

## Molecular cloning, characterization and expression analysis of *CpCBF2* gene in harvested papaya fruit under temperature stresses

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### Abstract

**Background:** C-repeat binding factors (CBFs) are transcription factors that regulate the expression of a number of genes related to abiotic stresses. Few *CBF* genes have been cloned from other plants but no report in papaya. In present study, a full-length cDNA, designated as *CpCBF2*, was cloned from papaya using *in silico* cloning and 5'- rapid amplification cDNA ends (RACE). Sequence analysis was performed to understand the gene function. The expression pattern of *CpCBF2* in papaya under low (7°C) and high temperature (35°C) stresses was examined using real-time quantitative polymerase chain reaction (RT-qPCR).

**Results:** The full-length cDNA of *CpCBF2* was 986-bp, with a 762-bp open reading frame (ORF) encoding a 254 amino acid polypeptide. *CpCBF2* contained several major highly conserved regions including the CBF-family signature PKRRAGRKKFQETRHP and FADSAW in its amino acid sequence. Phylogenetic tree and three-dimensional structure analysis showed that *CpCBF2* had a relatively close relationship with other plant CBFs. Gene expression analysis showed that high temperature stress had little effect on the expression of *CpCBF2* but low temperature repressed *CpCBF2* expression.

**Conclusion:** The results showed that *CpCBF2* may involve in different roles in temperature stress tolerance. This study provided a candidate gene potentially useful for fruit temperature stress tolerance, although its function still needs further confirmation.

**Keywords:** *CpCBF2*, gene expression, papaya, temperature stresses.

### INTRODUCTION

Papaya (*Carica papaya* L.) is an important fruit crop in tropical and subtropical areas. They have favourable flavor, rich nutrition and many pharmacological benefits (De Oliveira and Vitória, 2011). As a typical climacteric fruit, papaya suffers many problems including rapid ripening and susceptible to biotic and abiotic stresses. Inappropriate storage temperature is the most common problem for papaya fruit during postharvest storage and transportation, which always causes rapid ripening, skin freckles, pulp translucency, pericarp pitting and pulp water-soaking. These symptoms negatively affect its economic value throughout the chain of papaya production. Understanding how the papaya fruit respond to low storage temperature at molecular level will enable us to improve the control of stress tolerance and fruit quality of papaya fruit.

The C-repeat binding factors (CBFs) are transcriptional activators that belong to AP2/EREBP transcription factor family. They are known to induce cold-responsive (COR) transcription by binding to a C-repeat/dehydration-responsive element (CRT/DRE) in the regulatory regions of those genes related to low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994; Novillo et al. 2012), as well as other abiotic stresses such as drought, high temperature and high-salt stress (Campoli et al. 2009;

Wang et al. 2009). Over-expression of *CBF* gene can increase stress tolerance (Savitch et al. 2005) and a few *CBF* genes have been cloned from other plants such as *Arabidopsis* (Gilmour et al. 2004), rice (Ito et al. 2006), tomato (Zhang et al. 2004). However, no report has been made in papaya so far. In present study, a novel *CBF* gene in papaya (*CpCBF2*) was cloned and characterized and its expression was investigated as well. The objective of this study was to investigate the role of *CBF* in papaya fruit responding to temperature stresses and to possibly improve the quality of harvested papaya fruit during storage.

## MATERIALS AND METHODS

### Plant materials and experimental conditions

Papaya (*Carica papaya*) at colour break stage (5% < peel colour < 15% yellow) were harvested from a local commercial plantation near Guangzhou, South China, and then transported to the laboratory and sorted by size, shape and maturity. Uniform fruits free from visual symptoms of any disease or blemishes were randomly selected. The selected fruits were cleaned, dipped in 1% hypochloride solution for 10 min to astringe cut and then soaked in 0.2% (w/v) Sporgon solution (Bayer, Leverkusen, Germany) for 1 min to eliminate potential microbes. After air dried, fruit were placed into unsealed plastic bags (0.02-mm thick) and subsequently transferred to 7°C, 25°C and 35°C for storage, respectively. Samples stored at 25°C were collected at 0, 2, 4, 6, 8, 10 and 12 days after treatment; samples stored at 35°C were collected at 0, 2, 4, 6, 8 and 10 days after treatment; samples stored at 7°C were collected at 0, 2, 6, 10, 14, 18 and 22 days after treatment. Three independent biological triplicates were performed for each treatment. For all samples collected, the fruit core was removed and the flesh with peel was crushed in liquid nitrogen and stored at -80°C until use.

