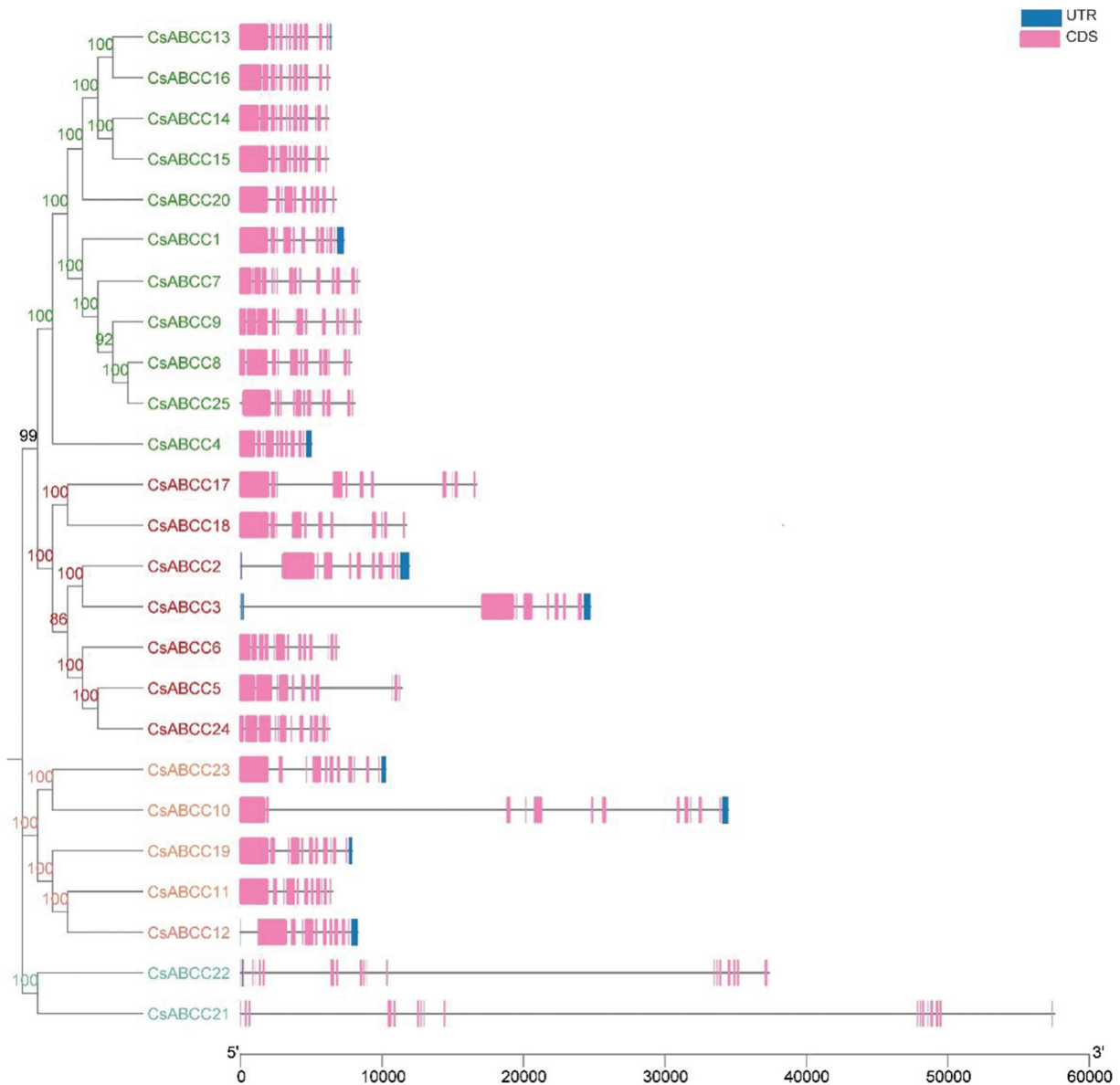


Figure 4: *CsABCC* phylogenetic tree of tea plant (left) gene structure (right), purple box, blue box and black line represent exons, UTR and introns, respectively

