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RESEARCH ARTICLE

Overexpression of *CsBRC*, an F-box gene from *Camellia sinensis*, increased the plant branching in tobacco and rice

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Abstract

Tea plant (*Camellia sinensis* [*L.*]) is one of the most important crops in China, and tea branch is an important agronomic trait that determines the yield of tea plant. In previous work focused on GWAS that detecting GWAS signals related to plant architecture through whole genome re-sequencing of ancient tea plants, a gene locus TEA 029928 significantly related to plant type was found. Sequence alignment results showed that this gene belonged to the F-box family. We named it *CsBRC*. CsBRC-GFP fusion proteins were mainly localized in the plasma membrane. By comparing the phenotypes of *CsBRC* transgenic tobacco and WT tobacco, it was found that the number of branches of transgenic tobacco was significantly higher than that of wild-type tobacco. Through RNA-seq analysis, it was found that *CsBRC* affects the branching development of plants by regulating the expression of genes related to brassinosteroid synthesis pathway in plants. In addition, overexpression of *CsBRC* in rice could increase tiller number, grain length and width, and 1,000-grain weight.

KEYWORDS
F-box protein, phenotypic analysis, RNA-seq, tea tree

1 | INTRODUCTION

Number of branching and shoot branch angle are two key traits of plant architecture. Plants with a large shoot branch angle have a spread-out architecture, which helps them avoid diseases caused by high humidity and reduce competition for resources, but such plants occupy a great deal of space and increase shading and lodging, consequently reducing the photo-synthetic efficiency of their leaves and grain yield per unit area (Xu & Sun, 2021; Zhang et al., 2018).

In higher plants, branching is a complex biological process that is influenced to some extent by external environment, plant hormones, and genetic factors. Among them, plant hormones play a key role in regulating branching development (Beveridge, 2006; Olszewski et al., 2002). Auxin is the first hormone found to participate in branching development and has been receiving considerable attention (Ongaro & Leyser, 2008). The primary bud tip is a major site for auxin synthesis, from where auxin is transported downward into the plant by polar transport. This movement of auxin inhibits branch branching, known as apical dominance (Ljung et al., 2001). However, auxin does not directly inhibit the growth of buds but rather is required for buds growth, and the level of auxin in buds will increase with the activation of buds (Leyser, 2003).

Brassinosteroids (BRs) are an important plant hormone, which exists in various organs of plants and involved in many growth and development processes such as seedling growth, cell elongation, and cell wall formation (Gendron & Wang, 2007). Rice mutants with *DLT* mutation were insensitive to brassinolide and exhibited dwarfing, reduced tiller number, and shortened grain length phenotype (Tong et al., 2009). In *Arabidopsis*, the functional mutation of the

transcription factor *BES1*, which regulates brassinolide synthesis, led to the increase of shoot branching (Wang et al., 2013).

F-box proteins are the largest family in plants and are a key component of the SCF complex in the ubiquitin-protein degradation pathway (Risseeuw et al., 2003). The SCF complex (Skp1–Cullin–F-box-protein) is a multi-subunit ubiquitin ligase that specifically transfers activated ubiquitin to target-protein substrates (Cardozo & Pagano, 2004). The N-terminus of the F-box is composed of about 50 conserved amino acids, and its function is to bind to SCF, while the C-terminus is a variable protein interaction domain that undertakes the function of recruiting target proteins and locating them near E2 for ubiquitination (Skaar et al., 2013).

F-box plays a role in hormone signal transduction pathways such as auxin, brassinolide, strigolactone, methyl jasmonate, ethylene, and gibberellin (Dharmasiri et al., 2005). In the auxin signaling pathway, *TIR1/AFB* encodes a subunit of SCF E3 ubiquitin ligase, which is an F-box protein that can specifically bind to the auxin transcriptional repressor AUX/IAA and degrade it through ubiquitination, thereby releasing the transcription regulatory factor AFR that binds to AUX/IAA (Dharmasiri et al., 2005; Prigge et al., 2016). In the BR signal transduction pathway, BR-sensitive 2 (*BIN2*) negatively regulates BR signaling by phosphorylating *BES1* (bri1 EMS suppressor 1) and *BZR1* (brassinazole-resistant 1) (Ye et al., 2012). In *Arabidopsis*, *KIB* encodes a F-box protein that could bind to BIN 2 and promote its degradation, directly blocking the binding of BIN 2 to its substrate BZR (Zhu et al., 2017).

