Comparative transcriptome and weighted correlation network analyses reveal candidate genes involved in chlorogenic acid biosynthesis in sweet potato

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Chlorogenic acids (CGAs) are important secondary metabolites produced in sweet potato. However, the mechanisms of their biosynthesis and regulation remain unclear. To identify potential genes involved in CGA biosynthesis, analysis of the dynamic changes in CGA components and RNA sequencing were performed on young leaves (YL), mature leaves (ML), young stems (YS), mature stems (MS) and storage roots (SR). Accordingly, we found that the accumulation of six CGA components varied among the different tissues and developmental stages, with YS and YL recording the highest levels, while SR exhibited low levels. Moreover, the transcriptome analysis yielded 59,287 unigenes, 3,767 of which were related to secondary-metabolite pathways. The differentially expressed genes (DEGs) were identified based on CGA content levels by comparing the different samples, including ML vs. YL, MS vs. YS, SR vs. YL and SR vs. YS. A total of 501 common DEGs were identified, and these were mainly implicated in the secondary metabolites biosynthesis. Additionally, eight co-expressed gene modules were identified following weighted gene co-expression network analysis, while genes in darkgrey module were highly associated with CGA accumulation. Darkgrey module analysis revealed that 12 unigenes encoding crucial enzymes (PAL, 4CL, C4H, C3H and HCT/HQT) and 42 unigenes encoding transcription factors (MYB, bHLH, WD40, WRKY, ERF, MADS, GARS, bZIP and zinc finger protein) had similar expression patterns with change trends of CGAs, suggesting their potential roles in CGA metabolism. Our findings provide new insights into the biosynthesis and regulatory mechanisms of CGA pathway, and will inform future efforts to build a genetically improved sweet potato through the breeding of high CGA content varieties.

Chlorogenic acids (CGAs) are phenolic compounds widely found in plants with a broad spectrum of biological activities, such as anti-inflammatory, antioxidant activity, antibacterial, anti-obesity, antiviral, antidiabetic, anti-microbial, hypolipidemic and anti-hypertension1-3. Three biosynthetic routes have been proposed for CGA production in plants4. The first pathway involves the caffeic acid coenzyme A and quinic acid catalysis by hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) to generate CGA5. In contrast, the second route entails generating CQAs with caffeoyl glucoside as an activated intermediate catalyzed by hydroxyl cinnamoyl D-glucose:quinate hydroxycinnamoyl transferase (HCGQT)6. Third, CGA is biosynthesised from p-coumaroyl quinic acid and catalyzed by hydroxyl cinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT) and p-coumarate 3'-hydroxylase (C3H)7. Notably, both HQT and HCT enzymes are members of plant-specific acyl-CoA-dependent acyltransferases BAHD superfamily8,9. Among the three distinct pathways, the HQT-mediated pathway is considered the principal route for CGA biosynthesis1-9. Moreover, various studies have confirmed that overexpression or suppression of HQT in many plants leads to significant changes in CGA levels in most

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plants. However, the specific mechanisms involved in CGA biosynthesis regulation in plants are still not well understood.

Sweet potato [Ipomoea batatas (L.) Lam.] is one of the most important crops globally mainly utilized as food, feed and industrial raw material. Several studies have reported that sweet potatoes contain many phenolic compounds, such as CGAs. These compounds have multiple physiological and health functions, including conferring the resistance against pests and diseases. CGA accumulation varies in different tissues and development stages of sweet potatoes. Therefore, it is critical to understand CGA biosynthesis patterns during development and their regulation at the transcriptional level. However, the underlying molecular basis and regulation of CGA biosynthesis in sweet potato remain unclear. In the present study, sweet potato CGA composition and content were investigated in different tissues and development stages, including young leaves (YL), mature leaves (ML), young stems (YS), mature stems (MS) and storage roots (SR). Additionally, RNA sequencing, differentially expressed gene (DEG) analysis and weighted gene co-expression correlation network analysis (WGCNA) were performed to examine and identify CGA biosynthesis-associated genes. This study provides valuable information for future research on understanding the mechanism of CGA metabolism pathway in sweet potatoes.

