Genome-wide identification and expression analysis of GRAS family transcription factors in tea plant (Camellia sinensis)

Yong-Xin Wang, Zhi-Wei Liu, Zhi-Jun Wu, Hui Li, Wen-Li Wang, Xin Cui & Jing Zhuang

GRAS proteins are important transcription factors that play multifarious roles in regulating the growth and development as well as stress responses of plants. Tea plant is an economically important leaf-type beverage crop. Information concerning GRAS family transcription factors in tea plant is insufficient. In this study, 52 CsGRAS genes encoding GRAS proteins were identified from tea plant genome database. Phylogenetic analysis of the identified GRAS proteins from tea plant, Arabidopsis, and rice divided these proteins into at least 13 subgroups. Conserved motif analysis revealed that the gene structure and motif compositions of the proteins were considerably conserved among the same subgroup. Functional divergence analysis indicated that the shifted evolutionary rate might act as a major evolutionary force driving subfamily-specific functional diversification. Transcriptome analysis showed that the transcriptional levels of CsGRAS genes under non-stress conditions varied among different tea plant cultivars. qRT-PCR analysis revealed tissue and development stage-specific expression patterns of CsGRAS genes in tea plant. The expression patterns of CsGRAS genes in response to abiotic stresses and gibberellin treatment suggested the possible multiple functions of these genes. This study provides insights into the potential functions of GRAS genes.

Tea is a non-alcoholic beverage accepted by an increasing number of people because of its beneficial functional components. The active substances, such as tea polyphenols (especially catechins), caffeine, theanine, and polysaccharides, have been reported being a stimulant and an antioxidant, and reduce the frequency of cancers, and neurodegenerative diseases. Tea plant (Camellia sinensis (L.) O. Kuntze) is an important perennial evergreen leaf-used economic crop that originated from subtropical regions. Thriving in the wild, tea plant is seriously affected in terms of growth, yield, and quality by various adverse environmental stresses, including salt, drought, cold, and heat.

With the advent of next-generation sequencing technology, a great number of genomics, transcriptomics, proteomics and metabolomics have been reported, which provide great opportunity for the development of metabolic engineering. Recently, the high quality and available of genome sequences of tea plant have been published, which greatly promoted the understanding the biological processes and molecular/cellular mechanisms of tea plant.

Transcription factors (TFs) are critical components in physiological processes and regulatory networks of higher plants; TFs serve as a switch of gene regulation, promote or inhibit specific functional gene expression, and maintain the growth and development as well as stress responses of plants. A large proportion of the plant genome is dedicated to TFs. For example, 7.5% of the Arabidopsis genome encodes for putative TFs. A recent tea plant transcriptome analysis has indicated that at least 2543 unigenes belong to 58 TF families.

The name GRAS family TFs is derived from the first three isolated members, namely, GAI, RGA, and SCR. It was initially taken as a plant-specific TF family. However, a recent study has identified the GRAS gene family in bacteria and classified it under the Rossmann fold methyltransferase superfamily. Typically, GRAS TFs share a widely conserved C-terminal GRAS domain and a variable N-terminal region, and total amino acids range from 400 to 770. The highly conserved C-terminus GRAS domain is constituted by five critical conserved motifs in the following order: LHR I (leucine heptad repeat I), VHIID, LHR II (leucine heptad repeat II), PFYRE,
and SAW\textsuperscript{33,34}. VHIIID along with two leucine heptad repeats (LHR I and LHR II), similar to the bZIP protein–DNA interaction, may be critical for protein–DNA or protein–protein interactions\textsuperscript{35}. Mutations in the SAW and PPFYRE regions of SLR1 and RGA proteins emerged in the dramatic phenotypic differences in Arabidopsis\textsuperscript{28–29}. The N-terminal of the GRAS domain family is diverse and may mainly decide the specificity of corresponding proteins. As an exception, DELLA proteins contain two conserved motifs (DELLA and TVHYNP) in their N-terminal region.

Whole-genome analysis of the GRAS family revealed 33 GRAS genes in Arabidopsis\textsuperscript{27}, 60 in rice\textsuperscript{27}, 46 in \textit{Prunus mume}\textsuperscript{30}, 48 in Chinese cabbage\textsuperscript{31}, 106 in populus\textsuperscript{32}, 53 in tomato\textsuperscript{33}, 48 in castor\textsuperscript{34}, and 52 in grapevine\textsuperscript{35}. Studies in several plants have indicated that GRAS family TFs serve diverse functions and participate in various physiological processes. For example, five widely known Arabidopsis GRAS proteins, GAI, RGA, RGL1, RGL2, and RGL3, which belong to the DELLA subfamily, negatively regulate GA signal transduction\textsuperscript{28,36,37}. Three GRAS homologous genes, \textit{MOCI} in rice, \textit{Ls} in tomato, and \textit{LAS} in Arabidopsis, participate in the formation of auxillary meristem\textsuperscript{38–40}. Two Arabidopsis GRAS genes, \textit{AtSCR} and \textit{AtSHR}, are mainly involved in root and shoot radial pattern formation\textsuperscript{41–43}. Two Arabidopsis GRAS proteins, SCL21 and PAT1, positively regulate phytochrome A signal transduction, which promotes etiolated to photomorphogenic transformation\textsuperscript{44,45}. Two \textit{Medicago truncatula} GRAS proteins, NSP1 and NSP2, are important for efficient nodulation, which could regulate the expression of nodulation genes by forming the NSP1–NSP2 complex\textsuperscript{46}.

Until recently, no reports have characterized GRAS family TFs in tea plant. In the present study, CsGRAS family genes were identified from previous tea plant genome database. Phylogenetic tree, sequence alignment, protein motif structural, and functional annotation analyses of CsGRAS TFs were performed. In addition, the expression patterns of CsGRAS genes among different tea plant cultivars were surveyed using available transcriptome data. Furthermore, tissue-specific expression patterns were investigated using qRT-PCR in tea plant cultivars ‘Huangjiyna’ and ‘Yingshuang’, and the expression patterns in response to diverse abiotic stresses and gibberellin (GA) treatment were detected. This research provides the first evidence about tea plant GRAS TFs, which may help elucidate the molecular mechanisms underlying stress responses in tea plant.

**Results**

**Identification of CsGRAS TFs in tea plant.** A total of 52 CsGRAS proteins were confirmed and used for further analyses (Table S1). Hidden Markov models (HMMs) of these CsGRAS proteins were determined using the Pfam database. All of these CsGRAS proteins contain a conserved GRAS domain (PF03514.13). However, five of the CsGRAS members (CsGRAS11, -12, -16, -24 and -48) possess a DELLA domain (PF12041.7) and thus were considered DELLA proteins (Table S2).

The physical and chemical characteristics of tea plant GRAS proteins were analyzed using ExPasy (Table S3). The length and molecular weight (Mw/Da) of the deduced GRAS proteins ranged from 416 to 1340 amino acids and 46291.14 Da to 146535.66 Da, respectively. The theoretical isoelectric point (pI) of most CsGRASs is slightly acidic (4.88–6.84), and two GRAS proteins are alkalines. In most CsGRAS proteins, the number of positive amino acids (Arg + Lys) is less than that of negative amino acids (Asp + Glu). The grand average of hydropathy (GRAVY) of all CsGRAS proteins varied from −0.618 to −0.074, indicating that these proteins are hydrophilic. The instability index of most CsGRAS proteins exceeded 40 (42.42–62.37), indicating that these proteins are unstable. No CsGRAS proteins were predicted to contain transmembrane helices by using the TMHHM server. Subcellular location analysis showed that most CsGRAS proteins were located in the nucleus (Table S4).

