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ORIGINAL ARTICLE

# Ectopic expression of the maize *ZmADF3* gene in *Arabidopsis* revealing its functions in kernel development

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**Abstract** Actin-depolymerizing factors (ADFs) are the members of actin-binding proteins, which play pivotal roles in various cellular processes in plant cells. In this study, we isolated an *ADF* gene *ZmADF3* from the kernels of a popcorn inbred N04. This gene had a 417 bp open reading frame (ORF) which encoded for 139 amino acids. Phylogenetic analysis showed that ZmADF3 had a high sequence identity with TaADF3 and OsADF4. Quantitative real-time PCR and western blotting showed that *ZmADF3* expressed variously according to tissues and developmental stages during kernel development both in RNA and in protein levels. A 1738 bp promoter for *ZmADF3* was obtained and the cis-acting regulatory elements were predicted. Furthermore, yeast two-hybrid screening revealed that ZmADF3 interacted with glyceradehyde-3-phosphate dehydrogenase (GAPDH). Heterologous overexpression of *ZmADF3* showed an increase in seed size in transgenic *Arabidopsis* plants mainly through the increase in cell size. In addition, the positive regulation of seed size genes *SHB1* and *IKU1* were up-regulated while the negative regulators *AP2* and *ARF2* were down-regulated in the transgenic lines. Our results showed that *ZmADF3* might play important roles in kernel development in maize. However,

further research should be done to thoroughly explore the mechanism of *ZmADF3* in playing these functions.

**Keywords** *ZmADF3* · Yeast two-hybrid · Glyceradehyde-3-phosphate dehydrogenase · Heterologous overexpression · Seed size

## Introduction

The intracellular cytoskeleton consists of microtubules, actin filaments and intermediate filaments, which is involved in various cellular organizations and processes such as cell shape, cell division, differentiation, expansion, communication and metabolism (Ishikawa 1990; Kost et al. 1999; Volkmann and Baluska 1999; Staiger 2000; Smith 2003). Actin filaments play a decisive role in these cell functions (Tiezzi 1991; Kost et al. 2002). Globular actin (G-actin) and filamentous actin (F-actin) are the two forms of actin in eukaryotic cells, and G-actin as an actin monomer is the basic subunit of the double-stranded helical actin filament (Staiger 2000; Blanchoin et al. 2014). The activity of actin filaments are modulated by numerous actin binding proteins such as profilin, actin depolymerizing factor (ADF)/cofilin, cyclase associated protein (CAP), actin interacting protein (AIP), Rho of plants (ROP) GTPases, gelsolin, myosin, formins, fibrin, Arp2/3 complex and villin (Hussey et al. 2006; Ren and Xiang 2007; Ketelaar 2013). ADFs/cofilin, as the members of the actin-binding proteins, have a low molecular mass (15–20 kD) and ubiquitously distribute in eukaryotes (Bowman et al. 2000). They can bind to G-actin and F-actin and play a pivotal role in actin filament dynamics in cells (Carlier et al. 1997; Maciver and Hussey 2002; Zheng et al. 2013). As a functional node in cell biology, *ADFs* can be

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regulated by phosphorylation and ubiquitination, $PIP_2$, pH, oxidation, and nuclear translocation (Bernstein and Bamburg 2010; Sun et al. 1995; Bowman et al. 2000).

There are much more *ADFs* in plants than in animals (Maciver and Hussey 2002). Quantities of *ADFs* had been isolated and characterized from plants, such as lily (Kim et al. 1993), *Brassica napus* (Kim et al. 1993), maize (Rozycka et al. 1995; Lopez et al. 1996), *Petunia* (Mun et al. 2000), *Arabidopsis* (Dong et al. 2001a; Bou et al. 2011), wheat (Ouellet et al. 2001), *Nicotiana tabacum* (Chen et al. 2002), rice (Feng et al. 2006) and cotton (Zhang et al. 2007; Li et al. 2010). In plants, *ADF* genes always exist in the form of large families (Mun et al. 2000; Feng et al. 2006). For example, there are 12 ADF genes have been isolated and characterized in *Arabidopsis* as well as in rice (Feng et al. 2006; Ruzicka et al. 2007). According to the tissue-specific expression and the subcellular localization, the *ADF* family in *Arabidopsis* was divided into four subclasses (Ruzicka et al. 2007).

Many relevant researches have been confirmed that *ADFs* were involved in plant growth and development (Huang et al. 2012). Overexpression of *AtADF1* was associated with the disappearance of thick actin cables and caused abnormal cellular and tissue morphogenesis, while antisense expression of *AtADF1* leaded to a delay in flowering, and promoted the cell expansion and organ growth (Dong et al. 2001b). Ectopic expression of *ADF1* of *Gossypium barbadense* in tobacco resulted in shorter hypocotyls and fewer root hairs (Chi et al. 2013). The downregulation of *GhADF1* increased fiber length and strength due to more abundant F-actin filaments in transgenic *Gossypium hirsutum* plants (Wang et al. 2009). Overexpression of *NtADF1* resulted in the disruption of the actin cytoskeleton and the inhabitation of pollen tube growth in elongating pollen tubes (Chen et al. 2002). Silence expression a rice *ADF* gene resulted in the suppressed expression of α-amylase gene, which gave rise to the decrease of seed germination and depressed the immune responses (Cheng et al. 2013). In addition, further studies had also indicated that plant *ADFs* could respond to biotic and abiotic stimuli, such as light, drought, cold, salt and pathogen attack (Dong and Hong 2013; Burgos-Rivera et al. 2008; Huang et al. 2012; Porter et al. 2012; Fu et al. 2014; Henty-Ridilla et al. 2014).

In maize, three *ADFs* have been studied. *ZmADF1* and *ZmADF2* expressed specifically in pollen, while *ZmADF3* expressed in all tissues except of pollen (Rozycka et al. 1995; Lopez et al. 1996). *ZmADF3* was a key regulator of actin dynamics in plant cells, which could reset the cytoskeletal network during root hair initiation and elongation (Jiang et al. 1997a). *ZmADF3* could be phosphorylated on serine Ser6 by a *calmodulin-like domain protein kinase* (*CDPK*), which was essential for the control of actin

dynamics (Smertenko et al. 1998; Allwood et al. 2001). In our previous study, the protein profiles of endosperm and pericarp at three key developmental stages in popcorn were identified using iTRAQ labeling coupled with LC–MS/MS (Dong et al. 2015). As one of the differentially expressed protein and due to the important role of the *ADF* family in plant, we isolated the full-length cDNA sequence of *ZmADF3* from a popcorn inbred and analyzed its expression patterns in the development of pericarp and endosperm. The promoter of *ZmADF3* was also obtained from the genomic DNA of the inbred N04. Yeast two-hybrid library screening was carried out. Heterologous overexpression of *ZmADF3* in *Arabidopsis* was used to investigate the possible functions of *ZmADF3* in kernel development. These results were useful in further research to explore the biological functions of *ZmADF3* in maize.

## Materials and methods

### Plant materials and growth conditions

Plant materials and sample collections had been described in our previous research (Liu et al. 2010). The popcorn inbred line N04 with small-sized grains was planted in the Scientific Research and Education Center of Henan Agricultural University in Zhengzhou, Henan, China in 2014. The rows were 4 m long with 0.67 m spacing between rows and standard cultivation management practices were performed. Each plant was self-pollinated by hand. Ears were harvested at 10, 20 and 33 days after pollination (DAP). Kernels were isolated from the middle part of the ears. At each time point, kernels were collected from at least three ears. Endosperm and pericarp were separated from the kernels, respectively. The collected samples were immediately frozen in liquid nitrogen and stored at −80 °C.

The Columbia ecotype of *Arabidopsis thaliana* was used for gene transformation. *Arabidopsis* seeds were sterilized by 6.25 % sodium hypochlorite solution with 0.01 % TritonX-100 for 15 min, washed three times with sterilized $H_2O$ and then grown on 0.8 % (w/v) agar plates containing 1/2 Murashige and Skoog medium (MS, Sigma) and 3 % sucrose. Seven-day-old seedlings were planted in square plates filled with soil mixture. The seedlings were cultured in a growth chamber under controlled conditions, 22 °C and 16 h light/8 h dark cycle.