Results

Chlorogenic acid content and composition analysis in sweet potato tissues. Six CGAs in different tissues and developmental stages were quantified and analyzed by HPLC to evaluate the dynamic accumulation of CGA in sweet potato (Fig. 1A). All the six CGAs were detected at distinct levels in different tissues and developmental stages (Fig. 1B). Concomitantly, 5-O-caffeoylquinic acid (5-CQA) accumulation was highest in YL with a maximum of ~ 2.62 mg/g dry weight, while the remaining CQAs, including 3-cafeoylquinic acid (3-CQA), 4-cafeoylquinic acid (4-CQA), 3,4-di-CQA, 3,5-diCQA, 3,5-diCQA and 4,5-diCQA and total CGA (the total sum of six CGAs) in different sweet potato tissues.

Transcriptome sequencing and assembly. Comparative transcriptome analysis of the YL, YS, ML, MS, and SR was performed in triplicates through RNA sequencing to understand the molecular mechanism of CGA biosynthesis in sweet potato and identify the associated genes. After eliminating the adapter and low-quality reads, a total of 99.92 Gb clean data was obtained from 15 sample, and each sample yielded up to 5.73 Gb of the data. The GC content of the 15 samples was 46.32 to 48.25%, with an average of 46.99%, and the Q30 base percentage in each sample was more than 92.12%. The mapped ratio of each sample ranged from 73.45% to 77.24% (Supplementary Table S1). The assembly yielded 167,860 transcripts with an average length and N50 value of 1,340 bp and 1,889 bp, respectively. After the processing by Trinity and TGICL software, 167,860 transcripts were further assembled into 59,287 unigenes, with an average length of 1,067 bp and an N50 value of 1,720 bp (Table 1).

Functional annotation and KEGG characterization. A total of 40,781 unigenes (68.78%) were annotated by searching against public databases. Further, the unigenes matched to NR, COG, GO, KEGG, KOG,
Identification of differentially expressed genes (DEGs). The gene expression levels were evaluated using fragments per kilobase of exon per million fragments mapped (FPKM) values. Furthermore, correlation analysis revealed that the 15 samples could be divided into five groups and that the replicates had a strong positive correlation ($r > 0.89$), suggesting high reproducibility and reliability of the transcriptome data (Fig. 2A and Supplementary Table S4). The sample transcriptome data exhibiting noticeable differences in CGA accumulation were compared to identify candidate genes involved in CGA accumulation in sweet potato. In total, 10,458 DEGs were obtained by comparing the five samples in pairs. For the pairs, ML vs. YL, MS vs. YS, SR vs. YL and SR vs. YS, the differentially expressed unigenes were 2,518, 4,920, 5,731 and 4,440, respectively (Fig. 2B). Specifically, the genes were up-regulated more than they were down-regulated. Additionally, 501 common unigenes were obtained by comparing the five samples in pairs. Functional GO and KEGG enrichment analyses were performed to evaluate the functional categories of the 501 common DEGs. Functional GO analysis showed that 314 common DEGs were categorized into 31 functional groups, and these were further divided into three categories: biological process, cellular component and molecular function (Supplementary Fig. S2 and Supplementary Table S6). For the biological processes classification, the most abundant groups were metabolic processes (181), cellular processes (147), and single-organism processes (136). The most abundant groups within the molecular function category were catalytic activity (197) and binding (121). Finally, the highest categories were membrane (138), followed by cell (155) and cell part (155) in the cellular components category. Meanwhile, KEGG enrichment results showed that common DEGs were mainly enriched in phenylpropanoid biosynthesis, flavonoid biosynthesis and phenylalanine metabolism (Supplementary Fig. S3 and Supplementary Table S7), indicating that these metabolic pathways might be highly correlated to CGA accumulation in sweet potato.

