PmCBFs synthetically affect PmDAM6 by alternative promoter binding and protein complexes towards the dormancy of bud for Prunus mume

Kai Zhao, Yuzhen Zhou, Sagheer Ahmad, Xue Yong, Xuehua Xie, Yu Han, Yushu Li, Lidan Sun & Qixiang Zhang

The survival in freezing temperature for woody plants is exclusively dependent on the perception of coldness and induction of dormancy. CBF/DREB1 transcriptional factors join cold-response conduits and the DAM genes, especially PmDAM6, are well-known regulators of dormancy. Despite the immense importance, little is documented on the association between CBF proteins and the complexity of the promoter region in PmDAM6 with the function of bud dormancy in P. mume. Therefore, this study was based on the cloning of PmDAM6 and six PmCBFs to evaluate their integral roles in the process of bud development. The consistency of expressions in either vegetative or reproductive buds provided a negative control from PmCBFs to PmDAM6 during the onset of dormancy. Besides, PmCBF5 could form heteromeric complexes with PmDAM1 and PmDAM6. PmCBF1, PmCBF3, and PmDAM4 recognized the promoter of PmDAM6 by the alternative binding sites. Therefore, the interactions of these genes formulated the base of an obvious model to respond to the coldness and engendered dormancy release. Findings of this study will further help the unveil the genetic control of bud dormancy and its augmentation in P. mume and may offer an explanation for the vernalization.

Formation of a bud for perennial plants is often concomitant with its ability to enter dormant state and prevailing cold climates challenge the buds in their capacity to retain the reproductive potential unless favorable conditions arrive. Therefore, dormancy helps plants to escape bad environmental circumstances and to keep their growth potential alive. Prunus genus is rich in fruit-bearing species like peach (Prunus persica), plum (Prunus domestica), apricot (Prunus armeniaca), Japanese apricot (Prunus mume), almond (Prunus dulcis) and cherry (Prunus avium). These species are capable of mending their growth habits in accordance with seasonal variations. P. mume has been cultivated in China for over 3000 years. The flower of this tree can bloom in low temperature, earlier than many other species in Prunus. However, the bud induction and floral organ differentiation appeared in the next year. Therefore, it is of immense importance to search out the genetic factors underlying the coldness perception and induction of dormancy. According to whole genome sequencing analysis, six PmDAM genes were identified, their tandem repeats were distributed in the genome, and six CBF binding sites were found in the upstream of PmDAM genes. It is supposed that the PmDAMs and their CBF binding sites may be the key factors controlling early dormancy release.

MADS-box gene family contains transcriptional factors which were found important to have applications in plant organogenesis covering flower organ development, determination of meristematic identity and the vegetative transformation into reproductive phase. Dormann associated MADS-box (DAM) genes play integral

1Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, National Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological Environment, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants of Ministry of Education, School of Landscape Architecture, Beijing Forestry University, 100083, Beijing, China. 2Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, 100083, Beijing, China. Correspondence and requests for materials should be addressed to Q.Z. (email: zqxbjfu@126.com)
roles in specifying the dormancy transitions during the growth curves\textsuperscript{8–11}. In \textit{P. persica}, the expression profiles of \textit{DAM} genes are associated with obvious seasonal changes of temperature\textsuperscript{12,13}. As for \textit{P. avium}, the expressions of \textit{DAM} genes are related to the quantification of the cold response and flowering date manipulation under varying environmental circumstances\textsuperscript{14–16}. \textit{PpDAM5} and \textit{PpDAM6}, showing the expressions closely related to cooling capacity and flowering date\textsuperscript{16,18}, can inhibit peach bud growth at low temperature\textsuperscript{17}. The expressions of \textit{PpDAM5} and \textit{PpDAM6} were up-regulated during internal dormancy, but down-regulated during dormancy release\textsuperscript{17}.

Low temperature significantly affects C-Repeat Binding Factor (CBF), a cold response signal factor isolated by Stockinger\textsuperscript{18}. In \textit{Arabidopsis}, there are three \textit{CBF/DREB1} genes guiding the signal pathway to low temperature response\textsuperscript{19}. \textit{CBFs} can function to enhance the cold resistance of plants by inducing the downstream genes\textsuperscript{20,21}. Benedict\textsuperscript{22} transferred the \textit{AtCBF1} gene into poplar, comparing it to the wild-type poplar, and found that the cold resistance of the transgenic poplar was highly enhanced. \textit{CBF} genes in flowering peach can increase the cold tolerance and can cause dormancy in short-day conditions\textsuperscript{23}. When a peach CBF gene was overexpressed in apple, both non-acclimated and acclimated freezing tolerance was observed and displayed these phenotypes\textsuperscript{24}.