### RNA isolation and cDNA synthesis

Total RNAs were extracted from the endosperm and pericarp at different development stages in maize, and the different tissues of transgenic *Arabidopsis* plants using a

Column Plant RNAout kit (Tiandz) according to the manufacturer's instructions. DNA was digested using RNAse-free DNAse (TaKaRa). The quantity and quality of the total RNA were verified by 1 % agarose gel electrophoresis and spectrophotometric measurement (ND-1000 NanoDrop Technologies). The first-strand cDNA was synthesized from 2 μg of DNA-free RNA using a Prime-Script RT reagent Kit (TaKaRa).

## Cloning and structural analysis of *ZmADF3*

We chose an important protein peptide (GRMZM2G060702_P02) from the isobaric tags for relative and absolute quantification (iTRAQ) protein libraries constructed in our laboratory as the objective protein in this study. The function of this protein was predicated as ADF3, and its expression tendency was greatly different between the two tissues and among the three developmental stages (Dong et al. 2015). Using TBLASTN in NCBI, the most highly homology sequences were assembled into an extended sequence by in silico cloning. The specific primers *ADF3*-F and *ADF3*-R (Table S1) were designed to amplify the assembled sequences. PCR was carried out using 1 μl of the obtained cDNA, 2.5 μl 10 × PCR buffer, 2.5 μl dNTPs mixture (2.5 mM each), 1 μl of each primer (10 μM), 0.125 μl TaKaRa La Taq, and ddH2O was added to make up the final volume of 25 μl. The PCR program was used as follows: 5 min at 95 °C, then 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 90 s at 72 °C, and a final extension of 10 min at 72 °C. PCR products were cloned into the pMD18-T vector (TaKaRa) and sequenced. The deduced protein of ZmADF3 and other ADF proteins were aligned using ClustalW. A phylogenetic tree based on the result of ClustalW protein alignments was constructed using the Neighbor-Joining method in the software MEGA 6.0. The Poisson correction method was applied to compute the evolutionary distances.

## Isolation of the promoter sequence of *ZmADF3*

The genomic DNA of inbred N04 was isolated from leaves using a Plant Genomic DNA kit (TianGen) according to the manufacturer's instructions. To isolate the promoter sequence of *ZmADF3* from inbred N04, a BLAST analysis with the mRNA sequence of *ADF* (X97726) was performed in MaizeGDB (www.maizegdb.org). The specific primers Pro-F and Pro-R (Table S1) were designed to amplify the promoter sequence of *ZmADF3* by PCR amplification of the N04 genomic DNA. The cis-elements in the promoter sequence were analyzed using the Plant-CARE (Lescot et al. 2002).

## Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed in ABI 7500 Real-time PCR System and using SYBR Premix ExTaq II (TaKaRa) and gene-specific primers (Table S1). The 25.0 μl reaction contained 12.5 μl 2 × SYBR mix, 1 μl PCR forward primer, 1 μl PCR reverse primer, 1 μl of a five times dilution of the cDNA as the template, and 9.5 μl sterilization of double distilled water. The amplification procedure was as follows: 95 °C for 3 min, then 40 cycles each of denaturation at 95 °C for 10 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s. The 18S rRNA gene was used as the endogenous control, and each sample was repeated for three times. The relative transcript levels of synthesis were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

## Western blot analysis

Total proteins were extracted by BPP method as described by Wang et al. (2007). Total proteins were electrophoresed in 12 % SDS-PAGE and the gels were transferred onto PVDF microporous membranes. After blocking for 2.5 h in TBST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05 % Tween 20) with 5 % nonfat dry milk at room temperature, membranes were incubated with special antibody of ZmADF3 at 1:1000 dilution for overnight at 4 °C. Following three times of washing with TBST, membranes were incubated with secondary antibodies (Goat anti-Rabbit IgG-HRP, Abmart) at 1:5000 dilution for 1.5 h at room temperature away from light. After three times of washing with TBST, signals were detected using an ECL Western Blotting Kit (Amersham) following the manufacturer's instructions. Anti-ZmADF3 rabbit polyclonal antisera were generated against the peptide of ZmADF3 by the Abmart biomedical company (Shanghai China).

## Yeast two-hybrid library screening

Yeast two-hybrid library screening was carried out using Matchmaker™Gold Yeast Two-Hybrid System (Clontech). The full-length ORF of *ZmADF3* was cloned into the pGBKT7 vector and expressed as a BD fusion protein and then transformed into the yeast strain Y2HGold. The cDNA library cloned inframe with the GAL4 activation domain in the pGADT7 vector constructed previously in our laboratory from the kernels of inbred N04 (Wang 2015) was used to screen for the interaction clones with ZmADF3. Two-hybrid screening was performed as described in the Clontech's Matchmaker™Gold Yeast Two-Hybrid user manual (Clontech). The positive clones were sequenced and characterized. For a further confirmation, the full-length ORFs

of the positive clones were cloned into the pGADT7 vector, respectively. Then, as the described by Cai et al. (2014), they were co-transformed with the pGBKT7-ZmADF3 vector into the Y₂HGold yeast strains. The mated culture was spread on DDO (SD/-Trp/-Leu), QDO/X (SD/-Trp/-Leu/-His/-Ade supplemented with X-α-gal) and QDO/X/A (SD/-Trp/-Leu/-His/-Ade supplemented with X-α-gal and Aureobasidin A) medium incubated at 30 °C for 5–10 d.

### Vector construction and genetic transformation

The ORF of *ZmADF3* with the termination codon was generated by PCR using the following primers: 5′ GC CCATGGATGGCAAACGCGAGATCGGGTG 3′ (NcoI site underlined) and 5′GCACTAGTCTAGCGAGCCCGA TCCTTGATCT 3′ (SpeI site underlined). After verifying by sequencing, the PCR fragment was inserted into pCAMBIA1302 under the control of the CaMV 35S promoter. The fused plasmid (35S::*ZmADF3*) and the empty plasmid were introduced into the Agrobacterium tumefaciens strain EHA105 by freeze-thawing with liquid nitrogen, and then transferred into *Arabidopsis* plants (Col-0) using the floral dip method (Clough and Bent 1998), respectively. *ZmADF3* transgenic *Arabidopsis* plants were selected on 1/2MS medium with hygromycin resistance and confirmed by PCR using specific primers (Table S1). Western blotting was used to confirm the expression of *ZmADF3* in the T3 transgenic *Arabidopsis*.

### Phenotypic analysis of seeds and mature embryos

The wild-type (WT) plants and three independent T3 homozygous progenies of transgenic lines were selected for morphological analysis. The average seed weight was determined by weighing the mature dry seeds in batches of 1000 using an electronic analytical balance (OHAUS DV215CD), and five sample batches were measured for each seeds lot. Seeds were observed and photographed under a stereoscopic microscope (Nikon SMZ1500), and the seed size was measured using the Image-Pro Plus6.0 software (http://www.mediacy.com).

The dried mature seeds were soaked in the water for 1 h and dissected under an SMZ-T2 microscope to isolate the mature embryos. The isolated embryos were treated as described by Ohto et al. (2005) and then photographed using a Leica differential interference contrast (DIC) microscope. About 30 transgenic and WT embryos were photographed to measure cell size and count the number of cells using Image-Pro Plus6.0 software.

## Results

### Cloning and characterization of *ZmADF3*

A 678 bp cDNA fragment was obtained via PCR from inbred N04, which contained an ORF of 417 bp encoded for 139 amino acids (Fig. 1). In addition, a 148 bp 5′ untranslated region (UTR) and a 113 bp 3′ UTR were predicted. The calculated molecular weight of the encoded protein was 15.91 kDa, with an isoelectric point (pI) of 5.47. The function domain for *ZmADF3* was predicted by software CDD of NCBI on line (http://www.ncbi.nlm.nih. gov/Structure/cdd/wrpsb.cgi). The result showed that the protein belonged to the ADF/cofilin-like subtribe of the ADF-gelsolin family, which had two putative actin monomer (G-actin) binding sites (Ser6 and Gly7) and five putative actin filaments (F-actin) binding sites (Lys82, Arg84, Lys98, Asp125 and Glu128).

The multiple sequence alignment and phylogenetic analysis were performed to investigate the evolutionary relationships among ZmADF3 and other ADF proteins. Comparisons among the ADF protein sequences from *Arabidopisis thaliana*, *Oryza sativa*, *Setaria italica* and *Triticum aestivum* showed their high identity in plants. The similarity for their amino acid sequences were above 64 % (Fig. 1). As shown in Fig. 2, ZmADF3 had the highest homology with SiADF3, OsADF4 and TaADF3. They occupied an independent branch in the tree, while other four maize ADFs were classified into three different branches. This implied the specialty of ZmADF3 compared with other ADFs in maize.