### Fruit evaluation under temperature stress

Fruit peel colour and colouring index were calculated as our previous study (Zhu et al. 2012b), where 1 = entirely green, 2 = ≤ 25% yellow, 3 = 25-50% yellow, 4 = 50-75% yellow, 5 = ≥ 75% yellow, and 6 = orange blush/yellow. Fruit colouring index was calculated on a daily basis as: colouring index =  $\sum$  (colouring grade x number of fruit)/total number of fruit.

### Total RNA isolation and first strand cDNA synthesis

Total RNA was extracted using hot borate method described by Wan and Wilkins (1994) and treated with DNase I digestion using RNase-free kit (TaKaRa, Japan) to eliminate the potential DNA contamination. The RNA concentration and purity were determined. Only those that met the criterion (260/280 ratio of 1.8-2.1, 260/230 ratio ≥ 2.0) were used for further analyses. To prepare cDNA for RT-qPCR, 2 µg of total RNA was reverse-transcribed using the ReverTra Ace qPCR RT kit (TOYOBO, Japan). The final cDNA products were diluted 150-fold with RNAase free water prior to use. The cDNA for RT-PCR was prepared by reverse transcribing 1 µg of total RNA using the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA).

### In silico cloning of *CpCBF2* and sequence analysis

To amplify the *CBF* gene from papaya, a homologous *CBF1* protein from *Arabidopsis* (GenBank accession number: AAV80413.1) was used as bait and seed sequence for retrieval in papaya translated nucleotide database using tblastn. A papaya *CBF* EST sequence (GenBank accession number: EX276366.1) was obtained, and found to be highly homologous to the *CBF* obtained from other plant species in NCBI BLAST program. A pair of primers (sense: 5'-TTCTTCTTTTTCCCTTTTCCC-3', antisense: 5'-TTCCACCACTAATCAACTCAAC-3') was designed based on this EST sequence. RT-PCR was conducted under the following condition: one cycle of 94°C for 3 min, 35 cycles (94°C for 1 min, 55°C for 30 sec, 72°C for 1 min), and then one cycle of 72°C for 10 min. The PCR products of the predicted size (about 928 bp in length) were purified and cloned into PMD20-T vector (TaKaRa, Japan) and then sequenced by Beijing Genomics Institute (BGI, China). The sequenced fragment information was then used to design primers for obtaining the full-length cDNA of *CpCBF2* by RACE using SMARTer™. RACE cDNA Amplification Kit (Clontech, USA). Two sets of specific primers for *CpCBF2* (5'-RACE: outer, TGGAGGTGGTGATAGCAGAAG; inner,

CTGTGGCAATCTCCAAGCA) were used for 5'-RACE. The PCR products were cloned and sequenced as described above (503 bp). After aligning and assembling the sequences of the internal conserved fragment and 5'-RACE product, the full-length cDNA sequence of the *CpCBF2* gene was deduced and subsequently obtained.

### Bioinformatics analysis

The identification of nucleotide sequences from RT-PCR clones was established using the NCBI Blast program (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequence alignment was conducted using the DNAMAN software and the phylogenetic tree was constructed by ClustalW program (<http://www.ebi.ac.uk/clustalw>). Open reading frame and protein prediction were made using NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The theoretical isoelectric point (pI) and mass values for mature peptides were calculated using the Peptide-Mass program (<http://us.expasy.org/tools/peptide-mass.html>). Protein subcellular localization was predicted using WoLF PSORT (<http://wolfpsort.org/>). The three-dimensional structure of the *CpCBF2* protein domain was predicted using the SWISS-MODEL workspace (<http://swissmodel.expasy.org>).

