Characterization and Functional Analysis of Five MADS-Box B Class Genes Related to Floral Organ Identification in *Tagetes erecta*

Ye Ai¹,², Chunling Zhang¹, Yalin Sun³, Weining Wang⁴, Yanhong He¹*, Manzhu Bao¹*

¹ Key Laboratory of Horticultural Plant Biology, Ministry of Education, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, Hubei, China, ² College of Landscape Architecture, Fujian Agriculture and Forestry University, Cangshan District, Fuzhou, Fujian, China, ³ Institute of Vegetable Science, Wuhan Academy of Agricultural Sciences, Wuhan, Hubei, China, ⁴ Gulf Coast Research and Education Center, Department of Environmental Horticulture, University of Florida, Wimauma, Florida, United States of America

* hyh2010@mail.hzau.edu.cn (YH); mzbao@mail.hzau.edu.cn (MB)

Abstract

According to the floral organ development ABC model, B class genes specify petal and stamen identification. In order to study the function of B class genes in flower development of *Tagetes erecta*, five MADS-box B class genes were identified and their expression and putative functions were studied. Sequence comparisons and phylogenetic analyses indicated that there were one *PI*-like gene—*TePI*, two *euAP3*-like genes—*TeAP3-1* and *TeAP3-2*, and two *TM6*-like genes—*TeTM6-1* and *TeTM6-2* in *T. erecta*. Strong expression levels of these genes were detected in stamens of the disk florets, but little or no expression was detected in bracts, receptacles or vegetative organs. Yeast hybrid experiments of the B class proteins showed that *TePI* protein could form a homodimer and heterodimers with all the other four B class proteins *TeAP3-1*, *TeAP3-2*, *TeTM6-1* and *TeTM6-2*. No homodimer or interaction was observed between the euAP3 and TM6 clade members. Over-expression of five B class genes of *T. erecta* in *Nicotiana rotundifolia* showed that only the transgenic plants of 35S::*TePI* showed altered floral morphology compared with the non-transgenic line. This study could contribute to the understanding of the function of B class genes in flower development of *T. erecta*, and provide a theoretical basis for further research to change floral organ structures and create new materials for plant breeding.

Introduction

*Tagetes erecta* is a commercial plant renowned for its significant ornamental, industrial and medicinal usage [1–3]. It is a member of the Asteraceae which is characterized by the structure of a terminal capitulum. Its unique inflorescence comprises hundreds of florets of two types, ray florets in the periphery and disk florets in the center. The ray florets have three whorls of
floral organs (sepal, petal, and carpel), while the disk florets have four whorls of floral organs (sepal, petal, stamen and carpel). Complexity of the capitulum structure greatly hinders manual emasculation, a necessity in plant breeding methodology. Male sterile plants with defective anthers and degenerated petals of ray and disc florets could provide a cost-effective alternative for plant breeders [4, 5]. Advancing technology of molecular biology has the potential to modify floral structure for easier artificial breeding, yet literature related to the molecular mechanisms of floral organ development in *T. erecta* is lacking.

Based on the study of homeotic mutants in the model plants *Arabidopsis thaliana* and *Antirrhinum majus*, the classical ABC model was proposed to explain the genetic regulation of floral organ development, in which the identity of each floral whorl component is determined by a combination of genes grouped into three homeotic functional classes, called class A, B and C genes, respectively [6, 7]. Class A genes specify sepals of the outermost whorl; class A and B genes collectively control petal identity in the second whorl; class B and C genes concomitantly specify stamens in the third whorl; class C genes determine formation of the carpels in the fourth whorl. Subsequent discoveries of the class D and E genes expanded the model to its contemporary form, the ABCDE model [8]. Class D genes regulate ovule development [9, 10], while class E genes specify ovule identity and are necessary for specification of sepal, petal, stamen and carpel identities when functioning together with class A, B, C, and D genes, respectively [11, 12].

In view of the original characterization in *A. thaliana* (Brassicaceae) and *A. majus* (Plantaginaceae), B class genes could be divided into *APETALA 3* (AP3) / *DEFICIENS* (DEF) and *PISTILLATA* (PI) / *GLOBOSA* (GLO) subfamilies [13, 14]. Kim et al. [15] studied the phylogeny and diversification of B-function MADS-box genes in angiosperms, suggesting that a duplication event happened approximately 260 million years ago was responsible for producing the PI/GLO and AP3/DEF lineages. The estimated timeframe placed the duplication event past the splitting of extant gymnosperms and angiosperms, but well before the oldest angiosperm fossils. In the higher eudicot species, the AP3/DEF subfamily experienced another significant duplication event and was divided into the euAP3 and *Tomato MADS-box gene 6* (TM6) lineages [16]. These three lineages can be distinguished according to the different C-terminal motifs. The PI/GLO-like proteins have a PI-motif, while the euAP3 and TM6-like proteins have the euAP3 and paleoAP3 motifs, respectively. Both the euAP3 and TM6-like proteins have a PI-motif-derived region [16–18].

Many studies on MADS-box genes indicate the ABC model is not only applicable to the eudicot plants but also to monocot plants [19]. In both eudicots and monocots, B class genes play similar roles in specifying petal and stamen development [20–26]. Functional analysis in *Gerbera hybrida* (Asteraceae) showed that the PI/GLO and euAP3 lineage genes encoded the classical B function and the TM6 lineage gene might act redundantly in stamen development. Down-regulating the expression of the PI/GLO (*GGLO1*) and euAP3 (*GDEF2*) lineage genes led to stamen and petal defects [27]. In *Helianthus annuus* (Asteraceae), the B class genes encoded the B function and were involved in the formation of petals and stamens. Furthermore, it was found that *HaPI* and *HaAP3* were preferentially expressed in petals of ray flowers but their expression levels were significantly weaker in petals and stamens of disk flowers [28].