We analyzed the distribution of exons and introns in the *CsABCC* genes sequence. The exons of *CsABCC* ranged from 8 to 18 (Fig. 4). Among them, *CsABCC22* and *CsABCC21* have the most exons, with 18 and 17 exons, respectively, while *CsABCC3* contains only 8 exons, which is the least. There were 17 *CsABCC* genes without UTR. To better understand the evolutionary relationship of *CsABCC*, we constructed a phylogenetic tree of ABCC proteins, and divided all ABCC proteins into four groups. As

shown in Fig. 4, there are 9 pairs of homologous pairs in *CsABCCs*, and all 9 pairs of Bootstrap values are 100, indicating that these 9 pairs of *CsABCCs* are closely related.

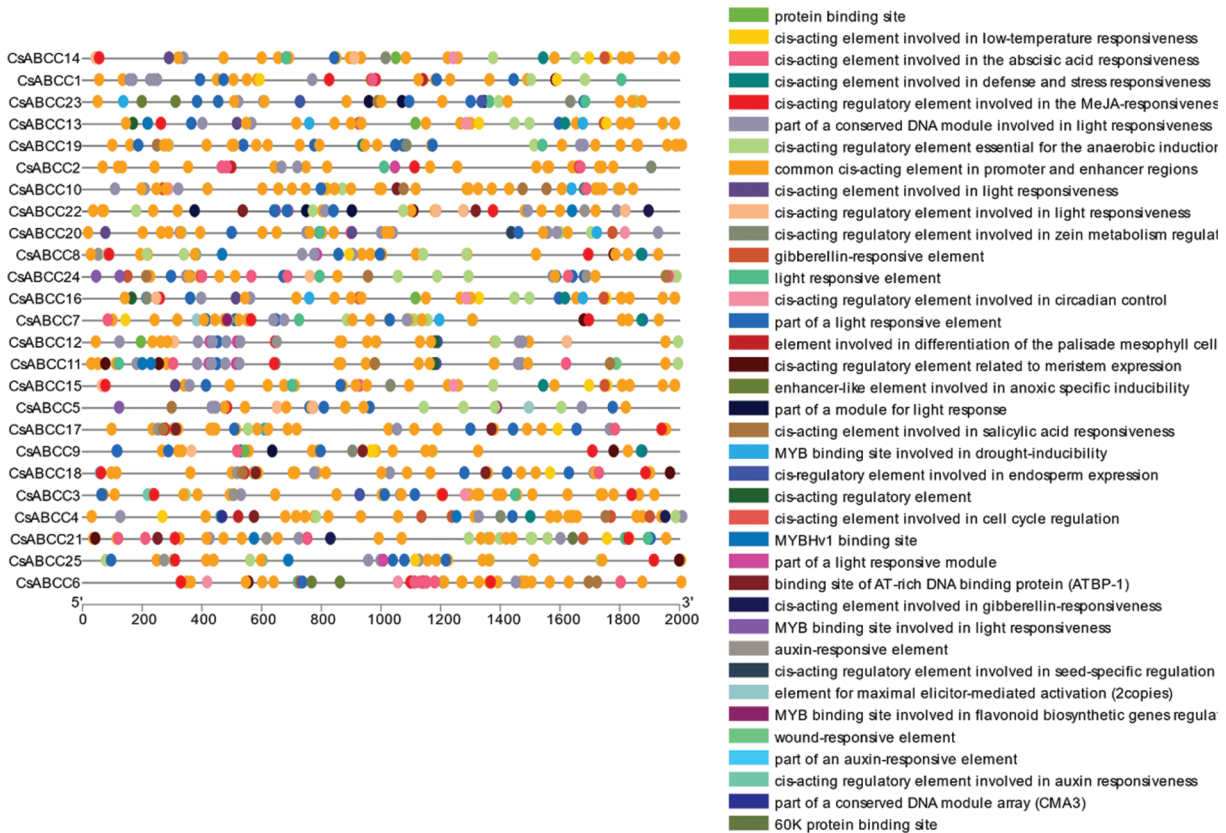


Figure 5: Analysis of *CsABCC* genes promoter region. The left circle represents the position of the cis-acting element in the promoter region, the right box represents the name of the cis-acting element

3.4 Analysis of Cis-Acting Elements of *CsABCC* Genes Promoter

In this study, 2000 bp upstream of the transcription start site ATG in the genome of 25 *CsABCC* genes was extracted and submitted to the online database plantCARE. The analysis results showed that the promoter region contained a large number of promoter cis-acting elements as shown in Fig. 5. Light response, plant hormone response, plant growth and development, adversity stress and so on. These results indicate that *CsABCC* genes plays a role in different tissues and stress environments.

