Genome-Wide Analysis of the *CCT* Gene Family in Chinese White Pear (*Pyrus Bretschneideri* Rehd.) and Characterization of *PbPRR2* in Response to Varying Light Signals

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Abstract

Background:

Canopy architecture is critical in determining the light environment, and subsequently the photosynthetic productivity of fruit crops. Numerous CCT domain-containing genes are crucial for plant adaptive responses to diverse environmental cues. Due to the biological importance of *CCT* genes, many researchers have focused on their functional characterization. However, little information was available about the *CCT* genes (*PbCCTs*) of pear, an important fruit crop.

Results:

Genome-wide sequence analysis identified 42 putative *PbCCTs* in the genome of pear (*Pyrus bretschneideri* Rehd.). Phylogenetic analysis indicated these genes were divided into five subfamilies, namely, COL (14 members), PRR (8 members), ZIM (6 members), TCR1 (6 members) and ASML2 (8 members). Analysis of exon-intron structures and conserved domains provided support for the classification. Genome duplication analysis indicated that segmental duplication events played a crucial role in the expansion of the CCT family in pear, and that the CCT family evolved under the effect of purifying selection. Expression profiles exhibited diverse expression patterns of *PbCCTs* in various tissues and in response to varying red and blue light. Additionally, transient overexpression of *PbPRR2* in *Nicotiana benthamiana* leaves resulted in inhibition of photosynthetic performance, suggesting that *PbPRR2* may be a negative regulator of photosynthesis.

Conclusions:

This study provides a comprehensive analysis of the *CCT* gene family in pear and will facilitate further functional investigations of the *PbCCTs* to uncover their biological roles in light response.

Background

CCT [CONSTANS (CO), CONSTANS-LIKE (COL) and TIMING OF CAB1 (TOC1)] transcription factors, which encode proteins with a conserved motif (CCT domain) of ~ 43 amino acids residues towards their carboxy-terminus, constitute one of the plant-specific families [1,2]. The CCT domain has important functions in nuclear localization, protein-protein interactions, transcriptional regulation and nuclear protein transport [3–5]. CCT family genes can be divided into five categories based on the sequencing information of *Arabidopsis*: COL, PRR (Pseudo-Response Regulator), ZIM (Zinc-finger Protein Expressed in Inflorescence Meristem), TCR1 (Tunicamycin-induced COL-Related 1) and ASML2 (Activator of Spo^{min}::LUC2) subfamilies [6,7]. The CCT family has been comprehensively analyzed in some plants, including *Arabidopsis*, rice, maize, brachypodium, sorghum, foxtail millet, barley, *Aegilops tauschii* and *Medicago truncatula* [7–11], but not yet in woody perennial fruit crops.

It is well known that CCT family members play diverse and important roles in flowering, circadian rhythms, development and abiotic stress tolerance [12]. The COL subfamily has been extensively studied and can be further subdivided into three smaller groups, according to the degree of conservation and number of B-box domains [13–15]. In addition to the CCT domain, COL proteins contain one or two zinc-finger B-box domains towards the amino terminus, thought to be involved in protein-protein interactions [16,17]. The first cloned CCT family gene in *Arabidopsis* is *AtCO* that required to promote photoperiodic flowering, at least partly by activating the expression of *AtFT* (*Flower Time*) and *AtSOC1* (*Suppressor of Overexpression of CO1*) genes [18–20]. Other CO homologs (*COLs*) also have been associated with abiotic stress tolerance, plant growth, development and metabolic processes, apart from playing a key role in the photoperiodic flowering induction [21–26]. PRR subfamily genes contain a Pseudo-receiver domain towards the amino-terminus, as well as a CCT domain at the carboxy-terminus [26]. They are key genetic components of the interconnected transcriptional-translational feedback loops, regulating circadian clock-output pathways [26–29]. In *Arabidopsis*, all PRR genes (*AtPRR1*, *AtPRR3*, *AtPRR5*, *AtPRR7*, and *AtPRR9*) which have redundant functions directly regulates the expression of genes implicated in abiotic responses, cell elongation, and photoperiodic flowering response [26–28,30]. In addition to the CCT domain, ZIM subfamily contains both a C2C2-GATA zinc-finger domain and a TIFY domain [31,32]. ZIM subfamily genes, including *ZIM* and *ZIM-like* (*ZML*) genes, are involved in hypocotyls and petiole elongation, cryptochrome 1-dependent response to excess light and wound-induced lignification [33–35]. TCR1 and ASML2 subfamilies represent two distinct classes of CCT family and both encode proteins possessing just a single CCT domain [7,36]. The function of TCR1 and ASML2 subfamily members are generally not well understood. *AtTCR1* gene has been shown to be transcriptionally induced by *Arabidopsis* endoplasmic reticulum stress [7]. Overexpression of *AtASML2* resulted in enhanced expression of a subset of sugar-inducible genes in *Arabidopsis* [36].