In this study, expression and putative functions of five *T. erecta* B class genes were studied. This work provides a better understanding of B class gene function in floral organ development of *T. erecta*, and provides a theoretical basis useful in modifying floral organ structure and generating new germplasm resources for plant breeders.
Materials and Methods

Materials

Inbred line TE-I-1 of *T. erecta* had been developed through 10 generations of self-crossing, which had one whorl of ray florets in the periphery of the capitulum. The plants were grown in 21 cm sized pots in natural conditions in fall 2012 at Huazhong Agricultural University, Wuhan, Hubei Province, China (lat. 30˚28’36.5” N, long. 114˚21’59.4” E).

RNA extraction of various tissues and organs

When the plants were in florescence phase, samples of roots, tender stems, fresh leaves, different sizes of flower buds (1 mm, 3 mm, 5 mm and 7 mm in diameter, respectively), sepals, petals and pistils of ray and disk florets, stamens of disk florets, receptacles, bracts, and ovaries of opened flowers were collected, frozen immediately in liquid nitrogen and stored at -80˚C for further RNA extraction. Total RNA was isolated using Trizol reagent (Tiangen, Shanghai, China) according to manufacturer’s instruction, and RNA content was quantified by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Only 1 μg of total RNA, after DNase I treatment (Boehringer Manheim), was used per sample for the synthesis of cDNA. First strand cDNA template was synthesized using Oligo-dT as primers and Multi-scribe reverse transcriptase (Takara).

Isolation of full-length MADS-box B class genes of *T. erecta*

Based on the transcriptome sequence (accession number SRP066084) [29], five partial cDNA clones with different sequences were selected as B class genes of *T. erecta*, named as TePI, TeAP3-1, TeAP3-2, TeTM6-1, and TeTM6-2, respectively. The protein coding sequence (CDS) was predicted by using NCBI blast 2.2.28+. In order to verify the accuracy of CDS, primers set for the clones with complete coding sequences were designed in the 3’ and 5’ terminal region using the Primer 5 software (S1 Table). The cDNA from flower buds 5 mm in diameter were used as material to isolate the CDS of B class genes. The reaction mixture contained 2.5 μl of the first strand cDNA as template, 100 μM of dNTPs, 0.2 μM primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 1 unit of Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China), and double-distilled H₂O to a total volume of 50 μl. The PCR procedure was initiated with pre-denaturation at 94˚C for 4 min, followed by 35 cycles of amplification at 94˚C for 30 s, 58˚C for 30 s and 72˚C for 1 min, and a final extension at 72˚C for 10 min. The PCR products were cut from the 1.2% agarose/EtBr gel, cloned in the pMD18-T Vector (TaKaRa Biotech, Dalian, China) and the fragments were sequenced using universal M13F primer (Shanghai Invitrogen Biological Technology Co., Ltd.). Sequenced data were analyzed using the DNAMAN software (Lynnon Corporation). The full-length sequences of TePI, TeAP3-1, TeAP3-2, TeTM6-1, and TeTM6-2 have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession number KU696419, KU696420, KU696421, KU696422, and KU696423, respectively.

Analysis of cDNA sequences and construction of phylogenetic tree

The cDNA sequences of five B class genes were used to search for homologous sequences via blast in the National Center for Biotechnology Information. A total of 37 corresponding nucleotide and amino acid sequences of class B MADS-box factors were downloaded from the NCBI GenBank. The cDNA sequences of the B class genes were analyzed using the DNAMAN software. The predicted amino acid sequences of the B class genes of *T. erecta* and other plant species were used for phylogenetic analysis. The multiple sequence alignment was performed
using software Clustal X 1.83. A phylogenetic tree was constructed using the maximum likelihood analysis method provided by the MEGA 3.0 software with the model generated by default. The phylogenetic tree was tested with a bootstrap of 1000 replicates to ascertain the reliability of a given branch pattern.

Expression of five MADS-box B class genes by quantitative Real-time PCR

To analyze the expression of five B class genes in *T. erecta*, first-strand cDNAs from the samples of roots, tender stems, fresh leaves, different sizes of flower buds (1 mm, 3 mm, 5 mm and 7 mm in diameter, respectively), sepal, petals and pistils of ray and disk florets, stamens of disk florets, receptacles, bracts, and ovaries of opened flowers were used as templates. Quantitative Real-time PCR was carried out using an SYBR Primix Ex Taq kit (TaKaRa, Dalian, China) according to manufacturer’s instructions and analyzed in the ABI 7500 Real-time System (Applied Biosystems, USA). The levels of gene expression were calculated by ABI Prism 7500 Sequence Detection System Software (Applied Biosystems, USA). Real-time PCR was performed with specific primers of target genes and primers of housekeeping gene (*beta-actin*) (S1 Table) and the products were amplified in 20 μl reaction volumes containing 2 μl template of the reaction mixture, 10 μl 2 × SYBR Green Master Mix, 2 μl forward primer, 2 μl reverse primer (10 μmol·μl⁻¹ for primers) and double-distilled water. The PCR was carried out with the following cycling parameters: heating at 95˚C for 2 min, 40 cycles of denaturation at 95˚C for 10 s, annealing at 56˚C for 20 s, and extension at 72˚C for 35 s. Real-time PCR was performed in four experimental replicates for each sample and the expression values obtained were normalized against *beta-actin*. Data are shown as mean values ± standard deviation. Analysis of the relative gene expression data was conducted using the 2⁻ΔΔCt method.

Yeast two-hybrid assays

The full length coding cDNA sequences of *TePI, TeAP3-1, TeAP3-2, TeTM6-1*, and *TeTM6-2* genes containing restriction sites at the 5’ and 3’ ends were amplified by PCR from the sequenced clones using primers in S2 Table. The PCR products were introduced into the GAL4-based yeast two-hybrid vectors pGBKT7 (Clontech) and pGADT7-Rec (Clontech) and co-transformed into the AH109 yeast strain by the LiAc/DNA/PEG method according to the Yeast Protocols Handbook from Clontech (http://www.clontech.com). Co-transformed yeast cells were placed on selection plates with synthetic defined (SD) media lacking leucine (Leu) and tryptophan (Trp). Yeast double transformants were tested for interaction/activation on selective SD—Leu—Trp media. Positive interactions were confirmed by yeast growth in SD selective medium without Leu, Trp, histidine (His) and adenine (Ade). X-gal assay was performed for yeast cells grown on SD—Leu—Trp—His—Ade plates following instructions.