Branching, as an important agronomic trait, plays an important role in plant yield (Jiao et al., 2010). However, the current research reports on tea plants mainly focus on the contents and processing techniques of tea leaves, and there are few reports on the configuration of tea plants. In our previous research group, a branch-related gene was found in ancient tea trees by GWAS mapping (Lu et al., 2021). Through bioinformatics analysis, it was found to belong to the F-box family, and we named it *BRANCHED* (*CsBRC*). Our results confirmed that overexpression of *CsBRC* could promote branching. In addition, we also found that *CsBRC* has a certain effect on rice yield. The RNA-Seq analysis results of transgenic tobacco show that *CsBRC* may affect plant branching development by regulating the expression of plant hormone-related genes. This study preliminarily explored the function of *CsBRC* in tea plant tea branching, laying a foundation for further revealing the molecular mechanism of tea plant branching.

2 | MATERIALS AND METHODS

2.1 | Sequence analysis of *CsBRC*

The isoelectric point and molecular weight of the CsBRC-deduced protein were predicted using ProtParam. Multiple sequence alignment was analyzed by DNAMAN. MEGA X was used to construct a phylogenetic tree comprising *CsBRC* from tea plants, *Arabidopsis*, rice, and poplar with the NI method (bootstrap = 1,000).

2.2 | Subcellular localization of the *CsBRC*

To identify the subcellular localization of CsBRC protein, the target gene (*CsBRC*) fragments were inserted to the pCAMBIA1300-GFP vector to obtain the PCAMBIA1300-*CsBRC*-35S-GFP recombinants. Then, these recombinant vectors were transformed into *Agrobacterium tumefaciens* strain GV3101, respectively. These vectors were transiently expressed in the tobacco (*Nicotina benthamiana*) leaves epidermal cells through the *A. tumefaciens* mediated infection, and GFP fluorescence observations were conducted using confocal laser scanning microscopy (Zeiss) after 48 h of dark incubation.

2.3 | Vector construction and genetic transformation

The full-length of *CsBRC* was ligated to the pSH737 vector to obtain the overexpression vectors of pSH737-*CsBRC*. Two types of tobacco, namely, *N. benthamiana* and *Nicotiana tabacum L. K326*, were used for genetic transformation. Tobacco transformation was harbored using the leaf disk transformation method. Transgenic tobacco plants that overexpressing *CsBRC* were identified by GUS staining and PCR (Ai et al., 2009).

The full-length *CsBRC* was ligated to the p BWA (V) HU vector to obtain the recombinant vector. Rice transformation was performed by the *A. tumefaciens*-mediated co-cultivation approach. Transformed calli were selected on hygromycin-containing medium. Homozygous T3 seeds were used for further study.

2.4 | Measurement of plant architecture parameters

After 60 days of transplantation, we observed and recorded the plant height, leaf number, leaf length, leaf width, and branch number of transgenic and WT plants. Ten plants from each transgenic rice line were randomly selected for agronomic traits measurement. When rice enters the tillering stage, the number of tillers is counted. After rice maturity, the main panicle of each rice plant is selected to measure traits such as panicle length, grain number per panicle, seed setting rate, 1,000 grain weight, grain length, and grain width.

2.5 | RNA sequencing analysis

Through qRT PCR analysis, the strains with the highest gene expression levels in 35S:: CsBRC transgenic tobacco were identified. The branch parts were collected from 10 *N. benthamiana* plants overexpressing *CsBRC* (35S:: CsBRC) and wild-type *N. benthamiana* plants, respectively. Samples for each group were divided into three parts as three biological replicates. A total of six samples were sequenced. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer

(Agilent Technologies, Palo Alto, CA). The samples with RNA integrity number (RIN) ≥ 7 were submitted to enrich mRNA and construct cDNA libraries using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, United States) according to the manufacturer's instructions. RNA was extracted and sequenced by a biotechnology company (Novogene, Beijing, China). After the library was constructed, the insert size and effective concentration of the library were detected to ensure the quality of the library. After the library inspection was qualified, different libraries were pooled according to the target sequencing data and sequenced. Differentially expressed genes (DEGs) between wilted leaves were identified by DESeq. The false discovery rate ≤ .05 and the absolute foldchange value ≥ 1.5 were used as the criteria for identifying DEGs.

2.6 | Statistical analysis

Excel2020 software was used for statistical analysis. SPSS 26.0 software was used for significance analysis. TBtools draw heat map. The data were analyzed by Kruskal–Wallis, one-way ANOVA, and Duncan's test in SPSS software. Produce graphics using MultiExperiment Viewer, OriginPro 2018, and Adobe Illustrator CC 2019. The data were expressed as mean ± standard deviation. Significant levels are as follows: ****P < .0001; ***P < .00; **P < .01; *P < .05.