As sessile organisms, plants need depend on their ability to adapt to the complex changes of surrounding environment [37,38]. As a consequence, they have evolved power mechanisms that integrate environmental cues such as light signaling (light quality, light intensity and photoperiod), and their endogenous regulators to optimize their growth and productivity [30,39]. Pear is one of the most important economic fruit crops in the world. In the field, canopy architecture of pear is critical in determining the light environment, and thereby indirectly affects source-sink relationship [40]. To understand the potential mechanism of pear photosynthetic variability responses with heterogeneous light environment within canopies, we have conducted physiological and transcriptomic surveys to capture progressive stages of photosynthetic differentiation between distinct canopy structures [41]. We found that the two orthologs (LOC103943360 and LOC103951583) of *AtPRR5* in pear were hub genes of the module positive correlated with pear photosynthetic rate, and may play key roles in photosynthetic performance under distinct canopy microclimate. There are increasing evidences that CCT genes are associated with light responses and/or photosynthetic capacity. *Ghd7*, the homolog of CCT gene in rice, is a major locus responsible for natural variation of chlorophyll content at heading stage [42]. Another CCT gene in rice, *CRCT* (CO₂ Responsive CCT Protein), controlled the capacity for starch synthesis, which can indirectly affect photosynthetic rate under elevated CO₂ conditions [43,44]. In *Arabidopsis*, *AtBBX4* is

a CCT domain protein whose abundance positively modulated by phyB in red light, thereby promoting photomorphogenic development [45]. Considering the potential value of *CCT* genes in light signal response and photosynthesis, we identified *CCT* genes (*PbCCTs*) in pear (*Pyrus bretschneideri* Rehd.) and analyzed the chromosomal locations, phylogenetic relationships and gene structure, as well as expression patterns of some members in various tissues and in response to different light signals. In addition, we performed functional analysis of one key *CCT* member, *PbPRR2*, in transgenic *Nicotiana benthamiana* plants. This comprehensive study of the CCT family may provide valuable information for the further research and utilization of the *PbCCTs*, helping to enhance our understanding of possible roles of *PbCCTs* in the adaptation of pear to changing ambient light signaling.

Methods

Genome-wide identification of CCT family genes

The Hidden Markov Model profile of the CCT domain (PF06203) downloaded from the Pfam database (<http://pfam.xfam.org/>) was used for identification of the *PbCCTs* from the downloaded database of the Pear Genome Project (<http://peargenome.njau.edu.cn/>) using HMMER program (version 3.1b2) with a threshold of $e\text{-value} < 10e^{-10}$ [46]. Using the same criterion, CCT family sequences from *Arabidopsis thaliana* and *Oryza sativa* were obtained from genome databases (ftp://ftp.ensemblgenomes.org/pub/plants/release-38/fasta/arabidopsis_thaliana/, ftp://ftp.ensemblgenomes.org/pub/plants/release-38/fasta/oryza_sativa/). To confirm the reliability of searched results, the obtained sequences were further examined based on the presence of conserved domain of CCT proteins using the InterProScan software package (version 5.25-64.0). Finally, a self-blast of protein sequences was performed to remove redundancy. Alternative splice variants were not considered. Any two protein sequences which showed a perfect match were deemed to be redundant gene pairs and the shorter sequence was removed from the potential *PbCCTs* list.

Analysis of chromosomal locations, synteny relationships, protein properties, gene structure and conserved motifs

The chromosome locations of *PbCCTs* were determined according to genome annotation data, and then plotted using MapChart software (<https://www.wur.nl/en/show/Mapchart.htm>). To detect gene duplication events, the Multiple Collinearity Scan toolkit (MCScan X) was applied [47]. The synteny relationship of the orthologous *CCT* genes between pear and *Arabidopsis* as well as between pear and rice was determined by using Dual Synteny Plotter software (<https://github.com/CJ-Chen/TBtools>). The KaKs_Calculator 2.0 was used to determine non-synonymous (Ka) and synonymous (Ks) substitution [48].

The molecular weights and theoretical isoelectric points of the *PbCCT* proteins were predicted by the compute pI/Mw tool in the ExPASy server (https://web.expasy.org/compute_pi/). The exon-intron structures of the *PbCCTs* were identified with the Gene Structure Display Server (GSDS,

<http://gsds.cbi.pku.edu.cn/>) program by the alignment of cDNA sequences with the corresponding genomic sequences. The InterProScan program (<http://www.ebi.ac.uk/interpro/>) was used to characterize the domains and motifs of the pear CCT family.