### Isolation of the promoter region for *ZmADF3*

Based on the B73 genome sequence from 293162401 to 293166106 on chromosome 1, a 1738 bp promoter of *ZmADF3* was obtained from the genomic DNA of inbred N04. Then the cis-acting regulatory elements were predicted using PlantCARE (http://bioinformatics.psb.ugent. be/webtools/plantcare/html). The results showed that the *ZmADF3* promoter contained several basic regulatory elements, including 18 TATA boxes and 9 CAAT boxes. Some known cis-elements were also contained, including light responsive element ACE, AE-box, AT1, G-box, GATA, GT1, I-box and Sp1, abscisic acid responsive element ABRE, gibberellin responsive element GARE, MeJA responsive element CGTCA and TGACG, heat stress responsive element HSE, fungal elicitor responsive element Box-W1, defense and stress responsive element TC-rich repeats, wounding and pathogen responsive element W
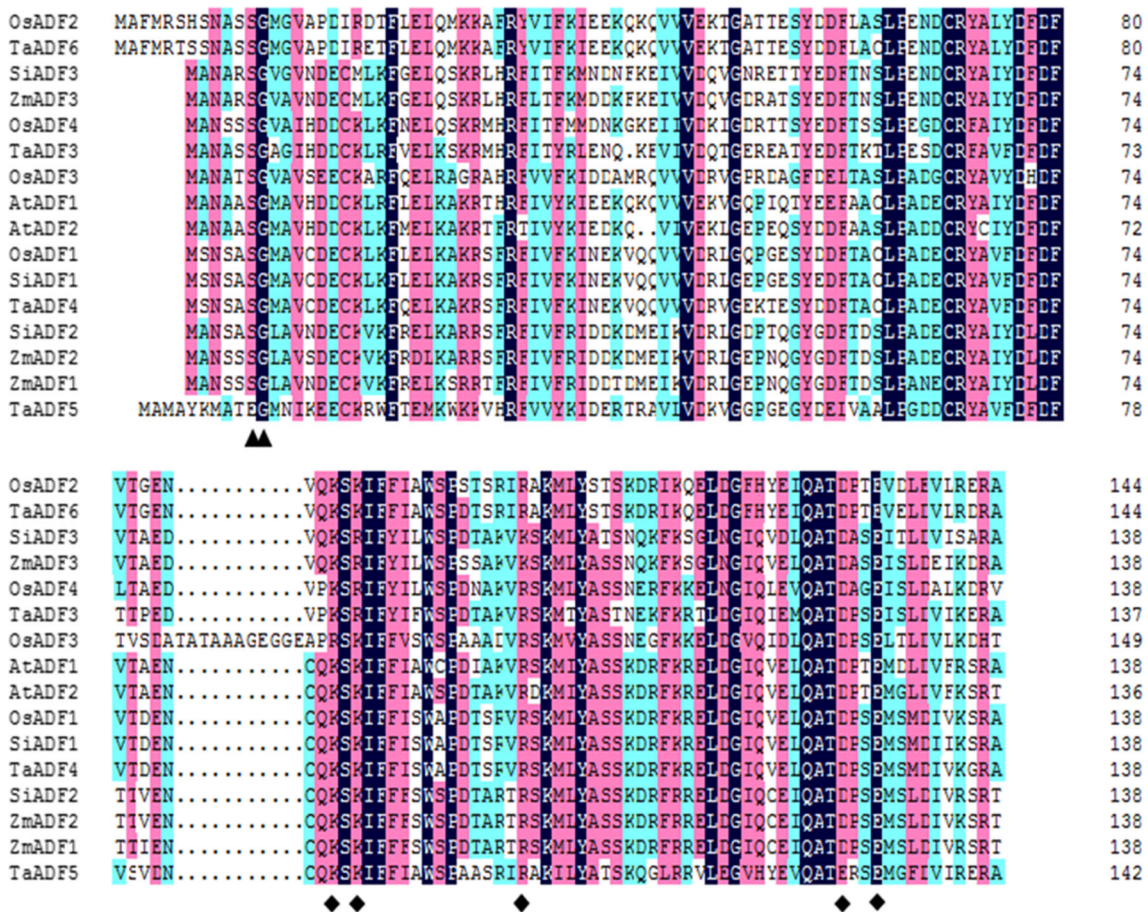
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OsADF2  MAFMRSHSNASSGMGVAPDIRDTFLELQMKKAFRYVIFKIEEKQKCVVVEKTGATTESYDDFLASLFENDCRYALYDFDF  80
TaADF6  MAFMRTSSNASSGMGVAPDIRETFLELQMKKAFRYVIFKIEEKQKCVVVEKTGATTESYDDFLAQLFENDCRYALYDFDF  80
SiADF3  MANARSGVGVNDECMLKFGEIQSKRLHREITFKMNDNFKEIVLCVGNRETTYEDFTNSLFENDCRYAIYDFDF          74
ZmADF3  MANARSGVAVNDECMLKFGEIQSKRLHRELTFKMDDKFKEIVLCVGDRATSYEDFTNSLFENDCRYAIYDFDF          74
OsADF4  MANSSSGVAIHDDCKLKFNEIQSKRMHREITFMMDNKGKEIVLDKIGDRTTSYEDFTSSLFEGDCRFAIYDFDF         74
TaADF3  MANASSGAGIHDDCKLRFVEILKSKRMHREITYRLENQ.KEVILDQTGEREATYEDFTKTLFESDCREAVFDFDF        73
OsADF3  MANATSGVAVSEECKARFQEIRAGRAHREVVFKIDDAMRCVVVDRVGFRDAGFDELTASLFADGCRYAVYDHDF         74
AtADF1  MANAASGMAVHDDCKLRFLELKAKRTHRFIVYKIEEKQKCVVVEKVGQPIQTYEEFAAQLFEADECRYAIYDFDF        74
AtADF2  MANAASGMAVHDDCKLKFMELKAKRTFRTIVYKIEDKQ..VIVEKLGEPEQSYDDFAASLFEADDCRYCIYDFDF        72
OsADF1  MSNSASGMAVCDECKLKFLEIKAKRSFREIVFKINEKVQCVVVDRIGQPGESYDDFTAQLFEADECRYAVFDFDF        74
SiADF1  MSNSASGMAVCDECKLKFLEIKAKRSFREIVFKINEKVQCVVVDRIGEPGESYEDFTAQLFEADECRYAVFDFDF        74
TaADF4  MSNSASGMAVCDECKLKFQEIKAKRSFREIVFKINEKVQCVVVDRVGEKTESYDDFTAQLFEADECRYAVFDFDF        74
SiADF2  MANSASGLAVNDECKVKFRELKAKRSFREIVFRIDDKDMEIKVDRIGDPTQGYGDFTDSLFEADECRYAIYDLDF        74
ZmADF2  MANSSSGLAVSDECKVKFRDIKARRSFREIVFRIDDKDMEIKVDRIGEPNQGYGDFTDSLFEADECRYAIYDLDF        74
ZmADF1  MANSSSGLAVNDECFVKFRELKSRRTFREIVFRIDDTDMEIKVDRIGEPNQGYGDFTDSLFEANECRYAIYDLDF        74
TaADF5  MAMAYKMATEGMNIKEECKRWFTEMKWKFVHREVVYKIDERTRAVIVDKVGGPGEGYDEIVAALFEGDDCRYAVFDFDF    78
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OsADF2  VIGEN..........VQKSKIFFIAWSESTSRIRAKMLYSTSKDRIKQELIGFHYEIQATDFTEVDLEVLRERA  144
TaADF6  VIGEN..........VQKSKIFFIAWSFDTSRIRAKMLYSTSKDRIKQELIGFHYEIQATDFTEVELIVLRDRA  144
SiADF3  VTAED..........VQKSKIFYILWSPDTAPVKSKMLYATSNQKFKSGLNGIQVDLQATDASEITLIVISARA  138
ZmADF3  VTAED..........VQKSKIFYILWSFSSAPVKSKMLYASSNQKFKSGLNGIQVELQATDASEISLDEIKDRA  138
OsADF4  LIAED..........VPKSKIFYILWSPDNAPVRSKMLYASSNERFKKELNGIQLEVQATDAGFISLDALKDRV  138
TaADF3  TIPED..........VPKSRIFYIFKSEDTAPVRSKMTYASTNEKFKRTLDGIQIEMQATDFSPISLIVIKERA  137
OsADF3  TVSDATATAAAGEGGEAPRSKIFFVSWSEAAAIVRSKMVYASSNEGFKKELDGVQIDLQATDFSFLTLIVLKDHT  149
AtADF1  VTAEN..........CQKSKIFFIAWCPDIAKVRSKMLYASSKDRFKRELDGIQVELQATDFTEMDLIVFRSRA  138
AtADF2  VTAEN..........CQKSKIFFIAWSPDTAPVRDKMIYASSKDRFKRELDGIQVELQATDFTEMGLIVFKSRT  136
OsADF1  VTDEN..........CQKSKIFFISWAEDTSFVRSKMLYASSKDRFKRELDGIQVELQATDFSPMSMDIVKSRA  138
SiADF1  VTDEN..........CQKSKIFFISWAEDTSFVRSKMLYASSKDRFKRELDGIQVELQATDFSPMSMDIIKSRA  138
TaADF4  VTDEN..........CQKSKIFFISWAEDTSFVRSKMLYASSKDRFKRELDGIQVELQATDFSPMSMDIVKGRA  138
SiADF2  TIVEN..........CQKSKIFFFSWSEDTARTRSKMLYASSKDRFRRELDGIQCEIQATDFSPMSLDIVRSRT  138
ZmADF2  TIVEN..........CQKSKIFFFSWSEDTARTRSKMLYASSKDRFRRELDGIQCEIQATDFSPMSLDIVRSRT  138
ZmADF1  TTIEN..........CQKSKIFFFSWSFDTARTRSKMLYASSKDRFRRELDGIQCEIQATDFSPMSLDIVRSRT  138
TaADF5  VSVDN..........CQKSKIEFIAWSEAASRIRAKILYATSKQGLRRVLEGVHYEVQATERSEMGFIVIRERA  142
               ◆  ◆            ◆              ◆  ◆
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**Fig. 1** Multiple sequence alignment of *ZmADF3* and other plant *ADFs*. Completely identical or partial conserved amino acids were *shaded in colors*. The putative actin monomers (G-actin) binding sites and actin filaments (F-actin) binding sites of *ZmADF3* were marked by triangle and rhombus, respectively. The sequences were derived from the following accession numbers: *AtADF1* (At3g46010), *AtADF2* (At3g46000), *OsADF1* (Os02g44470), *OsADF2* (Os03g56790), *OsADF3* (Os03g60580), *OsADF4* (Os03g60590), *TaADF3* (AIZ95472), *TaADF4* (AGW22223), *TaADF5* (AIT97056), *TaADF6* (AIT97055), *SiADF1* (XP_004953378), *SiADF2* (XP_004957704), *SiADF3* (XP_0049812564)

