

Identification and expression of C2H2 transcription factor genes in *Carica papaya* under abiotic and biotic stresses

Ling Jiang · Lin-jie Pan

Received: 29 September 2011 / Accepted: 24 January 2012 / Published online: 8 April 2012
© Springer Science+Business Media B.V. 2012

Abstract C2H2 proteins belong to a group of transcription factors (TFs) existing as a superfamily that plays important roles in defense responses and various other physiological processes in plants. The present study aimed to screen for and identify C2H2 proteins associated with defense responses to abiotic and biotic stresses in *Carica papaya* L. Data were collected for 47,483 papaya-expressed sequence tags (ESTs). The full-length cDNA nucleotide sequences of 87 C2H2 proteins were predicted by BioEdit. All 91 C2H2 proteins were aligned, and a phylogenetic tree was constructed using DNAMAN. The expression levels of 42 C2H2 were analyzed under conditions of salt stress by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Methyl jasmonate treatment rapidly upregulated *ZF*_{23.4} and *ZF*_{30.912.1} by 18.6- and 21.7-fold, respectively. *ZF*_{1.3}, *ZF*_{138.44}, *ZF*_{94.49}, *ZF*_{29.160}, and *ZF*_{20.206} were found to be downregulated after low temperature treatment at very significant levels ($p < 0.01$). *ZF*_{23.4}, *ZF*_{161.1}, and *ZF*_{30.912.1} were upregulated while *ZF*_{1.3}, *ZF*_{158.1}, *ZF*_{249.5}, *ZF*_{138.44}, *ZF*_{94.49}, *ZF*_{29.160}, and *ZF*_{20.206} were significantly downregulated by Spermine treatment. *ZF*_{23.4} was upregulated while *ZF*_{1.3}, *ZF*_{249.5}, *ZF*_{94.94}, *ZF*_{29.160}, *ZF*_{138.44}, and *ZF*_{20.206} were significantly repressed after SA treatment. *ZF*_{23.4} and *ZF*_{30.912.1} were significantly upregulated after sap

inoculation with papaya ringspot virus pathogen. *ZF*_{30.912.1} was subcellularly localized in the nucleus by a transgenic fusion of pBS-*ZF*_{30.912.1}-*GFP* into the protoplast of papaya. The results of the present study showed that *ZF*_{30.912.1} could be an important TF that mediates responses to abiotic and biotic stresses in papaya.

Keywords Cys₂/His₂ finger protein (C2H2) · Quantitative real time PCR (qRT-PCR) · Biotic stress · Abiotic stress · Papaya ringspot virus (PRSV) · *Carica papaya* L

Abbreviations

ABA	Abcisic acid
SA	Salicylic acid
C2H2	Cys ₂ /His ₂ finger protein
EAR	ERF associated amphiphilic repression
GFP	Green fluorescent protein
MeJA	Methyl-jasmonate
PEG	Polyethylene glycol
RNAi	RNA interference
Spm	Spermine
TF	Transcription factor
TFPs	Transcription factor proteins
VIGS	Virus induced gene silencing
ZF	Zinc finger
ZFP	Zinc finger protein
qRT-PCR	Real-time quantitative PCR

Electronic supplementary material The online version of this article (doi:10.1007/s11033-012-1542-y) contains supplementary material, which is available to authorized users.

L. Jiang (✉) · L. Pan
College of the Department of Horticulture and Forestry
of Huazhong Agricultural University, Key Laboratory
of Horticultural Plant Biology of Ministry of Education,
National Indoor Conservation Center of Virus-free Gemplasms
of Fruit Crops, Wuhan 430070, Hubei, China
e-mail: jiangling@mail.hzau.edu.cn

Introduction

Carica papaya L. is an important fruit in tropical and subtropical countries. Severe losses of production may

occur in *Carica papaya* L. under conditions of abiotic stressors, such as extremely low ($<-2^{\circ}\text{C}$) or high temperature ($>35^{\circ}\text{C}$), waterlogging, drought, and salt stress caused by excessive use of chemical fertilizers, and biotic stresses, such as papaya ringspot virus (PRSV) infection. Thus, efforts are continuously being made to develop papaya varieties that have better tolerance to these stresses.

The response of plants to abiotic and biotic stresses is more likely to be a polygenic cooperative defense response induced by cold, drought, salt, wounding, and disease rather than the single response of a single gene [1]. Therefore, in improving the stress tolerance of plants through molecular breeding, the traditional transformative method that applies a single resistance gene has limitations. Transcription factors (TFs) that regulate the expression of multiple genes are more promising candidates for manipulation [2]. TFs regulate gene expression in response to various external and internal cues by activating or suppressing downstream genes in the pathway. Zinc finger proteins (ZFPs) are a group of TFs that are expressed in different stressful conditions. Previous studies have indicated that some C2H2 ZFPs are induced by various stresses and may be important candidate genes. For example, the ZFP182 is markedly induced in paddy seedlings by cold, salt, and ABA treatments. The expression of ZFP182 in transgenic tobacco and overexpression in rice increases plant tolerance to salt stress [3]. C2H2 ZFP and pathogenic StZFP1 of potato are constitutively expressed in different organs of mature plants when subjected to salt, dehydration, and exogenously applied ABA. StZFP1 expression also occurs in response to infection by the late blight pathogen *Phytophthora infestans*, and the ectopic expression of StZFP1 increases plant tolerance to salt stress in transgenic tobacco [4].

C2H2 ZFPs constitute one of the largest TF families in plants [5], and their presence has been reported in several plant species, including *Arabidopsis*, petunia, rice, wheat, soybean, and pepper. In *Arabidopsis*, a total of 176 C2H2 ZFPs have been uncovered, constituting the abundant family of putative transcriptional regulators [6]. The genome of *Oryza sativa* codes for 189 C2H2 ZFP TFs [7]. The C2H2 protein is a major TF that regulates plant responses to various stresses and plant development [3, 4, 8]. In 2008, Ming and colleagues [9] compared gene numbers between the TF tribe and related tribes of *Arabidopsis* and papaya and reported that most TFs were represented by fewer genes in papaya than in *Arabidopsis*.

Previous studies have reported that C2H2 plays a very important role in the defense responses of many plants. C2H2 has been found to enhance the tolerance of plants to salt stress, some examples of which include TFIIIA-type ZFP with streptozotocin (STZ) [10], AtNAC2 [11], the EAR-motif of C2H2 in *Arabidopsis* [12], a R1R2R3-type

myeloblastosis (*MYB*) gene [13], ZFP179 [8], and ZFP DST in rice [14, 15]. ZFPs contribute significantly to the temperature stress tolerance of certain varieties of paddy [16–18], *Arabidopsis* [19], cotton [20], and soybean [21]. However, the role of papaya C2H2 in mediating defense responses to stresses has not been reported thus far.