**Evolution of GRAS TFs among different plant species.** A comparison analysis of GRAS TFs in different species was performed to understand the evolution of GRAS TFs among different plant species (Fig. 1). GRAS family members were only identified in higher plants; no members were found in lower plants and fungi (\textit{Volvox carteri}, \textit{Chlamydomonas reinhardtii}, \textit{Ostreococcus lucimarinus} and \textit{Saccharomyces cerevisiae}). Evolutionary analysis indicated that the GRAS proteins may have expanded since the divergence from lower plants to higher plants, and these GRAS factors are closely related to higher plant evolution. The number of GRAS TFs in tea plant (52) was similar with closely related species, \textit{Coffea canephora} (50), \textit{Solanum lycopersicum} (53). The number and genome size of the GRAS proteins had no evident regularity between monocots and eudicots as well as woody and herbal plants. Some species, including \textit{Populus trichocarpa}, \textit{Glycine max}, and \textit{Zea mays}, exhibited a relatively large number of GRAS family members (i.e., over 100). The density of the GRAS proteins to genome size was rather low in several plant species, which are particularly evident in two gymnosperms, namely, \textit{Pinus abies} (0.0015 number/Mb) and \textit{Pinus taeda} (0.0004 number/Mb). This occurrence is mainly due to the large genome size of these two species.

**Phylogenetic analysis of GRAS proteins in tea plant, Arabidopsis, and rice.** A phylogenetic analysis of GRAS proteins from tea plant, Arabidopsis, and rice was conducted to clarify the evolutionary relationships among tea plant GRAS proteins. We acquired 34 GRAS proteins in Arabidopsis and 60 in rice, which had been proven in a previous study\textsuperscript{32}. Meanwhile, some members possess atypical GRAS domains, and some of them are severely truncated. For example, the domain sequences of some GRAS proteins are less than 100 amino acids (e.g., \textit{ΨOsGRAS2}, \textit{ΨOsGRAS3}, \textit{ΨOsGRAS5}, \textit{ΨOsGRAS7}), whereas the approximate number of typical GRAS domain is 350 amino acids. The short or atypical sequences may be putative pseudogenes and derived from ancient pseudogenization events\textsuperscript{33}. Considering that incorporating these fragments results in low reliability in multiple sequence alignment and phylogenetic analysis, we excluded these fragments for later analyses. Finally, 33 GRAS members in Arabidopsis and 50 in rice were identified (Table S5). Similar to those in tea plant, these proteins possess a typical GRAS domain.

Phylogenetic trees were constructed with alignments of 135 full-length GRAS proteins, including 52 from tea plant, 33 from Arabidopsis, and 50 from rice, and the neighbor-joining (NJ), minimum evolution (ME), and
maximum likelihood (ML) algorithms were employed (Fig. 2 and Figs S1 and S2). The NJ and ME trees exhibited similar topologies. Compare with the NJ and ME trees, the ML tree exhibited only minor modifications. In this study, the NJ phylogenetic tree was used for further analysis. On the basis of the phylogenetic analysis and existing research, all GRAS proteins were grouped into 13 subfamilies, namely, HAM, DELLA, AtSCL3, DLT, AtSCR, AtLAS, AtSCL4/7, AtSHR, AtPAT1, Os4, Os19, Os43, and LISCL. The CsGRAS proteins were unevenly distributed. For instance, most of the CsGRAS proteins were distributed in the AtPAT1 (9 members), and LISCL (10 members) subfamilies, whereas no members were found in the AtSCL4/7 subfamily.

Estimation of functional divergence. To investigate the level of functional divergence between gene clusters of the GRAS subfamily, type I and type II functional divergence were estimated using DIVERGE (v2.0) software on the basis of the ML algorithm, which provides a sequence-based method to identify cluster-specific amino acid sites that are likely responsible for functional changes after gene duplication. Type I functional divergence between two duplicate clusters indicates that some amino acid residues are highly conserved in one duplicate cluster but highly variable in another cluster, suggesting that these sites may have experienced shifted functional constraints. Type II functional divergence between two duplicate clusters indicates that some amino acid residues are highly conserved in both duplicate clusters but exhibits distinct biochemical properties, suggesting that these sites may be responsible for functional specification in different subfamilies. On the basis of the GRAS protein NJ tree, GRAS subfamilies HAM, DELLA, AtSCL3, AtSCR, AtSHR, AtPAT1, Os4, and LISCL were used in the present study as input for the DIVERGE2 analysis, whereas Os19, Os43, and AtSCL4/7 were excluded because of their small size (Table 1).

Functional distance analysis was conducted and a star-like tree in terms of the functional branch length (bF) of each subfamily was generated (Fig. 3) to examine the pattern of type I functional divergence for each pair of subfamilies. The functional branch lengths of the subfamilies were measured and ranked as follows: bF (LISCL, 1.01) > bF (Os4, 0.83) > bF (HAM, 0.78) > bF (AtSHR, 0.52) > bF (AtSCL3, 0.43) > bF (AtPAT1, 0.35) > bF (ATLAS, 0.06) > bF (AtSCR, 0.01). LISCL exhibited the longest functional branch, indicating that the evolutionary conservation may have been changed at many sites for this subfamily and that the derived functional state may be far away from the ancestral state. By contrast, the bF for AtSCR was virtually zero, suggesting that the evolutionary rate of each site has not changed substantially since duplication and that this subfamily is likely to have inherited the ancestral function.

The coefficients of type I functional divergence (θI) between any two subfamilies were significantly greater than 0 (p < 0.05, Table 1), suggesting that site-specific rate shift after gene duplication is a common phenomenon in the evolution of the GRAS family. The coefficient of type II functional divergence (θII) was smaller than 0 between most pairs but showed relatively high standard errors. To further explore the pattern of functional divergence between GRAS subgroups, posterior analysis was conducted to identify the critical amino acid sites (CAASs) that may be highly relevant to functional divergence. To reduce possible false positives, CAASs between gene clusters were identified using posterior probability (Qk) > 0.95 as the cut-off. Results showed that the numbers of CAASs were considerably varied among each comparison (Table 1). Some CAASs were supposed to be responsible for type I functional divergence, of which 16, 24, 24, 11, 26, and 44 CAASs were identified for pairs AtPAT1/AtSHR, AtPAT1/HAM, AtSHR/LISCL, HAM/AtSCL3, HAM/LISCL, and DELLA/LISCL, respectively. In contrast to type I functional divergence, 52, 94, 54, and 56 type II-related CAASs were identified for pairs AtPAT1/AtLAS, AtSHR/HAM, HAM/DELLA, and DELLA/LISCL, respectively. No CAASs were identified for
type I functional divergence between AtSHR/HAM and DLT/Os4, whereas 94 and 44 respectively predicted sites existed for type II functional divergence. Thus, the rapid change in amino acid physiochemical properties can be mainly attributed to the evolutionary force driving the functional divergence between this pair and secondarily to the shift in evolution rate. This research indicated that site-specific shifts in evolutionary rate and changes in amino acid property are inconsistent acts on the GRAS subfamily over the course of evolution.

Analysis of the sequence alignment and conserved motifs of the GRAS proteins. Multiple alignments indicated that all GRAS proteins contain a widely and conserved C-terminal GRAS domain, which could be divided into five parts (LHRI, VHIID, LHRII, PFYRE, and SAW) (Fig. S3), and a highly variable N-terminal domain (data not shown) as described in previous studies24,25,27. The five conserved motifs could be further subdivided into small distinct units. For example, both LHRI and LHRII are composed of two repeat units (A and B); VHIID can be divided into three units (A, B, and C); PFYRE contains three distinct parts (P, FY, and RE); SAW is composed of four units (RVER, W-G, L-W, and SAW). Overall, the results are slightly similar to previous statements.