Candidate genes involved in the chlorogenic acid biosynthesis pathway. CGA biosynthesis has been proposed to occur via three alternative routes (Fig. 3A). A total of 56 potential genes, including eight PAL, twenty-five 4CL, thirteen C3H, thirteen HCT/HQT, four C4H, and three UGCT encoding putative enzymes involved in the CGA biosynthesis, were identified based on transcriptome data (Table 2 and Supplementary Table S8). However, no gene encoding HCGQT in the second route was detected in transcriptome data. Meanwhile, differential expression analysis showed that 28 unigenes were identified as DEGs (Fig. 3B and Supplementary Table S8). Among the DEGs, six PAL (c82584.graph_c0, c91820.graph_c1, c103655.graph_c0, c92674.graph_c0, c104837.graph_c0 and c91820.graph_c0), two 4CL (c100009.graph_c0 and c102274.graph_c0), two C4H (c98593.graph_c0 and c96439.graph_c0), one C3H (c94695.graph_c0) and two HCT/HQT (c90935.graph_c0, c95351.graph_c0) showed high expression levels in YL and YS, and relatively low expressed levels in SR. These findings were consistent with CGA accumulation (Fig. 3B), suggesting the probable implication of the 28 unigenes in the CGA biosynthesis. Interestingly, there was no correlation between the expression of UGCT genes and CGA accumulation.

Molecular and expression characterization of HCT/HQT. Considering the important roles of HCT/HQT in CGA biosynthesis, molecular characterization and expression of HCT/HQT genes were further inves-
Three out of thirteen unigenes were predicted as complete full-length ORFs, and the other partial unigenes were further blasted against the genome databases to obtain full-length sequences. Phylogenetic analysis showed that all characterized BAHD proteins were divided into five distinct clades (I–V) and HCT/HQT proteins belonged to group V (Fig. 4A), which was similar to results reported previously8. Four sequences (c85446.graph_c0, c125397.graph_c0, c97205.graph_c0 and c84565.graph_c0) belonged to clade IIIa and three sequences belonged to clade IIIb (c98234.graph_c1, c76240.graph_c0 and c100061.graph_c1), which were involved in the modification of alkaloid compounds and volatile ester biosynthesis8. Sequences of c90935.graph_c0 (IbHQT1) and c95351.graph_c0 (IbHQT2) clustered together with HQTs in clade Vb, while c99097.graph_c0 (IbHCT1) assembled with HCTs in clade Vb. Sequences of c99097.graph_c0 in clade Vb had a close evolutionary relationship with AtSHT in *Arabidopsis thaliana* that characterized as a spermidine hydroxycinnamoyl transferase22. Multiple alignments of the deduced amino acid sequences indicated that IbHQT1, IbHQT2 and IbHCT1 had typical structural characteristics of HCT/HQT proteins, exhibiting the conserved HXXXD and DFGWG motifs (Fig. 4B). Therefore, IbHQT1, IbHQT2, and IbHCT1 are postulated to be the HQT/HCT genes in sweet potatoes.

To investigate the correlation between the CQA content and HQT/HCT expression levels, we measured the expression profiles of IbHQT1, IbHQT2 and IbHCT1 using qRT-PCR. The results indicated that the IbHQT1 transcript levels in different tissues and developmental stages were significantly higher than those of IbHQT2 and IbHCT1 (Fig. 4C), and showed a significant correlation with CQA content, probably enhancing CQA biosynthesis.

**Candidate transcription factors involved in chlorogenic acid biosynthesis.** Plant phenylpropanoid metabolism is often regulated by transcription factors targeting the structural genes encoding enzymes. A gene co-expression network was constructed by WGCNA and compared with CQA contents in different tissues and developmental stages to screen the candidate transcription factors regulating CQA accumulation in sweet potato. In total, 9,408 DEGs were divided into eight distinct co-expression modules based on the similarity of expression profiles (Fig. 5A). Among the modules, the turquoise module was found to be highly positively related to 5-CQA accumulation (0.95); while the correlation coefficient of the darkgrey module and that of with
the other CGA components, such as 3-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and total CQA was highest, which were 0.96, 0.77, 0.85, 0.91, 0.86 and 0.94, respectively (Fig. 5B). This finding suggested that the darkgrey module was highly correlated with the CGA accumulation in sweet potatoes. Further analysis showed that darkgrey module contains 12 genes required for the CQA biosynthesis pathway. The genes included six PALs (c82584.graph_c0, c91820.graph_c1, c103655.graph_c0, c92674.graph_c0, c104837.graph_c0, and c91820.graph_c0), one 4CL (c102274.graph_c0), two C4Hs (c98593.graph_c0 and c96439.graph_c0), one C3H (c94695.graph_c0) and one HCT/HQT (c90935.graph_c0), further substantiating the positive correlation between the darkgrey module and CQA biosynthesis. Among the 730 genes of darkgrey module, 42 were regulatory transcription factors, including nine MYBs, eleven bHLHs, seven zinc finger proteins (ZFPs), six bZIPs, three ERFs, two GARSs, two MADs, one WRKY and one WD40 (Supplementary Table S10). These