Phylogenetic analysis

To investigate the phylogenetic relationship between pear and *Arabidopsis*, an unrooted neighbor-joining (NJ) trees was constructed by MEGA7 software with 1000 bootstrap replicates [49]. The numbers generated for each clade represent the bootstrap support values expressed as percentages. The same method was adopted to construct the NJ phylogenetic tree for the five subfamilies of pear CCT family.

Plant materials, growth conditions and treatments

To investigate the effects of light quality on *PbCCTs* gene expression, one-year-old grafted seedlings of 'Wonhwang' (*P. pyrifolia* Nakai cv. 'Wonhwang') pear cultivar were obtained from the experimental orchard (30.292°N, 114.143°E) of Research Institute of Fruit and Tea. Uniform and healthy plants were transplanted into plastic pots and cultured in an intelligent growth chamber maintained at 25°C and 70% relative humidity (RH). The commercially available light source used in this study was cool-red (R)/blue (B)/green (G) light-emitting diode (LED) panels. The distance between the lamps and pear leaves was 20 cm. For the R light gradient treatments, fixed B light (1800 lx) and G light (5000 lx) intensities were also provided. The gradual increase of R light included six light intensity treatments, i.e., 500 lx (R500), 1000 lx (R1000), 1500 lx (R1500), 2000 lx (R2000), 2500 lx (R2500) and 3000 lx (R3000). For the B light gradient treatments, the seedlings were sequentially incubated in six B light intensity gradients, i.e., 1000 lx (B1000), 1500lx (B1500), 2000 lx (B2000), 2500 lx (B2500), 3000 lx (B3000) and 3500 lx (B3500), while R light (2000 lx) and G light (5000 lx) were fixed light intensities. The fully expanded leaves from three individual plants were defined as three biological replicates. After light irradiation, leaf samples were harvested by rapid freezing in liquid nitrogen, and stored at -80°C until further use.

To test whether *PbPRR2* can regulate photosynthetic performance in response to varying R light signals, *N. benthamiana* leaves were transformed with a 35S:*PbPRR2* transgene. One day after infiltration, *N. benthamiana* plants started to receive LED light treatment. The distance between the lamps and samples was 30 cm. During the experiment, temperature was maintained at 25°C while RH was maintained at 70%. A gradient of R light was established, while B light (5000 lx) and G light (1800 lx) were relatively uniform. To determine the R light irradiance that each leaf was exposed to, light intensities (45-105 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were measured on the upper surface of each individual leaves using the LI-180 spectrometer (LI-COR Inc., USA).

Measurements of light environment among different pear tree canopy positions

To investigate light environment among different pear tree canopy positions, sunlight spectra was measured with LI-180 spectrometer at 5 cm above the surface of the leaves in specific location. The adult 'Wonhwang' pear trees (ten-year-old) were grown in the experimental orchard of Research Institute of Fruit

and Tea. The experiment was carried out in a randomized complete block design with three replications. Trees from each block were randomly selected, which represented biological replicates. Each tested tree was divided into four canopy positions, i.e., sunny side-interior part of the canopy, sunny side-exterior part of the canopy, shady side-interior part of the canopy, shady side-exterior part of the canopy. Interior and exterior parts of the canopy were approximately 0-1.0 m and more than 1.0 m away from the trunk, respectively. For each biological replicate of each canopy position, light quality parameters were measured on three independent positions (three technical replicates). All sunlight spectra measurements were performed every 2 hours from 08:00 to 16:00 on sunny and clear day (105 day after flowering). All data were analyzed by one-way ANOVA analyses (IBM SPSS Statistics 19 software), followed by the Duncan's multiple range tests with a significance level of $P < 0.01$.

Expression analysis of *PbCCTs*

To investigate the expression patterns of *PbCCTs* in different pear tissues, the normalized RPKM (reads per kilobase per million mapped reads) values of pear *CCT* genes were extracted from the previously published RNA-Seq data of leaf, ovary, petal, shoot, stigma, fruit (15 days after full bloom) [50]. The results were visualized by heat map with transformed $\log_2$ (RPKM+1) values using the 'pheatmap' R package (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

In order to investigate the possible functions of the *PbCCTs* in the varying light quality environments, *PbCCTs* with higher expression level (RPKM values > 10) in leaf were selected for further analysis using qRT-PCR. Total RNA was extracted from the frozen leaves using the RNeasy Pure Plant Kit (Polysaccharides & Polyphenolics-rich) (Tiangen, China) according to the manufacturer's protocol, followed by RNA integrity examination on 1.0% agarose gels stained with ethidium bromide. First strand cDNA synthesis and qRT-PCR were performed as described previously [40]. Primer sequences for qRT-PCR analysis were designed using the Primer Premier 5 (Additional file 1). Two reference genes, i.e. *PbSKD1* and *PbYLS8*, shown to be stably expressed in pear leaves [40], were used as internal controls to normalize the qRT-PCR data. Relative quantification was calculated according to the Ct method ($2^{-\Delta\Delta C_T}$). For each sample, three independent biological replicates were performed to acquire reliable results. Statistical analyses were performed as described above.