Production of transgenic plants and phenotypic analysis of the transformants

The cDNA fragments containing the complete coding sequences of the five MADS-box B class genes of *T. erecta* were amplified using primers shown in S1 Table and cloned in a pMD18-T vector (Takara). Sequence accuracy and insertion direction were confirmed by sequencing. After digestion with restriction enzymes (*BamH*I and *Sal*I for *TePI, TM6-1*, and *TM6-2; Kpn*I and *Sal*I for *TeAP3-1; Xba*I and *Sal*I for *TeAP3-2*), the amplified fragments were subcloned into a binary vector pBI2300 which contained the CaMV35S promoter and the gene conferring kanamycin resistance. The vectors containing 35S::*TePI, 35S::TeAP3-1, 35S::TeAP3-2,
35S::TeTM6-1 and 35S::TeTM6-2 were introduced into the Agrobacterium strain EHA105 by the heat shock method, respectively. Leaf disk method for Agrobacterium-mediated transformation of Nicotiana rotundifolia was performed as described by Horsch et al. [30]. Transgenic N. rotundifolia plants at T0 generation were screened out from the non-transgenic line, and the presence of the transgene was confirmed by genomic PCR with 35S-F and specific primers (S1 Table). Transgenic and non-transgenic plants were grown in a specially assigned greenhouse for morphological comparisons following anthesis.

Results
Isolation and sequence analysis of five MADS-box B class genes of T. erecta

Based on the transcriptome sequence (accession number SRP066084) [29], full-length cDNA clones of five B class genes of T. erecta were isolated from a cDNA library constructed from flower buds 5 mm in diameter. These clones were named TePI, TeAP3-1, TeAP3-2, TeTM6-1, and TeTM6-2, respectively. The open reading frame (ORF) of cDNA sequences and predicted amino acid sequences were aligned in S1 Fig and Fig 1. The TePI transcript was predicted to have a 591 bp ORF and encode a protein made up of 196 amino acids. Two copies of TeAP3, TeAP3-1 containing a 690 bp ORF and TeAP3-2 containing a 708 bp ORF, encoded proteins made up of 229 and 235 amino acids, respectively. Two copies of TeTM6, TeTM6-1 containing a 681 bp ORF and TeTM6-2 containing a 636 bp ORF, encoded 226 and 211 amino acid proteins, respectively. The putative amino acid sequence of TeAP3-2 gene was homologous to the TeAP3-1 gene with 80% homology. Besides, the TeTM6-2 was a partial fragment of TeTM6-1 and most of their sequences were identical except additional 45 nucleotides of cDNA sequence and fifteen amino acids in the K domain of amino acid sequence in TeTM6-1.

The predicted amino acid sequences demonstrated that the protein sequences of the five B class genes of T. erecta had the typical MIKC-type domain structure, namely, the MADS-domain, I-region, K-domain and C-region (Fig 1). Furthermore, TePI protein had the highly conserved PI-motif between amino acid residues 197 and 211 within the C-terminal region.

![Fig 1. Comparison of the putative amino acid sequences of five MADS-box B class genes of T. erecta. The predicted amino acid sequences demonstrated that protein sequences of all the isolated B class genes of T. erecta have the typical MIKC-type domain structure. The MADS and K domains are indicated by black lines.](#)
Moreover, TeAP3-1 and TeAP3-2 proteins had a PI-motif-derived region and a conserved euAP3 motif in the C-terminal region (Fig 2b). Similarly, TeTM6-1 and TeTM6-2 proteins shared a PI-derived motif and a characteristic ancestral paleoAP3 motif instead of the euAP3 motif at the C-terminal end. In addition to the five B class genes of *T. erecta*, GenBank accession numbers of other PI/AP3-homologous genes were included in Fig 3.

**Phylogenetic analyses**

To determine the phylogenetic relationship of the MADS-box B class genes of *T. erecta* with other species, a phylogenetic tree was constructed using full-length amino acid sequences of MIKC domains from 37 known PI/AP3-homologous genes and the five MADS-box B class genes of *T. erecta*. All sequences used with their GenBank accession numbers and respective species were listed in Fig 3. The maximum likelihood analysis demonstrated that the B class gene family could be divided into PI/GLO and AP3/DEF subfamilies (Fig 3). *T. erecta* belongs to the Asteraceae family and all members in this family were gathered in the core eudicots group, separating themselves from the monocot species. The phylogenetic tree revealed that the TePI gene was classified into the PI-like genes, while TeAP3-1, TeAP3-2, TeTM6-1, and TeTM6-2 were all grouped into the clade of AP3-like genes. More specifically, TeAP3-1 and TeAP3-2 were classified into the euAP3 clade, while TeTM6-1 and TeTM6-2 were classified into the TM6 clade. Furthermore, we found that TeAP3-1 and TeAP3-2 were divided into two paralogous groups in the Asteraceae-specific subgroup of the euAP3 clade.

**Expression analysis of five MADS-box B class genes in *T. erecta***

The expression patterns of the five B class genes in different floral organs and tissues of *T. erecta* were analyzed by quantitative Real-time PCR. The expression levels of these genes
Fig 3. Phylogenetic tree calculated from the amino acid sequence of forty-two MADS-box B class genes. A phylogenetic tree was constructed based on the amino acid sequences using the maximum likelihood analysis method. In addition to T. erecta, the full-length amino acid sequences of MIKC domains were selected from 37 known PI/AP3-homologous genes. The phylogenetic tree showed that the TePI protein was classified into the PI/GLO subfamily, TeAP3-1 and TeAP3-2 placed within the euAP3 clade, while TeTM6-1 and TeTM6-2 placed within the TM6 clade. Besides, TeAP3-1
and TeAP3-2 were divided into two paralogous groups in the Asteraceae-specific subgroup of euAP3 clade. I: PI/GLO subfamily; I-1: PI/GLO in eudicot species; I-2: PI/GLO in monocot species; II: AP3/DEF subfamily; II-1: AP3/DEF in eudicot species; II-2: TM6 clade in eudicot species; II-3: euAP3 clade in eudicot species.