**Figure 3.** Expression of CGA biosynthesis pathway structural genes in sweet potato. (A) The three proposed CGA biosynthesis routes in sweet potato marked I, II and III. The genes involved in CGA biosynthesis including L-phenylalanine aminometylase (PAL), 4-coumarate:CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), P-coumarate 3-hydroxylase (C3H), Shikimate O-hydroxycinnamoyltransferase (HQT/HCT), UDP-glucose: cinnamate glucosyltransferase (UGCT) and hydroxyl cinnamoyl D-glucose:quinate hydroxycinnamoyl transferase (HCGQT). (B) Analysis of CQA-associated DEGs in different sweet potato tissues. The expression pattern of each gene is shown in heat map based on Log2 of FPKM values. Heat maps were generated using the MultiExperiment Viewer software (MeV v4.9.0, http://www.tm4.org/).

**Table 2.** The numbers of unigene involved in CGA biosynthesis.

| Gene                                      | EC number  | Numbers |
|-------------------------------------------|------------|---------|
| L-phenylalanine aminometylase (PAL)       | EC:4.3.1.24| 8       |
| 4-Coumarate:CoA ligase (4CL)              | EC:6.2.1.12| 25      |
| Cinnamate 4-hydroxylase (C4H)             | EC:1.14.13.11| 3      |
| Shikimate O-hydroxycinnamoyltransferase (HQT/HCT) | EC:2.3.1.133 | 13  |
| P-coumarate 3-hydroxylase (C3H)           | EC:1.14.13.36| 4      |
| UDP-glucose: cinnamate glucosyltransferase (UGCT) | EC 2.4.1.177 | 3      |
| **Total**                                 |            | **56**  |
transcription factors showed similar expression patterns with those of the genes encoding CQA biosynthesis enzymes (Fig. 5C), suggesting they may participate in regulating CGA biosynthesis in sweet potato.

Validation of DEGs via qRT-PCR Analysis. Ten DEGs associated with CGA biosynthesis were chosen for qRT-PCR assay to validate the reliability of gene expression data obtained from RNA-seq. As a result, all the 10 genes exhibited a similar pattern as those displayed by the FPKM values (R^2 > 0.9) (Fig. 6). Therefore, the RNA-Seq results might provide reliable data for further research on CGA biosynthesis and its regulation in sweet potato.

Discussion
Chlorogenic acids (CGAs) are widely distributed in sweet potato, contributing to biotic and abiotic stress resistance and nutritional benefits. To date, most studies on sweet potato CGA mainly focus on extraction methods, and functional activities. However, very little is known about CGA biosynthesis mechanisms and their regulation in sweet potatoes. Although CGAs have been reported in sweet potatoes, previous studies have not quantified and characterized CGA content in different tissues and different developmental stages. In this study, CGAs were found to be most abundant in young leaves and young stems, whereas much lower levels were detected in storage roots. Therefore, this study attempted to determine the potential genes responsible for CGA biosynthesis through comparative transcriptome combined with CGA content analyses in different tissues and developmental stages.