Gene cloning, vector construction and transient transformation

The cDNA sequence of *PbPRR2* was amplified from 'Wonhwang' pear leaf cDNA using gene-specific primers (Additional file 1), and then cloned into the pDONR221 vector by BP reaction (Gateway, Invitrogen). Several independent clones were sequenced to confirm the correct sequence of *PbPRR2*, then transferred by Gateway LR reaction into the destination vector pHEX2. All Gateway reactions were performed as recommended by the manufacturer (ThermoFisher Scientific, <https://www.thermofisher.com>). Sequence alignment was performed using Clustal X2 program (<http://www.clustal.org/>) and GENEDOC. pHEX-*GUS*, pHEX-*PbPRR2* and pBIN61-p19 (the suppressor of gene silencing p19) constructs were separately introduced into the *Agrobacterium tumefaciens* strain

GV3101 by electroporation method. *A. tumefaciens* cultures carrying each expression vector were mixed with an equal volume of *A. tumefaciens* strain containing the pBIN61-p19, and co-infiltrated into *N. benthamiana* leaves as described in Hellens *et al.*, (2005) [51]. Three days after infiltration, photosynthetic measurements were taken on 20 leaves per construct (n=20) under different R light levels, using the portable CIRAS-3 photosynthesis system (PP. Systems Inc., USA). Data were expressed as mean±SEM and analyzed using the IBM SPSS Statistics 19 software. The differences of the means were determined by Student's *t*-test with a significance level of $P < 0.001$.

Results

Identification and genomic distribution of CCT family in pear

In total, 42 *PbCCTs* were identified in the pear genome (Additional file 2). CCT family members were classified into COL, PRR, ZIM, TCR1 and ASML2 subfamilies, then systematically named according to their family name and sequence similarity. Our analysis revealed that COL subfamily consisted of the highest number of *CCT* genes in pear, with 33.3% (14 *PbCOLs*) of the total *PbCCTs* (Additional file 2). Both PRRs and ASMLs constituted the second largest subfamily with 19.0% (8 *PbPRRs* and 8 *PbASML2s*) of the *PbCCTs*. The ZIM and TCR1 subfamilies were smallest, with 14.3% (6 *PbZIMs* and 6 *PbTCR1s*) of the *PbCCTs*. The molecular weight of these *PbCCT* proteins ranged from 22.8 kD to 93.6 kD, and their values of isoelectric point were between 4.27 and 9.45.

PbCCTs were unevenly distributed over 15 of the 17 pear chromosomes, with no *PbCCT* gene found on chromosome 2 and 4 (Fig. 1). Among these, chromosome 1, 3, 8 and 12 contained the fewest *PbCCTs* with only one member (2.4%) on each chromosome, while chromosome 17 possessed the highest number of *PbCCTs* with five (11.9%) of the 42 members. However, it should be noted, eight *PbCCTs* were remained on as yet unmapped scaffolds. Among all identified *PbCCTs*, a total of seven segmental duplication events were identified in the pear genome (Fig. 1 and Additional file 3), indicating that segmental duplication events were the major contributors to the expansion of the pear CCT family. With one exception (*PbTCR5-PbTCR6*), the Ka/Ks ratios of the other duplicated pairs were less than 0.26, implying that the pear *CCT* gene family had mainly undergone strong purifying selection (Additional file 3).

To further explore the synteny relationships of CCT family genes between pear and the other two representative species, *Arabidopsis* (dicot) and rice (monocot), we performed interspecies comparative synteny analysis in a pairwise manner (Fig. 2). A total of 109 and 35 collinear *CCT* gene pairs were identified in the pear/*Arabidopsis* and pear/rice pairs, respectively (Additional file 4). The synteny analysis also showed that multiple pear *CCT* genes that correspond to single other plant species gene, for examples, *PbCOL1/PbCOL6/PbCOL8-At5g24930* and *PbPRR1/PbPRR2/PbPRR3/PbPRR8-At2g46790* (Additional file 4). These results indicated that *CCT* genes may have a common evolutionary ancestor among these species. Some *CCT* collinear gene pairs of pear/*Arabidopsis* were anchored to highly

conserved collinear blocks, in which the number of syntenic gene pairs was up to 41, whereas none of counterparts of pear/rice pairs contained more than 28 genes (Additional file 4). The high level of syntenic conservation between the pear and *Arabidopsis* indicated that *CCT* genes in pear might share similar structures and functions with orthologous genes in *Arabidopsis*.