3.5 Prediction of Putative miRNA Targeting *CsABCC* Gene

As a class of non-coding small RNA (sRNA), microRNA (miRNA) is regulated at the post-transcriptional level by binding to target genes. Based on the miRNA of *Arabidopsis thaliana*, the miRNA targeting *CsABCC* genes was predicted (Table S2). The network diagram was drawn by online software (Fig. 6). Among them, the miRNA targeting *CsABCC7* gene was the least, with 12 miRNAs, and the miRNA targeting *CsABC19* gene was the most, with 49 miRNAs. There were 44 genes targeting *CsABCC11*. A large number of miRNAs binding to *CsABCCs* may regulate the accumulation of secondary metabolites in tea plants.

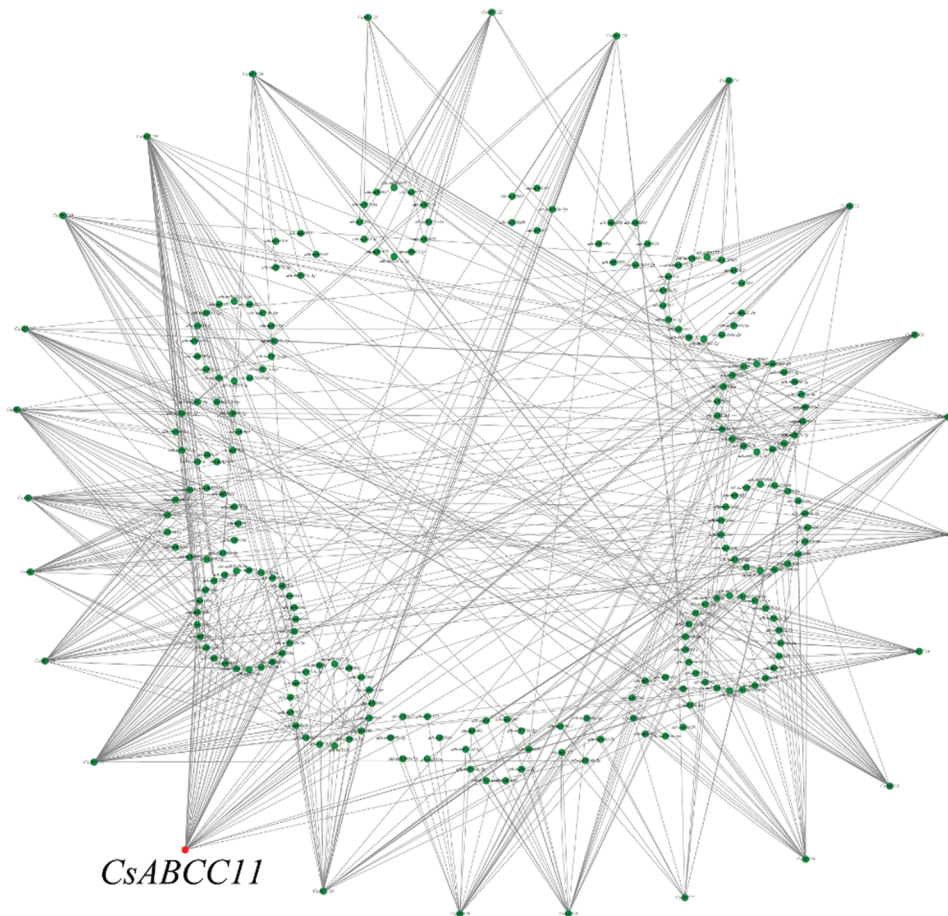


Figure 6: miRNA-*CsABCC* network diagram, the inner circle represents the name of miRNA, and the outer circle represents the name of *CsABCCs*