3 | RESULTS

3.1 | Identification and analysis of F-box family in tea plants

Based on the previous experimental work of the research group, the whole genome re-sequencing of ancient tea trees was carried out, and the gene locus TEA 029928 related to plant architecture was found (Jiao et al., 2010). Sequence alignment results showed that this gene belongs to the F-box family.

The full-length cDNA sequence of *CsBRC* was 1,122 bp, encoding 373 amino acids with a predicted molecular weight of approximately 42,690.57 Da. Phylogenetic tree analysis showed that the protein sequences encoded by *CsBRC* had high homology with the protein sequences poplar (PtBRC11, PtBRC8, and PtBRC9), which belonged to the same group (Figure 1a).

The BRC proteins of tea (*CsBRC*), poplar (PtBRC11, PtBRC8, and PtBRC9), all contain the same F-box domain (Figure 1b).

3.2 | Subcellular localization of CsBRC

In order to determine the subcellular localization of CsBRC, the PCAMBIA1300-CsBRC-GFP recombinant vector was transformed into *A. tumefaciens* strain GV3101. The *A. tumefaciens* cell (harboring the PCAMBIA1300-CsBRC-GFP construct) suspension was infiltrated in tobacco leaves (*N.benthamiana*). After 48 h of dark culture, the cells were observed by laser confocal microscopy. It was found that the fluorescence of CsBRC was distributed on the cell membrane of tobacco, indicated that CsBRC was localized on the cell membrane. (Figure 2).

3.3 | Cs*BRC* positively regulate the formation of tobacco branches

To investigate the function of *CsBRC*, we overexpressed this gene in two tobacco varieties. The GUS staining and RT-PCR results verified that *CsBRC* was successfully transformed into the transgenic tobacco, respectively (Figures S1 and S2).

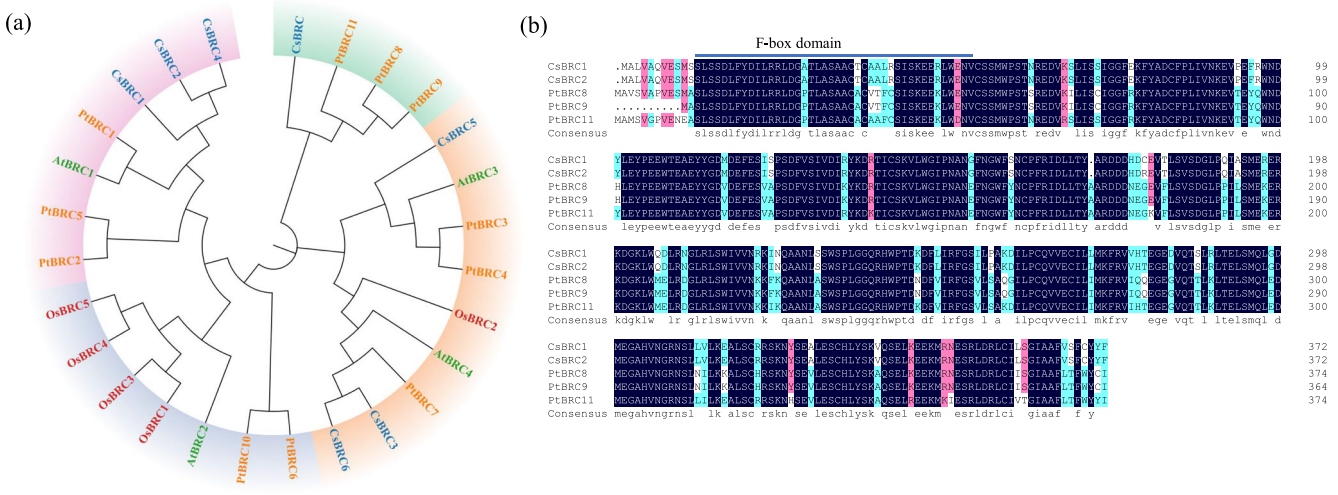

FIGURE 1 Bioinformatics analysis of tea plant *CsBRC*. (a) Phylogenetic analysis of *CsBRC*. (b) Protein alignment of homologous sequences. The conserved domain of *CsBRC* is indicated by the black line.