Phylogenetic analyses of CCT genes

To explore the phylogenetic relationship of the CCT family, an unrooted neighbor-joining phylogenetic tree was established based on the alignment of the full-length CCT protein sequences from the pear and *Arabidopsis* (Fig. 3). In most clades, internal nodes were supported by confidence values of at least 80%, indicative of good consistency in the topology, which further corroborates the reliability of the tree. In order to test the reliability of the tree topology, protein domain architecture was used to provide additional support for the proposed phylogeny. The majority of members belonging to the same phylogenetic group exhibited common motif compositions (Fig. 4D). For example, TIFY and C2C2-GATA zinc-finger domains are specifically shared by ZIM subfamily. Presence of the Pseudo-receiver domain is also clade dependent in the PRR subfamily. The conserved intron/exon structural characteristics also supported the fine structure of the phylogenetic trees. For example, all the coding sequences of the *PbPRRs* were interrupted by 5 or 7 introns, while the TCR1 subfamily contained no more than two introns.

According to the classification criteria of CCT family in *Arabidopsis*, *PbCCTs* were classified into seven major clades, named Clade I-VII (Fig. 3). 14 *PbCOLs* were categorized into three clades, with well-supported bootstrap values: five *PbCOLs* in Clade I, four *PbCOLs* in Clade II, and five *PbCOLs* in Clade III (Fig. 3 and Fig. 4A). Clade I comprised of five *PbCOLs* (*PbCOL1/4/5/8/9*), featuring a conserved CCT domain with two upstream zinc-finger B-box domains (Fig. 4D). *PbCOL* members (*PbCOL2/3/6/7*) in Clade II exhibited one B-box domain and a CCT domain. The gene structures of Clade I and II were highly conserved, containing two exons and one intron (Fig. 4C). The *PbCOL* homologs (*PbCOL10-PbCOL14*) were clustered with the Clade III of *Arabidopsis* COL subfamily that possesses a normal B-box domain, a second divergent B-box domain and a CCT domain. We found that this phylogenetic classification of *PbCOLs* in Clade I and Clade II was the same as the classification of *Arabidopsis* COL subfamily based on the difference of the B-box domain (Fig. 3 and Fig. 4D). However, *PbCOL10*, *PbCOL11* and *PbCOL12* in Clade III contained only one or no B-box domain. These patterns suggest that the corresponding genes may have lost the B-box type zinc finger domain.

The PRR subfamily was further divided into three main subgroups based on their phylogenetic relationship, named as Clade IVa, Clade IVb and Clade IVc (Fig. 3). Four members (*PbPRR1/4/5/6*) in Clade IVa and two members (*PbPRR2/3*) in Clade IVb were highly conserved, containing eight exons and seven introns (Fig. 4C). Clade IVc included two pear PRR genes (*PbPRR7/8*) that clustered with *AtPRR1* (*AtTOC1*) gene from the same branch (Fig. 3).

The TCR subfamily could be divided into two subgroups, i.e. Clade Va and Clade Vb (Fig. 3). For the Clade Va, *PbTCR1* and *PbTCR4* were clustered with two *AtTCR* genes (*At5g57180* and *At4g25990*). Clade Vb

contained four pear members (*PbTCR2/3/5/6*), which were clustered with two *AtTCR* genes (*AtTCR1* and *At5g14370*).

All pear *ZIM* genes were also divided into two subgroups (Fig. 3). In detail, Clade VIa was comprised of three pear *PbZIM* members (*PbZML4/5/6*), which clustered with *Arabidopsis* *ZIM* subfamily genes (*AtZIM*, *AtZML1* and *AtZML2*), while three pear *ZIM* homologs (*PbZML1/2/3*) were identified as a distinct subgroup (Clade VIb) that had no counterpart in *Arabidopsis*.

Pear *ASML2* members were classified into four subgroups and were characterized by only conserved CCT domain (Fig. 3 and Fig. 4D). Clade VIIa and Clade VIIb were divided from the same branch, and contained *PbASML3/4* and *PbASML7/8*, respectively. *PbASML1/2* were clustered to Clade VIIc and *PbASML5/6* belonged to Clade VIId.