For many woody plants, \textit{DAM} genes are involved in plant dormancy induction, and \textit{CBF} genes are related to cold-response pathway. On account of the participation of these genes in supervising the plant growth and development in cold climate, it is necessary to investigate their functions in plant growth and dormancy control. Therefore, studying the interactive influence of both the gene types as determinants of bud activity in \textit{P. mume} could be a nascent concept inviting plentiful directions for future studies. In this research, we cloned \textit{PmDAM6} and six \textit{PmCBFs}, ascertained their expression profiles in flower bud, flower, leaf bud, and leaf, and tested their roles in the simulated bud dormancy. In addition, multiple interaction experiments were carried out to investigate the protein-protein and protein-DNA associations among \textit{PmCBFs} and \textit{PmDAM6} in controlling bud growth and dormancy. This will invite future studies on molecular perceptive of bud control and cold response conduit.

**Results**

\textbf{PmCBFs bind to the promoter of \textit{PmDAM6}}. Based on the research of \textit{P. mume} genome, there exist more binding sites (C-repeat/DRE element) in the promoter of \textit{PmDAM6} than those of other \textit{PmDAMs}. In this research, we obtain the upstream fragment of \textit{PmDAM6}. Indeed, there are three potential binding sites in the 1 kb region of \textit{PmDAM6}'s promoter, and four in the 2 kb region. To uncover the role of \textit{PmCBFs} and \textit{PmDAM6} in induction and release of flower bud dormancy, we conducted yeast one hybrid (Y1H) experiments to explore the regulation between six \textit{PmCBFs} and the promoter of \textit{PmDAM6}.

As shown in Fig. 1A, the promoter of \textit{PmDAM6} (Supplementary Data S2) was separated into four potential binding sites based on the position of CCGAC: M1, –1971; M2, –706; M3, –648; M4, –294. Then, baits (length less than 100 bp) were designed as shown in Fig. 1B. A total of six bait vectors were constructed, M2 and M3 were designed in one bait, which got close to each other, the other two, M1 and M4, were independently performed as baits, then the original sequences of these three baits were duplicated respectively to form other three baits (Supplementary Data S3). Five of these six baits were tested negative to perform the following yeast one hybrid. The corresponding working concentrations of Aureobasidin A (AbA) were as follows: pAbAi-1-1, 400 ng/ml; pAbAi-1-3, 200 ng/ml; pAbAi-2-3, 1000 ng/ml; pAbAi-3-1, 400 ng/ml; pAbAi-3-3, 200 ng/ml. And the pAbAi-2-1 was not considered as a bait because of its normally growing under 1000 ng/ml AbA.

All yeasts with successful transformations showed normal growth on the SD/-Leu solid medium (Fig. 1C). The Y1H assays showed that \textit{PmCBF1} and \textit{PmCBF3} could associate with baits of pAbAi-1-2, pAbAi-3-1, and pAbAi-3-3; and \textit{PmCBF1} and \textit{PmCBF4} could bind to baits of pAbAi-2-3, pAbAi-3-1, and pAbAi-3-3. These results suggested that \textit{PmCBF1} and \textit{PmCBF3} recognized the promoter of \textit{PmDAM6} by the binding site M2 and M3. \textit{PmCBF1} and \textit{PmCBF4} could discern M1 and M4. However, \textit{PmCBF2}, \textit{PmCBF5}, and \textit{PmCBF6} failed to bind to the four C-repeat/DRE sites of \textit{PmDAM6} promoter.