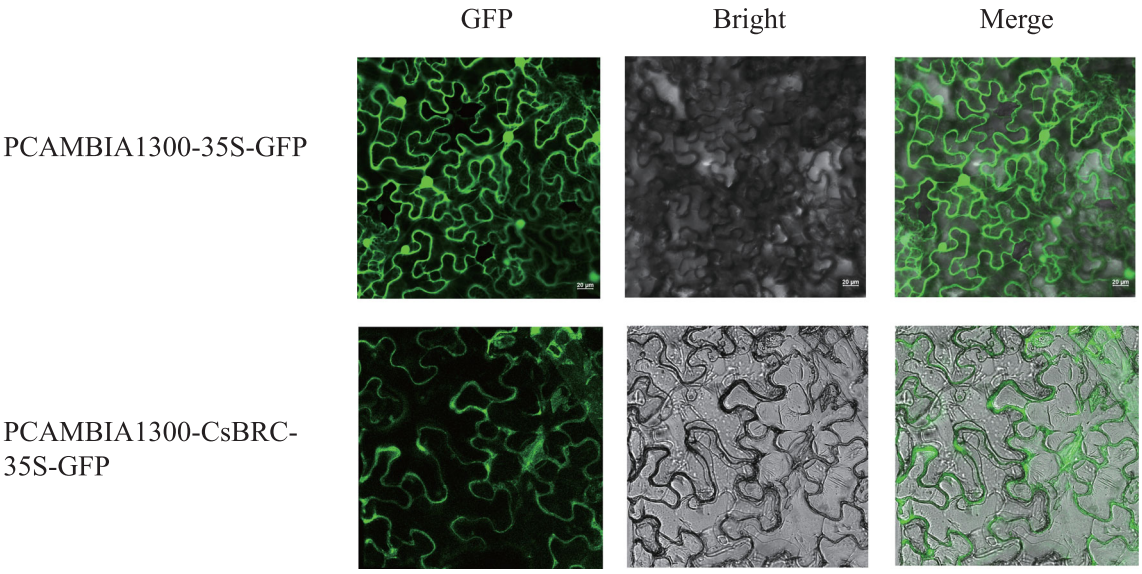

FIGURE 2 Subcellular localization of CsBRC in tobacco leaves. *Note*: Left to right are fluorescence channel, brightfield, and superposition field.