Salicylic acid (SA) and methyl jasmonate (MeJA) are essential signal molecules involved in many aspects of plant development, as well as the responses of plants to biotic stresses, especially to pathogen infection, wounding, and insect attack [22–25]. SA plays a positive role in plant defenses against biotrophic pathogens, whereas jasmonic acid/ethylene (JA/ET) appears to be important in the defense against necrotrophic pathogens [26]. Early studies have indicated that polyamine is involved in plant responses to different environmental stresses. In addition, there is genetic potential for modulation of the polyamine levels of plants to cope with abiotic stress conditions [27–29].

Identification and evaluation of C2H2 related to stress tolerance are essential for molecular enhancements in papaya breeding. The present study aimed to identify prominent C2H2 that evince rapid responses to stressors, such as Spm, SA, MeJA, salt, low temperature, and PRSV infection. Data of the papaya C2H2 superfamily were collected using an ExPasy proteomics server (<http://cn.expasy.org>), a BLAST local database of expressed sequences tags (ESTs) of papaya was built, and the exact nucleotide sequence of C2H2 in the papaya superfamily were obtained using Bioedit software. The phylogenetic tree of C2H2 was constructed using DNAMAN software. Real-time PCR was used to quantitate the expression level of ZF in response to stressors and determine TF that are potential candidates of interest for improving stress tolerance through transgenic modification.

Materials and methods

Plant materials and treatments

Seeds of *Carica papaya* L. ‘Hawaii’ were obtained from Shanya City in Hainan Province, China. Sprouts were grown in sterile vermiculite and incubated in illumination incubators at 28°C under a photoperiod of 14 h/d. Treatments were carried out on 35-day-old seedlings with 5–6 leaves. In preliminary experiments, several concentrations of spermine (Spm), salt (NaCl) solution, SA, and MeJA were separately tested, and the highest possible concentration for each stressor that did not induce necrosis was selected. Each treatment involved the leaves of five plants, and samples were taken from experiments conducted in triplicate. Plants treated only with water were used as controls.

Different groups based on treatments

In Group A, 200 mmol/L salt solution (10 mL/plant) was carefully dropped from the apical point of the plants and allowed to flow naturally, aided by gravity. Group B comprised tests where 0.5 mmol/L Spm was carefully sprayed over two cotyledons and two euphylla at a dose of 10 mL/plant. In Group C, 1 mmol/L SA was sprayed onto the cotyledons and two euphylla at a dose of 10 mL/plant. Group D experiments involved distribution of droplets of 1 mmol/L MeJA at a dose of 1 mL/plant. Group E plants were kept at 4°C for 1 h in the dark, and then moved to the growth incubator at a temperature of (25 ± 1)°C; samples were collected at different intervals. Plants with and without treatment were placed in different growth chambers. The euphylla were harvested and frozen immediately in liquid nitrogen and stored at -70°C until RNA extraction. Basal levels of C2H2 expression were assessed in leaves, stems, and roots of papaya. The C2H2 expression levels recorded were normalized against *ACTIN* transcript accumulation. The PRSV pathogen was inoculated and samples were collected after 24 h.

Database searches and gene annotation

Through superfamily 1.75 (HMM library and genome assignments server; <http://supfam.org>), the protein sequences corresponding to each papaya C2H2 unigene were predicated. The specific ZF C2H2-type domain signature and QALGGH motif were investigated by searching the ExPasy proteomics server (<http://cn.expasy.org>). Weak hits were subsequently excluded. The C2H2 of papaya were aligned and a phylogenetic tree was constructed by using DNAMAN software (gap opening 10, gap extension 2, Blossum weight matrix). A BLAST local database of the papaya ESTs was constructed with BioEdit software using several EST sequences (EX227656–EX303501) for comparison, and the nucleotide sequence of C2H2 ZF were confirmed. The EAR motifs in ZFP proteins were identified according to known EAR-motifs and the DLN-box [3, 30, 31].

RNA extraction and qRT-PCR

Total RNA was extracted from papaya leaf, root, and stem samples using TRIzol reagent (Invitrogen) and subjected to DNase I digestion for purification. RNA samples were detected using an Ultraspec2100 UV/Visible spectrophotometer (Amersham GE Healthcare, USA), and 1% agarose gel was run to visualize the quality of the RNA. First-strand cDNA was synthesized from 2 µg of total RNA at a final volume of 20 µL using TransScript first-strand cDNA synthesis superMix (Transgen) and oligo-dT₍₁₈₎ primers according to the manufacturer's instructions.

A qRT-PCR assay of gene expression-specific primers was designed from the papaya cDNA sequences using Primer Express 3.0 software, with temperatures in the range of 58–60°C, primers lengths of 19–24 bp, and amplification fragment lengths measuring 150–200 bp. Where possible, primers were designed outside the conserved ZF C2H2-type domain to ensure gene-specific primers; primer pairs are listed in Supplementary Table 1. The primers were synthesized by Sangon Shanghai Biology Technology Ltd., at a final concentration of 0.2 µmol/L. qRT-PCR was performed using a Line Gene K system (Bioer Technology, Japan), and the software program for comparative quantitation PCR was run for 1.3 ng cDNA in a 20 µL amplification mixture containing SYBR Green (Invitrogen). The cycling conditions are as follows: 2 min at 50°C, followed by 3 min polymerase activity at 95°C and 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Each assay was conducted in triplicate and included a no-template control. The threshold cycle (Ct) of the primary amplification curve was used for calculations. The *ACTIN* gene was selected as the internal constitutively expressed control (normalization), according to the following formula:

$$\Delta\Delta Ct = (C_{t,\text{target}} - C_{t,\text{Actin}})_{\text{time}_x} - (C_{t,\text{target}} - C_{t,\text{Actin}})_{\text{time}_0}$$

Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [32]. In general, genes whose expressions changed more than twofold in response to stress treatment were specifically noted. Dilutions of cDNA (1:10–1:1,000) from a reference sample were used to construct a relative standard curve. For all primer pairs tested, efficiencies varied from 0.9 to 1.10 (data not shown). The specificity of PCR results was checked by melting-curve analyses, and only primer sets producing a single sequence-specific peak were conserved. Data were analyzed using the FQDpqr software package to obtain relative expression levels of the papaya gene based on the comparative Ct method; data are either expressed as fold changes relative to the calibrator average or as log (base 2)-fold changes relative to the calibrator average. Data were analyzed using Microsoft Excel.