Expression profiles of CCT genes in different tissues and under varying light signals environments

To investigate the tissue expression profiles of the *PbCCTs* in pear, we analyzed their transcript levels based on a publically available RNA-seq data of different tissues, including leaf, ovary, petal, shoot, stigma and fruit (Fig. 4B). In general, the candidate *PbCCTs* showed variation in tissue expression patterns. Many *PbPRRs* and *PbCOLs* exhibited high transcript abundance level in all six tissues, whereas most *PbASMLs* were expressed at relatively lower levels in multiple tissues. On the other hand, several *PbCCTs* exhibited tissue-specific expression. For example, *PbCOL6* and *PbTCR3* were mainly expressed in leaf, whereas *PbCOL9* showed the highest transcript abundance in the petal. Some duplicated gene pairs also showed divergent transcript levels. For instance, *PbZML3* showed very low expression in six different tissues; whereas its duplicated gene, *PbZML2*, was highly expressed in all tested tissues. These results suggest that duplicated genes may evolve to have diverse functions.

We investigate the environmental light spectrum changes among different pear tree canopy positions. Compared with the exterior part of the canopy, the levels of R and B light decreased significantly in the interior part of the canopy, indicating that intensity changes of light quality are important signatures in fruit orchards (Additional file 5). Partial genes from *CCT* family have showed to regulate growth and development by responding to light signals; therefore, the response of the *PbCCTs* induction to light quality treatments was further characterized by qRT-PCR. Overall, most of selected *PbCCTs* showed highly diverse expression patterns under the enhancement of R/B light radiation (Fig. 5 and Fig. 6). These results suggested that they are sensitive to light quality signals, thus, intriguing different responses according to the external light conditions. Under the R light treatments, the expression of three genes (*PbCOL6*, *PbTCR1*, *PbTCR2*) reached a peak at the early stage (R1000), and then down-regulated during subsequent increased exposure to R light (Fig. 5). Additionally, *PbCOL11* and *PbTCR3* presented an increasing trend with the increasing R light process. It was found that one homeologous pair (*PbPPR2* and *PbPPR3*) displayed strong rhythmic expression patterns, suggesting that these genes could respond to R light changes during their regulation on pear growth and development. Noteworthy, compared with the control (R500), the abundance of *PbPPR2* and *PbTCR3* transcripts dramatically increased more than 13.5-fold at R2500 stage and 107.6-fold at R3000 stage, respectively. Meanwhile, we also analyzed the

expression pattern of the *PbCCTs* in the B light treatment (Fig. 6). Among them, the transcript levels of four genes (*PbPPR2*, *PbPRR3*, *PbTCR1* and *PbTCR3*) were induced by enhanced B light, some of which decreased markedly at the highest abundance (B3500). Notably, the relative expression level of *PbPPR2* approximately increased 208.9-fold at B3000 stage relative to control and that of *PbTCR3* approximately increased 172.0-fold at B3500 stage. In addition, two *PbCCTs* (*PbCOL5* and *PbTCR4*) showed the decreased expression levels in response to progressively increasing B light signals.

PbPRR2 is a candidate for negatively regulating photosynthetic performance

Combining the previous transcriptomic study, present bioinformatics analysis and expression analysis [41], *PbPRR2* (LOC103943360), a close homologue of *Arabidopsis* circadian clock gene *AtPRR5*, was chosen as a strong candidate for functional verification. Because *AtPRR5* are implicated photomorphogenesis in R light that is considered as the most efficient wavelength for driving photosynthesis [52–55], these facts prompted us to examine the photosynthetic performance of plants overexpressing *PbPRR2* under a broad range of R light intensity. *PbPRR2* has a 2013-bp open reading frame and encodes a protein of 670 amino acids (GenBank accession number: MZ826141). The amino acid sequences encoded by *PbPRR2* and *AtPRR5* (AT5G24470) - the ortholog of *PbPRR2* from *Arabidopsis* - are 40.12% identical (Additional file 6). *PbPRR2* protein featured a PR domain at the N terminus and a CCT motif at the C terminus.

To further test the role of *PbPRR2* in the regulation of photosynthetic properties under the changing R light signals, *PbPRR2* was transiently over-expressed in *N. benthamiana* leaves and compared with the control leaves. Fluctuating profiles of net photosynthetic rate, stomatal conductance and internal CO₂ were observed in both *PbPRR2* infiltrated and control leaves with the increasing R light intensity (Fig. 7). However, significantly reduced levels of these photosynthetic parameters were observed in leaves inoculated with the pHEX2-*PbPRR2* construct compared to the pHEX2-GUS control. These observations indicate that *PbPRR2* may suppress the red light-dependent enhancement of photosynthetic performance.