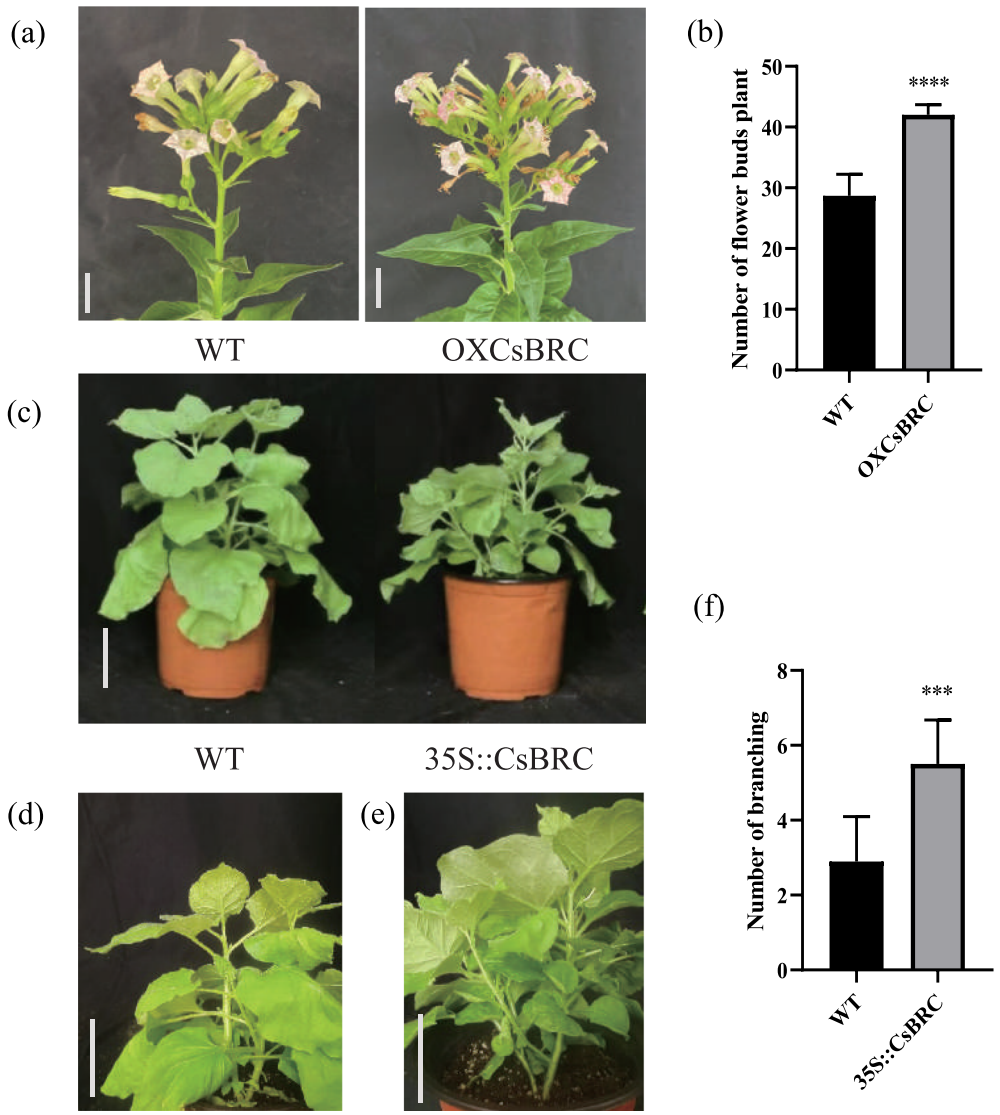

FIGURE 3 Phenotypic characterization of *CsBRC*-transgenic plants. (a) Phenotypes of transgenic "K326" tobacco (OXCsBRC) and WT. (b) The number of flower buds of transgenic "K326" tobacco (OXCsBRC) and WT. (c) Phenotypic comparison of transgenic *N. benthamiana* (35S::CsBRC) and WT. (d) Branching phenotype of WT. (e) Branching phenotype of transgenic *N. benthamiana* (35S::CsBRC). (f) Branch number of transgenic *N. benthamiana* (35S::CsBRC) and WT. Bar = 5 cm. *P* values were determined using two-tailed Student's *t* tests. Significant levels: $^{****}P < .0001$; $^{***}P < .001$. Data are presented as mean SD ($n = 10$).

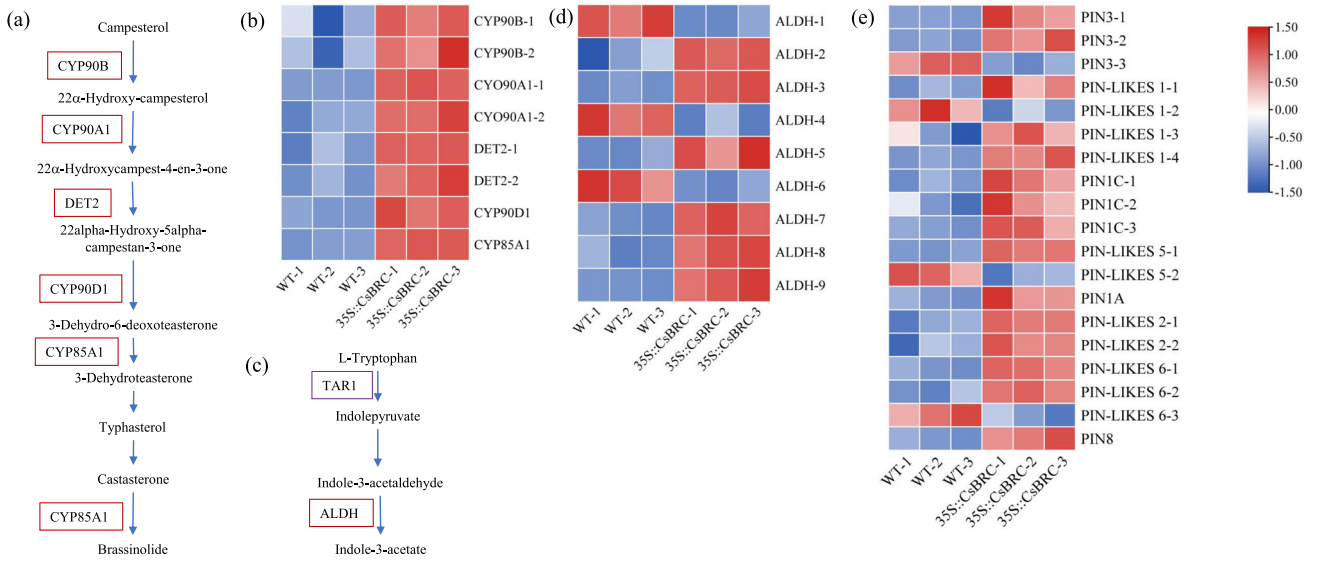

FIGURE 4 Analysis of differential gene expression levels in Brassinolide and auxin synthesis and signaling pathways. (a) Brassinosteroid KEGG synthesis pathway. (b) Differential gene expression in brassinosteroids synthesis pathways of WT and transgenic tobacco. (c) Auxin KEGG synthesis pathway. (d) Differential gene expression in auxin synthesis pathways of WT and transgenic tobacco. (e) Differential gene expression of auxin transporters PIN in WT and transgenic tobacco. In (a) and (c), the up-regulated genes are in the red box, and the purple box is the non-differential gene.