Increased significantly as flower buds grew bigger (Fig 4). Relative expression levels showed that these MADS-box B class genes were expressed at significant levels exclusively in stamens of the disk florets, but little or no expression was detected in bracts, receptacles or vegetative organs (Fig 4). Taking the example of TePI, strong expression was detected in stamens of disk florets, weak expression was detected in petals of disk and ray florets, little expression was detected in sepals of ray florets and no expression was detected in pistils of ray florets, sepals of disk florets, bracts, receptacles and vegetative organs. This result indicated that TePI was predominantly involved in the formation of stamen and petal in T. erecta. TeAP3-1 was strongly expressed in sepals and petals of disk and ray florets, stamens of disk florets and ovaries, but weakly expressed in the pistils of ray florets. Besides, the transcript signals were hardly detected in pistils of disk florets, bracts, receptacles and vegetative organs. Expression pattern of TeAP3-2 was similar to TeAP3-1, but its expression level in pistils of ray and disk florets was higher than that of TeAP3-1, no expression was detected in ovaries. Strong expression of TeTM6-1 was detected in stamens of disk florets, weak expression was detected in sepals of disk and ray florets, ovaries, petals of disk florets, whereas no expression could be detected in petals of ray florets. As most of the sequences of TeTM6-2 were identical to that of TeTM6-1, it was difficult to distinguish the expression level of TeTM6-2 from TeTM6-1. Instead, we detected the expression levels of TeTM6-1 and TeTM6-2 in one Real-time PCR reaction and found that no expression of TeTM6-2 could be detected in petals of ray florets.

Protein interactions of five MADS-box B class proteins

To determine the protein interactions of the B class proteins in vitro, yeast two-hybrid assays were performed between the B class proteins. The results of two-hybrid analysis revealed that the TePI protein could interact with all of the four AP3-type proteins (Fig 5) and homodimerization could be observed in TePI proteins. No homodimer or other interaction between the AP3-type members was observed in T. erecta.

Constitutive expression of five MADS-box B class genes of T. erecta in N. rotundifolia

To determine the potential role of B class genes of T. erecta in the floral development, the identified five class B genes were ectopically expressed in N. rotundifolia through Agrobacterium-mediated transformation. At least 21, 17, 19, 18, and 17 independently transformed N. rotundifolia lines of 35S::TePI, 35S::TeAP3-1, 35S::TeAP3-2, 35S::TeTM6-1 and 35S::TeTM6-2 were obtained by PCR detection, respectively. The non-transgenic plants and transformants of the T0 generation were selected for morphological comparisons. Results showed that none of the transgenic N. rotundifolia lines of 35S::TeAP3-1, 35S::TeAP3-2, 35S::TeTM6-1 or 35S::TeTM6-2 showed any obvious morphological changes from the non-transgenic line. For transgenic lines of 35S::TePI, five out of 21 N. rotundifolia plants showed altered floral morphology as compared with the non-transgenic line (Fig 6). There was a decrease in plant height of the transformants compared with the non-transgenic plants (Fig 6a, Table 1), but no significant differences were observed in terms of crown size, leaf length and leaf width (Table 1). The most obvious floral changes were detected in the second, third and forth whorls of the flower. The petal (Fig 6b), stamen (Fig 6c) and style (Fig 6d) of transformants were significantly shorter than the non-
Fig 4. Expression levels of B class genes in different tissues, organs and different development stages of flower buds of *T. erecta*. The relative expression level showed that strong expression of *TePI* was detected in stamens of disk florets, and weak expression in petals of disk and ray florets along with little expression in sepals of ray florets and no expression was detected in pistils of ray florets, sepals of disk floret, bracts, receptacle and vegetative organs. *TeAP3-1* was expressed in sepals and petals of ray and disk.
transgenic line, whereas the ovaries of transformants became larger (Table 1). In addition, in the non-transgenic plants, the ovary was conical shaped and the surface was smooth, but in transgenic plants, the ovary was football-like shaped and the surface was rough (Fig 6d).

Discussion

The B class genes were divided into AP3/DEF and PI/GLO subfamilies [21, 22, 31, 32]. Based on the completely divergent C-terminal motifs, the euAP3 and TM6 lineages were recognized as the clades in the AP3/DEF subfamily in a number of higher eudicot species [18]. In this study, five MADS-box B class genes were isolated and characterized in T. erecta. Sequence comparisons and phylogenetic analyses indicated that there were two euAP3-like genes in T. erecta, which was consistent with findings in many other Asteraceae species [28, 33]. Broholm et al. [27] and Barker et al. [34] concluded that the paralogous pair of euAP3 lineage genes was a common origin, possibly from the genome-wide duplication at the base of the Asteraceae

![Yeast two-hybrid interactions of five MADS-box B class proteins of T. erecta](https://example.com/yeast_two-hybrid_interactions.png)

**Fig 5. Yeast two-hybrid interactions of five MADS-box B class proteins of T. erecta.** The PCR products were introduced into the GAL4-based yeast two-hybrid vectors pGADT7 vector (AD) and pGBK7 vector (BK) and co-transformed into the AH109 yeast strain. (a) Co-transformed yeast cells were plated on selection plates SD / -Trp-Leu- solid medium. (b) Yeast growth on selection plates SD / -Trp-Leu-His-Ade / X-ar-GAL solid medium showed that TePI protein could interact with other four AP3/TM6-like proteins. No homodimer or interaction between the AP3/TM6-like proteins was observed in T. erecta. Besides, we observed homodimerization in TePI proteins.