Subcellular localization of the ZF_{30912.1}-GFP fusion protein

RNA was used as the reverse transcription element for the ZF_{30,912.1} and cDNA was the template. The gene fragment was cloned, recovered, and constructed into the pMD-T19 vector and confirmed by sequencing. The forward primer was 5'-ATGAAGAGAAGTAATTTG-3' and the reverse primer was 5'-GAAGAAGCAATCCAC-3'. To confirm that the ZF_{30,912.1} protein was indeed the target in the nucleus, the ZF_{30,912.1} was selected for analysis in vivo. The coding regions of ZF_{30,912.1} were fused to the N-terminus of synthetic green fluorescent protein (GFP) and expressed in

papaya under the control of the constitutive cauliflower mosaic virus 35S promoter. The fusion protein-expression vector pBS-ZF_{30,912.1}-GFP was constructed. Papaya protoplasts were isolated and either the pBS-GFP or the pBS-ZF_{30,912.1}-GFP plasmid was transformed using the PEG method. Previous reports have proven that pBS-GFP is expressed in the nucleus and cytomembrane [33, 34]. GFP expression and subcellular localization were observed using a confocal laser scanning microscope (LSM 510 META; Zeiss, Germany) with excitation and emission wavelengths of 475 and 520 nm, respectively (pixel depth: 12 bit, objective, plan-A; Pochromat 63×/1.4 oil DIC), and imaged using the associated software (Carl Zeiss Image Microscopy Release 4.0).

Results

Identification of C2H2 transcription factor

To assess the characteristics of the C2H2 superfamily proteins in *Carica papaya*, data were collected on a set of 91 expressed sequences of protein. Data of 47,483 papaya ESTs were collected and a BLAST local database was built with the BioEdit software. A total of 87 C2H2 were identified, and their full-length nucleic acid sequences were obtained from six reading frames using the tblastn process, according to the percentage of homology, GenBank accession number, and corresponding position of nucleotide sequence; details of these 87 C2H2 are shown in Supplementary Data Tables 2-1, 2-1, and 2-3.

Conserved ZF C2H2-type domain signatures and motifs were analyzed. Supplementary Table 2 shows 91 amino acid sequences with the typical family structure, including the diagnostic signatures of C-X_{2,4}-C-X₃-Φ-X₅-Φ-X₂-H-X_{3,4}-H, and with the corresponding genes encoding 1–5 C₂H₂ domains. Among these, 38 ZFPs had a ZF QALGGH motif, five ZFPs had a two-ZF QALGGH motif, and one ZFP had a three-ZF QALGGH motif. 11 C2H2 proteins possessed the HKH motif-containing C2H2 ZF, three C2H2 proteins comprised a BED-type ZF, and 39 contained the classic ZF motif, of which six contained two types of ZF. Among the 91 ZFP subfamily members, 15.4% contained an EAR-motif while 17.6% contained nucleus location signatures (DLN-box). The protein name, size (amino acids), GenBank accession number, frame, corresponding position of available nucleotide sequences, location of C2H2 domain signature, EAR motif, and DLN-box of each protein are listed in supplementary data Tables 2-1, 2-1, and 2-3.

Phylogenetic analysis of C2H2 proteins

An unrooted tree is shown for 91 ZF motifs to evolutionary relationships among different types of C2H2 ZF motifs of

papaya. The number of ZFPs in the proteins of this superfamily varied from one to five domain signature(s): Φ-X-C-X_{2,4}-C-X₃-Φ-X₅-Φ-X₂-H-X_{3,4}-H. The C2H2 domain sequences of the papaya proteins were aligned. An unrooted plot tree was also made, exclusively comprising all ZF domains from the first cysteine to the last histidine. In this unrooted plotted phylogenetic tree comparing the C2H2 proteins of papaya, proteins containing classic ZF motifs are shown in Supplementary Fig. 1a, d. Q-type C2H2 proteins with the QALGGH motif were arranged together in order, as shown in Supplementary Fig. 1b. Proteins contained two classic ZFs (Supplementary Fig. 1c); those containing one or more classic ZFs, or those with one or more HKH motifs, are shown in Supplementary Fig. 1e.

C2H2 TFs are involved in the early response of papaya to salt stress

The effects of salt stress were determined relative to *ACTIN* housekeeping genes in leaves 12, 24, and 48 h after treatment of 42 ZF analyzed genes with 200 mmol/L salt (Fig. 1a). According to the *t*-test analysis of the average value of the samples and control in pairs, the expression levels of ZF_{9.152}, ZF_{30,912.1}, ZF_{48.203}, ZF_{92.92}, ZF_{190.29}, and ZF_{1.3} were downregulated after 12 h of salt stress treatment at very significant levels ($p < 0.01$), those of ZF_{30.114}, ZF_{9.152}, ZF_{48.203}, ZF_{92.92}, ZF_{190.29}, and ZF_{1.3} were downregulated after 24 h of salt stress treatment at very significant levels ($p < 0.01$), those of ZF_{30,912.1} and ZF_{45.58} decreased at significant levels ($p < 0.05$), and those of ZF_{9.152}, ZF_{30,912.1}, ZF_{45.58}, ZF_{92.92}, ZF_{158.1}, and ZF_{190.29} were downregulated after 48 h at very significant levels ($p < 0.01$). The salt solution significantly activated the expressions of ZF_{29.160}, ZF_{20.206}, ZF_{9.185}, ZF_{138.44}, and ZF_{169.30} after 24 h and decreased thereafter, while the expression levels of ZF_{28.115} and ZF_{4.50} were upregulated very significantly after 48 h of the same treatment (Fig. 1c). The expression levels of other ZF were changed but not significantly, as shown in Fig. 1a–c.

C2H2 ZFs are involved in early response to low temperature in papaya

After subjecting papaya plants to low temperature, leaves were harvested after 1, 4, and 24 h. The expression level of ZF_{23.4} was significantly induced after 24 h ($p < 0.05$) while that of ZF_{161.1} was induced at 4 and 24 h at significant levels ($p < 0.05$). The expressions of ZF_{1.3}, ZF_{94.49}, and ZF_{20.206} were found to be downregulated at 1 h, those of ZF_{1.3}, ZF_{138.44}, ZF_{29.160}, and ZF_{20.206} were found to be downregulated at 4 h, and those of ZF_{1.3}, ZF_{94.49}, and ZF_{29.160} were found to be downregulated at 24 h; all