Three possible routes have been reported for the CGA biosynthesis (Fig. 2A). Putative genes encoding the key enzymes associated with the first and third routes of CGA biosynthesis were identified in the assembly. Phenylalanine ammonia-lyase (PAL), the first rate-limiting enzyme of the phenylpropanoid metabolism, has been...
demonstrated to play a crucial role in CGA synthesis. Furthermore, we found that all the six differentially expressed \( \text{PAL} \) genes exhibited similar expression patterns that positively correlated changes in with CGA levels, suggesting an essential role of \( \text{PAL} \) in CGA accumulation. Recently, it has been confirmed that \( \text{IbPAL1} \) overexpression in sweet potato significantly enhanced CGA accumulation. C4H and 4CL (the early enzymes of phenylpropanoid metabolism) in CGA biosynthesis are yet to be understood. However, our findings showed that two \( \text{C4H} \) genes (c98593.graph_c0 and c96439.graph_c0) and one 4CL (c102274.graph_c0) exhibited a similar expression pattern with CGA concentrations, implying that they may be involved in CGA biosynthesis. The function of \( \text{C3H} \) in CGA biosynthesis has been determined by enzyme assays and gene overexpression in various plants. Similarly, this study, expression of a \( \text{C3H} \) gene (c94695.graph_c0) was correlated with CQA content, which may be involved in CGA biosynthesis. Additionally, \( \text{HQT/HCTs} \) have been shown to play vital roles in CGA biosynthesis. In the present study, three out of 13 potential \( \text{HQT/HCTs} \) sequences (HbHQT1, HbHQT2 and HbHCT1) presented HXXXD and DFGWG conserved motifs, and were identified as \( \text{HCT/HQT} \) proteins. Among the three \( \text{HCT/HQT} \) genes, \( \text{HbHQT1} \) exhibited relatively high abundance in different tissues and developmental stages than \( \text{HbHQT2} \) and \( \text{HbHCT1} \). Moreover, \( \text{HbHQT1} \) expression significantly correlated with

Figure 5. Gene co-expression networks associated with CGA metabolites generated by WGCNA. (A) Dendrogram showing modules identified by the weighted gene co-expression network analysis (WGCNA) and expressed genes clustering dendrogram. (B) Module-CGA weight correlations and corresponding \( P \)-values. The left panel shows the eight modules, while the right panel shows positive (red, 1) and negative (blue, −1) correlations. Values close to −1 or 1 indicate a strong positive or negative linear relationship, while values close to 0 indicate weak correlation. (C) Heat map for the expression of transcription factor genes in the darkgrey module based on Log2 of FPKM values. Heat maps were generated using the MultiExperiment Viewer software (MeV v4.9.0, http://www.tm4.org/).
CQA content, suggesting its implication in CQA biosynthesis. Although the three genes encoding UDP-glucose: cinnamate glucosyltransferase (UGCT) in the second route of CQA biosynthesis were identified, their expression did not correlate with CQA levels in different tissues and developmental stages of sweet potato. HCGQT, catalyzing caffeoyl-D-glucose and quinic acid to form CQA, was postulated to be the key enzyme of the second CQA biosynthesis pathway. However, no corresponding HCGQT gene has been identified in plants to date. In addition, chlorogenic acid: glucaric acid caffeoyltransferase (CQT) in tomato was shown to catalyze the transfer of caffeic acid from CQA to glucaric and galactaric acids, indicating a possible CQA recycling route in plants. Thus, the first and third routes may be the main CQA biosynthesis pathways in sweet potato. Nonetheless, more research is needed to understand the significance of the second CQA biosynthesis route.