To further examine the sequence features of GRAS TFs from tea plant, a comparative analysis of the conserved motifs was performed between tea plant and Arabidopsis GRAS proteins. Thirty motifs were predicted to reveal the details of the GRAS protein structure using the MEME program (Table 2 and Fig. S4). In general, GRAS proteins clustered in the same subgroups share similar motif compositions (Fig. 4), indicating functional similarities among members of the same subgroup. Nearly all of the GRAS members contained motifs 1, 3, 5, 7, 9, and 10, suggesting the important roles of these motifs in the GRAS gene family. Motifs 17, 20, 21, 23, 25, and 27 were only found in subgroup LISCL; 26 was only found in subgroup HAM; 2 was completely lost in subgroups AtSCR, Os19, and HAM; 15, 18, 22 were only found in subgroup DELLA; 24 and 28 were only found in subgroup AtPAT1; 30 was only found in subgroup AtSCR; 6 was found and 11 was completely lost in subgroups AtPAT1 and LISCL. The differences in motif distribution among the subgroups of GRAS genes revealed that the functions of these genes may have diverged during evolution.

Functional identification and interaction network of the GRAS proteins in tea plant. Annotations of the CsGRAS factors were retrieved from tea plant transcriptome databases and were predicted from integrated function annotation databases (COG, GO, KEGG, Swissprot, TrEMBL, Nr, and Nt) (Table S6).
Table 1. Analysis of functional divergence between subfamilies of GRAS proteins among tea plant, Arabidopsis, and rice.

The predicted CsGRAS genes possibly participate in diverse development processes, such as "floral organ morphogenesis", "regulation of seed dormancy process", "post-embryonic development", "seed germination", "post-embryonic morphogenesis", "reproductive structure development", "regulation of post-embryonic development", "organ development", "petal formation", "sepal formation", "radial pattern formation" and "leaf development". CsGRAS genes may be involved in various abiotic and biotic stresses, such as, "response to xenobiotic stimulus", "hypersomatic response", "response to salt stress", "response to temperature stimulus", "response to osmotic stress", "response to red or far red light" and "defense response to bacterium". As transcriptional regulators, CsGRAS genes may be associated with the biosynthetic and signaling pathways of multi-hormones, such as GA, jasmonic acid, abscisic acid, salicylic acid, auxin, and ethylene.
Expression profiles of CsGRAS genes in four tea plant cultivars. Using RNA sequence transcriptome data, we investigated the transcript levels of the putative GRAS genes among different tea plant cultivars under normal condition. A heat map was exhibited to assess the transcription profile of the CsGRAS genes among four tea plant cultivars on the basis of the reads per kilobase per million (RPKM) values (Fig. 6 and Table S7). All of the 20 CsGRAS genes were widely expressed across a variety of cultivars, apart from CsGRAS20, which was not detected in Tea_T3. This gene may have lost or weakened its functions in Tea_T3 during evolution that its expression became either too low for detection or had spatial and temporal patterns. Overall, most CsGRAS genes (except for CsGRAS19 and CsGRAS20) exhibited a relatively high level (RPKM > 1) in all of the four tea plant cultivars. Some genes, such as CsGRAS7, 11, and 12, were stable and highly expressed. Some obviously exhibited cultivar specificity. For example, CsGRAS16, 19, and 20 displayed higher expressions in Tea_T4 than in the three other cultivars; on the contrary, CsGRAS2 and CsGRAS8 showed a lower expression in Tea_T4 than in the others. In addition, CsGRAS1, 4, 6, 7, 9, 10, 13, 14, 15, and 18, displayed higher expression levels in Tea_T1 and Tea_T2 than in Tea_T3 and Tea_T4. Among the three genes encoding DELLA proteins, CsGRAS11 and CsGRAS12 were stable and highly expressed in all tea plant cultivars, whereas CsGRAS16 was variable. The diversity of expression indicates that the CsGRAS genes may show functional differentiation among different tea plant cultivars.

Expression profiles of CsGRAS genes in different tissues of tea plant. The expression patterns of CsGRAS genes in different tissues or different development stages (root, stem, bud, first leaf, second leaf, and third leaf) of two tea plant cultivars (‘Huajingyin’ and ‘Yingshuang’) were analyzed using qRT-PCR to explore the potential functions of GRAS genes during the vegetative development of tea plant. Eighteen CsGRAS genes were investigated. The 18 genes were representative and can explain the expression profiles of genes from different subgroups. All of the designed primers were suitable for qRT-PCR based on the corrected sizes of PCR amplified band and the validated by PCR paired-end sequencing (Figs S5 and S6).

Results indicated that these CsGRAS genes were detected in all of the tissues of the two tea plant cultivars and exhibited variable transcript levels (Fig. 7). CsGRAS1, 3, 5, 7, 9, 11, 15, 16, 17, and 18 were expressed at relatively high levels, whereas the other genes were expressed at low levels. Most of the CsGRAS genes presented similar tissue-specific expression patterns between two tea plant cultivars. For example, in two tea plant cultivars, CsGRAS2 and CsGRAS7 showed relatively high expression levels in the roots; CsGRAS16 and CsGRAS19 were abundantly expressed in the buds; CsGRAS3 was highly expressed in the third leaf; CsGRAS8 was highly expressed in the stems and leaves and weakly expressed in the roots and buds. The transcript levels of CsGRAS1 and CsGRAS13 gradually increased in the first, second, and third leaves. Some differences also existed. For
Conserved motifs identified from tea plant and Arabidopsis GRAS proteins by MEME software.

Table 2. Conserved motifs identified from tea plant and Arabidopsis GRAS proteins by MEME software.

Expression analysis of CsGRAS genes under GA treatment in two tea plant cultivars. Considering that many GRAS proteins are involved in regulating GA response, we examined the response of CsGRAS genes to GA treatment in ‘Huangjinya’ and ‘Yingshuang’ through qRT-PCR. Under GA treatment (Fig. 8), three CsGRAS genes (CsGRAS1, 3, and 17) were obviously upregulated in both tea plant cultivars, whereas CsGRAS2, 6, 7, and 15 were obviously downregulated. Three CsGRAS genes (CsGRAS11, 13, and 14) were mainly upregulated in ‘Huangjinya’ but downregulated in ‘Yingshuang’. CsGRAS8 and CsGRAS10 were mainly upregulated in ‘Yingshuang’ and showed a relatively stable expression level in ‘Huangjinya’.

Expression analysis of CsGRAS genes under different abiotic treatments in two tea plant cultivars. The responses of CsGRAS genes to salt (200 mM NaCl), drought (20% PEG 6000), cold (4 °C), and heat (38 °C) treatments in ‘Huangjinya’ and ‘Yingshuang’ were examined through qRT-PCR to elucidate further the role of GRAS genes in tea plant during diverse environmental stresses. Except for CsGRAS3, all of the analyzed genes responded to at least one abiotic stress treatment. Although most CsGRAS genes exhibited a similar tendency in the two tea plant cultivars, some notable exceptions were also observed.

Salt treatment. Under salt treatment (Fig. 9), 10 CsGRAS genes (CsGRAS1, 2, 6, 7, 10, 15, 16, 17, 18, and 19) showed obvious increases in both tea plant cultivars. Some CsGRAS genes (CsGRAS4, 5, 8, 9, 11, 13, and 14) were only increased in one tea plant cultivar. CsGRAS1, 10, and 14 were the most highly induced genes. CsGRAS1 rapidly reached the peak at 2 h and then decreased at 24 h in both tea plant cultivars. CsGRAS14 was highly induced in ‘Huangjinya’, whereas CsGRAS17 was highly induced (approximately fortyfold) in ‘Yingshuang’.

example, CsGRAS10 showed the highest expression in the second leaf in ‘Yingshuang’ but was highly abundant in the third leaf in ‘Huangjinya’.