\textbf{In vivo protein-protein interactions among \textit{PmCBFs} and \textit{PmDAM6}}. Based on yeast two hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assay, a further exploration about \textit{PmCBFs} and \textit{PmDAMs} was made. According to Yeast two-hybrid experiments, \textit{PmCBF5} showed a strong interaction with \textit{PmDAM1} and a general interaction with \textit{PmDAM6} (Fig. 2A). Moreover, protein-protein interactions among \textit{PmDAMs} and \textit{PmCBFs} were established by BiFC with a yellow fluorescent protein. Fluorescence released by these yellow fluorescent proteins was positioned at nucleus, suggesting the strong inclination among \textit{PmCBF5} and \textit{PmDAM1}–\textit{PmDAM6}. In this research, we cloned \textit{PmDAM6} and these two \textit{PmDAMs}. \textit{PmCBF5} could form heteromeric complexes with \textit{PmDAM1} and \textit{PmDAM6} (Fig. 2B). As shown in Supplementary Fig. S3, there were no interactions in \textit{PmDAM1}-\textit{YFP}\textsuperscript{C} and \textit{YFP}\textsuperscript{N}, \textit{PmDAM5}-\textit{YFP}\textsuperscript{C} and \textit{YFP}\textsuperscript{N}, \textit{PmDAM5}-\textit{YFP}\textsuperscript{C} and \textit{YFP}\textsuperscript{N}, and \textit{YFP}\textsuperscript{C}/\textit{PmDAM6}-\textit{YFP}\textsuperscript{C}. In order to exclude the false positives, a member from the same protein family can be chosen to execute a negative control\textsuperscript{25}. Therefore, the interactions of \textit{PmDAM2}-\textit{YFP}\textsuperscript{C} and \textit{PmDAM1}-\textit{YFP}\textsuperscript{C}, \textit{PmDAM6}-\textit{YFP}\textsuperscript{C} and \textit{YFP}\textsuperscript{N}, \textit{PmDAM6}-\textit{YFP}\textsuperscript{C} and \textit{YFP}\textsuperscript{N}, and \textit{YFP}\textsuperscript{N}/\textit{PmDAM4}-\textit{YFP}\textsuperscript{C} were used as the negative controls of \textit{PmDAM1}-\textit{YFP}\textsuperscript{C} and \textit{PmDAM4}-\textit{YFP}\textsuperscript{C}. \textit{PmDAM6}-\textit{YFP}\textsuperscript{C} and \textit{PmDAM5}-\textit{YFP}\textsuperscript{C} were used as the negative controls of \textit{PmDAM5}-\textit{YFP}\textsuperscript{C} and \textit{PmDAM6}-\textit{YFP}\textsuperscript{C}. Fluorescent was not detected in these negative tests.

\textbf{Crossover of the expressions between \textit{PmCBFs} and \textit{PmDAM6} in flower bud dormancy}. In \textit{Beijing}, \textit{P. mume} 'SanlunYudie' begins to form buds from June to July, undergoes flower bud differentiation from July to November, steps into dormancy from September to October, retains dormancy from November to January, and initiates dormancy breaking from January to February (Fig. 3A). From July to October, \textit{PmDAM6} showed up-regulated in the chilling environment with a temperature under 20 °C and down-regulated in the coldness deepened weather. However, the expression tendencies of six \textit{PmCBFs} during different periods of flower bud development were basically similar with high positive correlations (Fig. 3B) and were induced
vastly under low temperature before freezing. The transcript levels of *PmCBFs* were low during July-October and January-February, but were high during November-December. The high expression of *CBF* genes seemed to inhibit the expressions of *DAM* genes. Together with all expression data, we can conclude that *PmDAM6* and *PmCBFs* function in two temperature regions, respectively. This means *PmDAM6* actively respond in the chilling temperature (below 20 °C) and *PmCBFs* dominate in the freezing zone (below 0 °C), and the regulations in this biological process between these genes are continuous with a suppressive tendency.

**Figure 1.** *PmCBFs* bind the promoter of *PmDAM6* in vivo. (A) The genomic structure and promoters of *PmDAM6* and the CBF-binding sites in were marked by red blocks. (B) The designed baits in Y1H, the whole promoter was split into three part about 100 bp. (C) The results of Y1H assays between *PmCBFs* and the baits.
Expression patterns of $PmDAM6$ and $PmCBFs$ in different blooming stages and different flower structures. When the temperatures return to chilling, the flower bud started to grow and bloom. To explore the expression patterns of $PmDAM6$ and $PmCBFs$ genes in the different blooming stages and different floral structures.
structures, the blooming process was divided into four stages. On the whole, the expression trends of \( PmDAM6 \) also declined from F1 to F4. In addition, \( PmDAM6 \) were expressed in all the floral structures, and the expression of \( PmCBFs \) kept low levels and got lower in the flower blooming process (Fig. 4A). The expression tendencies of the \( PmCBFs \) in the flower blooming stages were basically the same, the transcript levels were relatively high in F1 and F2, but low in F3 and F4. According to the expression patterns of \( PmCBFs \) in different flower structures, \( PmCBFs \) were divided into two groups. \( PmCBF1-4 \) showed high expression specificity in sepals. The other type genes, including \( PmCBF5-6 \) were expressed in all of the floral structures without significant differences.