### Real-time quantitative PCR

RT-qPCR was conducted to investigate the expression pattern of *CpCBF2* under temperature stresses. Eukaryotic initiation factor 4A (*EIF*) was selected as reference gene under different storage temperatures according to our previous study on the selection of reliable reference genes for expression study by qPCR in papaya fruit (Zhu et al. 2012a). The primer pair of *CpCBF2* was listed as: Forward: 5'-GGAGGATGAAGAATCTAGTGATGT-3', Reverse: 5'-CCACCACTAATCAACTCAACAAC-3'. Specificity of the amplifications was confirmed by both agarose gel electrophoresis and melting curve analysis. Each assay using the gene-specific primers amplified a single product of the correct size with high PCR efficiency (90-110%). All RT-qPCR was carried out in 96-well plates with Bio-Rad CFX96 Real-Time PCR System and Bio-Rad CFX96 Manager Software (Bio-Rad, USA) using SYBR Green-based PCR assay. Each reaction mix containing 5  $\mu$ l diluted cDNAs, 10  $\mu$ l of THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan), 0.25  $\mu$ M of each primer to a final volume of 20  $\mu$ l was subjected to the following condition: 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 35 sec. The melting curves were analyzed at 65-95°C after 40 cycles. In addition, reverse transcription negative control was included to check for potential genomic DNA contamination. Each RT-qPCR analysis was performed in triplicates and the mean value was used for RT-qPCR analysis. The relative expression of *CpCBF2* was calculated according to the method of  $2^{-\Delta\Delta C_t}$ . The  $C_T$  values for both the target and the reference genes were the means of triplicate independent PCRs.

### Statistical analysis

Experiments were arranged using a completely randomized design. Data were analyzed by analysis of variance (ANOVA) using SPSS version 16.0. The Duncan's multiple range tests ( $P < 0.05$ ) were used to compare differences among mean values.

## RESULTS

### Fruit evaluation under temperature stress

Fruit coloring index was calculated on a daily basis as our previous study (Zhu et al. 2012b) (Figure 1a). The results showed that high temperature accelerated the fruit color change and firmness decline, while low temperature completely blocked the coloration (Figure 1). Chilling injury symptoms such as pericarp pitting and pulp water-soaking were observed for fruit stored at 7°C for 20 days after storage (Figure 1b). The selection of 7°C and 35°C for low and high temperature stresses was based on the previous studies that storage temperature below 10°C or above 30°C can cause stresses to papaya fruit (An and Paull, 1990).

## Isolation and characterization of full-length cDNA of *CpCBF2*

One fragment of a *CpCBF2* homolog approximately 928 bp in length was cloned from papaya by RT-PCR using specific primers based on a papaya EST sequence. The corresponding full-length sequence, designated as *CpCBF2*, was subsequently amplified by RACE and deposited into GenBank (accession number: JX047553). The nucleotide sequence of this cDNA was 986 bp in length, containing a 762-bp open reading frame, a 106-bp 3'-UTR and a 112-bp 5'-UTR. It encoded a predicted polypeptide of 254 amino acids, with the predicted molecular weight of 28.78 kDa and pI of 5.45.

A BLAST search in the GenBank showed that the putative amino acid sequence of *CpCBF2* exhibited higher identity with *Citrus trifoliata* (62%), *Prunus mume* (61%), *Manihot esculenta* (60%), and *Populus trichocarpa* (62%). The multiple sequence alignment of *CpCBF2* protein with other plant species was presented in Figure 2. The translated proteins ranged from 209 to 254 amino acids in length and shared conserved motifs typical of other CBFs (Figure 2), including the AP2 DNA-binding domain flanked by CMIII-3 (PKK/RPAGRxKFxETRHP) and CMIII-1 (DSAWR) (Jaglo et al. 2001), and the LWS sequence near the C-terminal (Dubouzet et al. 2003). CBF/DREB family genes had valine at position 14 and glutamine acid at position 19 (Sakuma et al. 2002), and *CpCBF2* also had the conserved valine at position 80 and glutamine acid at position 85 (Figure 2). As a result, *CpCBF2* belonged to the CBF family of papaya. A phylogenetic tree was constructed to exhibit the evolutionary relationship among an array of plant CBFs (Figure 3). As shown in Figure 3, two major groups were distinguished within the tree. *CpCBF2* belonged to the CBF group which exhibited a relative distant relationship with ERF group (Figure 3). *CpCBF2* was grouped with MtDREB1F, indicating that they have a relatively close evolutionary relationship.

## Predicted cellular localization and three-dimensional structure of the *CpCBF2* protein

WoLF PSORT program was used to predict the cellular localization of the *CpCBF2* protein. It was predicted that the indicated protein was mainly located on nucleus, which agreed with its role as a nuclear transcriptional factor. A predicted three-dimensional structure of the *CpCBF2* domain was obtained by homology modeling based on template of solution NMR structure of the complex of GCC-BOX binding domain of AtERF1 and GCC-BOX DNA (Figure 4a). The identity of *CpCBF2* and AtERF1 are 29.032%. The three-dimensional structure of an *Arabidopsis thaliana* CBF1 protein (AAV80413.1) was also predicted (Figure 4b), showing some different structures with *CpCBF2* but with some conserved domains.