Expression analysis of CsGRAS genes under GA treatment in two tea plant cultivars. Considering that many GRAS proteins are involved in regulating GA response, we examined the response of CsGRAS genes to GA treatment in ‘Huangjinya’ and ‘Yingshuang’ through qRT-PCR. Under GA treatment (Fig. 8), three CsGRAS genes (CsGRAS1, 3, and 17) were obviously upregulated in both tea plant cultivars, whereas CsGRAS2, 6, 7, and 15 were obviously downregulated. Three CsGRAS genes (CsGRAS11, 13, and 14) were mainly upregulated in ‘Huangjinya’ but downregulated in ‘Yingshuang’. CsGRAS8 and CsGRAS10 were mainly upregulated in ‘Yingshuang’ and showed a relatively stable expression level in ‘Huangjinya’.

Expression analysis of CsGRAS genes under different abiotic treatments in two tea plant cultivars. The responses of CsGRAS genes to salt (200 mM NaCl), drought (20% PEG 6000), cold (4 °C), and heat (38 °C) treatments in ‘Huangjinya’ and ‘Yingshuang’ were examined through qRT-PCR to elucidate further the role of GRAS genes in tea plant during diverse environmental stresses. Except for CsGRAS3, all of the analyzed genes responded to at least one abiotic stress treatment. Although most CsGRAS genes exhibited a similar tendency in the two tea plant cultivars, some notable exceptions were also observed.

Salt treatment. Under salt treatment (Fig. 9), 10 CsGRAS genes (CsGRAS1, 2, 6, 7, 10, 15, 16, 17, 18, and 19) showed obvious increases in both tea plant cultivars. Some CsGRAS genes (CsGRAS4, 5, 8, 9, 11, 13, and 14) were only increased in one tea plant cultivar. CsGRAS1, 10, and 14 were the most highly induced genes. CsGRAS1 rapidly reached the peak at 2 h and then decreased at 24 h in both tea plant cultivars. CsGRAS14 was highly induced in ‘Huangjinya’, whereas CsGRAS17 was highly induced (approximately fortyfold) in ‘Yingshuang’.

Table 2. Conserved motifs identified from tea plant and Arabidopsis GRAS proteins by MEME software.
Drought treatment. Under drought treatment (Fig. 10), 10 CsGRAS genes (CsGRAS1, 16, 17, and 19) were induced in both tea plant cultivars. Three CsGRAS genes (CsGRAS9, 10, and 14) were obviously upregulated in 'Huangjinya' but downregulated in 'Yingshuang'. CsGRAS4, 5, 6, 7, 11, 15, and 18 were increased in 'Huangjinya' but showed relatively consistent expression profiles in 'Yingshuang'. CsGRAS1, 10, 14, and 17 were the most highly induced genes. CsGRAS11 and CsGRAS14 were highly induced in 'Huangjinya', whereas CsGRAS17 was highly induced (over 300-fold at 24 h) in 'Yingshuang'.

Low-temperature treatment. Under low-temperature treatment (Fig. 11), six CsGRAS genes (CsGRAS1, 2, 9, 10, 16, and 19) showed obvious increases in both tea plant cultivars. Six CsGRAS genes (CsGRAS9, 10, and 14) were mainly upregulated in 'Huangjinya' but showed a relatively stable expression level in 'Yingshuang'. CsGRAS4, 5, 8, 11, 14, and 16 were obviously increased in 'Huangjinya' but decreased in 'Yingshuang'. CsGRAS1 and CsGRAS10 were the most highly induced genes. CsGRAS1 and CsGRAS10 were upregulated in both tea plant cultivars, of which CsGRAS10 rapidly reached the peak at 2 h and then decreased at 24 h in both cultivars (over 75- and 20-fold, respectively).

High-temperature treatment. Under high-temperature treatment (Fig. 12), six CsGRAS genes (CsGRAS2, 4, 10, 15, 17, and 19) were evidently upregulated in both tea plant cultivars. CsGRAS1, 5, 8, 11, 14, 16, and 18 were increased in one tea plant cultivar. CsGRAS2, 10, and 14 were the most highly induced genes. CsGRAS2 was rapidly increased at 2 h and then decreased at 24 h in 'Yingshuang', whereas it reached the peak at 24 h in 'Huangjinya'.

Figure 4. Motif analysis of CsGRAS proteins from tea plant and Arabidopsis. A phylogenetic tree was constructed by MEGA5.0. Different subfamilies are marked with different color backgrounds. Motifs in the GRAS proteins were elucidated by MEME. Different motifs are represented by different colored numbered boxes.
CsGRAS10 and CsGRAS14 were mainly upregulated in ‘Huangjinya’ but showed a relatively stable expression level in ‘Yingshuang’.

In general, most CsGRAS genes were affected by drought, salt, and high and cold stresses. CsGRAS1, 10, and 17 from subfamily AtPAT1 and CsGRAS14 from subfamily LISCL were identified as highly induced genes.

**Discussion**

In recent years, a large number of TFs have been identified and analyzed in various species through bioinformatics technology. Compared with that of other TFs families, the research progress of the GRAS family is relatively slower. To date, insufficient information is available about GRAS TFs in tea plant. In the present study, 52 CsGRAS genes were identified from tea plant genome database.

Gene loss and duplication events are the dominant driving forces in species evolution, supplying the raw materials for the generation of novel gene functions and promoting the formation of gene families. No GRAS family members were found in lower plants. However, a recent study has identified the GRAS gene family in bacteria and classified it under the Rossmann fold methyltransferase superfamily26. Although methyltransferase activity is possibly lacking in most GRAS proteins in plants, the GRAS proteins in plants still bind a similar substrate to those in bacteria32. Exon–intron organization analysis on several species, such as *Prunus mume*, Arabidopsis, rice, populous, and grapevine, reported that most GRAS genes either lack introns or possess only a single intron27,30,32,35. Thus, horizontal gene transfer from ancient prokaryote genomes of bacteria may explain the origin of plant GRAS genes.

Gene duplication and differentiation are the major pathways of origin for new genes and for the differentiation of gene function32. Members clustered in similar subgroups commonly exhibit functional similarities. Therefore, phylogenetic analysis could facilitate functional genomics. In the present study, we identified 135 GRAS proteins with full-length domain sequences from tea plant, Arabidopsis, and rice, and classified them into 13 subfamilies on the basis of sequence structures and phylogenetic relationships. All of the five predicted tea plant DELLA (CsGRAS11, -12, -16, -24, and 48) proteins together with other DELLA proteins (AtGAI, AtRGA, AtRGL1, AtRGL2, AtRGL3, and OsSLR1) were grouped into the DELLA subfamily. Some rice GRAS members (OsGRAS1 and OsGRAS27) that were similar to the other DELLA proteins but lacked the DELLA domain were also grouped into the DELLA subfamily. Previously, Liu et al. grouped OsGRAS1 and OsGRAS27 together with the other DELLA proteins into the DELLA subfamily32. The results are largely consistent with previous
reports. Considerable bootstrapping value supports existed for many of the defined subfamilies in the unrooted tree, although some poor supporting values remained for several clusters. This result was expected because the GRAS protein sequences used in this study had an average of approximately 600 amino acid-lengths, a constraint imposed by a large number of substitutable residues.