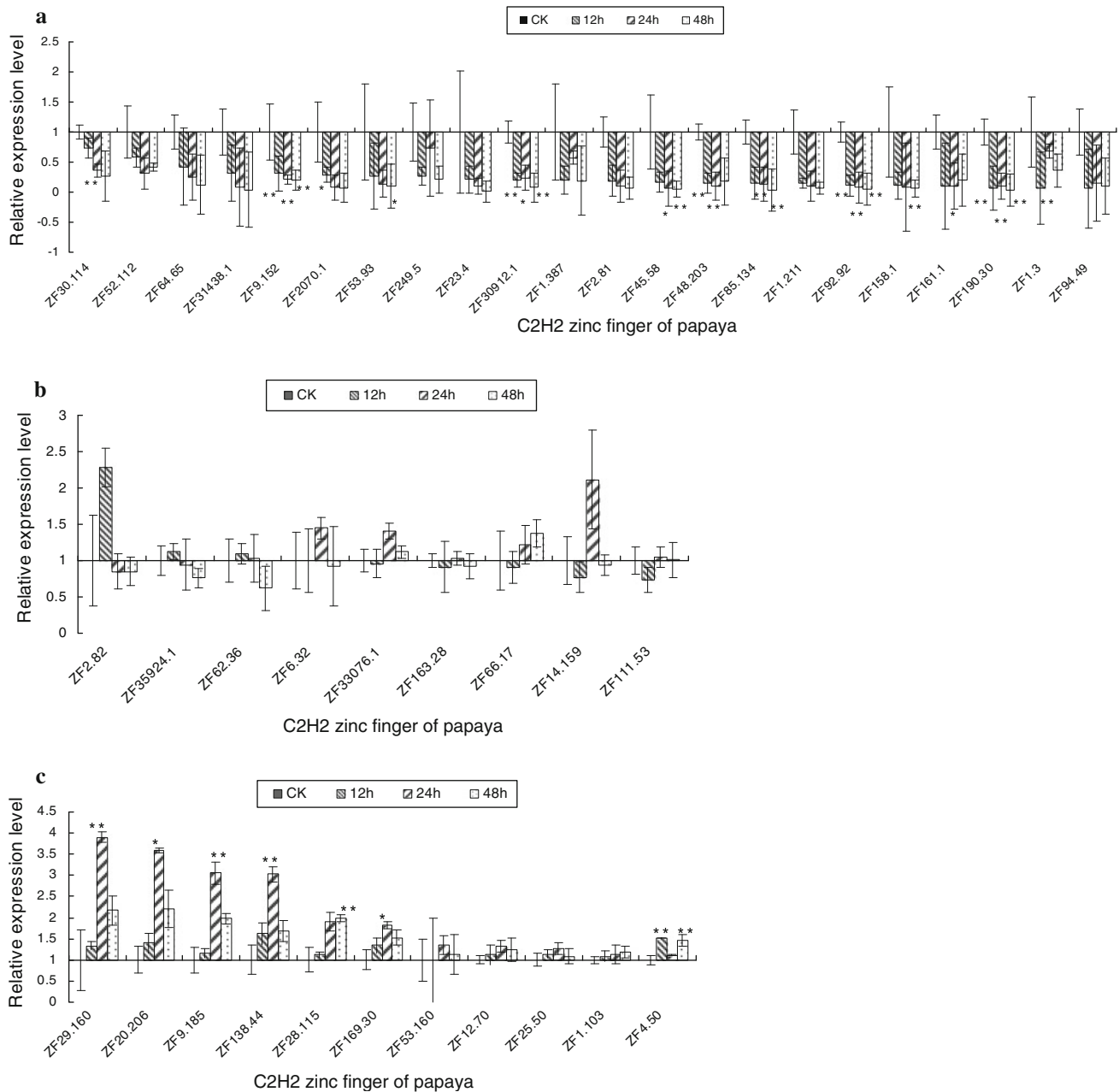


Fig. 1 Gene expression analysis by quantitative RT-PCR for the salt stress test. The code of *evm.TU.supercontig* C2H2 transcription factors is shown in the x axis. Expression levels were calculated relative to ACTIN expression. Expression levels are presented as fold-induction relative to the control. Mean \pm SE of triple

experiments are shown. **a** Expression levels of all 22 ZFs were relatively downregulated compared with ACTIN and calculated by the formula $2^{-\Delta\Delta C_t}$. **b** Different ZFs were upregulated and downregulated by salt treatment. Expression levels of ZFs increased after salt stress treatment

difference analysis results showed significant levels of variation ($p < 0.01$) (Fig. 2).

C2H2 ZFs are involved in early response of papaya to spermine

Spm plays an essential role in the response of plants to abiotic stress. After Spm treatment, ZF_{23.4} was

upregulated by 2.3- and 4.4-fold after 4 and 12 h, respectively, ZF_{161.1} was upregulated by 2.8-fold after 4 h at very significant levels ($p < 0.01$), and ZF_{30,912.1} was upregulated by more than twofold after 4 h at significant levels ($p < 0.05$). The expression levels of ZF_{1.3}, ZF_{138.44}, ZF_{94.49}, ZF_{29.160}, and ZF_{20.206} were downregulated at very significant levels ($p < 0.01$) after 12 h. ZF_{1.3}, ZF_{158.1}, ZF_{249.5}, ZF_{138.44}, ZF_{94.49}, ZF_{29.160}, and