We first overexpressed *CsBRC* in the common tobacco variety "K326" to obtain transgenic "K326" tobacco OXCsBRC. Phenotypic statistics showed that the number of flower buds of OXCsBRC was higher than that of WT and there was no significant difference in the number of buds between these two transgenic plants (Figure 3a,b), but the branches and plant height of OXCsBRC were not different from those of WT.

Since no branching phenotype was observed in OXCsBRC, *CsBRC* were further overexpressed in *N. benthamiana* to obtain transgenic *N. benthamiana* 35S::CsBRC. The phenotypes of 35S::CsBRC transgenic tobacco at 60 d after transplanting were statistically analyzed. Compared with WT, the number of branching in transgenic tobacco that overexpressing *CsBRC* was increased, respectively (Figures 3c–f and S3).

3.4 | Transcriptome sequencing analysis of transgenic tobacco and wild-type tobacco

Studies have shown that plant branching is regulated by genes related to hormone biosynthesis and signal transduction (Durbak et al., 2012; Umehara et al., 2008). To clarify the potential mechanism by which *CsBRC* regulates plant branching, we performed transcriptome sequencing to identify DEGs between the transgenic tobacco (35S::CsBRC) and WT in branched stem segments.

Among the DEGs between transgenic tobacco (35S::CsBRC) and WT tobacco, there were eight DEGs involved in the brassinolide synthesis pathway, including *CYP90B1-1*, *CYP90B1-2*, *CYP90A1-1*, *CYP90A1-2*, *DET2-1*, *DET2-2*, *CYP90D1*, and *CYP85A1*, were up-regulated with expression level increasing by 1.08–3.5 times in transgenic tobacco (35S::CsBRC) (Figure 4a,b). A total of nine DEGs (belonged to the *ALDH* family) related to auxin synthesis pathway were identified. Among them, the expression levels of *ALDH-2*, *ALDH-3*, *ALDH-5*, *ALDH-7*, *ALDH-8*, and *ALDH-9* were up-regulated by 1.61–3.9 times, and the other three genes (*ALDH-1*, *ALDH-4*, and *ALDH-6*) decreased by .39–.95 times in transgenic tobacco (35S::CsBRC) (Figures 4c,d). The *PIN* family is an auxin efflux protein that functions to excrete auxin out of cells. Therefore, we further analyzed the expression changes of *PIN* family between transgenic (35S::CsBRC) and WT plants and identified 19 DEGs belonging to the *PIN* family. In transgenic tobacco (35S::CsBRC), except for a decrease (.18–.89 times) in the expression levels of four genes (*PIN3-1*, *PIN-LIKES 1-2*, *PIN-LIKES 5-2*, and *PIN-LIKES 6-3*), the expression levels of the other 15 genes showed varying degrees of increase (.8–3.2 times) (Figure 4e).

3.5 | Overexpression of *CsBRC* affects tillering and grain yield in rice

In order to further verify whether there are differences in the function of *CsBRC* among different species, *CsBRC*-overexpressing transgenic rice were generated. Statistical analysis of plant phenotypic traits showed that the effective spike number per plant of *CsBRC* overexpressing plants (pUbi::CsBRC) was higher than those of the wild type control, respectively (Figure 5a,d); the tiller number of the *CsBRC* overexpressing plants was both higher than that of the wild type control, respectively (Figure 5b,c). These results indicated that overexpression of *CsBRC* gene could increase the tiller number, effective panicle number per plant. Considering the above results, we further