3.6 Analysis of *CsABCC* Genes Expression in Different Tissues and under Different Stress Conditions

The differential expression of 25 *CsABCC* genes in eight different tissues of tea plant and their potential role in stress response were studied. The *CsABCCs* expression data of 8 different tissues of tea plant and cold acclimation, drought stress and salt stress were obtained. The 25 *CsABCC* genes showed different expression patterns in eight different tissues. Most *CsABCC* genes were lowly expressed in apical buds. Only *CsABCC7* gene was significantly expressed in flowers. *CsABCC5* and *CsABCC24* genes were significantly expressed in fruits. The expression of *CsABCC3* gene was higher in young leaves. Most *CsABCC* genes were highly expressed in mature and old leaves. *CsABCC13* gene was significantly expressed in roots, while *CsABCC9* and *CsABCC23* genes were highly expressed. *CsABCC24* gene was significantly expressed in stems (Fig. 7A). Among them, *CsABCC11* was expressed in different degrees in terminal buds, young leaves, old leaves, roots, flowers and fruits, indicating that the expression of *CsABCC11* may play a significant role in the transport of substances in tea plants. The changes in expression patterns indicate that different *CsABCCs* play a key role in the growth and development of tea plants.

Under drought stress, the expression of three *CsABCC* genes (5,17,19) was significantly up-regulated at 24 h, the expression of four *CsABCC* genes (9,18,21,24) was significantly up-regulated at 48 h, and the expression of seven *CsABCC* genes (1,2,6,11,12,17,23) was up-regulated at 72 h, and the expression of

CsABCC6 and *CsABCC23* was continuously up-regulated (Fig. 7B). Under cold conditions, most *CsABCC* genes were significantly up-regulated in CA1 and CA3 states (Fig. 7C). Under salt treatment, the expression of three *CsABCC* genes (1,7,17) was significantly up-regulated at 24 h, the expression of 10 *CsABCC* genes (2,5,6,9,11,12,18,19,21,23) was up-regulated at 48 h, and the expression of three *CsABCC* genes (11,12,17) was up-regulated at 72 h (Fig. 7D). These results indicate that *CsABCC* gene plays an important role in different stress environments.

3.7 The Expression Level of *CsABCC11* Was Closely Related to the Content of EGCG in Tea Buds

To explore the relationship between *CsABCC11* and EGCG content in buds of Kingbird, according to the EGCG content from low to high, the EGCG content of 108 strains of Kingbird was divided into 7 groups for qRT-PCR expression analysis. The primer sequence of *CsABCC11* gene is shown in Table S3. It can be seen from Fig. 8 that with the increase of EGCG content, the expression of the *CsABCC11* gene in tea plants increased gradually, indicating that EGCG content was positively correlated with *CsABCC11* expression, which also indicated that *CsABCC11* transporter promoted the synthesis of EGCG in the buds of Kingbird.

3.8 Subcellular Localization Results

ABC transporters are usually membrane localization proteins [36]. In order to find the subcellular location of *CsABCC11* protein, we fused the GFP empty protein with the *CsABCC11* protein transiently expressed in tobacco leaves. As shown in the Fig. 9, the empty vector PCAMBIA1300-35S-GFP can detect green fluorescence signals in tobacco epidermal cell membrane and nucleus, and the plant fusion expression vector PCAMBIA1300-*CsABCC11*-35S-GFP can detect green fluorescence signals in tobacco epidermal plasma membrane, which proves that *CsABCC11* protein is located in the plasma membrane.