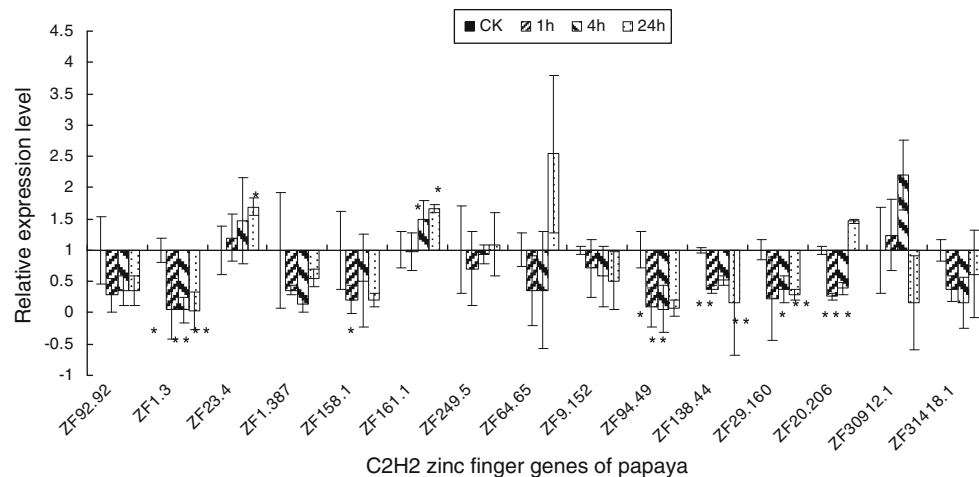


Fig. 2 C₂H₂ ZF expression when subjected to cold (4°C) stress

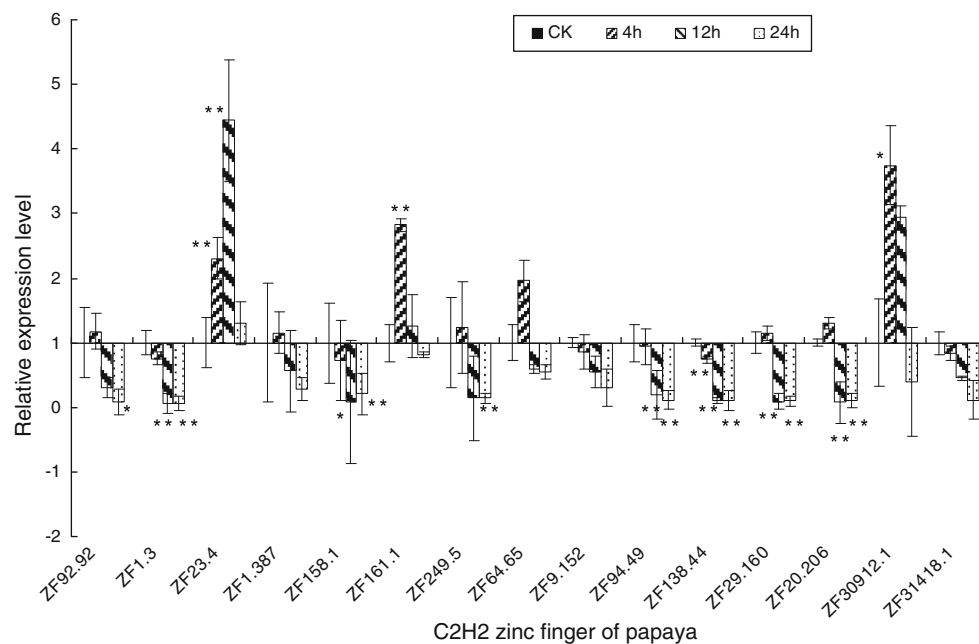


Fig. 3 The expression pattern of 15 ZF in papaya treated with and without Spm. The *first column* represents the expression levels of ZF compared with those of ACTIN without Spm treatment as the control.

The *three other columns* represent the expression levels of ZF in Spm-treated plants. ZF23.4, ZF161.1, and ZF30912.1 were upregulated by more than twofold compared with the control

ZF_{20.206} were also downregulated at very significant levels ($p < 0.01$) after 24 h (Fig. 3).

C₂H₂ ZFs are involved in the early response to SA

The expression patterns of ZFs were examined in response to SA. The ZF_{23.4} was upregulated 2.4-fold at 4 h after SA treatment but decreased thereafter. The expression levels of ZF_{23.4} were upregulated at 4, 12, and 24 h at very significant levels ($p < 0.01$) compared with the control (Fig. 4). ZF_{92.92}, ZF_{1.3}, ZF_{138.44}, ZF_{29.160}, and ZF_{20.206} were rapidly repressed after 4 h at very significant levels ($p < 0.01$),

ZF_{1.3}, ZF_{249.5}, ZF_{94.94}, ZF_{29.160}, and ZF_{20.206} were rapidly repressed after 12 h at very significant levels ($p < 0.01$), and ZF_{1.3}, ZF_{249.5}, ZF_{138.44}, ZF_{94.94}, ZF_{29.160}, and ZF_{20.206} were rapidly repressed after 24 h at very significant levels ($p < 0.01$).

ZF_{23.4} and ZF_{30912.1} are involved in the early response of papaya to MeJA

To further characterize the expression patterns of ZF in response to MeJA, a time course expression pattern was determined by qRT-PCR. The ZF displayed different

Fig. 4 The expression patterns of C2H2 following SA treatment. $ZF_{23.4}$ was upregulated rapidly by SA to 2.4-fold at 4 h; the expression levels of 11 other ZF were repressed rapidly at 4, 12, and 24 h after SA treatment. The first column represents the control

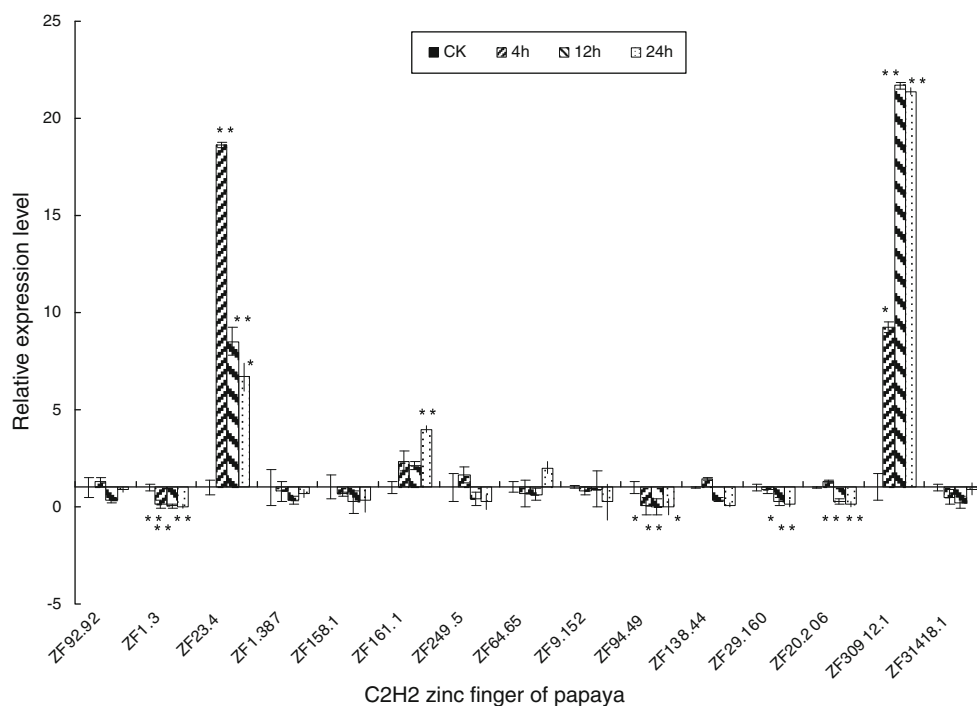
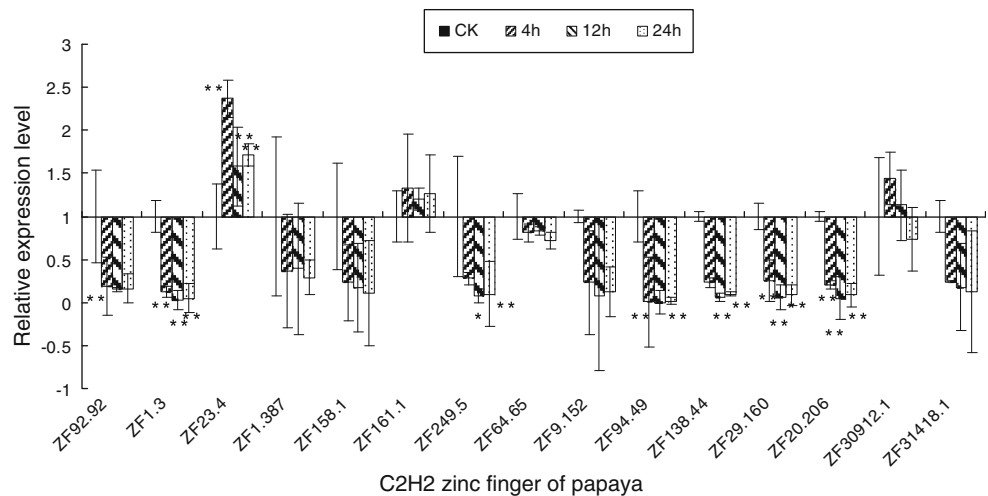