## Expression profiling analysis of *CpCBF2* gene under temperature stresses

The expression profiles of *CpCBF2* gene were evaluated at three temperatures (7°C, 25°C, 35°C) at seven time points. As shown in Figure 5, *CpCBF2* transcript level showed a similar expression trend and level in the control fruit (25°C) and fruit under high temperature stress (35°C) except at day 8. It showed an irregular expression and fluctuated up and down. High temperature had little effect on the expression of *CpCBF2* throughout the storage period excepted at day 8, where it significantly repressed the expression of *CpCBF2* (Figure 5). However, low temperature (7°C) strongly inhibited the expression of *CpCBF2*. The expression of *CpCBF2* was dramatically decreased at 2 days and remained at a low level thereafter. The results indicated that temperature stresses could not induce the expression of *CpCBF2*; on the contrary, low temperature could repress *CpCBF2* expression.

## DISCUSSION

It is well known that an important component of cold acclimation is the C-repeat binding factor (CBF) induced cold response pathway (Thomashow, 2001). Some CBF genes have been cloned and studied in several plant species but none for papaya. In present study, a novel CBF gene, *CpCBF2*, was cloned from papaya fruit by silico cloning for the first time. Sequence analysis showed that the deduced *CpCBF2* contained the typical motifs of CBFs (AP2 DNA-binding domain PKK/RPAGRxKFxETRHP and DSAWR), as well as the conserved valine and glutamine acid (Figure 2). Phylogenetic analysis and three-dimensional structure prediction showed that *CpCBF2* had a close evolutionary relationship with other CBFs. As shown previously, CBF/DREB family genes had valine at position 14 and glutamine acid at position 19. Conversely, ERF family genes were found to contain conserved alanine

(A) at position 14 and aspartic (D) acid at position 19 (Sakuma et al. 2002). The conserved valine at position 80 and glutamine acid at position 85 of *CpCBF2* revealed that it belonged to the CBF/DREB family. Phylogenetic analysis also confirmed the result (Figure 3).

Gene expression analysis showed that high temperature had little effect on *CpCBF2* expression while low temperature repressed the expression of *CpCBF2* (Figure 5). Most previous studies showed that low temperature can induce the expression of *CBF* genes (Mboup et al. 2012; Novillo et al. 2012). However, *CBFs* are transcriptional activators belonging to the AP2/EREBP transcription factor family, which involved in diverse functions. In *Triticeae*, ten *ScCbf* genes showed different expression patterns and *CBF* genes were divided into different groups based on their expression pattern (Campoli et al. 2009). *VvcCBF1* and *VvcCBF4* were also found not to be induced by low temperature but by other stresses (Fernández-Caballero et al. 2012). In *Arabidopsis*, *CBF1* and *CBF3* mRNAs did not accumulate under cold conditions but *CBF2* did and *CBF2* negatively regulates *CBF1* and *CBF3* expression (Novillo et al. 2007). Novillo et al. 2004 revealed that the function of *CBF2* is different from that of *CBF1* and *CBF3*. They found that *Arabidopsis* mutant plants carrying a T-DNA insertion that inactivated the expression of *CBF2* showed higher levels of *CBF1* and *CBF3* transcripts and had a greater freezing tolerance than wild-type plants. Down-regulation of *CBF2* also found resulting in an increase in freezing tolerance (Novillo et al. 2004). However, in *G. barbadense L.*, *GbCBF1* was induced by ABA, drought, and salt in addition to coldness (Guo et al. 2011). All the results indicated that *CBFs* have different functions in plant stress tolerance and that the precise function of each *CBF* gene needs to be further defined. However, whether *CpCBF2* played a role as *CBF2* in *Arabidopsis*, which enhanced the freezing tolerance (Novillo et al. 2004) or played a negative role in cold stress coordinated with other genes needs further investigations.