![Diagram of flower parts](https://example.com/diagram_flower_parts.png)

**Fig 6. Diagram of flower parts.** The diagram shows the relative expression levels of B class genes in different sizes of flower buds revealed that the expression level increased with the size of flower buds in T. erecta. RS: sepal of ray floret; RP: petal of ray floret; RPi: pistil of ray floret; Se: sepal of disk floret; Pe: petal of disk floret; St: stamen of disk floret; Pi: pistil of disk floret; Br: bract; Re: receptacle; Ov: ovary; Le: leaf; Sm: stem; Rt: root; FB: size of flower bud; 1 mm: flower buds 1 mm in diameter; 3 mm: flower buds 3 mm in diameter; 5 mm: flower buds 5 mm in diameter; 7 mm: flower buds 7 mm in diameter.

The relative expression levels of B class genes in different sizes of flower buds revealed that the expression level increased with the size of flower buds in T. erecta.
lineage. Furthermore, there were two TM6-like genes in *T. erecta*, and the TeTM6-2 was a partial fragment of TeTM6-1 and there seemed to be no difference between their protein interaction capacities, indicating that TeTM6-1 or TeTM6-2 was completely functionally redundant. Meanwhile, our study proved that there was only one TePI gene belonging to the PI/GLO subfamily in *T. erecta*. This result agreed with the finding in *G. hybrida* [27] but contrasted with findings in *H. annuus* [28] and *Chrysanthemum x morifolium* [35] in which two and three PI/GLO-like genes were found, respectively.

According to the classical ABC model, B class genes are essential for the development of the second and third whorls of flowers [21, 22, 32, 36]. Thus, it was expected that the B class genes would predominantly expressed in petals and stamens [37]. However, expression studies of MADS-box B class genes in several eudicot species revealed that B-function regulation varied within the eudicot lineages [18, 37]. In this study, apart from petals and stamens, TeAP3-1 was also expressed in sepals, ovaries, and pistils of ray florets, and TeAP3-2 was also expressed in
genes may have different effects in the floral organ development of transformants. Over-
could affect floral organ development in the surface of the abnormally shaped ovary was rough, indicating that the over-expression of the petal, stamen and pistil were significantly shorter than the non-transgenic plants and the contrast, the transgenic plants of 35S::TeAP3 through Agrobacterium five B class genes connected with the 35S promoter were transformed into floral whorls in disk flowers but not in the petals of ray flowers at comparable developmental expression of and TeTM6 in sepals of disk and ray florets, ovaries and petals of disk florets. No expression of anthesis [44].

Calcium piloselloides and petioles. [22] and Micro-Tom [41]. Besides, the eu in A not only expressed in petal and stamen, but also in sepal, carpel and all the other floral organs [31, 38], the expression of eu AP3 genes were also expressed in the ovules in A. thaliana [22] and Medicago truncatula [42]. In G. hybrida, GDEF2 and GDEF3 transcripts were detected throughout all of the four whorls of floral organs, as well as in vegetative organs such as leaves and petals. GDEF2 was also expressed in scapes, ovules, leaves and roots [27, 43]. In Hieracium piloselloides, the expression of HpDEF was detected along with ovule development before anthesis [44]. TeTM6-1 was strongly expressed in the stamens of disk florets, weakly expressed in sepals of disk and ray florets, ovaries and petals of disk florets. No expression of TeTM6-1 and TeTM6-2 were detected in the petals of ray florets. Similar results were reported in the expression of GDEF1 in G. hybrida where GDEF1 transcripts were detected in all of the four floral whors in disk flowers but not in the petals of ray flowers at comparable developmental stages [27, 43]. Similar phenomenon was also reported in P. hybrida, where PhTM6 was mainly expressed in the developing stamens but played no role in petal development [45]. Vandenbussche et al. [37] concluded that PhTM6 behaved more like a C-function gene than a B-function gene. Besides, the TeAP3-1, TeTM6-1 and TeTM6-2 genes were expressed in the ovary, indicating that these genes might have a role in ovule development.

To further investigate the function of TePI, TeAP3-1, TeAP3-2, TeTM6-1 and TeTM6-2, the five B class genes connected with the 35S promoter were transformed into N. rotundifolia through Agrobacterium-mediated transformation. None of the transgenic plants of 35S::TeAP3-1, 35S::TeAP3-2, 35S::TeTM6-1 or 35S::TeTM6-2 showed obvious morphological changes from the non-transgenic line. Similar phenomena occurred in many other species, such as Rosa rugosa [46], Narcissus tazetta var. Chinensis [47], Chimonanthus praecox [48] and Prunus mume [49]. It seemed that TeAP3-1, TeAP3-2, TeTM6-1 and TeTM6-2 could not regulate floral organ development independently, when ectopically expressed in N. rotundifolia. By contrast, the transgenic plants of 35S::TePI showed obvious changes in floral morphology as the petal, stamen and pistil were significantly shorter than the non-transgenic plants and the surface of the abnormally shaped ovary was rough, indicating that the over-expression of TePI could affect floral organ development in N. rotundifolia. The ectopic expression of PI-like genes may have different effects in the floral organ development of transformants. Over-

Table 1. Phenotypic analysis of non-transgenic and transgenic N. rotundifolia lines of 35S::TePI unit: cm.

| Traits           | Non-transgenic lines | Transgenic lines of 35S::TePI |
|------------------|----------------------|-----------------------------|
| Number of plant  | 15                   | 5                           |
| Plant height     | 20.61±1.43*          | 15.83±1.13                  |
| Crown size       | 18.02±1.27           | 17.20±1.56                  |
| Leaf length      | 9.30±0.71            | 8.74±0.57                   |
| Leaf width       | 3.70±0.34            | 3.53±0.23                   |
| Flower height    | 1.27±0.14*           | 1.03±0.11                   |
| Flower diameter  | 0.66±0.03            | 0.62±0.03                   |
| Stamen length    | 1.08±0.04*           | 0.80±0.07                   |
| Style length     | 0.87±0.06*           | 0.40±0.05                   |
| Ovary height     | 0.25±0.03            | 0.32±0.02*                  |

*Indicated significant difference by Student t test (P<0.001)  
doi:10.1371/journal.pone.0169777.t001
expression of *Platanus acerifolia* PaPI2a gene in transformed *Nicotiana tabacum* led to elongated sepals, shorter pistils and abnormal ovaries covered with trichomes [50]. Over-expression of PeMADS6 of *Phalaenopsis equestris* in *A. thaliana* resulted in petaloid sepals [51].