box, and meristem expression element CAT-box and CCGTCC-box (Table 1). In addition, endosperm expression element GCN4 and Skn-1, zein metabolism regulation element O2-site and MYB binding site element MBS and CCAAT-box were also identified (Table 1).

## The expression profiles of *ZmADF3* during kernel development in maize

The expression profiles of *ZmADF3* in the kernels of inbred N04 were measured using qPCR. The results showed that *ZmADF3* expressed differently in the two tissues and across three developmental stages. For the two tissues, the expression levels were much higher in pericarp than in endosperm at all stages. For the expression tendency among different stages, the overall trend was on the decline during the developmental periods (Fig. 3a). Comprehensively speaking, *ZmADF3* expressed abundantly in pericarp at the early developmental stages.

## Western blot assay of ZmADF3 protein

Western blot was performed for the same two tissues at three developmental stages to explore the expression of ZmADF3 in the protein levels. The ZmADF3 proteins were detected for all samples (Fig. 3b). The result showed that the expression levels in pericarp increased at first and decreased subsequently and reached the highest at 20 DAP, while the expression tendency in endosperm were completely contrary with in pericarp and had the highest expression level at 33 DAP.

## ZmADF3 interacted with GAPDH in yeast two-hybrid system

Protein–protein interaction assays were performed to investigate the interacting proteins with ZmADF3. The ORF of ZmADF3 was designed as the bait to screen the prey cDNA library prepared from maize kernels at
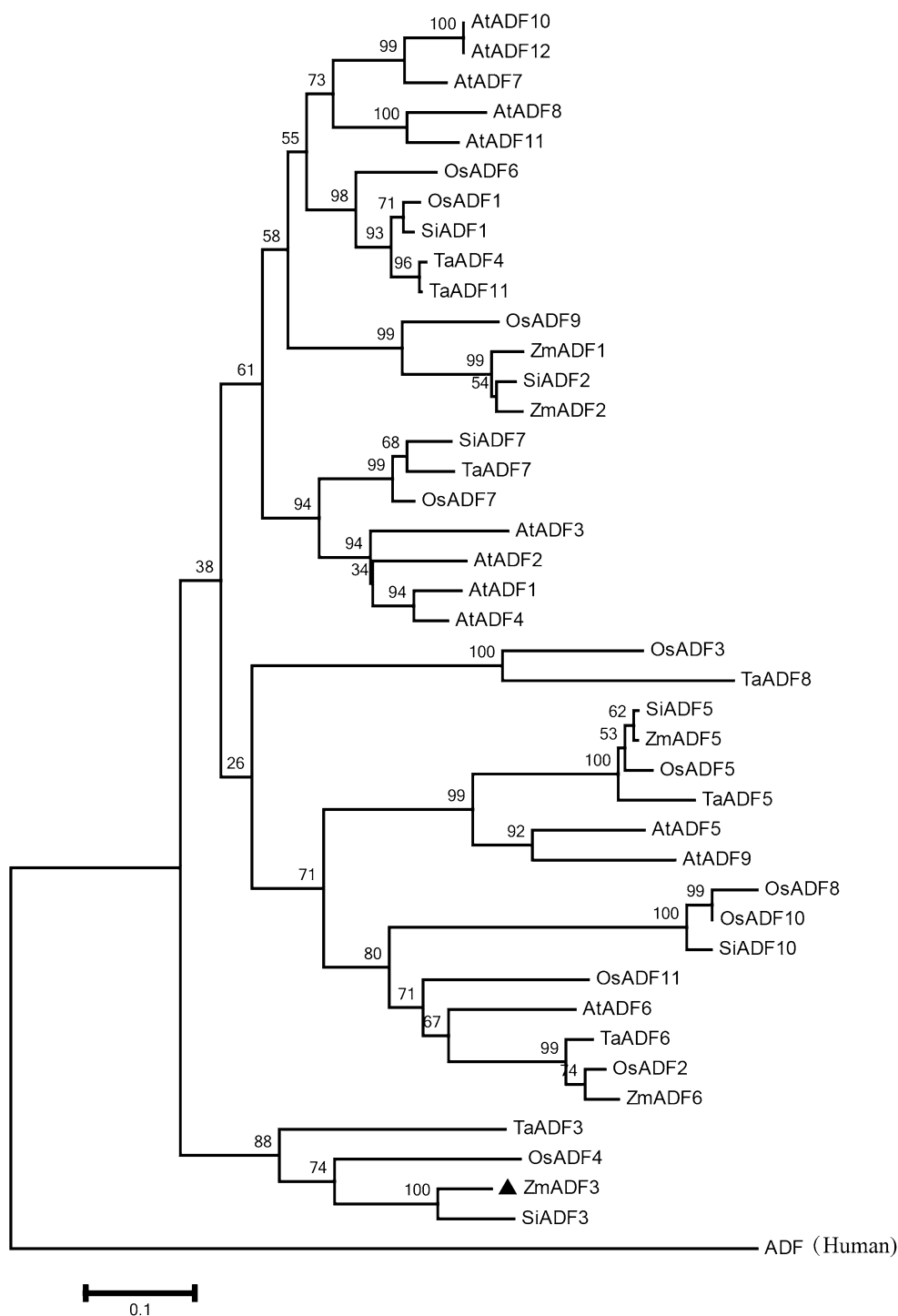
**Fig. 2** Phylogenetic analysis of the ADF sequences from *Arabidopisis thaliana*, *Oryza sativa*, *Setaria italica* and *Triticum aestivum* using the Neighbor-Joining method. The percentages of replication trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown above the branches. The Poisson correction method was applied to calculate the evolutionary distances and a *ADF* gene (AAB28361) of human was used as an outgroup. The sequences were derived from the following accession numbers: *AtADF1* (At3g46010), *AtADF2* (At3g46000), *AtADF3* (At5g59880), *AtADF4* (At5g59890), *AtADF5* (At2g16700), *AtADF6* (At2g31200), *AtADF7* (At4g25590), *AtADF8* (At4g00680), *AtADF9* (At4g34970), *AtADF10* (At1g01750), *AtADF11* (At3q45990), *AtADF12* (At5g52360), *OsADF1* (Os02g44470), *OsADF2* (Os03g56790), *OsADF3* (Os03g60580), *OsADF4* (Os03g60590), *OsADF5* (Os03g13950), *OsADF6* (Os04g46910), *OsADF7* (Os05g02250), *OsADF8* (Os07g20170), *OsADF9* (Os07g30090), *OsADF10* (Os10g37670), *OsADF11* (Os12g43340), *TaADF3* (AIZ95472), *TaADF4* (AGW22223), *TaADF5* (AIT97056), *TaADF6* (AIT97055), *TaADF7* (AFS49701), *TaADF8* (AIZ95473), *TaADF11* (AIT97057), *ZmADF1* (NP_001105463), *ZmADF2* (NP_001105590), *ZmADF5* (NP_001167686), *ZmADF6* (NP_001148357), *SiADF1*(XP_004953378), *SiADF2*(XP_004957704), *SiADF3* (XP_004981256), *SiADF5* (XP_004985039), *SiADF7* (XP_004960504), *SiADF10* (XP_004983892)