Definite roles of the transcription factors in phenylpropanoid biosynthetic pathway regulation have been well established; however, less is known about the transcription factors regulating CQA biosynthesis. Previous works have shown that the transcription factors involved in flavonoids biosynthesis might also regulate CQA biosynthesis. Following the DEG and WGCNA analyses, we identified 42 transcription factor genes from nine families (MYB, bHLH, WD40, WRKY, ERF, GRAS, MADS, ZFP and bZIP) in the darkgrey module that might be involved in CQA biosynthesis (Supplementary Table 10 and Fig. 5C). These genes had a similar expression pattern across the different tissues and developmental stages and were co-expressed with CQA biosynthesis-related structural genes. Among the genes, c89342.graph_c0 was homologous to the Arabidopsis AtPAP1/AtMYB75 gene encoding an MYB transcription factor implicated in anthocyanin biosynthesis. In addition, overexpression of AtPAP1/AtMYB75 in Platycodon grandiflorum and Leonurus Sibiricus can increase CQA content. Conversely, c93698.graph_c0 showed high similarity to the Arabidopsis AtMYB12, which was identified as a flavonol-specific transcriptional activator in Arabidopsis. Arabidopsis gene AtMYB12 also regulated CQA biosynthesis in tomato. Moreover, bHLH (c104813.graph_c0, encoding GLABRA 3) and bZIP (c95202.graph_c3, encoding HY5) genes were detected by darkgrey module, their homologous were considered to be key transcription factors controlling anthocyanin biosynthesis. In addition, LjbZIP8 could act as a transcriptional repressor in regulating PAL2 expression and CQA content in Lonicera japonica. Recent studies have revealed that the WRKY transcription factors acting as HCT2 activators in poplar might also play an important role in CQA biosynthesis. These studies showed the importance of transcription factors in regulating CQA biosynthesis. Although no reports on CQA biosynthesis regulation by WD40, ERF, GRAS, MADS and ZFP transcription factors have been published so far, these transcription factors have been reported to regulate other phenylpropanoids such as flavonoids and lignins, suggesting their possible participation in CQA biosynthesis regulation. Therefore, understanding the specific functions of the 42 transcription factors in regulating sweet potato CQA biosynthesis need further studies.

In summary, comparative transcriptome and CQA changes in different sweet potato tissues and developmental stages were systematically investigated. The CQA accumulation varied among the tissues and developmental stages, and was abundant in YL and YS. Moreover, 59,287 unigenes were obtained, 3,767 of which were involved in secondary metabolism pathways. A darkgrey module associated with CQA accumulation was identified by differential expression analysis and WGCNA. In this module, 12 unigenes encoding crucial enzymes (PAL, 4CL, C4H, C3H and HCT/HQT) and 42 unigenes encoding transcription factors (MYB, bHLH, WD40, WRKY, ERF, MADS, GARS, bZIP and zinc finger protein) in darkgrey module may play essential roles in CQA biosynthesis. These results provide a basis for future research on the biosynthesis and regulation of the CQA metabolism pathway in sweet potatoes.
Methods

Plant materials. Sweet potatoes (QS80-12-11) used in this study were grown on a plantation in Hainan Academy of Agricultural Sciences, Hainan, China. The samples from different tissues and developmental stages (young leaves, mature leaves, young stems, mature stems, and storage roots) were collected at 90 days after planting for RNA extraction and CGA content analysis (Fig. 1A). Each sample was collected from at least three individual plants, and all experiments were conducted in triplicate. A portion of the samples was immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. The other samples portion was dried in a blast drier (ZHICHENG, Shanghai, China) at 60 °C and ground for CGA measurement analysis.

Estimation of chlorogenic acid content. The ground samples (0.2 g) were mixed with 1.5 ml of ethanol (70%, v/v) and then incubated in a water bath at 60 °C for 1 h. The mixture was then centrifuged at 5,000 g rpm for 10 min. Thereafter, the supernatant liquid was blown dry using nitrogen, re-dissolved in 1 ml methanol, and then filtered through 0.22 μm membrane filter for CGA analysis. Additionally, HPLC analysis was performed using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) with a Waters Acquity HSS T3 column (2.1 × 100 mm, 1.8 μm) and a mixture of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% aqueous solution of formic acid) as the mobile phase. The column oven temperature was maintained at 40 °C, and the flow rate was set at 0.3 ml/min. Typically, the linear gradient elution was programmed as follows: 10% A from 0 to 2 min; 10% to 60% A from 2 to 10 min; holding at 60% A from 10 to 15 min; 60% to 10% A from 15 to 15.1 min; holding at 10% A from 15.1 to 20 min. The eluting compounds were detected by monitoring at 326 nm. Subsequently, the CGA compounds were identified by comparing with the standard reagents, including 3-cafeoylquinic acid (3-CQA), 4-caeoylquinicacid (4-CQA), 5-O-caffeooylquinicacid (5-CQA), 3, 4-di-O-cafeoylquinic acid (3, 4-diCQA), 3, 5-di-O-cafeoylquinic acid (3, 5-diCQA) and 4, 5-di-O-cafeoylquinic acid (4, 5-diCQA) (Sigma, St. Louis, MO, USA).