Fig. 5 The expression pattern of C2H2 ZF, with/without MeJA treatment in the papaya

induction patterns when subjected to MeJA treatment. As shown in Fig. 5, of the 15 genes analyzed, the expression of two genes were upregulated prominently: $ZF_{23.4}$ was rapidly induced by 18.6-, 8.5-, and 6.7-fold while $ZF_{30912.1}$ was rapidly induced by 9.3-, 21.7-, and 21.4-fold after 4, 12, and 24 h of MeJA treatment, respectively. Peak expression levels of these genes occurred after 4 and 12 h, respectively. The two genes showed exceptionally sensitive responses to MeJA treatment at very significant levels ($p < 0.01$). The expression level of $ZF_{161.1}$ was induced by 2.4-, 2.1-, and 4-fold after 4, 12, and 24 h, respectively. Otherwise, the expression level of $ZF_{1.3}$ was downregulated after 4, 12, and 24 h at very significant levels

($p < 0.01$). Furthermore, the expression level of $ZF_{138.44}$ was decreased after 12 h at very significant levels ($p < 0.01$) and the expression levels of $ZF_{29.160}$ and $ZF_{20.206}$ were downregulated after 12 and 24 h at very significant levels ($p < 0.01$).

C2H2 expression in different plant organs and leaves inoculated with PRSV pathogen

Basal expression levels of leaves, stems, and roots varied for different genes. The mRNA expression pattern of six C2H2 ZF in different organs was further characterized in this study. The expression level of $ZF_{92.92}$ in the stem was

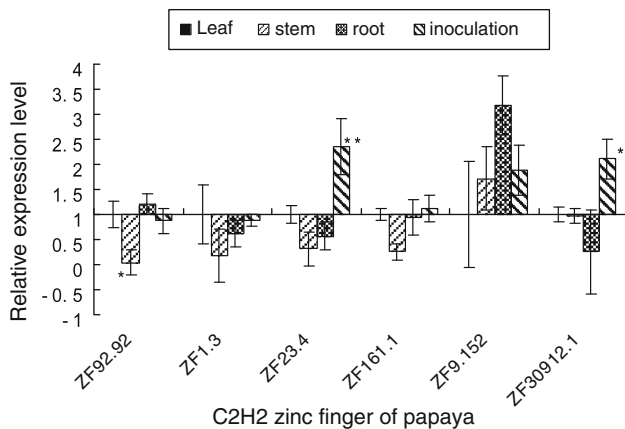


Fig. 6 Expression levels of six C2H2 in leaves, stems, and roots of papaya after inoculation with the PRSV pathogen

lower by 0.03-fold, corresponding to the expression of the *ACTIN* gene. The experiment revealed stem tissue-specific accumulation. The expression levels of *ZF*_{23.4} and *ZF*_{30,912.1} were induced by 2.4- and 2.1-fold 24 h after sap inoculation with PRSV pathogen at significant levels ($p < 0.05$) (Fig. 6).

Sequencing of *ZF*_{30,912.1} and localization of *ZF*_{30,912.1} C2H2 proteins

After 600 bp fragment amplification, the *ZF*_{30,912.1} was cloned and constructed into the pMD-T19 vector. Sequencing and analysis indicated that the 600 bp sequence shared 100% homology with the sequence from GenBank, gil1861364051, frame (−1), for the full length from 2116 to 1517 bp (Supplementary Table 2; Fig. 2a). The amino acid sequence of the C₂H₂-type *ZFP*_{30,912.1} were compared with

ZF domains of 12 proteins, which involved in environmental stress and organ development. The results are shown in Fig. 2b. *ZFP*_{30,912.1} was homologous to CAZFP1, SCOF1, AZF1, BCZFP1, ZPT2-1, ZPT2-2, AZF3, OSZFP34, ZPT3-3, ZPT2-10, and ZPT3-2 by 61.9, 61.9, 57.2, 57.1, 37.7, 61.9, 66.7, 61.9, 76.2, 76.2, and 57.1%, respectively.

Analysis of the *ZFP*_{30,912.1} amino acid sequence indicated that it contained two canonical Cys2/His2 ZF motifs comprising 21 amino acids and separated by 39 amino acids. In addition to these two well-conserved motifs, three other more or less complete regions were observed: a hydrophobic leucine-rich region (L-box), probably involved in protein–protein interaction, a C-terminal region DLN-box, and a residue conserved among all 13 sequences (Supplementary Fig. 2b).

Transient expression analysis of the *ZF*_{30,912.1}-GFP fusion protein revealed that *ZFP*_{30,912.1} was localized preferentially in the nucleus. Subcellular localization of the C2H2 *ZF*_{30,912.1} was examined using GFP. The control plasmid pBS-*GFP* and the *GFP* fusion gene pBS-*ZF*_{30,912.1}-*GFP* were separately transformed into the protoplasts of papaya. Under a confocal laser scanning microscope, the control plasmid pBS-*GFP* was observed to express GFP in the nucleus and cytomembrane (Fig. 7a–c), whereas the *GFP* fusion protein gene of pBS-*ZF*_{30,912.1}-*GFP* was transformed and expressed only in the nucleus (Fig. 7d–f).

Discussion

C2H2 ZFs have been reported to regulate gene expression under biotic and abiotic stresses in several plant species

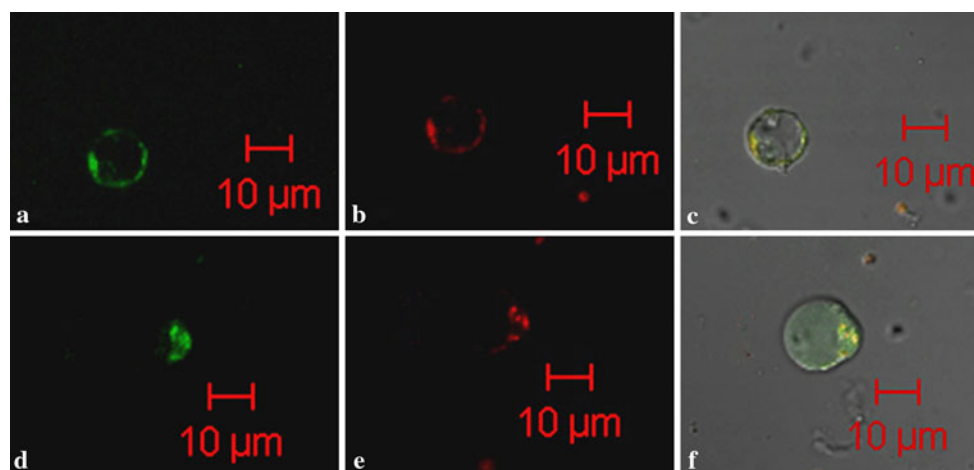


Fig. 7 Nuclear localization of the *ZF*_{30,912.1} protein in the protoplast of papaya in 25-days-old leaves. In the present study, 25-days-old leaves were observed by confocal microscopy. **a–c** The control vector pBS-*GFP* was transformed. **d–f** The subcellular localization of pBS-