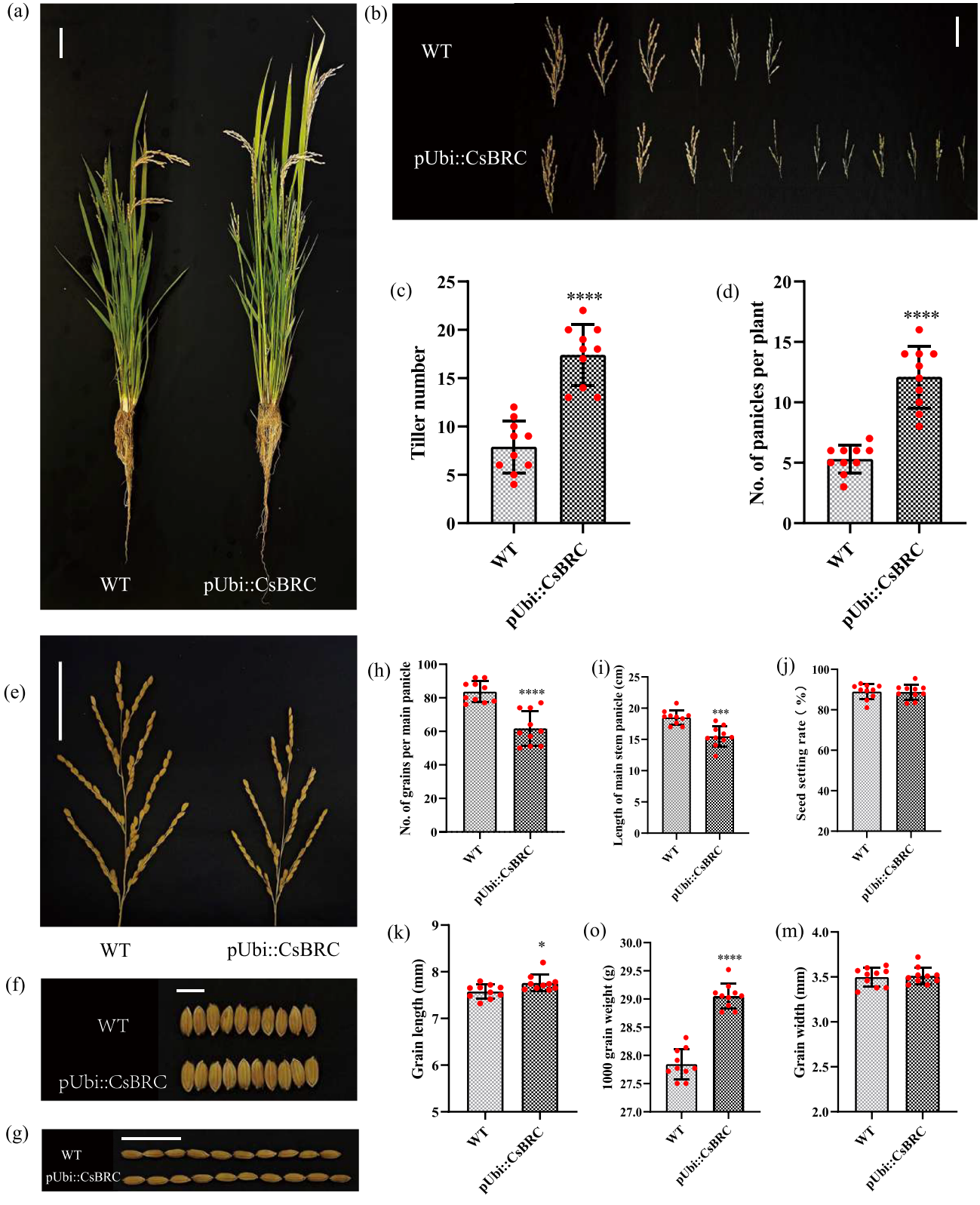

FIGURE 5 Phenotype of transgenic rice and statistical analysis of transgenic rice yield. (a) Spike number per plant. Bar = 5 cm. (b) Panicle phenotype. Bar = 5 cm. (c) Comparison of tiller number. (d) Comparison of spike number per plant. (e) Main stem panicle comparison. Bar = 5 cm. (f) Grain width. Bar = 2 cm. (g) Grain length. Bar = 2 cm. (h) Comparison of spike length of main stem. (i) Comparison of grain number per spike of main stem. (g–m) Seed setting rate, 1,000-grain weight, grain length comparison, and grain width comparison. *P* values were determined using two-tailed Student's *t* tests. Significant levels: $^{****}P < .0001$; $^{***}P < .001$; $^{**}P < .01$; $^{*}P < .05$. Data are presented as mean SD ($n = 10$).

conducted statistics on the yield traits of transgenic and wild type rice to investigate the effects of *CsBRC* overexpression on rice yield. The panicle length and grain number of the main stem of pUbi::CsBRC plants were lower than those of the wild type control (Figure 5e,h,i). The results showed that the grain length of the overexpression plants was longer than that of wild type rice (Figure 5g,h). There was no difference in grain width between wild type and mutant (Figure 5f,m). The 1,000-grain weight of pUbi::CsBRC transgenic rice was higher than that of wild-type rice (Figure 5k), but the seed setting rate was not significantly different from that of wild-type rice (Figure 5j).