Phylogenetic analysis revealed the following several pairs of closely related orthologous GRAS proteins between tea plant and Arabidopsis/rice: CsGRAS8 and AtSCL15, CsGRAS11 and AtRGA, and AtGAI, CsGRAS2 and AtSCL3, CsGRAS19 and AtSCL28, CsGRAS20 and AtSCR, CsGRAS3 and AtSCL1, CsGRAS5 and AtSCL1, CsGRAS4 and AtSCL9, and CsGRAS10 and CsGRAS17 with AtSCL13. Orthologs generally retained similar functions. AtSCL3, which shows a high similarity to CsGRAS2, functions as a repressor of DELLAs and controls root development through the GA pathway. CsGRAS19, which shares a high similarity to OsGRAS29 (also known as DLT), is involved in brassinosteroid signaling. CsGRAS3 and CsGRAS20 show high degrees of similarity to AtSHR and AtSCR, respectively, which are associated with root and shoot radial pattern formation. CsGRAS10 and CsGRAS17 show high degrees of similarity to AtSCL13, which is a positive regulator of phytochrome B signaling. The results suggest the possible functions of GRAS genes in tea plant.

The structural divergence of gene sequences has played vital roles in the evolution of gene families, which is an adaptive process for speciation and leads to the efficient use of natural resources or adaptation to unfavorable conditions. Comparisons of GRAS proteins between tea plant and Arabidopsis were conducted to characterize the GRAS sequences. Most GRAS proteins clustered into similar subgroups sharing similar motifs, suggesting their group-specific functions. Significant differences were observed among the different groups. For example, six motifs (motifs 17, 20, 21, 23, 25, and 27) were only present in subgroup LISCL; two motifs (motifs 24, and 28) were only found in subgroup AtPAT1, indicating their specific functions to other subgroup members. Among them, the DELLA (motif 15) and TVHVNP (motif 18) domains function in GA perception. The distributions of conserved motifs also reflect the relations of different subgroups. For example, motif 6 was found and 11 was completely lost in subgroups AtPAT1 and LISCL. This result indicates the close evolutionary relationships between subgroups AtPAT1 and LISCL. Therefore, structural analysis also provides a clue to locate which subgroup of GRAS genes is the ancient origin.

Type I and type II functional divergence between subfamilies of GRAS genes were estimated. Significant type I functional divergence was detected between any two subfamilies, suggesting that site-specific rate shift after gene duplication is the main force driving the functional divergence in the evolution of the GRAS family. Type II functional divergence between most subfamily pairs was smaller than 0, whereas the standard errors were relatively high, suggesting that the GRAS gene family may adopt type II in different degrees. In the present study, the functional divergence of GRAS genes was not closely correlated with expression profiling in different organs and samples under different abiotic stresses. It may contribute potentially to gene expression profiling accumulation.

The functional annotations of CsGRAS proteins were obtained from the well-characterized biological function of similar genes. It provided a functional context for the CsGRAS genes in tea plant with modules. CsGRAS genes were predicted to have diverse functions and involved in various physiological processes, such as responses to abiotic and biotic stresses, hormones, tissue development, and secondary metabolites.
The gene expression levels could provide critical clues to assess their possible functions. On the basis of previous transcriptome sequencing data, the transcript levels of the CsGRAS genes among different tea plant cultivars under normal conditions were investigated. In consideration these cultivars were obtained from different regions of China, the transcription levels were varied among the four tea plant cultivars. CsGRAS7 and two DELLA genes (CsGRAS11 and CsGRAS12) were stable and highly expressed in the four cultivars, indicating their critical roles in tea leaf development and metabolite biosynthesis.

Tissue-specific genes promote the development of particular organs or tissues, and tissue-specific expressions are helpful in defining the precise nature and function of genes. Eighteen tested CsGRAS genes widely exist in the tissues (the root, stem, bud, and 1st, 2nd and 3rd leaf) between the two tea plant cultivars ('Huangjinya' and 'Yingshuang'). Some results showed great error bars in expression levels, which may mainly because the differences among biological individuals. The expression patterns of different genes varied greatly; some genes showed similar tissue-specific expression patterns between two tea plant cultivars. The transcript levels of CsGRAS1 and CsGRAS13 gradually increased in the 1st, 2nd and 3rd leaf, indicating those two genes may function in the later development of different tissues of tea plant. It is observed that the expression levels of most of the CsGRAS genes in 1st, 2nd and 3rd leaf were higher for 'Yingshuang' compared to 'Huangjinya'. That may mainly because CsGRAS genes play greater roles in 'Yingshuang' compared to 'Huangjinya' during leaf development. The DELLA gene CsGRAS11 showed high expression levels in all tested tissues, which is consistent with DELLA genes that serve as the main signaling hub in the regulation of multifarious growth and developmental processes. The DELLA gene CsGRAS16 and DLT gene CsGRAS19 showed similar expression patterns and relatively higher expression levels in the buds than in the other tissues, indicating that these genes play a role in bud development. CsGRAS2 and CsGRAS7 showed high transcript levels in the roots. Previous studies reported that the CsGRAS2 homolog AtSCL3 regulates root development through the GA pathway.

GA is involved in multiple aspects of plant growth and development, such as seed development and germination, stem elongation, and flower development. The prediction of the functional interaction network indicated that the GRAS proteins act as important components of the GA signaling pathway. Expression analysis indicated that most GRAS genes were induced or inhibited in at least one tea plant cultivar. In Arabidopsis, DELLA proteins...
act as repressors of GA-responsive plant growth. Two DELLA genes (CsGRAS11 and CsGRAS16) were affected by GA treatment in tea plant. AtSCL3 represses DELLA and controls root development through the GA pathway. CsGRAS2 is an orthologous gene in tea plant, and it is evidently downregulated under GA treatment.

Harmful environmental conditions, such as salt, drought, cold, and heat, result in irreversible damages to the growth and development of tea plant. GRAS genes reportedly play potential regulatory roles in plant response to abiotic stresses (Fig. 13) [33,61–64]. However, information about this family in tea plant is currently lacking. In the present study, expression analysis indicated that most GRAS genes in tea plant could be affected by one or more various stress treatments, suggesting that GRAS family genes play important roles in the response to adversity stresses. Some expression trend differences exist between the cultivars 'Huangjinya' and 'Yingshuang'. Most of CsGRAS genes studied have shown higher levels of expression in 'Huangjinya' compared to 'Yingshuang' under salt treatment, indicating CsGRAS genes may have more important roles in 'Huangjinya' than that in 'Yingshuang' under salt stress. The expression pattern of CsGRAS6 under heat treatment has shown exactly opposite pattern in the two tea plant cultivars. Those different trends of expression of CsGRAS genes maybe involved in the response to abiotic stress in the two tea plant cultivars. Several highly induced genes were identified in subfamilies AtPAT1 (CsGRAS1, 10, and 17) and LISCL (CsGRAS14). Two members of the AtPAT1 subfamily genes from rice, CIGR1 and CIGR2, are involved in GA and stress response [65]. AtSCL13, an AtPAT1 subfamily gene from Arabidopsis, responds to various abiotic stresses and phytohormones [56]. The rice gene CIGR1 is orthologous to CsGRAS1, and the Arabidopsis gene AtSCL13 is orthologous to CsGRAS10 and CsGRAS17. Likewise, CsGRAS14 shares a strong sequence similarity to AtSCL14, which plays vital roles in the activation of stress-inducible promoters [66]. A previous expression analysis on Castor Beans indicated that LISCL and AtPAT1 subfamily genes respond to all of the four abiotic stresses (drought, salt, cold, and heat) [34]. These genes could be preferentially utilized for further plant functional studies. Several other genes respond to abiotic stresses. OsGRAS23, an AtSCL3 subfamily gene from rice, is involved in drought stress response through regulating the expression of stress responsive genes [67]. CsGRAS2, also an AtSCL3 subfamily gene in tea plant, is evidently upregulated under drought, salt, heat, and cold treatments, especially under heat stress. DELLA proteins participate in multiple abiotic stresses, such as low temperature and phosphate starvation [60,69]. The DELLA member CsGRAS16 is upregulated under all of the

---

**Figure 8.** Expression patterns of CsGRAS genes in response to GA treatment in tea plant cultivars 'Huangjinya' and 'Yingshuang'.
four abiotic treatments in both tea plant cultivars, whereas the expression of the \textit{CsGRAS11} genes dramatically changes under the four abiotic stress treatments in ‘Huangjinya’ but is relatively stable in ‘Yingshuang’.