Expression patterns of \( PmDAM6 \) and \( PmCBFs \) in leaf buds and leaves. The leaf buds experienced a different development process compared to flower bud. They formed in the same period with flower bud, but only enlarged after the flower blooming after dormancy in the cold winter. \( DAM \) genes were specifically expressed in leaf buds at different developmental stages. \( PmDAM1-3 \) exhibited high expression levels in September, and expression levels of \( PmDAM4-6 \) were the highest in October (Fig. 5A). Furthermore, the expression trends of \( PmDAMs \) in leaf bud were largely consistent at different development stages and showed significant expressions from September to October which then gradually declined from October to March in the next year. However, the expression levels of \( PmCBFs \) were the highest in November and December. Above all, the expression patterns of \( DAM \) and \( CBF \) genes between leaf bud and flower bud were largely consistent at different development stages.
The expressions of PmCBFs manifested two types of similar patterns with high levels. The first group (PmCBF1-3) showed peak expressions in August, up-regulated from April to August, and down-regulated from September to November (Fig. 5B). The second group (PmCBF4-6) showed peak expressions in June and July, then declined till November. But all the PmCBFs own high levels from June to August.

### Simulated bud dormancy and dormancy release.
In order to study the functions of PmCBFs and PmDAMs in the bud dormancy, we detected the expressions of all six PmCBFs and six PmDAMs in the simulated condition of bud dormancy and dormancy release.

One year old branches with flower buds were sampled on October 1, 2015. The growing branches were treated with 6 °C ± 2 °C to induce dormancy. The expressions of PmCBFs were shown in Fig. 3C, and were of two types. One category of PmCBFs, including PmCBF2-5, showed rising expressions, while the other category, containing PmCBF1 and PmCBF6, showed higher expressions in 10-40 days as compared to 0 day, suggesting the low temperature significantly affect the expressions of PmCBFs. However, the expression of PmDAM6 increased in 0–20 days but dropped in the following days. On first day of low temperature exposure, PmDAM6 presented a high expression. During the process of dormancy, PmCBFs accumulated in low temperature, while the expression of PmDAM6 declined.

Warm temperature of spring was simulated at 12 °C ± 2 °C to release the bud dormancy with one year old branches on December 10, 2015. After warm temperature treatment, flower buds turned to expand within 10 days, and after 20 days, 95% flower buds showed white petals. As shown in Fig. 4B, the expressions of PmCBFs and PmDAM6 reduced overall. The expressions of PmCBF1, PmCBF3, PmCBF4 and PmDAM6 keep stable, but declined remarkably during 30–40 days. In contrast, PmCBF2, PmCBF6 and PmDAM6 rapidly declined in 10 days. All results suggested that in the release of dormancy, PmCBFs and PmDAM6 showed a down regulation.

### Discussion

**PmCBFs suppress overexpression of PmDAM6 in flower bud dormancy.** Acquisition of protective strategies for reproductive organs in plants possesses vital importance for the continuation of life, however, the uncertain environment changes, like low-temperature stress, remains the major hurdle to the success of plants
inhabiting the cold regions. Regulatory pathways and transcriptional factors driving these routings are central to the studies about the success of these plants in fluctuating climatic conditions. DAM genes have been shown to be involved in the formation of terminal buds and also intervene growth dynamics in perennial plants like kiwifruit (*Actinidia spp*.), leafy spurge (*Euphorbia esula*), raspberry (*Rubusidaeus*), *Populustrichocarpa*, and peach17,26–32.