Discussion

Photosynthetic performance is an important agricultural trait vital to the adaptation of horticultural crops to ambient light conditions [56]. Increasing evidences suggest that *CCT* genes play important roles in light-regulated developmental processes [10,45,53,57,58]. Understanding the structural characteristics of pear *CCT* family genes and their specific responses to different light environments will help to find some important candidates that may be involved in canopy light signal regulation. Here, we performed a comprehensive analysis of the *PbCCT* family genes. A total of 42 *PbCCT* genes were identified from pear genome, accounting for 0.098% of all predicted protein-coding genes (42,812 genes) [46], which was less than that in *Arabidopsis thaliana* (36 *AtCCT* genes out of the 25,498 predicted genes, accounting for 0.141%) [7,59], but higher than that in *Oryza sativa* (41 *OsCCT* genes out of the 53,398 predicted gene, accounting for 0.076%) [8, 60, 61]. These results suggested that the *CCT* family members may not be

directly related to the genome sizes in different plants, since the sizes (512 Mb) of the pear genomes are larger than that of rice (403 Mb) and even about fourfold of that of *Arabidopsis* (125 Mb) [46, 59, 60]. In the process of genome evolution, gene duplication has generally been viewed as a necessary source of gene family expansion [62, 63]. In this study, none of the *PbCCTs* were located in tandem, and seven segmental duplication events were identified in the *PbCCTs*, suggesting that the segmental duplication was the main contributor to the expansion of *PbCCTs*; similar results were also observed for other gene families in pear [64–67]. After gene duplication, some duplicates may undergo functional divergence. The Ka/Ks ratios of *PbCCT* duplication pair indicated that purifying selection was a major force driving the evolution of new functions for *PbCCTs*.

Phylogenetic analysis showed that most of the *CCT* family clades contained both pear and *Arabidopsis* proteins, suggesting that the two species display relatively conserved evolution (Fig. 3). Gene expression patterns of *PbCCTs* in response to varying light quality could provide important clues regarding gene functions during light response process. For instance, two *PbCCTs* (*PbCOL5* and *PbCOL7*), showed progressively decreasing expression levels with increasing R light intensity (Fig. 5). *AtCO1*, an *Arabidopsis* ortholog of *PbCOL5* (Fig. 3), is a key player in the induction of flowering [18]. AtCO protein was degraded under R light by Phytochrome B [68]. Our result showed that shaded light had lower intensity of R/B light (Additional file 5). These findings suggest that increased *PbCOL5* expression induces early flowering in response to the lower R light level to which pear shoots are exposed when shaded by neighboring vegetation. Also the expression of *AtCOL7*, which is a homolog gene of *PbCOL7* in *Arabidopsis* (Fig. 3), was rapidly down-regulated in response to high R:FR (Red:far-red light) [58]. Overexpression of *AtCOL7* was shown to enhance branching number under high R:FR conditions. It would be interesting to determine if *PbCOL7* could increase branching by perceiving decreasing R light signaling.

In *Arabidopsis*, the *AtCOL13* was shown to act as a positive regulator of R light mediated inhibition of hypocotyl elongation [69]. The transcription level of *PbCOL11*, a homolog gene of *AtCOL13*, was up-regulated robustly under the enhanced R light environment (Fig. 3 and Fig. 5), implying that *PbCOL14* might act in response to R light signaling to regulate photomorphogenesis in pear.

Our results provided evidence indicating that *PbPRR2* may have a novel role in the regulation of photosynthetic performance under varying light signals environment. Our phylogenetic analysis showed that the homeologous pair (*PbPRR2/PbPRR3*) clustered together with *Arabidopsis AtPRR5* (Fig. 3). The functions of *AtPRR5* were implicated in the mechanisms underlying the control of flowering time and photomorphogenesis, as well as the circadian rhythm [52, 70]. Moreover, *AtPRR5-ox* plants are shown to be highly sensitive to continuous R light, and also seemed to be slightly hypersensitive to B light [70]. “Red or far-red light signaling pathway” was found to be an enriched category in *AtPRR5* direct-targets [26]. Here, we noticed that the expression of *PbPRR2* and *PbPRR3* showed robust free-running rhythms under continuously increasing R light (Fig. 5). Our previous study indicated that these two genes, which involved in ‘Circadian rhythm-plant’ pathway, were strongly correlation with photosynthetic performance [41]. A key piece of evidence supporting the role of *PbPRR2* in light response is that it resulted in inhibition of photosynthetic performance as compared with those of the control plants (Fig. 7). Therefore,

PbPRR2 (together with *PbPRR3*) should be taken into consideration for a better understanding of the molecular links between circadian rhythms and light signaling-controlled photosynthetic performance.