*ZF*_{30912.1}-*GFP*. In each case, the left panel shows *GFP* fluorescence, the middle panel shows red fluorescence, and the right panel represents overlays of bright-field and fluorescence images

[35, 36]. C2H2 ZFPs in papaya, however, have not been functionally characterized thus far. The present study is the first to provide data of the induced expression of C2H2 ZF TFs under biotic and abiotic stresses in papaya. Nucleotide sequence information of C2H2 was obtained using a conventional bioinformatics method. Conserved ZF C2H2-type domain signatures and motif characteristics were described, and the phylogenetic relationships among 91 major members were analyzed. Expression levels of some C2H2 ZF in response to abiotic stresses, such as salt and cold (4°C), and biotic stresses or their signal molecules, such as Spm, SA, and MeJA, as well as PRSV infection, were investigated. The present study provides information that may help facilitate cloning and further functional studies of C2H2 in papaya.

Abiotic stresses involve the negative impacts of non-living factors on living organisms that occur within a specific environment, while biotic stresses are caused by living organisms, such as fungi, bacteria, viruses, and nematodes, among others. In the present experiment, two common abiotic stresses, salt and cold (4°C), were artificially induced, and SA, MeJA, and Spm were used as biotic signal molecules, in addition to PRSV infection, to study abiotic and biotic stresses. ZFP_{30,912.1} was transiently upregulated by MeJA, Spm, and PRSV infection by 21.7-, 3.8-, and 2.1-fold, respectively. ZFP_{23.4} expression levels were enhanced by 18.6-, 2.4-, 4.4-, 2.4-, and 1.7-fold after MeJA, SA, Spm, PRSV, and cold treatments. However, salt stress downregulated the expressions of ZFP_{23.4} and ZFP_{30,912.1} by 0.01- and 0.08-fold, respectively. ZFP_{30,912.1} and ZFP_{23.4} were considered the top-priority genes for function validation.

Studies have previously reported that all C₂H₂ responses to stresses in plants have a double ZF structure [37, 38]. ZFP_{30,912.1}, a typical ZFP, has two C₂H₂ ZF motifs, each having a conserved QALGGH sequence; this QALGGH motif has so far been reported only in plants [5]. However, ZFP_{23.4} contains three classic ZFs and no QALGGH motifs or EAR-box. It is possible that the ZFP_{23.4}, and ZFP_{30,912.1} C₂H₂ play diverse roles in modulating defense responses against multiple biotic and abiotic stresses. Further, ZFP_{161.1} comprises four classic ZFs, the expression levels of which were upregulated by 2.8- and 4-fold after either Spm or MeJA biotic treatment. ZFP_{1.3}, ZFP_{138.44}, ZFP_{29.160}, and ZFP_{20.206} contained a QALGGH sequence and were downregulated very significantly by cold stress; thus, these genes are likely related to cold stress. The results of the present study indicate that the responses of C2H2 to stresses in plants are not restricted to the double ZF structure.

Researchers have previously reported that TF genes function in various ways to confer stress tolerance to plants [39–41]. SA and MeJA are key signal molecules involved in plant disease-defense responses [42]. ZFP_{23.4} and

ZFP_{30,912.1} were strongly induced by 18.6- and 21.7-fold after MeJA treatment, while the ZFP_{23.4} was induced by 2.4-fold and ZFP_{30,912.1} was induced by less than twofold after SA treatment. These findings indicate that MeJA is a more sensitive signal molecule than SA for ZFP_{23.4} and ZFP_{30,912.1} expression under stress.

A previous report demonstrated that the EAR domain of a C2H2 ZFP plays an important role in the defense response of *Arabidopsis* to abiotic stress [12]. In the present experiment, all ZFPs containing the DLN-box/EAR motif, including ZFP_{6.32}, ZFP_{14.159}, ZFP_{20.206}, ZFP_{53.160}, ZFP_{92.92}, ZFP_{138.44}, ZFP_{163.28}, ZFP_{190.29}, ZFP_{249.5}, ZFP_{30,912.1}, ZFP_{207.1}, ZFP_{33,076.1}, and ZFP_{1.3}, were distributed in Group B of the phylogenetic unrooted tree; only ZFP_{1.103} was located in Group E. ZFP_{29.160} and ZFP_{12.70} only contained the DLN-box and were located in Group B. All ZFPs in Group B belong to Q-type C2H2 proteins with the QALGGH motif. The ZFPs analyzed were distributed in the B, C, D, and E positions of the phylogenetic tree relative to one or more stress conditions, as shown in Supplement Fig. 1. Further functional validation of these genes may help identify candidate genes that could contribute to the defense responses of transgenic papaya plants.

Proteins with similar domains may have identical or similar biologic functions [43]. The ZFP_{30,912.1} was cloned and subcellular localization assay indicated that ZFP_{30,912.1} localizes in the nucleus. These results prove that ZFP_{30,912.1} may function in the nucleus. The results of organic expression analysis indicated that ZFP_{30,912.1} is constitutively expressed in roots, stems, and leaves of papaya. The sequence of ZFP_{30,912.1} was compared with those of 12 ZFPs with known functions, including improvements in disease resistance and drought, salt, and cold tolerance, as well as a number of development-related events. ZFP_{30,912.1} shares homologies with 12 ZFPs of between 37.7 and 76.2%. Whether or not ZFP_{30,912.1} possesses functions identical or similar to these 12 ZFPs can be verified by gene transformation and gene silencing techniques, such as RNAi and VIGS. The data generated in the present experiments will facilitate further functional genomics studies to understand the precise role of C2H2 in modulating stress responses in papaya and other plants.

Acknowledgment The project was supported by the National Natural Science Foundation of China (grants No. 30771477 and No. 30571277).