different developmental stages of inbred N04 using yeast two-hybrid assay. There were 18 possible interacting proteins were detected with putative functions, which were involved in oxidoreductase, isomerase, nutrient reservoir, GTP binding, ATP binding, NADP binding and nucleotide binding activities (Table. S2). Three independent clones encoding a maize glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified. To confirm the interaction, the full-length ORF of *GAPDH* was cloned into the pGADT7 vector. The Y$_2$HGold yeast strains co-transformed with pGADT7-*GAPDH* and pGBKT7-*ZmADF3* vectors could grow on the DDO, QDO/X, and QDO/X/A medium incubated at 30 °C for 5-10 d (Fig. 4). This result showed that ZmADF3 could interact with GAPDH in yeast two-hybrid system.

## Expression pattern of *ZmADF3* in transgenic *Arabidopsis* plants

The heterologous overexpression transgenic plants in *Arabidopsis* were generated, and the hygromycin resistant T0 transformants were transferred into soil and grown in a growth chamber to obtain the segregating T1 progeny for genetic analysis. Among the transformants, six overexpression lines showed a 3:1 segregation pattern for hygromycin resistance. The relative expression levels of the exogenous *ZmADF3* in the transgenic and WT *Arabidopsis* plants were analyzed by both qPCR and western blotting (Fig. 5). Three independent T3 homozygous progenies of transgenic lines with higher expression for *ZmADF3* were selected for the phenotype assays. In addition, the expression tendency of *ZmADF3* in root, stem, leaf, silique and inflorescence of the transgenic plants were determined by using both reverse transcription-polymerase chain reaction (RT-PCR) and qPCR. As shown in Fig. 6, the results showed that *ZmADF3* expressed with no tissue specificity and the expression abundance in stem and inflorescence was significantly higher than that in root, leaf and silique.

## Overexpression of *ZmADF3* increased seed size in transgenic *Arabidopsis* plants

The morphological analysis of mature seeds was performed to investigate the possible function of *ZmADF3* in seed development. Compared with the WT plant, the 1000-seed weights of the three independent transgenic lines (3#, 7# and 8#) increased 7.37, 29.97 and 21.16 %, respectively (Fig. 7a, b, c). Meanwhile, the seed area (+6.66, +23.16, +14.32 %) and length (+6.82 %, +20.27 %, +7.10) also increased significantly than the WT (Fig. 7d, e). Although the seed width of transgenic lines 3# and 8# had some increase (+0.44, +3.74 %), only line 7# reached the

significant level (+5.42 %) compared with the WT plant (Fig. 7f).

We also isolated and visualized the mature embryos from mature seeds and found that the cotyledon areas of the three transgenic lines were 1.15, 1.26 and 1.23-fold larger than the WT plant (Fig. 8a, c). The cell area was also determined by measured at least 30 cells in the central region of cotyledon epidermis (Fig. 8b). The average sizes of the cotyledon cells for the three transgenic lines were 1.16, 1.28 and 1.22-fold than that the WT plant (Fig. 8d). However, there were no significant differences for the average cell numbers of cotyledon epidermis in the mature embryos between the three transgenic lines and the WT plant (Fig. 8e).

## Expression analysis of seed size-associated genes between transgenic lines and the WT plant in *Arabidopsis*

The expression patterns of seed size-associated genes *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*), *APETALA2* (*AP2*), *AUXIN RESPONSE FACTOR2* (*ARF2*) and *HAIKU1* (*IKU1*) of *Arabidopsis* were analyzed by qPCR between 35S::*ZmADF3* and the WT plants to further investigate the underlying regulation mechanism of *ZmADF3* in increasing seed size in transgenic *Arabidopsis*. The results showed that the expression levels of *SHB1* and *IKU1* were significantly increased in overexpression plants, whereas the expression of *AP2* and *ARF2* were observably higher in four-week-old WT plant leaves (Fig. 9a) and 7 DAP siliques (Fig. 9b).

## Discussion

*ADFs* have been identified and cloned from a variety of plants. In Maize, three *ADFs* have been cloned and characterized (Rozycka et al. 1995; Lopez et al. 1996). *ZmADF3* was firstly identified and cloned based on an EST collected from a leaf cDNA library by Lopez et al. (1996). Herein, we isolated *ZmADF3* from a popcorn inbred N04 based on the protein sequence of GRMZM2G060702_P02 from the protein libraries for pericarp and endosperm at key developmental stages of the inbred constructed in our laboratory (Dong et al. 2015).

Histodifferentiation, reserve deposition and maturation drying are the three sequential stages during seed development (Wang et al. 2015). Cell division and expansion, as well as storage matter accumulation mainly occurred in the first two stages (Wang et al. 2015). In previous studies, *ADFs* showed high expression in the histodifferentiation stage and at the early stage of reserve deposition in the seeds of rice and *Arabidopsis* (Xu et al. 2008; Hajduch

**Table 1** Predicted cis-elements of the *ZmADF3* promoter with database analysis

| Element | Element core sequence | Element number | Function |
|---|---|---|---|
| A-box | CCGTCC | 1 | Cis-acting regulatory element |
| ABRE | GACACGTGGC | 1 | Cis-acting element involved in the abscisic acid responsiveness |
| ACE | CTAACGTATT | 1 | Cis-acting element involved in light responsiveness |
| AE-box | AGAAACTT | 2 | Part of a module for light response |
| ARE | TGGTTT | 1 | Cis-acting regulatory element essential for the anaerobic induction |
| AT1-motif | AATTATTTTTATT | 1 | Part of a light responsive module |
| Box-W1 | TTGACC | 1 | Fungal elicitor responsive element |
| CAT-box | GCCACT | 1 | Cis-acting regulatory element related to meristem expression |
| CCAAT-box | CAACGG | 1 | MYBHv1 binding site |
| CCGTCC-box | CCGTCC | 1 | Cis-acting regulatory element related to meristem specific activation |
| CGTCA-motif | CGTCA | 1 | Cis-acting regulatory element involved in the MeJA-responsiveness |
| G-box | GACACGTAGT | 2 | Cis-acting regulatory element involved in light responsiveness |
| GARE-motif | TCTGTTG | 1 | Gibberellin-responsive element |
| GATA-motif | GATAGGG | 1 | Part of a light responsive element |
| GC-motif | CCCCCG | 1 | Enhancer-like element involved in anoxic specific inducibility |
| GCN4-motif | CAAGCCA | 1 | Cis-regulatory element involved in endosperm expression |
| GT1-motif | GGTTAA | 1 | Light responsive element |
| HSE | AAAAAATTTC | 1 | Cis-acting element involved in heat stress responsiveness |
| I-box | GATAGGG | 2 | Part of a light responsive element |
| MBS | CGGTCA | 2 | MYB binding site |
| O2-site | GTTGACGTGA | 1 | Cis-acting regulatory element involved in zein metabolism regulation |
| Skn-1_motif | GTCAT | 1 | Cis-acting regulatory element required for endosperm expression |
| Sp1 | CC(G/A)CCC | 7 | Light responsive element |
| TC-rich repeats | ATTTTCTTCA | 1 | Cis-acting element involved in defense and stress responsiveness |
| TGACG-motif | TGACG | 1 | Cis-acting regulatory element involved in the MeJA-responsiveness |
| W box | TTGACC | 1 | Elicitation; wounding and pathogen responsiveness. Binds WRKY type transcription factors |

et al. 2010). Herein, *ZmADF3* also had a similar expression tendency in these stages and the expression levels in pericarp were higher than that in endosperm, which implied that *ZmADF3* might play a vital role in kernel development.