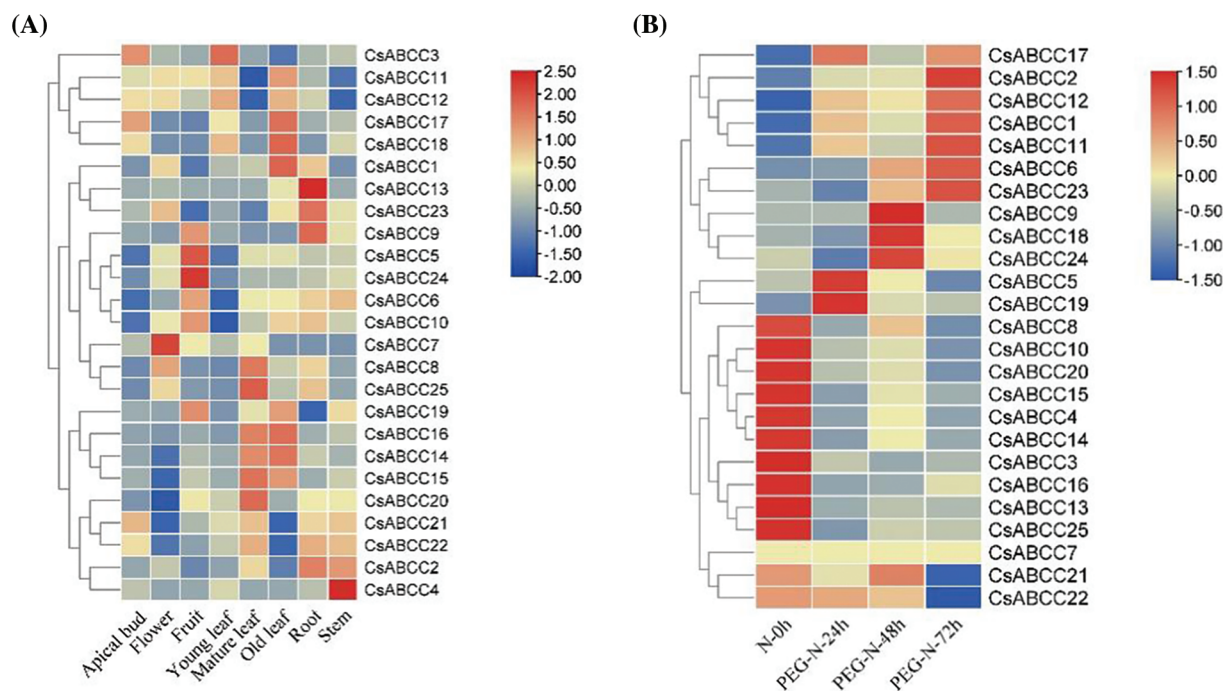


Figure 7: (Continued)