According to the previous studies, AP3/DEF and PI/GLO proteins are functional partners and interact with each other to form obligate heterodimers for DNA binding *in vitro* and to regulate gene expression by binding to the CArG motif of their promoters [8, 52]. In the present study, TePI protein could form heterodimers with all the other four B class proteins TeAP3-1, TeAP3-2, TeTM6-1, and TeTM6-2. No homodimer or other interaction was observed between the euAP3 or TM6 clade members. But interestingly, TePI protein could form a homodimer. B class proteins have been shown to form homodimers or heterodimers in monocot species and function as AP3/PI heterodimers in eudicot species. Some of the DEF/AP3-like MADS-box proteins from monocots have the ability to form homodimers, such as LMADS1 of *Lilium longiflorum* [53], PeMADS4 and PeMADS6 of *P. equestris* [54], OMADS5, OMADS3 and OMADS9 of *Oryza sativa* [55]. For eudicot species, Roque et al. [42] observed homo-dimerization of MtPI protein in *M. truncatula*, but the underlying mechanism is not clear and requires further studies.

Genetic male sterile lines have been utilized in the F1 hybrid production of *T. erecta* for many years [56, 57]. So far, however, the molecular mechanism of the male sterility trait in *T. erecta* remains unclear. Many reports indicated that the male sterility trait in *T. erecta* was controlled by a recessive gene [58, 59], but no related functional gene has been characterized yet. He et al. [59] found that the petals of the ray and disk florets of the male sterile plant of *T. erecta* developed to sepal-like, while the stamens developed to carpel-like structures, they concluded that the spontaneously derived genetic male sterility trait was the result of homeotic conversion of floral organs and assumed that the homeotic conversion in *T. erecta* might be due to the mutation of B class MADS-box genes. In this study, only the *TePI* gene was predominantly expressed in the petals of ray and disk florets and stamens of disk florets, while the other four *AP3* and *TM6* genes were not only expressed in petals and stamens, but also in other floral organs. And among all transgenic *N. rotundifolia* plants of five B class genes of *T. erecta*, only transgenic plants of 35S::*TePI* showed altered floral morphology. Thus, it is assumed that the male sterility traits of *T. erecta* might be associated with *TePI*, which is worthy of further investigation.

**Supporting Information**

S1 Fig. Sequence alignment of cDNA of five MADS-box B class genes of *T. erecta*. (TIF)

S1 Table. List of primers in this study. (DOCX)

S2 Table. Specific primers of five MADS-box B class genes of *T. erecta* containing restriction sites for the yeast two-hybrid assay. (DOCX)

**Acknowledgments**

We thank all past and present colleagues in our lab for constructive discussion and technical support.

**Author Contributions**

Conceptualization: YHH MZB YA.
Data curation: YA.
Formal analysis: YA CLZ.
Funding acquisition: YHH.
Investigation: YA CLZ YLS WNW.
Methodology: YHH MZB YA.
Project administration: YHH MZB.
Resources: YA CLZ YLS WNW YHH MZB.
Software: YA CLZ.
Supervision: YHH MZB.
Validation: YA CLZ YLS WNW.
Visualization: YA YHH MZB.
Writing – original draft: YA YHH MZB.
Writing – review & editing: YA YHH MZB WNW.

References
1. Vasudevan P, Kashyap S, Sharma S. (1997) Tagetes: a multipurpose plant. Bioreour. Technol. 62: 29–33.
2. Bhatt BJ. (2013) Comparative analysis of larvicidal activity of essential oils of Cymbopogon flexuosus (Lemon grass) and Tagetes erecta (Marigold) against Aedes aegypti larvae. Euro. J. Exp. Bio. 3: 422–427.
3. Ayyadurai N. (2013) Evaluation of cytotoxic properties of Curcuma longa and Tagetes erecta on cancer cell line (Hep2). Afr. J. Pharm. Pharma. 7: 736–739.
4. Liu Z, Cai X, Seiler GJ, Jan CC. (2014) Interspecific amphiploid-derived alloplasmic male sterility with defective anthers, narrow disc florets and small ray flowers in sunflower. Plant Breeding 133:742–747.
5. Ai Y, Zhang Q, Pan C, Zhang H, Ma S, He Y, et al. (2015) A study of heterosis, combining ability and heritability between two male sterile lines and ten inbred lines of Tagetes patula. Euphytica 203: 349–366.
6. Coen ES, Meyerowitz EM. (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353, 31–37. doi: 10.1038/353031a0 PMID: 1715520
7. Weigel D, Meyerowitz EM. (1994) The ABCs of floral homeotic genes. Cell 78: 203–209. PMID: 7913881
8. Theissen G. (2001) Development of floral organ identity: stories from the MADS house. Curr. Opin. Plant Biol. 4: 75–85. PMID: 11163172
9. Colombo L, Franken J, Koetje E, van Went J, Dons HJM, Angenent GC, et al. (1995) The petunia MADS box gene FBP11 determines ovule identity. Plant Cell 7: 1859–1868. PMID: 8535139
10. Favaro R, Immink RG, Ferioli V, Bemascconi B, Byzova M, Angenent GC, et al. (2002) Ovule-specific MADS-box proteins have conserved protein-protein interactions in monocot and dicot plants. Mol. Genet. Genomics 268: 152–159. doi: 10.1007/s00438-002-0746-6 PMID: 12395189
11. Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF. (2000) B and C floral organ identity functions require SEPALLATA MADS-box genes. Nature 405: 200–203. doi: 10.1038/35121013 PMID: 10821278
12. Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF. (2004) The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr. Biol. 14: 1935–1940. doi: 10.1016/j.cub.2004.10.028 PMID: 15530395
13. Purugganan MD, Rounsley SD, Schmidt RJ, Yanofsky MF. (1995) Molecular evolution of flower development: Diversification of the plant MADS-box regulatory gene family. Genetics 140: 345–356. PMID: 7635298
14. Theissen G, Kim JT, Saedler H. (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J. Mol. Evol. 43: 484–516. PMID: 8875863
15. Kim S, Yoo MJ, Albert VA, Farris JS, Soltis PS, Soltis DE. (2004) Phylogeny and diversification of B-function MADS-box genes in angiosperms: evolutionary and functional implications of a 260-million-year-old duplication. Am. J. Bot. 91: 2102–2118. doi: 10.3732/ajb.91.12.2102 PMID: 21652358