References

- Zhang James Z, Creelman Robert A, Zhu Jian-Kang (2004) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 135:615–621

2. Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr Opin Plant Biol* 9:436–442
3. Huang J, Yang X, Wang MM, Tang HJ, Ding LY, Shen Y, Zhang HS (2007) A novel rice C2H2-type zinc finger protein lacking DLN-box/EAR-motif plays a role in salt tolerance. *Biochim Biophys Acta* 1769:220–227
4. Tian ZD, Zhang Y, Liu J, Xie CH (2010) Novel potato C2H2-type zinc finger protein gene, StZFP1, which responds to biotic and abiotic stress, plays a role in salt tolerance. *Plant Biol (Stuttg)* 12(5):689–697
5. Kubo K, Sakamoto A, Kobayashi A, Rybka Z, Kanno Y, Nakagawa H, Takatsuji H (1998) Cys2/His2 zinc-finger protein family of petunia: evolution and general mechanism of target-sequence recognition. *Nucleic Acids Res* 26:608–615
6. Englbricht CC, Schoof H, Bohm S (2004) Conservation diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. *BMC Genomics* 5:39
7. Agarwal P, Arora R, Ray S, Singh AK, Singh VP, Takatsuji H, Kapoor S, Tyagi AK (2007) Genome-wide identification of C2H2 zinc-finger gene family in rice and their phylogeny and expression analysis. *Plant Mol Biol* 65:467–485
8. Sun SJ, Guo SQ, Yang X, Bao YM, Tang HJ, Sun H, Huang J, Zhang HS (2010) Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice. *J Exp Bot* 61(10):2807–2818
9. Ming R, Hou S, Feng Y et al (2008) The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature* 452:991–996
10. Xu SM, Wang XC, Chen J (2007) Zinc finger protein 1 (ThZF1) from salt cress (*Thellungiella halophila*) is a Cys-2/His-2-type transcription factor involved in drought and salt stress. *Plant Cell Rep* 26:497–506
11. He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. *Plant J* 44:903–916
12. Cifrci-Yilmaz S, Morsy MR, Song L, Coutu A, Krizek BA, Lewis MW, Warren D, Cushman J, Connolly EL, Mittler R (2007) The EAR-motif of the Cys2/His2-type zinc finger protein Zat7 plays a key role in the defense response of *Arabidopsis* to salinity stress. *J Biol Chem* 282:9260–9268
13. Dai X, Xu Y, Ma Q, Xu W, Wang T, Xue Y, Chong K (2007) Overexpression of a R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*. *Plant Physiol* 143:1739–1751
14. Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. *Genes Dev* 23:1805–1817
15. Oh S, Song S, Kim Y, Jang H, Kim S, Kim M, Kim Y, Nahm B, Kim J (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol* 138:341–351
16. Li G, Tai FJ, Zheng Y, Luo J, Gong SY, Zhang ZT, Li XB (2009) Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. *Biochem Biophys Res Commun* 389(3):556–561
17. Cuevas JC, Lopez-Cobollo R, Alcazar R, Zarza X, Koncz C, Altobelli T, Salinas J, Tiburcio AF, Ferrando A (2009) Putrescine as a signal to modulate the indispensable ABA increase under cold stress. *Plant Signal Behav* 4:219–220
18. Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol* 47:141–153
19. Vogel JT, Zarka DG, Buskirk VHA, Fowler SG, Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J* 41(2):195–211
20. Kim JC, Lee SH, Cheng YH, Yoo CM, Lee SI, Chun HJ, Yun DJ, Hong JC, Lee SY, Lim CO, Cho MJ (2001) A novel cold inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plant. *Plant J* 25:247–259
21. Li G, Tai FJ, Zheng Y, Luo J, Gong SY, Zhang ZT, Li XB (2010) Two cotton Cys2/His2-type zinc-finger proteins, GhDi19-1 and GhDi19-2, are involved in plant response to salt/drought stress and abscisic acid signaling. *Plant Mol Biol* 74:437–452
22. Lorenzo O, Solano R (2005) Molecular players regulating the jasmonate signalling network. *Curr Opin Plant Biol* 8:532–540
23. Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* 16:1938–1950
24. Koornneef A, Pieterse CM (2008) Cross talk in defense signaling. *Plant Physiol* 146:839–844
25. Wang Z, Cao GY, Wang XL, Miao J, Liu XT, Chen ZL, Qu LJ, Gu HY (2008) Identification and characterization of COI1-dependent transcription factor genes involved in JA-mediated response to wounding in *Arabidopsis* plants. *Plant Cell Rep* 27:125–135
26. Yang B, Jiang YQ, Rahman MH, Deyholos MK, Kav NN (2009) Identification and expression analysis of *WRKY* transcription factor genes in canola (*Brassica napus* L.) in response to fungal pathogens and hormone treatments. *BMC Plant Biol* 9:68
27. Groppa MD, Benavides MP (2008) Polyamines and abiotic stress: recent advances. *Amino Acids* 34:35–45
28. Alonso-Blanco C, Aarts MGM, Bentsink L, Keurentjes JJB, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? *Plant Cell* 21(7):1877–1896. www.plantcell.org/cgi/doi/10.1105/tpc.109.068114
29. Gill SS, Tuteja N (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal Behav* 7:5(1)
30. Kam J, Gresshoff PM, Shorter R, Xue GP (2008) The Q-type C2H2 zinc finger subfamily of transcription factors in *Triticum aestivum* is predominantly expressed in roots and enriched with members containing an EAR repressor motif and responsive to drought stress. *Plant Mol Biol* 67:305–322
31. Wang GF, Wang H, Zhu J, Zhang J, Zhang XW, Wang F, Tang YP, Mei B, Xu ZK, Song RT (2010) An expression analysis of 57 transcription factors derived from ESTs of developing seeds in Maize (*Zea mays*). *Plant Cell Rep* 29:545–559
32. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408
33. Yoo Sang-Dong, Cho Young-Hee, Sheen Jen (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* 2(7):1565–1572
34. Jiang L, Wang J, Liu Z, Wang L, Zhang F, Liu GC, Zhong Q (2010) Silencing induced by inverted repeat constructs in protoplasts of *Nicotiana benthamiana*. *Plant cell tissue organ cult* 100:139–148
35. Tian LM, Huang CL, Zhang XH, Zhang LS, Wu ZY (2005) Advances of plant zinc finger proteins involved in abiotic stress. *Biotechnol Bull* 6:13–16

36. Chen WQ, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou GZ, Whitham SA, Budworth PR, Tao Y, Xie ZY, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dang JL, Wang X, Zhua T (2002) Expression profile matrix of arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* 14:559–574
37. Huang J, Wang JF, Zhang HS (2004) Structure and function of plant C2H2 zinc finger protein. *Hereditas (Beijing)* 26(3):414–418
38. Miller J, Mclachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor 111A from xenopus oocytes. *EMBOJ* 4:1609–1614
39. Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in arabidopsis and grasses. *Plant Physiol* 149:88–95
40. Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low temperature, or high-salt stress. *Plant Cell* 6:251–264
41. Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* 53:247–273
42. Dong XN (1996) SA, JA, ethylene, and disease resistance in plants. *Curr Opin Plant Biol* 1:316–323
43. Lin R, Zhao W, Meng X, Wang M, Peng Y (2007) Rice gene OsNAC19 encodes a novel NAC-domain transcription factor and responds to infection by *Magnaporthe grisea*. *Plant Sci* 172:120–130. doi:101016/j.plantsci.2006.07.019