Till now, tea plant transgenic system was not efficient. It was difficult to obtained transgenic tea plants. For further verify the function of those \textit{CsGRAS} genes of tea plant, we shall carry out the \textit{CsGRAS} genes overexpression and gene knockout using tea plant system, Arabidopsis or other model plants by transgenic method. In the future, using new and modified Sanger and next-generation sequencing techniques, more quality tea plant genome data shall be performed. We could integrate various genomics, transcriptomics, proteomics, and metabolomics to understand the \textit{CsGRAS} genes function of tea plant.

Conclusion

The GRAS TF family has been characterized in several plant species and associated with various critical development and physiological processes. However, information about this gene family in tea plant is lacking. In this work, 52 genes encoding \textit{CsGRAS} family TFs were identified from tea plant. The phylogenetic relationship, conserved motif composition, and functional divergence of these \textit{CsGRAS} genes were systematically analyzed and compared, which provided insights into the functional diversity of the GRAS gene family. The expression analyses of \textit{CsGRAS} genes among different tissues (root, stem, bud, first leaf, second leaf, and third leaf), GA treatment, and abiotic stresses (salt, drought, heat, and cold) provide valuable information about the gene functions of this TF family in tea plant. This study could serve as a reference for future function investigations and molecular breeding of tea plant.

Materials and Methods

Retrieval of putative GRAS genes of tea plant. All candidate \textit{CsGRAS} genes were derived from the tea plant genome database\(^{18}\). The acquired amino acid sequences were analyzed using the BLASTP search at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only the sequences with full-length GRAS domain were considered as \textit{CsGRAS} proteins and used for further analyses. HMMs of the selected \textit{CsGRAS}s were analyzed using the Pfam database (http://pfam.sanger.ac.uk)\(^{19}\). The chemical and physical characteristics of the \textit{CsGRAS} proteins were

Figure 9. Expression patterns of \textit{CsGRAS} genes in response to salt treatment in tea plant cultivars ‘Huangjinya’ and ‘Yingshuang’.
predicted by ProtParam (http://web.expasy.org/protparam/). To confirm the subcellular localization of the identified CsGRAS proteins, protein sequences were predicted using WoLF PSORT (http://wolfpsort.org). TMHHM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the membrane-bound CsGRAS members. The GRAS family TF databases of other species were downloaded from the plant TFDB database (http://planttfdb.cbi.edu.cn/)71.

**Phylogenetic and evolutionary analyses of the GRAS TFs.** Multiple sequence alignment of the identified GRAS protein sequences was performed using the ClustalX1.83 program with default parameters and adjusted manually72. The sequence alignments were also used for the subsequent phylogenetic analysis. Phylogenetic trees were generated with MEGA5.02 software by using the NJ, ME, and ML algorithms, and the reliability of the obtained trees was assessed with a bootstrap value of 100073,74. Functional divergence analysis was performed using the DIVERGE (v2.0) software with the algorithm based on ML procedures47,50. MEME v4.11.1 (http://meme-suite.org/tools/meme) was employed to identify the conserved motifs with default parameters, except for the maximum number of motifs (30). The functional interacting networks of proteins were constructed using STRING software with the confidence parameter set at 0.15 threshold75.

**RNA-seq data analysis.** The expression profiles of CsGRAS genes in the four tea plant cultivars were assessed using transcriptome sequencing data, which was previously generated and analyzed by Wu et al.7. The tea plant samples used included mid-leaf 'Yunnanshilixiang' (Tea_T1) from Yunnan Province, small-leaf 'Chawansanhao' (Tea_T2) from Jiangsu Province, large-leaf 'Ruchengmaoyecha' (Tea_T3) from Hunan Province, and small-leaf 'Anjibaicha' (Tea_T4) from Zhejiang Province. These four tea plant samples exhibited different environmental adaptation modes and leaf sizes7. RPKM values were retrieved and normalized to estimate gene expression values. Heat maps were generated, and hierarchical clustering was conducted using HemI1.0 software (http://hemi.biocuckoo.org/faq.php).
Tea plant materials and stress treatments. In China, ‘Huangjinya’ and ‘Yingshuang’ are important tea plant cultivars that share several identical traits, such as high yield and good quality. ‘Huangjinya’ is a light-sensitive albino cultivar that shows potential for processing high-quality green tea even in summer but exhibits weak stress resistance. ‘Yingshuang’ possesses appropriate phenol ammonia content and good resistance to abiotic stresses, especially cold stress. Two-year-old vegetative propagated cuttings of tea plants, such as ‘Huangjinya’ and ‘Yingshuang’, were grown in a chamber at the Tea Science Research Institute of Nanjing Agricultural University (Nanjing, China). The seedlings were grown in pots containing a mixture of perlite, vermiculite, and sphagnum (ratio, 1:2:3). The chamber conditions were 23 °C temperature, 14/10 h day/night period, and 70% relative humidity for subsequent stress treatments. To determine the tissue-specific expression patterns of CsGRAS genes, the roots, stems, buds, and first, second, and third leaves were collected from ‘Huangjinya’ and ‘Yingshuang’ under normal conditions, rapidly frozen in liquid nitrogen, and then stored at −80 °C for RNA extraction.

RNA isolation and qRT-PCR analysis. The total RNA was isolated from plant samples using the Quick RNA Isolation Kit (Huayueyang, Beijing, China) in accordance with the manufacturer’s protocol. The purified RNA (1.0 μg) was reverse-transcribed into cDNA in 20 μL of reaction volume using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). The cDNA reaction mixture was diluted 20-fold using deionized water for fluorescence detection.

The specific primers for tea plant CsGRAS genes were designed from the non-conserved N-terminal region using Primer Premier 5.0 software. Primer specificity was confirmed by the melting curve with a single sharp peak.

Figure 11. Expression patterns of CsGRAS genes in response to cold treatment in tea plant cultivars ‘Huangjinya’ and ‘Yingshuang’.
Figure 12. Expression patterns of CsGRAS genes in response to heat treatment in tea plant cultivars ‘Huangjinya’ and ‘Yingshuang’.

Figure 13. A potential model of transcriptional regulation of GRAS TFs in tea plant.
Table 3. Sequences of primers used in qRT-PCR.