16. Kramer EM, Dorit RL, Irish VF. (1998) Molecular Evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. Genetics 149: 765–783. PMID: 9611190

17. Kramer EM, Holappa L, Gould B, Jaramillo MA, Setnikov D, Santiago PM. (2007) Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot Aquilegia. Plant Cell 19:750–766. doi: 10.1105/tpc.107.050385 PMID: 17400892

18. Kramer EM, Irish VF. (2000) Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. Int. J. Plant Sci. 161: S29–S40.

19. Kyozuka J, Kobayashi T, Morita M, Shimamoto K. (2000) Spatially and temporally regulated expression of rice MADS box genes with similarity to Arabidopsis class A, B and C genes. Plant Cell Physiol. 41: 710–718. PMID: 10943540

20. Bowman JL, Smyth DR, Meyerowitz EM. (1989) Genes directing flower development in Arabidopsis. Plant Cell 1:37–52. doi: 10.1105/tpc.1.1.37 PMID: 2535466

21. Goto K, Meyerowitz EM. (1994) Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Gene. Dev. 8: 1548–1560. PMID: 7958839

22. Jack T, Brockman LL, Meyerowitz EM. (1992) The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell 68: 683–697. PMID: 13467556

23. Angenent GC, Colombo L. (1996) Molecular control of ovule development. Trends Plant Sci. 1: 228–232.

24. Kang HG, Jeon JS, Lee S, An G. (1998) Identification of class B and class C floral organ identity genes from rice plants. Plant Mol. Biol. 36: 1021–1029. PMID: 9869408

25. Ambrose BA, Lerner DR, Ciceri P, Padilla CM, Yanofsky MF, Schmidt RJ. (2000) Molecular and genetic analyses of the Silky 1 gene reveal conservation in floral organ specification between eudicots and monocots. Mol. Cell 5: 569–579. PMID: 10882141

26. Nagasawa N, Miyoshi M, Sano Y, Sato H, Hirano H, Sakai H, et al. (2003) SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. Development 130: 705–718. PMID: 12506001

27. Broholm SK, Pöllänen E, Ruokolainen S, Tähtiharju S, Kotilainen M, Albert VA, et al. (2010) Functional characterization of B class MADS-box transcription factors in Gerbera hybrida. J. Exp. Bot. 61: 75–85. doi: 10.1093/jxb/erp279 PMID: 19767305

28. Dezar CA, Tioni MF, González DH, Chan RL. (2003) Identification of three MADS-box genes expressed in sunflower capitulum. J. Exp. Bot. 54: 1637–1639. doi: 10.1093/jxb/erg163 PMID: 12730268

29. Ai Y, Zhang Q, Wang W, Zhang C, Cao Z, Bao M, et al. (2016) Transcriptomic analysis of differentially expressed genes during flower organ development in genetic male sterile and male fertile Tagetes erecta by digital gene-expression profiling. PLoS one 11: e0150892. doi: 10.1371/journal.pone.0150892 PMID: 26939127

30. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. (1985) A simple and general method for transferring genes into plants. Science 227: 1229–1231. doi: 10.1126/science.227.4691.1229 PMID: 17757866

31. Sommer H, Beltrán JP, Huijser P, Pape H, Lönnig WE, Saedler H, et al. (1990) DEFICIENS, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J. 9: 605–613. PMID: 1968830

32. Tröbner W, Ramirez L, Motte P, Huijser P, Pape H, Lönnig WE, et al. (1992) GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. EMBO J. 11: 4693–4704. PMID: 13611166

33. Shulga OA, Shchennikova AV, Angenent GC, Skryabin KG. (2008) MADS-box genes controlling inflorescence morphogenesis in sunflower. Russ. J. Dev. Biol. 39: 2–5.

34. Barker MS, Kane NC, Matvienko M, Kozik A, Michelmore RW, Knapp SJ, et al. (2008) Multiple polyploidizations during the evolution of the Compositae reveal parallel patterns of duplicate gene retention after millions of years. Mol. Biol. Evol. 25: 2445–2455. doi: 10.1093/molbev/msn187 PMID: 18728074

35. Liu H, Sun M, Du D, Pan H, Cheng T, Wang J, et al. (2016) Whole-transcriptome analysis of differentially expressed genes in the ray florets and disc florets of Chrysanthemum morifolium. BMC Genomics 17: 398. doi: 10.1186/s12864-016-2733-z PMID: 27225275

36. Krizek BA, Meyerowitz EM. (1996) The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. Development 122: 11–22. PMID: 8565821
37. Vandenbussche M, Zethof J, Roayaert S, Weterings K, Gerats T. (2004) The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in *Petunia hybrid* flower development. Plant Cell 16: 741–754. doi: 10.1105/tpc.019166 PMID: 14973163

38. Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H. (1990) Genetic control of flower development by homeotic-box genes in *Antirrhinum majus*. Science 250: 931–936. doi: 10.1126/science.250.4983.931 PMID: 17746916