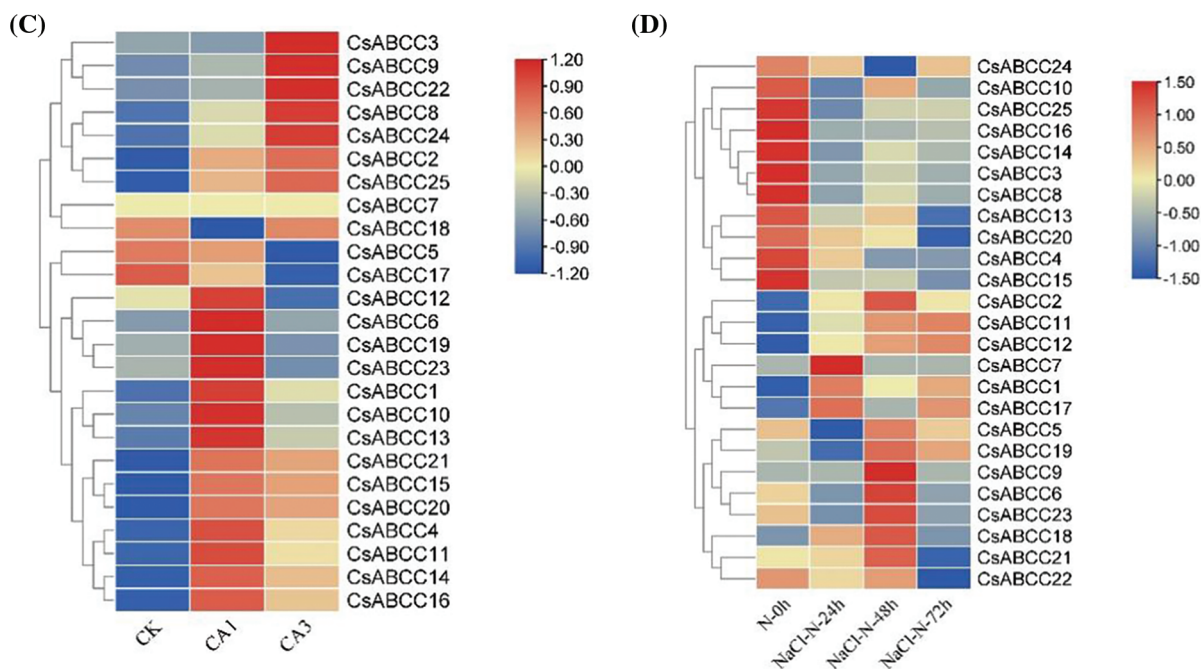


Figure 7: Expression profiles of *CsABCC* genes in 8 tissues and under different stress conditions. (A) Heat map of *CsABCC* expression in different tissues of tea plant. (B) Expression heat map of *CsABCC* under drought treatment. (C) Expression heat map of *CsABCC* under cold stress. (D) Expression heat map of *CsABCC* under salt stress

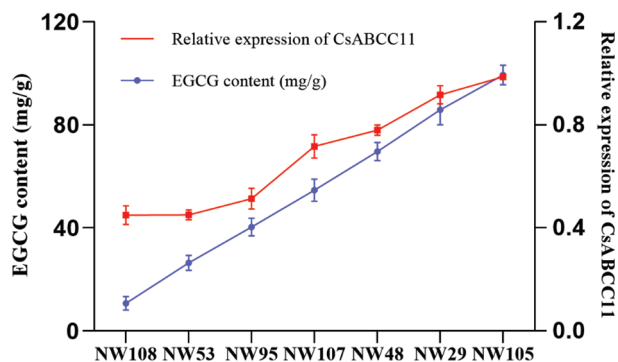


Figure 8: Correlation analysis of EGCG in 108 strains of *CsABCC11* Kingbird. The blue line represents the EGCG content, the red line represents the relative expression of *CsABCC11*, and NW represents the number of Kingbird. SPSS was used for correlation analysis, $r = 0.9767$, $p = 0.0002 < 0.05$

3.9 The EGCG Content of Fudingabai Tea Buds Decreased after *CsABCC11* Silencing

The latest established VIGS technology [35] was used to detect the change of EGCG content in tea plant cuttings, and the relative expression level of *CsABCC11* was verified by qRT-PCR (Fig. 10A). The relative expression of *CsABCC11* was significantly decreased after silencing. The results showed that *CsABCC11* was silenced in tea cuttings, and VIGS results showed that *CsABCC11* gene silencing inhibited the accumulation of EGCG in tea buds. Compared with WT and cuttings infected with pTRV1 + pTRV2, the

EGCG content of cuttings infected with pTRV2-*CsABCC11* was significantly reduced, and pTRV2-*CsABCC11* was about 7.0 times lower than that of WT and no-load (Fig. 10B). Silencing *CsABCC11* significantly inhibited the accumulation of EGCG. These results indicate that *CsABCC11* is required for EGCG intracellular transport in tea plants.