Obtaining mutations by overexpression or misexpression is an effective approach for exploring biological functions in plants (Prelich 2012; Liu et al. 2015; Su et al. 2015). *ADFs* regulated by light, drought, cold, salt and pathogen attack had been confirmed in the previous studies through this way (Dong et al. 2013; Burgos-Rivera et al. 2008; Huang et al. 2012; Porter et al. 2012; Fu et al. 2014; Henty-Ridilla et al. 2014). In this study, the promoter region of *ZmADF3* was obtained from the genomic DNA of the inbred N04, in which many cis-acting regulatory elements associated with light were found. Further study on the influence of light to the seedlings of 35S::*ZmADF3* and

WT plant showed that the length of hypocotyl of ectopic expression seedlings increased 55.8 % than that of the WT plant after 8 days darkness cultivation. Clearly, light had an important influence on the regulation of *ZmADF3* in plant growth and development. Furthermore, the endosperm expression element GCN4 and Skn-1 (Takaiwa et al. 1991; Wu et al. 1998), zein metabolism regulation element O2-site (Schmidt et al. 1992) and MYB Binding Site element MBS and CCAAT box (Edwards et al. 1998) identified in the promoter region of *ZmADF3* implied that *ZmADF3* might have special functions in endosperm, meristem and zein metabolism. These needed to be studied in further research.

The yeast two-hybrid system is a useful and powerful tool for studying protein–protein interactions (Finley and Jr 2007). In this study, 18 interacting proteins with ZmADF3 were detected by using yeast two-hybrid library screening
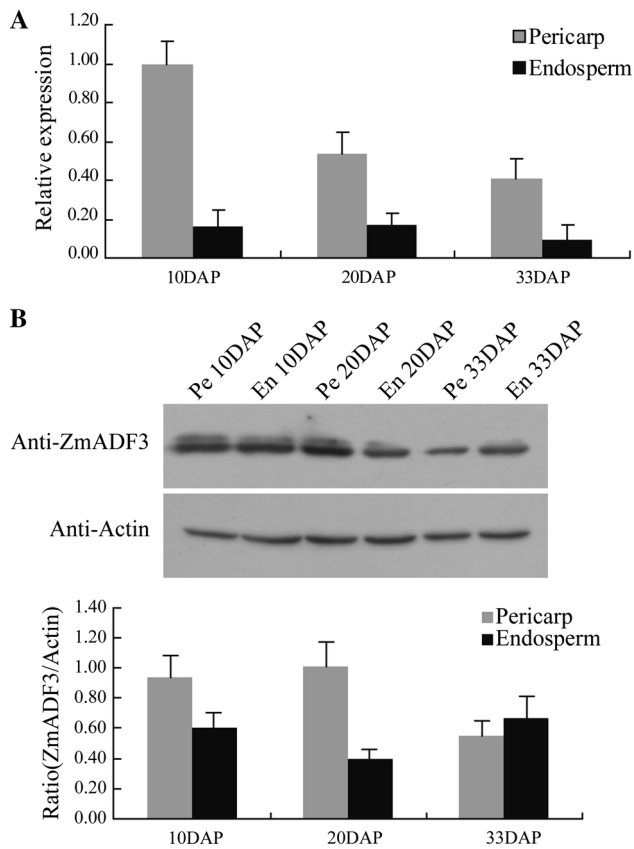
Fig. 3 The expression tendency of *ZmADF3* for all samples by quantitative RT-PCR analysis (**a**) and western blot analysis (**b**). The total proteins were extracted from pericarp (Pe10, Pe20, Pe33) and endosperm (En10, En20, En33) at 10, 20 and 33DAP, respectively. 18S rRNA was used as the endogenous control for qRCR and the immunizing mice with *Arabidopsis* ACT11 full-length protein (Abmart China) as the endogenous control for western blot. The protein expression was estimated by integrated optical density (IOD). Data were represented as the mean ± SE (n = 3)
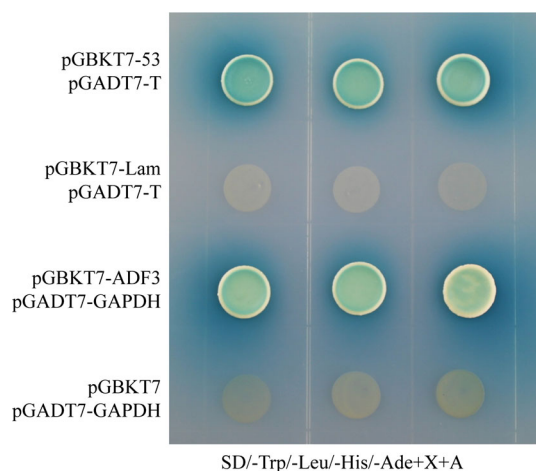


Fig. 4 ZmADF3 interacted with GAPDH in yeast two-hybrid system

method. Their molecular functions were predicted by Gene Ontology terms via the InterPro database (Mitchell et al. 2015). In addition, the interaction between ZmADF3 with GAPDH was confirmed also using the yeast two-hybrid system (Fig. 4). *GAPDH* was always used as internal control for transcription quantification experiments due to its relatively constant expression level (Lin et al. 2014). In previous studies, *GAPDH* had been shown to be involved in many cellular processes and played important roles in plant development and environmental stress response (Nicholls et al. 2012; Munoz-Bertomeu et al. 2010; Rius et al. 2008; Zhang et al. 2011; Cho et al. 2014). This implied that there might be a possible function contact between *ZmADF3* and *GAPDH*. Due to the intrinsic defect of yeast-two hybrid screening system (Sprinzak et al. 2003), the verification for the interaction between them should be done using other methods.

Many recent studies confirmed that *ADFs* related to the plant growth and development particularly in the tip tissue (i.e. root hairs, pollen tube) growth, and majority of the observed phenotypes might be ascribed to altered cytoplasmic cytoskeletal activities affecting the meristem development (Jiang et al. 1997b; Chen et al. 2002; Daher and Geitmann 2012; Clement et al. 2009; Augustine et al. 2008; Burgos-Rivera et al. 2008; Allwood et al. 2002; Dong et al. 2013). Herein, our results showed that *ZmADF3* had high expression in the inflorescence of transgenic plants in *Arabidopsis* (Fig. 6), which was in accordance with the previous results. In higher plants, seed size, as an important component of seed yield, is also a vital agronomic trait in crop domestication, and to improve seed yield is a key target for crop breeders (Xia et al. 2013). In this study, the possible functions of *ZmADF3* in kernel development were studied by means of heterologous overexpression of *ZmADF3* in *Arabidopsis*. The seed weight, seed area, seed length and seed width in transgenic lines all showed significant increase than in the WT plants. In dicotyledons, the endosperm is eventually replaced by the growing embryo, which then constitutes the majority of the mature seed (Sundaresan 2005). Accordingly, we isolated and visualized the mature embryos from the mature seeds. Our result showed that the cotyledon areas of transgenic lines were larger than that of the WT plant. The organ size was primarily determined by cell proliferation and cell expansion at the cellular level (Mizukami 2001). To reveal which was the determinant factor in this study, we measured the cells in the central region of cotyledon epidermis, and the average cell numbers of cotyledon epidermis. The result indicated that *ZmADF3* enlarged seed size through the regulation of cell expansion in transgenic *Arabidopsis*.