39. Davies B, Di Rosa A, Neve T, Saedler H, Sommer H. (1996) Alteration of tobacco floral organ identity by expression of combinations of *Antirrhinum* MADS-box genes. Plant J. 10: 663–677. PMID: 8893543

40. Tsuchimoto S, Mayama T, van der Krol A, Ohtsubo E. (2000) The whorl-specific action of a petunia class B floral homeotic gene. Genes Cells 5: 89–99. PMID: 10672040

41. de Martino G, Pan I, Emmanuel E, Levy A, Irish VF. (2006) Functional analyses of two tomato APE-TAL-A3 genes demonstrate diversification in their roles in regulating floral development. Plant Cell 18:1833–1845. doi: 10.1105/tpc.106.042978 PMID: 16844904

42. Roque E, Serwatowska J, Cruz-Rocha M, Wen J, Mysore KS, Yenush L, et al. (2013) Functional specialization of duplicated AP3-like genes in *Medicago truncatula*. Plant J. 73: 663–675. doi: 10.1111/tpj.12068 PMID: 23146152

43. Yu D, Kotilainen M, Mehto M, Albert VA, Teeri TH. (1999) Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). Plant J. 17: 51–62. PMID: 10069067

44. Guerin J, Rossel JB, Robert S, Tsuchiyao T, Koltunow A. (2000) A *MADS-box* gene reveals functional divergence within the DEF/AP3 lineage. Plant Cell 11: 1093–1099. doi: 10.1105/tpc.109.109065 PMID: 11028175

45. Rigpikema AS, Roayaert S, Zethof J, van der Weerden G, Gerats T, Vandenbussche M. (2006) Analysis of the petunia TM6 MADS-box gene reveals functional divergence within the DEF/AP3 lineage. Plant Cell 18: 1819–1832. doi: 10.1105/tpc.106.042937 PMID: 16844905

46. Hibino Y, Kitahara KS, Hirai S, Matsumoto S. (2006) Structural and functional analysis of rose class B *MADS-box* genes ‘MASAKO BP, euB3, and B3’: Paleo-type AP3 homologue ‘MASAKO B3’ association by expression of combinations of B function proteins. J. Biol. Chem. 279: 10747–10755. doi: 10.1074/jbc.M310546200 PMID: 16761882

47. Li XF, Xu J, Yang R, Jia LY, Deng XJ, Xiong LJ, et al. (2013) Analysis of B-Class Genes NAP3L3 and NAP3L4 in *Narcissus tazetta* var. chinensis. Plant Mol. Biol. Rep. 31: 255–263.

48. Zhang Q, Wang BG, Duan K, Wang LG, Wang M, Tang XM, et al. (2011) The paleoAP3-type gene CpaP3, an ancestral B-class gene from the basal angiosperm *Chimonanthus praecox*, can affect stamina and petal development in higher eudicots. Dev. Genes Evol. 221: 83–93. doi: 10.1007/s00427-011-0361-9 PMID: 21505842

49. Xu Z, Zhang Q, Sun L, Du D, Cheng T, Pan H, et al. (2014) Genome-wide identification, characterisation and expression analysis of the MADS-box gene family in *Prunus mume*. Mol. Genet. Genomics 289: 903–920. doi: 10.1007/s00438-014-0863-z PMID: 24859011

50. Zhang J, Guo C, Liu G, Li Z, Li X, Bao M. (2011) Genetic alteration with variable intron/exon organization amongst five *Ph*-homoeologous genes in *Platanus acerifolia*. Gene 473: 82–91. doi: 10.1016/j.gene.2010.11.005 PMID: 21112379

51. Tsai WC, Lee PF, Chen HI, Hsiao YY, Wei WJ, Pan ZJ, et al. (2005) *PeMADS6*, a *GLOBOSA/PISTIL-LATA*-like gene in *Phalaenopsis equestris* involved in petaloid formation, and correlated with flower longevity and ovary development. Plant Cell Physiol. 46: 1125–1139. doi: 10.1093/pcp/pci125 PMID: 15890679

52. Theissen G, Saedler H. (2001) Plant biology: floral quartets. Nature 409: 469–471. doi: 10.1038/35054172 PMID: 11206529

53. Tseng TY, Liu HC, Yang CH. (2004) The C-terminal sequence of *LMADS1* is essential for the formation of homodimers for B function proteins. J. Biol. Chem. 279: 10747–10755. doi: 10.1074/jbc.M311646200 PMID: 14676188

54. Tsai WC, Pan ZJ, Hsiao YY, Jeng MF, Wu TF, Chen W, et al. (2008) Interactions of B-class complex proteins involved in tepal development in *Phalaenopsis* orchid. Plant Cell Physiol. 49: 814–824. doi: 10.1093/pcp/pcn059 PMID: 18390881

55. Chang YY, Kao NH, Li JY, Hsu WH, Liang YL, Wu JW, et al. (2010) Characterization of the possible roles for B class MADS-box genes in regulation of perianth formation in orchid. Plant Physiol. 152: 837–853. doi: 10.1104/pp.109.147116 PMID: 20018605

56. Singh B, Swarup V. (1971) Heterosis and combining ability in African marigold. Indian J. Genet. Pl. Br. 31: 407–415.

57. Sreerakala C, Raghava SPS. (2003) Exploitation of heterosis for carotenoid content in African marigold (*Tagetes erecta*) L. and its correlation with esterase polymorphism. Theor. Appl. Genet. 106: 771–776. doi: 10.1007/s00122-002-1143-6 PMID: 12596009
58. He YH, Ning GG, Sun YL, Qi YC, Bao MZ. (2009) Identification of a SCAR marker linked to a recessive male sterile gene (Tems) and its application in breeding of marigold (Tagetes erecta). Plant Breeding 128: 92–96.

59. He YH, Ning GG, Sun YL, Hu Y, Zhao XY, Bao MZ. (2010) Cytological and mapping analysis of a novel male sterile type resulting from spontaneous floral organ homeotic conversion in marigold (Tagetes erecta L.). Mol. Breeding 26: 19–29.