## CONCLUDING REMARKS

A new *CpCBF2* gene was cloned, isolated and characterized in harvested papaya fruit. Sequence analysis showed that *CpCBF2* not only contained the highly conserved regions of *CBFs* but also displayed some differences. Gene expression analysis revealed that *CpCBF2* was not induced but suppressed by low temperature. Together, these results indicated that *CpCBF2* may play a role in papaya fruit which was different from the other *CBFs* in other plants. This newly isolated *CpCBF2* gene may be an important candidate for functional characterization and manipulation to improve stress tolerance and quality control of papaya fruit.

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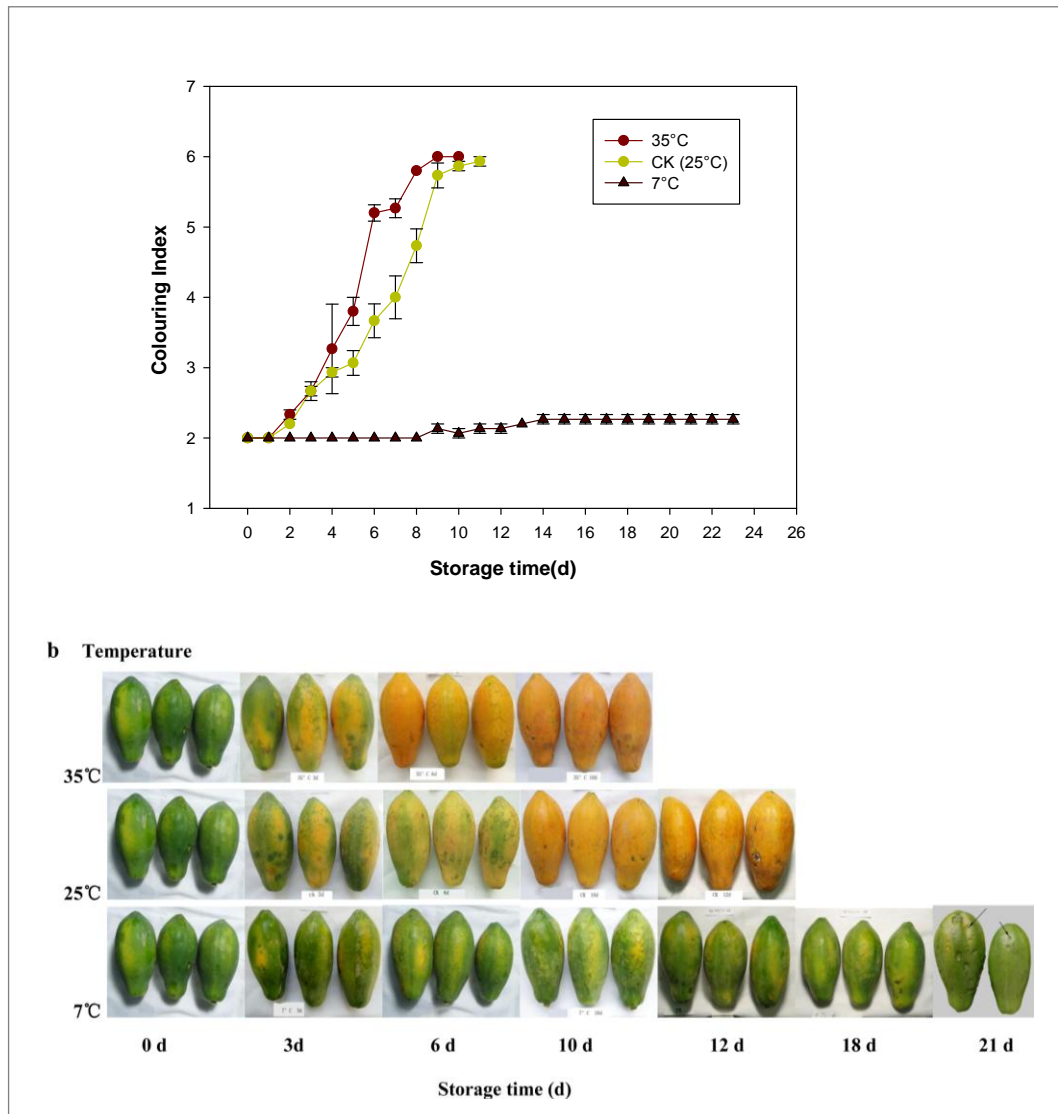
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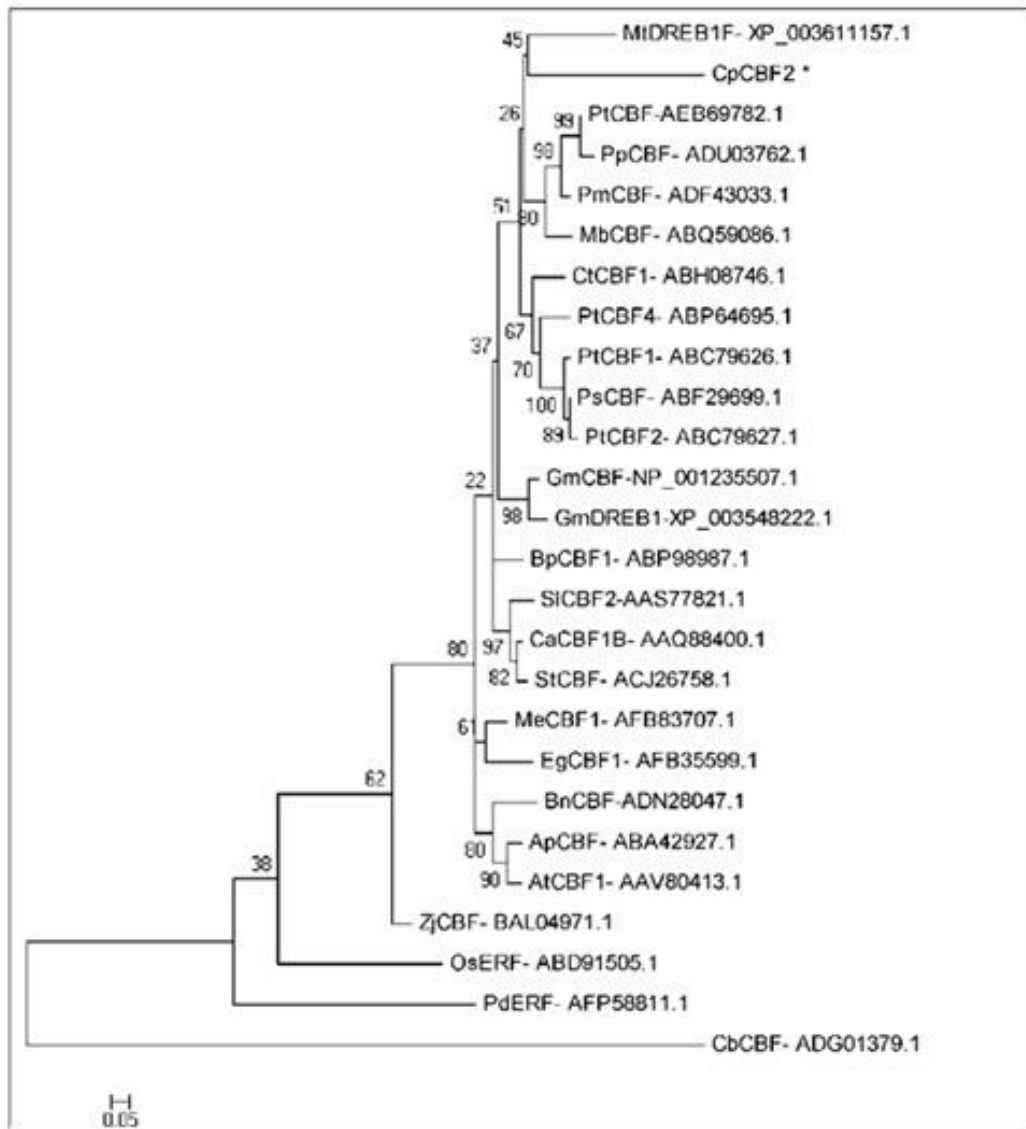
## Figures