RNA-Seq and gene co-expression analysis. Total RNA was extracted using RNAprep pure Plant Kit (Tiangen, Beijing, China) and subsequently used for cDNA library construction according to the Illumina manufacturer’s instructions. Furthermore, transcriptome sequencing was performed on the Illumina HiSeqTM 4000 platform at Biomarker Technologies Corporation (Beijing, China). Clean reads were obtained by eliminating the reads containing sequencing adapters, poly-N, and low-quality reads using SeqPrep (https://github.com/jstjohn/SeqPrep). De novo assembly of high-quality clean reads was performed using Trinity v2.5.1 (http://trinityrnaseq.sourceforge.net/)59, and then further processed with TGICL software to generate unigenes60. The BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) was employed for functional annotation of the unigenes against eight databases. These databases included NCBI non-redundant protein (NR) (http://www.ncbi.nlm.nih.gov)51, Gene Ontology (GO) (http://www.geneontology.org/)52, eukaryotic Orthologous Groups (KOG) (http://www.ncbi.nlm.nih.gov/COG/new/shokog.cgi)53, eggno g (http://eggno g5.embl.de/#/ app/home)54, Protein family (Pfam) (http://www.sanger.ac.uk/Software/Pfam/)55, Swiss-Prot protein (http://www.expasy.ch/sprot)56, Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg)57 and Clusters of Orthologous Groups of proteins (COG) (http://www.ncbi.nlm.nih.gov/COG)58. In addition, the gene expression level was calculated and normalized to fragments per kilobases of transcript per million fragments mapped (FPKM) values. Differentially expressed genes (DEGs) from different samples were identified by the DESeq2 software (version 1.12.4) based on the criteria of log2 [Fold Change]> 2, FDR < 0.001 and FPKM values > 2. Nonetheless, co-expression analysis was performed using the “Weighted Correlation Network Analysis (WGCNA)” package in R60. The data used in WGCNA analysis were components of the six CGA types and total CGA (sum of the six CGA components) compared with all RNA-seq genes.

Bioinformatics analysis of HCT/HQT proteins. The open reading frames (ORFs) of HQT/HCT genes were predicted using the ORF finder (http://www.ncbi.nlm.nih.gov/), and full length of HCT/HQT unigenes with incomplete ORFs were obtained by scanning the unigenes against the sweet potato genome databases. All candidate HCT/HQT sequences were confirmed by Pfam (http://pfam.sanger.ac.uk/) and NCBI conserved domains database (CDD) (http://www.ncbi.nlm.nih.gov/cdd) databases. Eventually, a phylogenetic tree was established by MEGÀ 9.0 and Clustal X2.0 based on the candidate HCT/HQT protein sequences from sweet potato and other plants using the Neighbor-Joining method with 1000 bootstrap replicates.

qRT-PCR analysis. Quantitative real-time PCR (qRT-PCR) was employed to validate the reliability of gene expression using the RNA-seq data. The assay was performed in a Real-Time System Thermocycler using FastFire qPCR PreMix (Tiangen, Beijing, China). The conditions of the qRT-PCR amplification were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles for 10 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C. All reactions were performed in triplicates with specific primers (Supplementary Table S11), and β-actin was used as the reference gene. The relative gene expression level was calculated using the delta-delta Ct (2-ΔΔCt) method61.

Statement on plant guidelines. Collection of plant material complies with relevant institutional, national, and international guidelines and legislation.

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Author contributions
J.X., J.H.Z. and X.N.W. conceived and designed the experiments. J.X., J.H.Z., Y.H.L., H.L.Z., L.Q.T. and X.H.W. performed the experiments. J.X., J.H.Z. and X.N.W. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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