In peach, *PpDAMs* has an association with seasonal dormancy transitions in flower buds26. *SVP* genes, orthologs of *DAM*, in *Arabidopsis* show regulatory effect during floral transitions and play a significant role in specifying the floral meristems33. In the flower bud of *Prunus mume*, *PmDAM6* exhibited a special expression leading to the dormancy under decreasing temperature (Fig. 3A). Moreover, the appearance of high CBF expression levels enhanced the tolerance of coldness in freezing climates and inhibited the expression of *PmDAM6*. This may lead to a different phenomenon that flowers buds are easier to sense the changes in environment and devise the mechanism of self-protection34,35. Nevertheless, this happens only if plants are shocked by cold. When plants were released from cold weathers, *PmDAM6* and *PmCBFs* just relaxed with all decreasing trends and relatively lower levels (Fig. 4). This must be an integrated result from regulation beyond CBFs and DAMs.

In *P. persica*, DAMs have shown in vegetative and non-vegetative plant parts development including bud12. Here, the protection mechanism prefers to appear in young tissues facing coldness. Annually sampled leaf buds gave important clues about potent roles of *DAM6* and *CBF* genes in *P. mume* wherein *PmDAM6* showed high expressions in September to October and the CBFs appeared from November to December (Fig. 5A). Besides, *MdCBF1*, 2 and 4 were discerned in the leaf tissues of apple subjected to cold conditions, and *VrCBF* exhibited a short expression (1/2 h-2 days) both in young and old leaves, while *VrCBF1*, 2 and 3 expressed only in younger tissues36. Some reports have indicated a reverse relation between *DAM* and *CBF* during growth curves, and dormancy control, wherein *FT* can be down-regulated by the high expression of *DAM*30. This may provide an explanation for the growth and dormancy of young tissues.

In the mature leaf aging process, *PmDAM6* also showed a negative correlation with *PmCBFs* especially for *PmCBF4-6*, though this happened under a warm environment. However, it is confirmed that CBFs function in heat and drought as well as cold response in soybean37. The ectopically expressing *PpCBF1* induced aging in leaf and also delayed bud opening in the spring24.

Under artificial control environment, *PpDAM5* and *PpDAM6* exhibited an upregulation under sustained low temperature and showed a downregulation by warm temperature in peach38. The growth of peach buds are negatively correlated with the expression of *PpDAM5* and *PpDAM6*3. In addition, the sustained low temperature could satisfy the needs of a chilling requirement in buds to promote the release of dormancy with *PpDAM5* and *PpDAM6* down regulated3. In *P. mume*, high expression of *PmDAM6* hastened the flower buds into dormancy. With the accumulation of coldness in winter, the expressions of *PmCBFs* increased, and that of *PmDAM6* decreased, indicating that high amount of *PmCBFs* seemed to depress the expression of *PmDAM6*. When

![Figure 5. Expression patterns of *PmDAMs* and *PmCBFs* in leaf buds and mature leaves. (A) Gene expressions in the leaf buds. (B) Gene expressions in the leaves. (Warm: higher than 20 °C, Chilling: lower than 20 °C, Freezing: lower than 0 °C).](image-url)
immersed in liquid nitrogen and were stored at 80 °C for RNA extraction. Total RNA was extracted by TRIzol respectively. In these simulation tests, the flower buds were sampled every 10 days. All samples were immediately 14 hours at night), thirty branches were cut to simulate the tests on October 1, 2015 and December 10, 2015, and were kept a relative stabilization, which drove us to hypothesize that the interaction from protein to protein and protein to DNA conducted a feedback loop in the deep dormancy condition (Fig. 6). However, there were still mysteries because of the multiple CRT/DRE core sites that whether the number of binding sites superpose or subduct the function of downstream gene, all the hypotheses need further researches.

In our study, we observed the crossover of the expression between PmCBFs and PmDAM6, and provided the evidences that besides multiple recognitions to the promoter of PmDAM6, PmCBFs also could form protein complex with PmDAM6, which act as a center role in dormancy induction. This multiplex regulation might reflect the evolvement of plant towards more rapidly changing environment. Moreover, the expression patterns in vegetative bud and simulated dormancy test of bud convince the negative control in the dormancy formation. We believe this novel research will help direct future discussion on the interactive role of DAM and CBF genes as essential regulators of bud dormancy.