| Target Gene | Forward primer sequence (5′→3′) | Reverse primer sequence (5′→3′) |
|-------------|---------------------------------|---------------------------------|
| CsGRAS1     | GCCAGATTTCCTGGATACGG            | ACTCAATGTGTAAGGGTGCTCA         |
| CsGRAS2     | AGAGATCATCATCATAGTTGCTCG        | CCAAGGAAATGGTGAAACC           |
| CsGRAS3     | ATACGTCCCTCAACCTCCAGGC         | GGTTGAAGGGATAGTGCT            |
| CsGRAS4     | CAGATTAGACCTCCATGAGTGTA        | TTCCCTAGGTTGGAAGTCCAT         |
| CsGRAS5     | AGGCGGATTTAGATGCCCGGA          | GATACCCATCCCTGATGCTTCTT       |
| CsGRAS6     | CTCCAAACCCCATCCCA              | CAAACCTGACGACTCTCCCT          |
| CsGRAS7     | CAGAAGACAGACAGGGGGA            | CAATTAGGATCTAAAGGGA           |
| CsGRAS8     | GAAGTGTCGTTTATTCGACGGC         | CGACTGTAACAAAGACCTCGGA        |
| CsGRAS9     | GCTCTACAAAGAATCTGGAAGA         | AGTCAATGCAACATGCGGA           |
| CsGRAS10    | ACATCGCCGAGAATACCAAG           | TCCGAAGGATAGCTGAGAAGC         |
| CsGRAS11    | AGCATCACCAGCACTACCCG           | TGTCCAGCTTCTGAGCAC            |
| CsGRAS13    | GTGGGCGCTGGTGCTTAAATG          | AAGATGGTGTGTAAGGGGTGT          |
| CsGRAS14    | AGCACTGACCGATTTTATGGA          | ACTTCCGTGTTGTGCTGCTTCT        |
| CsGRAS15    | GGCAGAGGTTGAGTGTCTCTACCC      | GTCCGCTCAAGGAAATCCCTC         |
| CsGRAS16    | GTGGGAGCCCGCATTAGAT            | ACCGATAACTCCTGGAGGA           |
| CsGRAS17    | AAGGGAGCCACAGCAACC             | GACTGAGACCTTGTGGTGAA          |
| CsGRAS18    | GGACTCATTTCACGCAGAAGC          | AAGTCAAGCCAGCGTGGTGCT         |
| CsGRAS19    | CCTGTAGAAGAGGGCTAAGAG          | CTCAAAACTTGGCGTGAACC          |
| CsActin     | GATTCCGCTTGGCCTGAGTCT         | CTTGCTCTACAGGGCTTGCCAT        |

and the electrophoresis pattern with a single amplicon and the correct predicted length. The accuracy of PCR amplified bands were validated by PCR paired-end sequencing, and completed by the GenScript Corporation (Nanjing, China) (Figs S5 and S6). The Csactin gene served as an internal control to normalize the expression levels89,90. All primer sequences are presented in Table 3. qRT-PCR assays were completed on the Bio-Rad CFX96 fluorescence quantitative PCR platform with the following program: 95 °C for 3 min; 40 cycles of 95 °C for 5 s for denaturation and 58 °C for 30 s for annealing and extension; and 61 cycles 65 °C for 10 s for melting curve analysis. Each reaction was conducted in a 20 µL reaction mixture containing 2 µL of diluted template cDNA, 0.4 µL of each specific primer, 10 µL of SYBR Premix Ex-Taq (TaKaRa, Dalian, China), and 7.2 µL of ddH2O. The experiments were independently repeated thrice, and relative expression levels were measured using the 2-ΔΔCt method88. Significant differences were detected using Duncan’s multiple-range test at the 0.05 level with SPSS 17.0 software.

References
1. Mondal, T. K., Bhattacharya, A., Laxmikumaran, M. & Ahuja, P. S. Recent Advances of Tea (Camellia Sinensis) Biotechnology. Plant Cell Tissue & Organ Culture 76, 195–254 (2004).
2. Weidong Wang, H. X. et al. Transcriptomic Analysis Reveals the Molecular Mechanisms of Drought-Stress-Induced Decreases in Camellia sinensis Leaf Quality. Front. Plant Sci., https://doi.org/10.3389/fpls.2016.00385 (2016).
3. Mukhopadhyay, M., Mondal, T. K. & Chand, P. K. Biotechnological advances in tea (Camellia sinensis [L.] O. Kuntze): a review. Plant Cell Reports 35, 235–287 (2016).
4. Weng, H. et al. Tea Consumption and Risk of Bladder Cancer: A Dose-Response Meta-Analysis. Frontiers in Physiology 7, 693 (2017).
5. Fang, W. P. et al. Varietal identification of tea (Camellia sinensis) using nanofluidic array of single nucleotide polymorphism (SNP) markers. Horticulture. Research 1, 14035 (2014).
6. Chen, Y., Yu, M., Jie, X., Chen, X. & Shi, J. Differentiation of eight tea (Camellia sinensis) cultivars in China by elemental fingerprint of their leaves. Journal of the Science of Food & Agriculture 89, 2350–2355 (2009).
7. Wu, Z., Li, X. H., Liu, Z. W., Xu, Z. S. & Zhuang, J. De novo assembly and transcriptome characterization: Novel insights into catechins biosynthesis in Camellia sinensis. BMC Plant Biology 14, 277 (2014).
8. Das, A., Das, S. & Mondal, T. K. Identification of Differentially Expressed Gene Profiles in Young Roots of Tea [Camellia sinensis (L.) O. Kuntze] Subjected to Drought Stress Using Suppression Subtractive Hybridization. Plant Molecular Biology Reporter, 1088–1101 (2012).
9. Li, X. W. et al. A novel cold-regulated gene from Camellia sinensis, CsCOR1, enhances salt- and dehydration-tolerance in tobacco. Biochemical & Biophysical Research Communications 394, 354 (2010).
10. Vyas, D. & Kumar, S. Tea (Camellia sinensis (L.) O. Kuntze) clone with lower period of winter dormancy exhibits lesser cellular damage in response to low temperature. Plant Physiology & Biochemistry 43, 383–388 (2005).
11. Zhou, Y. et al. Molecular cloning and characterization of galactinol synthases in Camellia sinensis with different responses to biotic and abiotic stressors. Journal of Agricultural & Food Chemistry 65, 2753 (2017).
12. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradation analysis. BMC Plant Biology 14, 271 (2014).
13. Xin-Chao, W. et al. Global transcriptome profiles of Camellia sinensis during cold acclimation. BMC Genomics 14, 415 (2013).
14. Li, C. F. et al. Biochemical and transcriptomic analyses reveal different metabolite biosynthesis profiles among three color and developmental stages in a ‘Anji Baicha’ (Camellia sinensis). BMC Plant Biology 16, 195 (2016).
15. Li, C. F. et al. Global transcriptome and gene regulation network for secondary metabolite biosynthesis of tea plant (Camellia sinensis). BMC Genomics 16, 560 (2015).
16. Zhang, Q., Liu, M. & Ruan, J. Integrated Transcriptome and Metabolic Analyses Reveals Novel Insights into Free Amino Acid Metabolism in Huangyin Tea Cultivar. Frontiers in Plant Science 8 (2017).
17. Ji, H. G. et al. Metabolic phenotyping of various tea (Camellia sinensis) cultivars and understanding of their intrinsic metabolism. Food Chemistry 233, 321 (2017).
18. Xia, E. H. et al. The Tea Tree Genome Provides Insights into Tea Flavor and Independent Evolution of Caffeine Biosynthesis. *Molecular Plant* **10**, 866–877 (2017).

19. Yamaguchishinozaki, K. & Shinozaki, K. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology* **57**, 781–803 (2006).

20. Zhuang, J., Zhang, J., Hou, X.-L., Wang, F. & Xiong, A.-S. Transcriptomic, Proteomic, Metabolic and Functional Genomic Approaches for the Study of Abiotic Stress in Vegetable Crops. *Critical Reviews in Plant Sciences* **33**, 225–237, https://doi.org/10.1080/07352689.2014.870420 (2014).

21. Silva, D. C. D. et al. Transcriptome analyses of the DoF-like gene family in grapevine reveal its involvement in berry, flower and seed development. *Horticulture Research* **3**, 16042 (2016).

22. Slobodan, R., Diego, R. P., Ingo, D. & Bernd, M. R. PhTfD8: an integrative plant transcription factor database. *BMC Bioinformatics* **8**, 42 (2007).

23. Wu, Z. L. Transcriptome-based discovery of AP2/ERF transcription factors related to temperature stress in tea plant (*Camellia sinensis*). *Functional & Integrative Genomics* **15**, 741–752 (2015).

24. Pysh, L. D., Wysokcakldiller, J. W., Camilleri, C., Bouchez, D. & Benfey, P. N. The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the 93 text SCARECROW-LIKE genes. (1999).