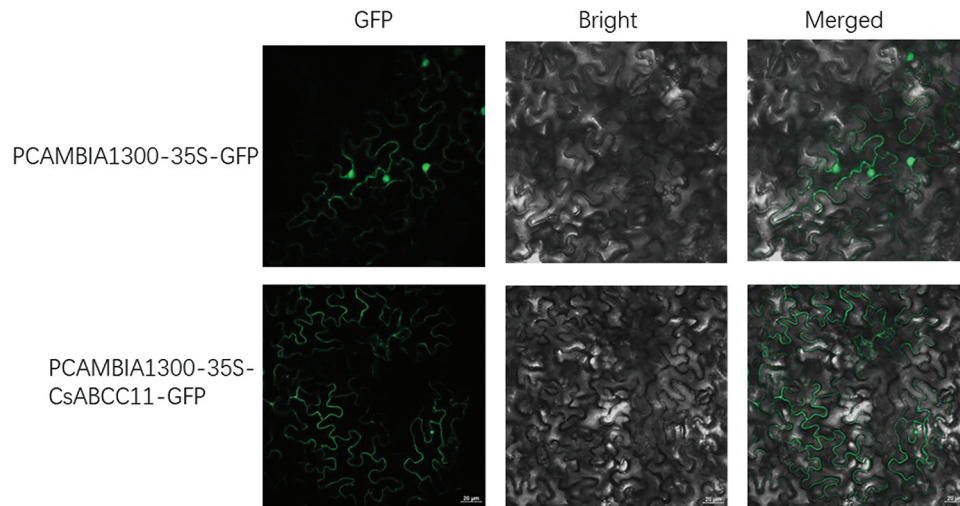


Figure 9: Subcellular localization of *CsABCC11* protein. Confocal microscopy was used to obtain the *CsABCC11*-GFP protein image in *N. benthamiana* mesophyll cells, and the empty GFP was used as a control. Bright represents the bright field picture, and Merged represents the superimposed field picture

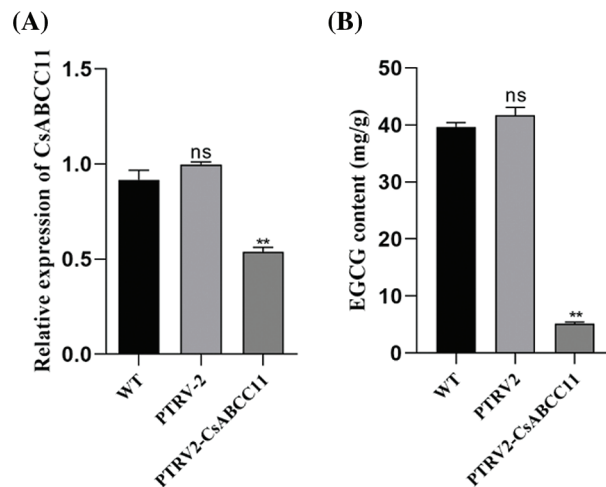


Figure 10: Using the VIGS system to silence *CsABCC11* in tea leaves. A shows the expression of *CsABCC11* after VIGS. B shows the content of EGCG, TRV, tobacco brittle fracture virus after VIGS; WT, wild type. pTRV2, pTRV1 + pTRV2 agrobacterium-infected tea cuttings; pTRV2-*CsABCC11*, pTRV1 + pTRV2-*CsABCC11* agrobacterium-infected tea cuttings. “***” indicates that there are significant differences between different samples. “ns” indicates that there is no significant difference

4 Discussion

There are many reports on the study of *ABCC* gene in other species, but there are few reports on tea plants. EGCG is the main component that determines the color, aroma and taste of tea. EGCG is the highest catechin content in green tea, which can reach 6%–10% of the dry weight of tea. We used bioinformatics analysis, correlation analysis, subcellular localization, qRT-PCR and VIGS-mediated gene silencing strategy to clarify that *CsABCC11* is involved in the transport of EGCG in tea plant buds.

4.1 Bioinformatics Analysis of *CsABCC* Genes Subfamily and Its Relationship with Environmental Response

We identified 25 *CsABCC* genes at the genomic level of tea plant (Table 1). According to the conserved domain of ABCC transporter protein, it contains a common domain (MRP), which is divided into four main phylogenetic groups (Fig. 4). It can be seen that the homology between each group is very high, which also indicates that the functions of the same subfamily may be similar. Multi-species phylogenetic tree analysis shows that the *ABCC* gene family is evolving in multiple directions (Fig. 1). *CsABCC* protein showed highly identical motif composition in the same subfamily (Fig. 2). It means that the evolution of these motifs is conservative.

The diversity of gene structure can provide important information about the evolutionary history of gene families and provide additional help for phylogenetic classification. The number of introns of *CsABCC* genes is between 7 and 17 (Fig. 4), which is much more than that of other species such as *Arabidopsis* [37]. This phenomenon indicates that *CsABCC* family genes exhibit high genetic structure diversity. The members of the same subgroup showed closely related intron-exon distribution patterns; this also proves the functional similarity between members of the same evolutionary group.