4 | DISCUSSION

Plant branching plays an important role in plant architecture and yield, but there are few studies on tea plant branching (Zhao et al., 2015). In our previous research group, a branching related gene from ancient tea trees was identified through GWAS mapping (Jiao et al., 2010). Sequence alignment revealed that this gene belongs to the F-box family, so we named it *CsBRC*. F-box proteins are involved in a variety of biological processes in plants, including flower development, hormone perception and signal transduction, circadian rhythm, defense response, and branching development (Arif et al., 2020). Transcriptional activation of F-Box DUF protein-encoding genes in rice improved stress resistance and tiller number (Raja et al., 2023). In this study, our results showed that the transgenic tobacco overexpressing *CsBRC* had more branches than the WT.

In order to reveal the mechanism of *CsBRC* regulating branching, transcriptome analysis was performed. Among DEGs, eight genes related to brassinolide synthesis pathway were identified, and all genes were upregulated in *CsBRC* overexpressing plants. Brassinolide plays a positive role in plant branching development (Fichtner et al., 2021). CYP90B1 is a key enzyme in the synthesis pathway of BRs. It converts oleyl alcohol (CN) into 6-deoxyoctadecanone, a steroid C-22 hydroxylase (Fujita et al., 2006). Overexpression of *CmDWF4* (*CYP90B1*) in chrysanthemum significantly increased the growth rate, number, and length of lateral buds (Fu et al., 2023). As a bifunctional cytochrome P450 monooxygenase, CYP85A1 catalyzes the conversion of ricinosterone to brassinolide and mediates the conversion of castasterone to brassinolide in the brassinolide synthesis pathway (Bancoş et al., 2002; Kim et al., 2005). Overexpression of *CYP85A3* (a homolog of *CYP85A1*) in *Populus trichocarpa* and tomato increased the shoot fresh weight and fruit yield of transgenic plants, while increasing the number of branches (Jin et al., 2017). In this study, the expression levels of *CYP90D1* and *CYP85A1* in *CsBRC* transgenic *N. benthamiana* were up-regulated. In addition, the genes *CYP90A1*, *CYP90D1*, and *DET2* that promote BR synthesis were also up-regulated. In our transcriptome analysis, there were also DEGs that involved in the auxin synthesis pathway. Auxin is necessary for the development of lateral buds (Hillman et al., 1977). Overexpression of *TaHST 1 L*, a gene that promotes auxin synthesis, showed an increase in the number of tillers in winter wheat and spring wheat, while *TaHST 1 L* silenced plants showed a decrease in the number of tillers (Zhao et al., 2023). *ALDH* is an important gene involved in auxin biosynthesis, which converts indole-3-acetaldehyde to IAA (Guo et al., 2019). The expression of *ALDH* in our *CsBRC* transgenic tobacco was up-regulated relative to the WT. PINs play a rate-limiting role in catalyzing auxin efflux from cells, and their asymmetric cellular localization determines the direction of intercellular flow, which is critical for auxin-regulated growth processes (Petrásek et al., 2006). In this study, *PIN* family gene transcript levels were increased *in CsBRC* overexpressing plants. Previous studies have shown that overexpression of *OsPIN2* increased the number of tillers in transgenic rice (Chen et al., 2012). These results indicated that the increase of branches in *CsBRC* overexpressing plants may be achieved by regulating the synthesis of brassinolide and auxin and up-regulating the expression of *PIN* family genes.

Studies have shown that F-box can promote the number of branches and yield in rice. Overexpression of F-box protein gene, *OsAFB*, could increase the number of tillers and yield of transgenic rice (He et al., 2018). Ectopic expression of *TaFBA-2A*, a F-box protein, in rice improved the salt tolerance of transgenic rice and also increased the number of tillers and yield (Gao et al., 2022). We also overexpressed *CsBRC* in rice to further demonstrate the function of *CsBRC*, and the results showed that transgenic rice harboring *CsBRC* exhibited an increased tillering and yield phenotype.

In conclusion, this study reports the function of *CsBRC* from tea plants in regulating the branching of plants. The overexpression of *CsBRC* in tobacco (*N.benthamiana*) and rice improved the branches and crop yield by promoting the biosynthesis of auxins and BRs. These results will help to provide potential candidate genes for improving the shoot branching or high-yield breeding.

AUTHOR CONTRIBUTIONS

Litang Lu conceptualized the study. Bokun Zhou and Qi Shen performed the experiments and analyzed the data. Xinzhuan Yao conducted the investigation. Tong Li and Bokun Zhou did the writing. Litang Lu acquired the funding. All authors read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

The data is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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