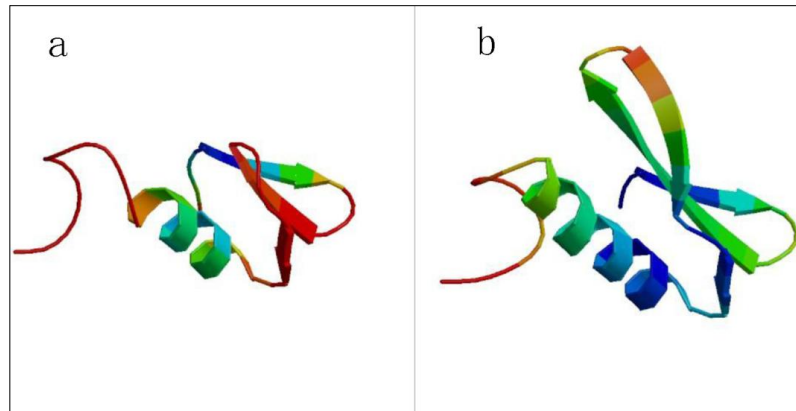
**Fig. 1 Papaya fruit stored at three different temperatures.** (a) Change in coloring index of papaya fruit during storage under different temperatures. (b) Papaya stored at high temperature (35°C), control temperature (25°C), and low temperature (7°C). The arrows indicate chilling injury symptoms. In Fig. 1a, the data for 35°C and 7°C treatments have been published in reference (Zhu et al. 2012b).