**Fig. 5** Overexpression of *ZmADF3* in *Arabidopsis*. **a** RT-PCR and qRT-PCR results for six homozygous T3 transgenic plants (3#, 7#, 8#, 14#, 31#, 33#) and wild-type *Arabidopsis* using the specific primers for *ZmADF3*. **b** Western blot analysis of ZmADF3 protein expression in the leaves of three homozygous T3 transgenic lines and wild-type plant. Data are represented as the mean ± SE
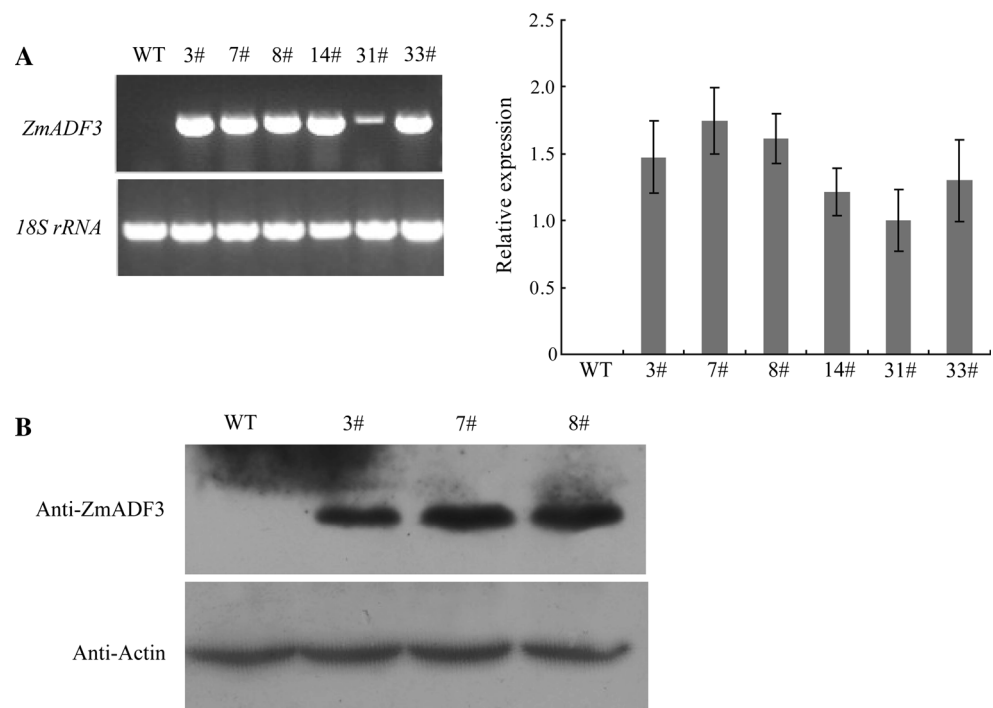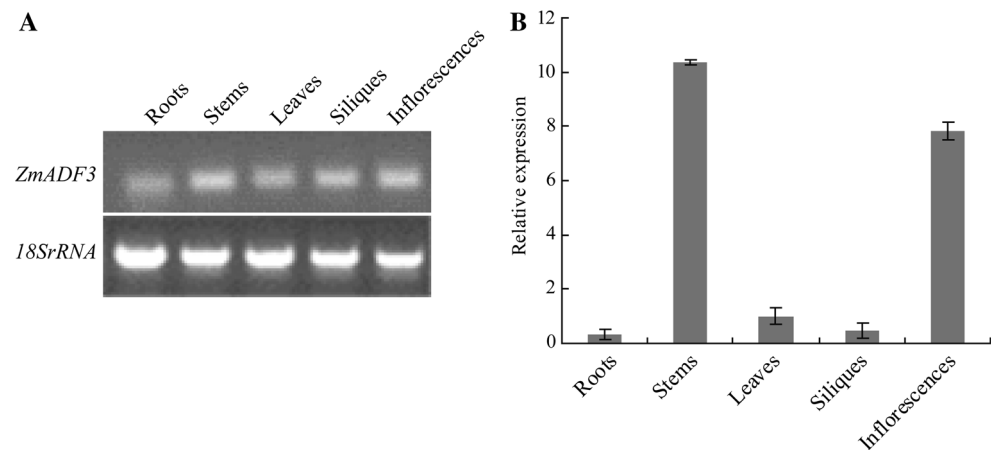
**Fig. 6** Expression pattern of *ZmADF3* in transgenic plants. Total RNA was isolated from root, stem, leaf, silique and inflorescence of transgenic plants at 8DAF. **a** RT-PCR and **b** qRT-PCR results for different tissues of transgenic plants. Data were represented as the mean ± SE (n = 3). 18S rRNA was used as the endogenous control

In *Arabidopsis*, *SHB1, IKU1, MINISEED3 (MINI3)* and *HAIKU2 (IKU2)* can positively regulate seed size by promoting endosperm growth in the early stage of seed development, and both *MINI3* and *IKU2* are under the control of *SHB1* and *IKU1* in different pathway (Jiang et al. 2013; Luo et al. 2005; Zhou et al. 2009; Wang et al. 2010). *IKU2* is regulated by *MINI3* through the binding of W-box motif in the promoter region of *IKU2* (Luo et al. 2005). In order to investigate the underlying regulation mechanism of *ZmADF3* in increasing seed size in transgenic *Arabidopsis*, the expression patterns of seed size-associated genes *SHB1, AP2, ARF2* and *IKU1* in *Arabidopsis* were

analyzed by qPCR between 35S::*ZmADF3* and WT plants in our present study. *SHB1* and *IKU1* showed up-regulated expression in the transgenic lines at 7 DAP siliques, which implied that the increased mature seed size in the *ZmADF3* heterologous overexpression lines might be also due to the endosperm proliferation in the early stage. In addition, a W-box motif (TTGACC) also was predicted in the promoter region of *ZmADF3*. Whether there is an internal relationship between the heterologous *ZmADF3* and the *SHB1-MINI3-IKU2* pathway on positive regulating seed size of *Arabidopsis* need to be proved in further research. In *Arabidopsis*, other pathways via maternal tissues to

**Fig. 7** Morphological analysis of the mature seeds in *Arabidopsis* of 35S::*ZmADF3* and wild-type. Morphology of seed size (**a**) and mature embryo size (**b**). Statistics of seed weight (**c**), seed area (**d**), seed length (**e**) and seed width (**f**). Seed weight was determined by weighing the mature dry seeds in batches of 1000. The weights of five sample batches were measured for each seed lot (n = 5). Seeds were observed and photographed under a stereoscopic microscope and 20 randomly selected seeds were used to measure seed area, seed length and seed width (n = 20). 3#, 7#, 8# represented three independent T3 progenies of transgenic lines. Data were represented as the mean ± SE. *t* test compared with the wild-type control: ** and *referred to significances at $P < 0.01$ and $P < 0.05$, respectively. *Bars* = 250 μm in (**a**) and (**b**)
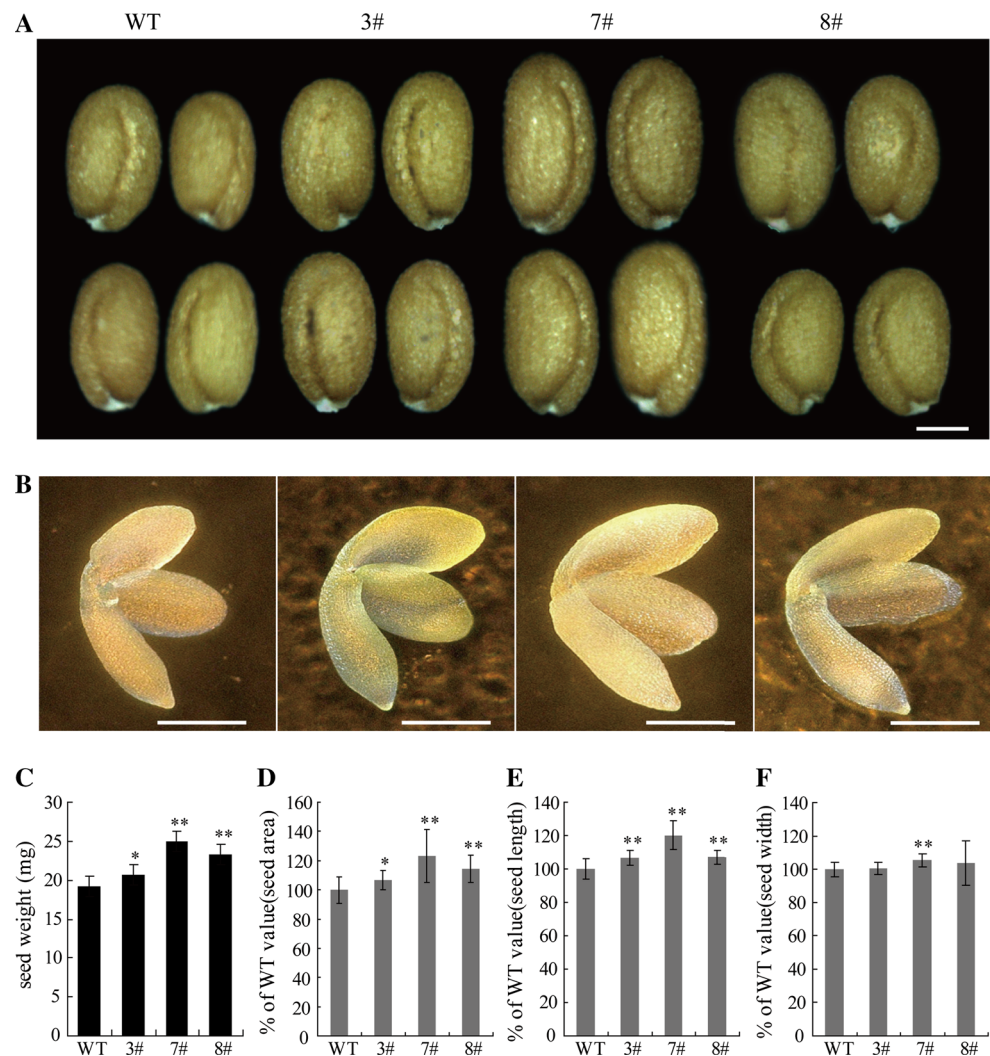