ABCC gene is involved in a variety of plant abiotic stress (such as salt, metal stress) has been widely studied [38]. In *Arabidopsis thaliana*, *Sedum alfredii*, etc., *ABCC* genes have been identified to respond to various abiotic stresses. We analyzed the cis-elements of *CsABCC* genes, the promoter region contained a large number of promoter cis-acting elements such as Light response, plant hormone response, plant growth and development, adversity stress and so on. As a class of non-coding small RNA (sRNA), microRNA (miRNA) is regulated at the post-transcriptional level by binding to target genes. The miRNAs have been shown to be involved in the regulation of plant secondary metabolites [39]. The miRNA-*CsABCC* network diagram (Fig. 6) showed that a large number of miRNA binding to *CsABCCs* may regulate the connotation of tea plant substances. The expression pattern study found that *CsABCC* had important contributions to the growth and development of tea plants and different stress environments (Fig. 7). These results suggest that *CsABCC* genes may have a response in the above pathways, and those that also typically induce the synthesis of EGCG for the growth or resistance to stress of the tea plant itself.

4.2 *CsABCC11* Were Related to the Accumulation of EGCG in Tea Plant Buds

The tea plant is the most EGCG-containing plant in nature. EGCG has a very special stereochemical structure and strong antioxidant activity [40]. EGCG has a broad-spectrum anti-viral effect. It can not only inhibit coronavirus, but also has obvious inhibitory effects on influenza virus, adenovirus, HIV (AIDS virus), HPV (human papillomavirus), EB virus and other viruses [41,42]. It is expected to become a potential broad-spectrum anti-viral drug. Through correlation analysis, we found that the EGCG content of 108 strains of the Kingbird was positively correlated with the expression of *CsABCC11* (Fig. 8). This shows that the content of EGCG is highly correlated with the expression of *CsABCC11*.

In plants, ABCC transporters are involved in the transport of secondary metabolites. Many studies have shown that MRP is located in the plasma membrane and participates in the accumulation of secondary metabolites, suggesting that MRP is involved in the vacuolar transport of anthocyanins. The *bz2* mutant showed that MRP (ABCC) was involved in the vacuolar transport of phenolic glycosides [43]. Such as

Arabidopsis, and carnation. Gao et al. provided further strong evidence for the involvement of MRP protein in anthocyanin accumulation through reverse genetics research. The ZmMRP3 is localized to the vacuolar membrane [13]. It is necessary for the accumulation of anthocyanin in maize. Interestingly, the above plant ABCC proteins were localized on the tonoplast, while the subcellular localization of CsABCC11 protein in our study was predicted to be in the plasma membrane, and the subcellular localization of CsABCC11 protein was confirmed by tobacco subcellular (Fig. 9) localization experiments.

To verify that *CsABCC11* gene is involved in the accumulation of EGCG content in tea plant buds, we used the newly developed virus-mediated gene silencing (VIGS) technology to silence *CsABCC11*. The findings indicated a significant reduction in EGCG content in silent branches compared to non-silent branches. qRT-PCR further showed that *CsABCC11* was silenced. These findings confirm the involvement of *CsABCC11* in the accumulation of EGCG in tea buds. Virus-induced gene silencing led to a significant decrease in EGCG accumulation (Fig. 10). *CsABCC11* appears to play a pivotal role in the accumulation of EGCG in tea plants. This may be due to ABCC transporters are known to facilitate the transport of multiple substrates across biofilms, which is a very important ability for the biosynthesis of EGCG in tea plant. Our study laid a foundation for studying the biological function of *CsABCC* and provided a new candidate molecular marker gene for further EGCG-related variety breeding, which will be of great interest to breeders.

5 Conclusion

In the early stage of this study, a gene *CsABCC11* was located by GWAS of EGCG content in 108 strains of bird king species, and we identified 25 *CsABCC* genes at the genomic level of tea plant. Bioinformatics analysis and expression pattern analysis were performed. Correlation analysis showed that the expression of *CsABCC11* was positively correlated with EGCG content. Subcellular localization experiments confirmed that the CsABCC11 protein was localized on the plasma membrane. Silencing *CsABCC11* in tea plants by VIGS strategy reduced the content of EGCG in tea buds. In summary, this study found that *CsABCC11* was closely related to EGCG content in tea plant buds, which provided a solid groundwork for further research into the function of *CsABCC* and the biosynthesis and transportation mechanism of EGCG.

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