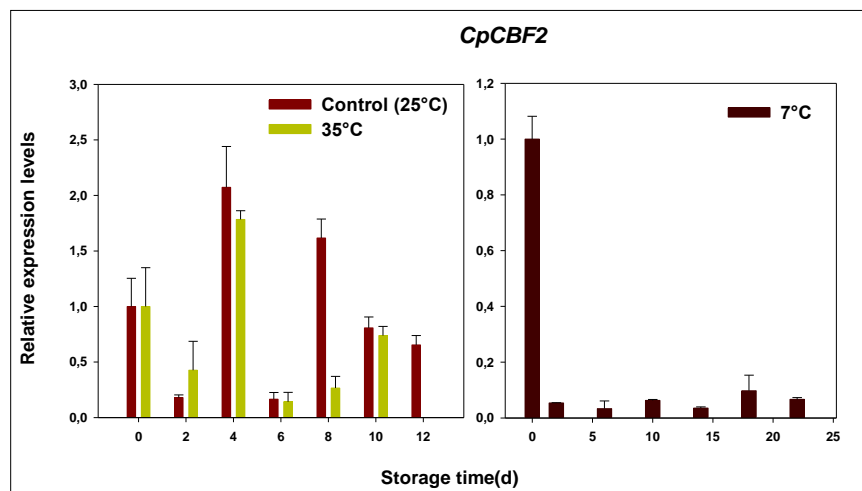




**Fig. 3 Phylogenetic tree of CBFs from different plants.** *Prunus tenella* (PtCBF-AEB69782.1), *Glycine max* (GmCBF-NP\_001235507.1, GmDREB1- XP\_003548222.1), *Arabidopsis thaliana* (AtCBF1- AAV80413.1), *Malus baccata* (MbCBF- ABQ59086.1), *Brassica napus* (BnCBF-ADN28047.1), *Populus deltoids* (PdERF- AFP58811.1), *Populus tomentosa* (PtCBF1- ABC79626.1,PtCBF2- ABC79627.1), *Populus trichocarpa* (PtCBF4- ABP64695.1), *Populus suaveolens* (PsCBF- ABF29699.1), *Capsicum annuum* (CaCBF1B- AAQ88400.1), *Oryza sativa Indica Group* (OsERF-ABD91505.1), *Zoysia japonica* (ZjCBF-BAL04971.1), *Solanum lycopersicum* (SICBF2-AAS77821.1), *Arabidopsis thaliana* (AtCBF1- AAV80413.1), *Medicago truncatula* (MtDREB1F- XP\_003611157.1), *Manihot esculenta* (MeCBF1- AFB83707.1), *Prunus mume* (PmCBF- ADF43033.1), *Solanum tuberosum* (StCBF- ACJ26758.1), *Betula pendula* (BpCBF1- ABP98987.1), *Citrus trifoliata* (CtCBF1- ABH08746.1), *Prunus persica* (PpCBF- ADU03762.1), *Eucalyptus grandis* (EgCBF1-AFB35599.1), *Clostridium botulinum F str. 230613* (CbCBF- ADG01379.1).



**Fig. 4** Predicted three-dimensional structure of CpCBF2 domain based on template of Solution NMR structure of the complex of GCC-BOX binding domain of AtERF1 and GCC-BOX DNA. (a) CpCBF2; (b) AtCBF1. Estimated per-residue inaccuracy visualized using a colour gradient from blue (more reliable regions) to red (potentially unreliable regions).



**Fig. 5** Relative quantification of CpCBF2 expression using selected reference gene EIF for normalization under different storage temperatures. The means are generated from three independent measurements and the bars indicate the standard errors.