25. Bolle, C. The role of GRAS proteins in plant signal transduction and development. *Planta* **218**, 683–692, https://doi.org/10.1007/s00425-004-1203-z (2004).

26. Zhang, D., Iyer, L. M. & Aravind, L. Bacterial GRAS domain proteins throw new light on gibberellic acid response mechanisms. (Oxford University Press, 2012).

27. Tian, C., Wan, P., Sun, S., Li, J. & Chen, M. Genome-Wide Analysis of the GRAS Gene Family in Rice and Arabidopsis. *Plant Molecular Biology* **54**, 519–532 (2004).

28. Silverstone, A. L., Ciampaglio, C. N. & Sun, T. The Arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant Cell* **10**, 155 (1998).

29. Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M. & Matsuoka, M. The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *Plant Cell* **14**, 57–70 (2002).

30. Lu, J., Wang, T., Xu, Z., Sun, L. & Zhang, Q. Genome-wide analysis of the GRAS gene family in *Prunus mume*. *Molecular Genetics and Genomics* **290**, 303–317 (2015).

31. Song, X. M. et al. Genome-wide analysis of the GRAS gene family in Chinese cabbage (*Brassica rapa* sp. pekinensis). *Genomics* **103**, 135–146 (2014).

32. Liu, X. & Widmer, A. Genome-wide Comparative Analysis of the GRAS Gene Family in Populus, Arabidopsis and Rice. *Plant Molecular Biology Reporter* **32**, 1129–1145 (2014).

33. Huang, W., Xian, Z., Xia, K., Tang, N. & Li, Z. Genome-wide identification, phylogeny and expression analysis of GRAS gene family in tomato. *BMC Plant Biology* **15**, 209 (2015).

34. Wei, X. et al. Genome-Wide Identification, Evolutionary Analysis, and Stress Responses of the GRAS Gene Family in Castor Beans. *International Journal of Molecular Sciences* **17**, 1004 (2016).

35. Jérôme, G., Patricia, A. R., Texeira, R. T., Martinez-Zapater, J. M. & Fortes, A. M. Structural and Functional Analysis of the GRAS Gene Family in Grapevine Indicates a Role of GRAS Proteins in the Control of Development and Stress Responses. *Frontiers in Plant Science* **7**, 353 (2016).

36. Peng, J. et al. The Arabidopsis GI1 gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes & Development* **11**, 3194 (1997).

37. Ren, C. K. & Chang, C. Arabidopsis RGL1 encodes a negative regulator of gibberellin responses. *Plant Cell* **14**, 87 (2002).

38. Li, X. et al. Control of tillering in rice. *Nature* **422**, 618 (2003).

39. Schumacher, K., Schmitt, T., Rossberg, M., Schmitz, G. & Theres, K. The Lateral suppressor (Ls) gene of tomato encodes a new member of the VHIID protein family. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 290 (1999).

40. Greb, T. et al. Molecular analysis of the LATERAL SUPPRESSOR gene in Arabidopsis reveals a conserved control mechanism for axial meristem formation. *Genes & Development* **17**, 1175–1187 (2003).

41. Di, L. L. et al. The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. *Cell* **86**, 423–433 (1996).

42. Helariutta, Y. et al. The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. *Cell* **101**, 555–567 (2000).

43. Sabatini, S., Hedrasta, R., Wildwater, M. & Scheres, B. SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. *Genes & Development* **17**, 354 (2003).

44. Bolle, C., Konec, C. & Chua, N. H. PAT1, a new member of the GRAS family, is involved in phytocrome A signal transduction. *Genes & Development* **14**, 1269–1278 (2000).

45. Torres-Galera, P., Hirtetre, B. & Bolle, C. Two GRAS proteins, SCARECROW-LIKE21 and Phytocrome A Signal Transduction1, function cooperatively in phytocrome A signal transduction. *Plant Physiology* **161**, 291–304 (2013).

46. Hirsch, S. et al. GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in Medicago truncatula. *Plant Cell* **21**, 545–557, https://doi.org/10.1105/tpc.108.064501 (2009).

47. Gu, X. Statistical methods for testing functional divergence after gene duplication. *Molecular Biology & Evolution* **16**, 1664–1674 (1999).

48. Gu, X. Maximum-likelihood approach for gene family evolution under functional divergence. *Molecular Biology & Evolution* **18**, 453–464 (2001).

49. Gu, X. & Velden, K. V. DIVERGE: phylogeny-based analysis for functional–structural divergence of a protein family. *Bioinformatics* **18**, 500–501 (2002).

50. Gu, X. A Simple Statistical Method for Estimating Type-II (Cluster-Specific) Functional Divergence of Protein Sequences. *Molecular Biology & Evolution* **23**, 1937–1945 (2006).

51. Dill, A. & Sun, T. Synergistic derepression of gibberellin signaling by removing RGA and GI1 factor in *Arabidopsis thaliana*. *Genetics* **159**, 777–785 (2001).

52. Song, A. et al. Transcriptome-Wide Identification and Expression Profiling of the DOF Transcription Factor Gene Family in *Chrysanthemum morifolium*. *Frontiers in Plant Science* **7** (2016).

53. Heo, J. O. & Estelle, M. Funnelling of gibberellin signaling by the GRAS regulator transcrarecrow-like 3 in the Arabidopsis root. *Proceedings of the National Academy of Sciences* **108**, 2160–2165 (2011).

54. Zhang, Z. L. et al. SCARECROW-Like 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in Arabidopsis. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **108**, 2160–2165, https://doi.org/10.1073/pnas.1012232108 (2011).

55. Tong, H. et al. Dwarf and Low-Tillering, a new member of the GRAS family, plays positive roles in brassinosteroid signaling in rice. *Plant Journal for Cell & Molecular Biology* **58**, 803–816 (2009).

56. Torrellas, P., Huang, L. E., Chua, N. H. & Bolle, C. The GRAS protein SCL13 is a positive regulator of phytocrome-dependent root light signaling, but can also modulate phytochrome A responses. *Molecular Genetics and Genomics* **276**, 13–30 (2006).

57. Fu, X. & Harberd, N. P. Gibberelin-mediated proteasome-dependent degradation of the barley DELLA protein SLN1 repressor. *Plant Cell* **14**, 3191–3200 (2002).
73. Saitou, H. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425 (1987).

79. Wu, Z. J. & Nagaraj, H. S. The Arabidopsis DELLA protein RGA-LIKE3 is a direct target of MYC2 and modulates jasmonate signaling responses. Plant Physiology 177, 307–319 (2018).

61. Achard, P. A., Zheng, H., Kong, L., Gao, G. & Luan, J. PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors and is essential for the activation of stress-inducible promoters. Plant Cell Reports 20, 2117 (2008).

66. Fode, B., Siemsen, T., Thurow, C., Gatz, C., Weigel, R. & Weigel, D. The Arabidopsis GRAS protein SCL7 confers salt and drought tolerance in Arabidopsis thaliana. Journal of Experimental Botany 61, 4011–4019 (2010).

51. Jin, L., Zhang, H., Kong, L., Gao, G. & Luan, J. PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors and is essential for the activation of stress-inducible promoters. Plant Cell Reports 20, 2117 (2008).

Acknowledgements
The research was supported by the National Natural Science Foundation of China (31570691).

Author Contributions
Conceived and designed the experiments: J.Z. Y.X.W. Performed the experiments: Y.X.W., Z.W.L., Z.J.W., H.L., W.L.W., X.C., J.Z. Analyzed the data: Y.X.W. Contributed reagents/materials/analysis tools: J.Z. Wrote the paper: Y.X.W. Revised the paper: J.Z., Y.X.W. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-22275-z.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018