Materials and Methods

Plant Material.  *P. mume* 'Sanlun Yudie', an early flowering cultivar, was selected as plant material from the Beijing Forestry University, in Beijing, China (40° 00′ N, 116° 18′ E). These samples were taken from flower bud (from July, 2015 to February, 2016; collected a sample every 30 days; Supplementary Fig. S1), flower (early flowers in three different morphologies, full blooming, sepal, petal, stamen, and pistil, in March, 2016; Supplementary Fig. S2), leaf bud (from September, 2015 to March, 2016; collected a sample every 30 days), leaf (from April, 2016 to November, 2016; collected a sample every 30 days). For the dormancy tests (8 °C for 10 hours during daytime and 10 °C for 14 hours at night), thirty branches were cut to simulate the tests on October 1, 2015 and December 10, 2015, respectively. In these simulation tests, the flower buds were sampled every 10 days. All samples were immediately immersed in liquid nitrogen and were stored at −80 °C for RNA extraction. Total RNA was extracted by TRIzol reagent (Aidlab, China).

**Figure 6.** Molecular regulation model of PmDAMs and PmCBFs during flower bud dormancy. PmCBF4 and PmDAM6 could form homodimers, respectively (displayed in larger circles). PmCBF5 could form heteromeric complexes with PmDAM1 and PmDAM6. Meanwhile, PmCBF1 and PmCBF3 recognized the promoter of PmDAM6 by the binding site M2 and M3, PmCBF1 and PmCBF4 could discern M1 and M4.
Real-time quantitative PCR. Full length cDNA sequences of six PmDAMs and six PmCBFs (Supplementary Data S1) were amplified through PCR using the primers in Supplementary Table S1. PikoReal
time PCR system (Thermo Fisher Scientific, Germany) was used to investigate the expressions of PmDAMs and 
PmCBFs in different organs. The primers of RT-qPCR experiments were shown in Supplementary Table S2 and the experiments were carried out using previous method\textsuperscript{40}. PmPP2A (protein phosphatase 2A) was considered as reference gene\textsuperscript{41}. Three biological replicates were performed to calculate the standard deviation. The correlations 
between gene expressions were done by spearman method, and significant was analysed with kruskal-wallis 
test in R.

Gene cloning and Yeast 2 Hybrid assays. Full length cDNA sequences of PmDAMs and PmCBFs were 
amplified through PCR using specific primers (Supplementary Table S3). These sequences were cloned into 
pGBeT7 bait vector and pGADT7 prey vector at the EcoRI and BamHI sites, respectively, using an In-Fusion HD 
Cloning Kit System (Clontech, USA). The Y2H assays were performed by former method\textsuperscript{45}. Each interaction 
analysis was performed three times.

BiC Assays. Specific primers were used for BiFC assessment (Supplementary Table S4). Full length cDNA 
sequences of PmDAMs and PmCBFs were cloned, pair-wise, into pCambia1300-YFP-C and pCambia1300-YFP-N 
to get BiFC constructs. Coexpression analysis was carried out on the leaves of Nicotiana benthamiana according 
to the procedure stated by Schutze et al\textsuperscript{45}. Chimeric fluorescence, emitted by fusion proteins, was examined 
under Leica TCS SP8 Confocal Laser Scanning Platform with YFPs being motivated at 514 nm.

Promoter cloning and Yeast 1 Hybrid assays. The genomic DNA of P. mume was isolated from flower 
bud using DNAsecure Plant Kit (DP320-02, TianGen, China). 2 kb up-stream promoter sequence of PmDAM6was 
extracted by PCR using specific primers (Supplementary Table S5) and the plasmid of pMDTM18-T-proDAM6 was 
obtained by former method\textsuperscript{46}. In 2 kb promoter of PmDAM6, there are four CCGAC sequences called CBF binding 
site. According these four CBF binding sites, six baits were designed. Three of them were obtained from the original 
genome, then each of them was duplicated to form the follow three baits. All six fragments were imported to the 
pAbAi-bait vectors. The special primers related to cloning these fragments were shown in Supplementary Table S6. 
The plasmids of pGADT7-PmCBFs were obtained in Y2H assays. These plasmids were transformed into the Y1H 
Gold strains containing pAbAi-bait, respectively, and these tests were screened on SD/-Leu/AbA plates. All trans-
formations and screenings were observed in triplicate. These Y1H assays were executed by a Matchmaker Gold Yeast 
One-Hybrid System kit (Clontech, America) following its user manual and correlation steps.

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Author Contributions
K.Z. and Q.Z. designed the experiments; Y.Z. completed the experiments; K.Z. and Y.Z. wrote the manuscript; S.A. improved the manuscript. K.Z., Y.Z., X.Y., X.H., Y.L. and L.S. analyzed the data. All authors read and approved the final manuscript.

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