Conclusions

In short, a total of 42 *PbCCTs* were identified in pear and divided into five subfamilies, as supported by phylogenetic relationship, intron-exon structures and conserved motifs. Expression analysis indicated that *PbCCT* family might have diverse functions and some members were sensitive to light induction, indicating that *PbCCT* genes were involved in light environmental adaptation. One *CCT* gene named *PbPRR2* was indicated to be a negative regulator of photosynthetic performance under the enhanced R light environment. All the results presented in this study provide comprehensive information on the *CCT* family in pear and lay a foundation for further research on the function of *PbCCT* gene.

Abbreviations

Ka: non-synonymous substitution; Ks: synonymous substitution; NJ: neighbor-joining; RH: relative humidity; R: red; B: blue; G: green; LED: light-emitting diode; RPKM: reads per kilobase per million mapped reads; FR: far-red; R500/1000/1500/2000/2500/3000: six red light intensity gradients, i.e., 500 lx, 1000 lx, 1500 lx, 2000 lx, 2500 lx and 3000 lx; B1000/1500/2000/2500/3000/3500: six blue light intensity gradients, i.e., 1000 lx, 1500 lx, 2000 lx, 2500 lx, 3000 lx and 3500 lx.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZL and TW conceived and designed the research. ZL, JLL and LA performed the experiments. ZL, LY, YSC and XSN analyzed the data. ZL wrote the paper. ZQQ revised the paper. All authors have read and approved the manuscript for publication.

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Figures

Figure 1

Chromosomal locations and regional duplication of pear CCT genes. The chromosomal position of each CCT gene was mapped to the pear genome. The chromosome number is indicated at the top of each chromosome. Duplicated gene pairs are linked by dotted lines.

Figure 2

Gene duplication and synteny analysis of the CCT genes between pear and Arabidopsis/rice. Gray lines in the background indicate the collinear blocks within pear/Arabidopsis genomes (A) and pear/rice genomes (B), respectively. The red lines highlight the syntenic CCT gene pairs.

Figure 3

Phylogenetic relationships and subfamily designations in CCT proteins from pear and Arabidopsis based on the neighbor-joining method. The reliability of the predicted tree was tested by bootstrapping with 1000 replicates. The percentage of neighbor-joining bootstrap replications (>40%) is shown above each node. These proteins were divided into seven Clades, and were represented by different colors, respectively.

Figure 4

Phylogenetic relationships, expression profiles, gene structure and protein structure of pear CCT genes. (A) Neighbor-joining trees constructed for CCT genes from the ASML2, ZIM, PRR, COL and TCR1 families. (B) Heatmap showing the expression profiles of CCT genes in different tissues, including leaf, ovary, petal, shoot, stigma and fruit. Color gradient from red-to-green indicates expression values change from high to low. (C) Structure of CCT genes with exon(s) in green, UTR regions in blue, and solid lines between the colored boxes indicating introns. The number indicates the phases of the corresponding introns. (D) Structures of CCT proteins with the CCT DNA binding domains represented by orange boxes, the Zinc-finger B-box domain in royalblue, Pseudo-receiver domain in aerugo, TIFY domain in green, C2C2-GATA zinc-finger domain in purple and Leucine rich repeat N-terminal domain represented by sky-blue boxes.

Figure 5

Expression profiles of 16 selected PbCCT genes under varying red light signals environments. Different capital letters above the bars indicate significant difference at $P < 0.01$.

R500/1000/1500/2000/2500/3000 (x-axis) indicate six red light intensity gradients, i.e., 500 lx, 1000 lx, 1500 lx, 2000 lx, 2500 lx and 3000 lx.

Figure 6

Expression profiles of 16 selected PbCCT genes under varying blue light signals environments. Different capital letters above the bars indicate significant difference at $P < 0.01$.

B1000/1500/2000/2500/3000/3500 (x-axis) indicate six blue light intensity gradients, i.e., 1000 lx, 1500 lx, 2000 lx, 2500 lx, 3000 lx and 3500 lx.

Figure 7

Photosynthetic performance of PbPRR2 transiently expressed in tobacco with the increasing R light intensity. Leaves were infiltrated with PbPRR2 (red line) or the control pHEX2-GUS (blue line). Data for three key photosynthetic parameters, i.e., net photosynthetic rate (A), stomatal conductance (B) and internal CO_2 (C) are presented as mean $\pm$ SEM ($n=20$). Asterisks indicate significant difference at $P < 0.001$.

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