regulate seed size have been confirmed (Xia et al. 2013). *AP2* and *ARF2* are two representative genes in this pattern and both of them reduce seed size by restricting cell expansion and proliferation in the integuments of seeds (Ohto et al. 2005, 2009; Jofuku et al. 2005; Schruff et al. 2006). In this study, the ectopic expression of *ZmADF3* in *Arabidopsis* promoted cell expansion in the cotyledon epidermis of mature embryo and the expression of *AP2* and *ARF2* were down-regulated in transgenic lines. Therefore, *ZmADF3* could promote cell expansion through the influence of the expression of seed size-associated genes. However, further work still be required to uncover the underlying mechanism.

In conclusion, we isolated and characterized *ZmADF3* from a popcorn inbred line N04. The promoter of *ZmADF3* was obtained from the genomic DNA of N04 and then the cis-acting regulatory elements were predicted. 18 possible interacting proteins with ZmADF3 were screened using yeast two-hybrid system. Heterologous overexpression in *Arabidopsis* showed that *ZmADF3* might increase seed size through promoting cell expansion and regulating the expression of seed size regulated genes. However, the underlying regulation mechanism of *ZmADF3* in increasing seed size in transgenic *Arabidopsis* and the relationship between *ZmADF3* and the seed size-associated genes was still unclear. Further research should be done to thoroughly

**Fig. 8** Phenotypic analysis of the mature embryos. The dissected mature embryos (**a**) and the epidermal cell layer from the central region of cotyledon (**b**). Statistics of cotyledon area (**c**), epidermal cell area (**d**) and average cell numbers (**e**). The isolated embryos after treated and then photographed using a Leica differential interference contrast (DIC) microscope (×300). The epidermal cell of the cotyledon were photographed using a DIC microscope (×700). 3#, 7#, 8# represented three independent T3 progenies of transgenic lines. Data were represented as the mean ± SE (n = 30). *t* test compared with the wild-type control: ** and * referred to significances at *P* < 0.01 and *P* < 0.05, respectively. *Bars* = 250 μm in (**a**) and 100 μm in (**b**)
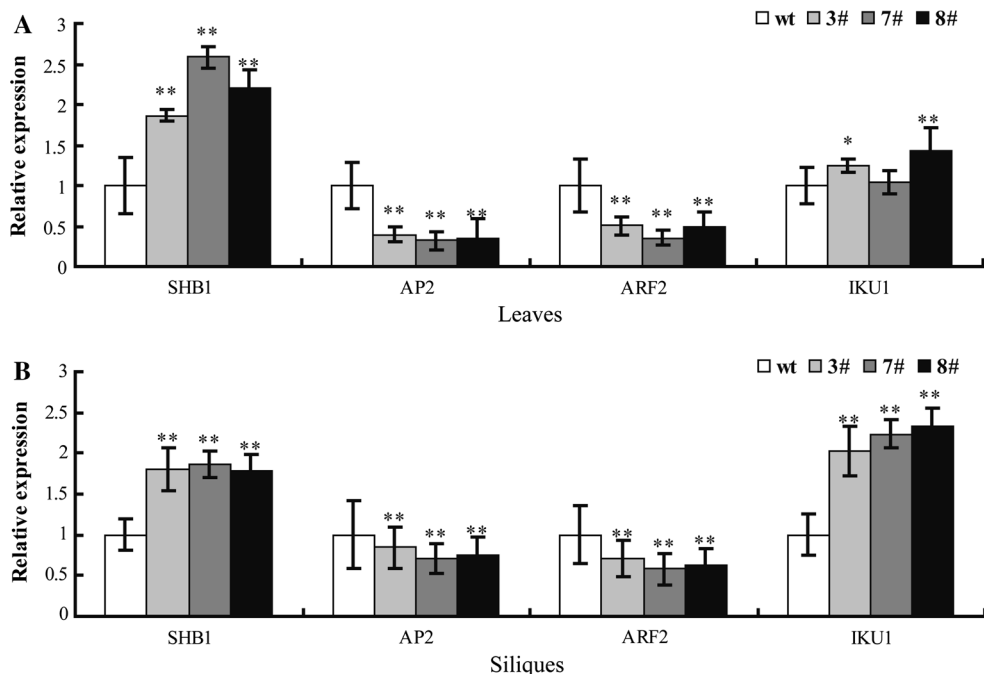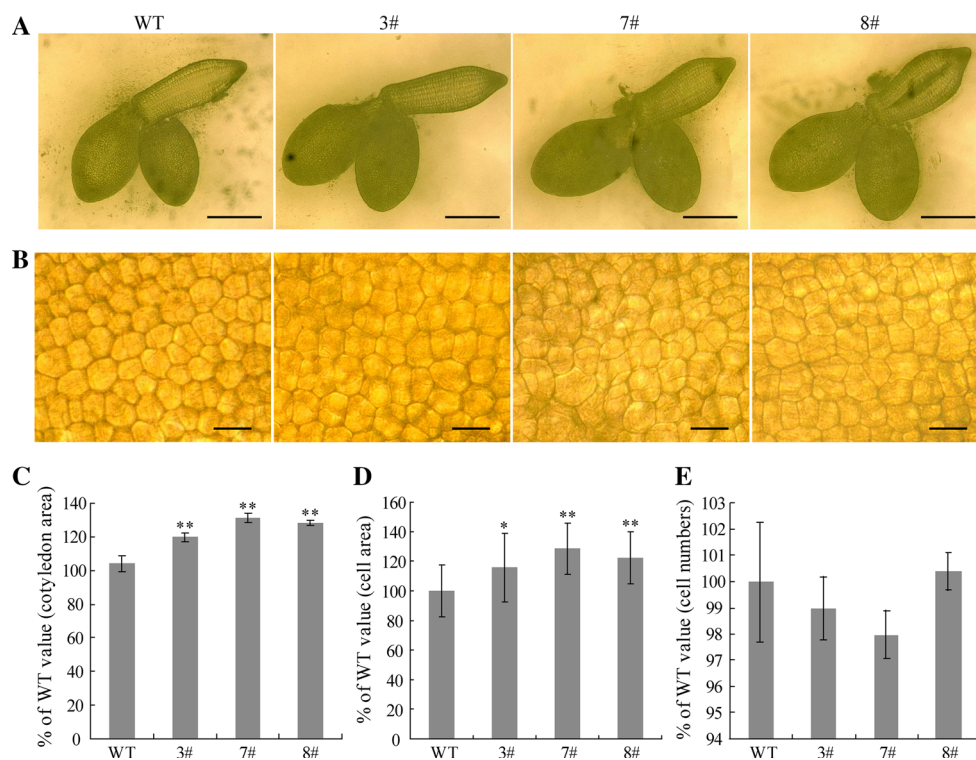


**Fig. 9** Expression analysis of seed size-associated genes between 35S::*ZmADF3* and wild-type plants. Total RNA was isolated from four-week-old leaves (**a**) and 7 DAP siliques (**b**). The primers for qRT-PCR analysis were referred to Jiang et al. (2013). 3#, 7#, 8# represented three independent T3 progenies of transgenic lines. Data were represented as the mean ± SE (n = 3). *t* test compared with the wild-type control: ** and * referred to significances at *P* < 0.01 and *P* < 0.05, respectively

explore the mechanism of *ZmADF3* in playing these functions in maize.

**Author contribution statement** D. Qiao performed most experiments, data analyses and manuscript preparation. Y. Dong contributed to some supervision of experiments. L. Zhang assisted in some data analysis and manuscript preparation. Q. Zhou, C. Hu and Y. Ren took part in the *Arabidopsis* planting and management. Y. Li was responsible for the whole study and the manuscript preparation.

**Compliance with ethical standards**

**Conflict of interest** All authors declared that they had no conflict of interest.

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