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1 Title

2 **Characterization of *CYCLOIDEA*-like genes in controlling floral zygomorphy in**
3 **the monocotyledon *Alstroemeria***

4

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1 **ABSTRACT**

2 The *CYCLOIDEA* (*CYC*) gene controls the development of zygomorphic flowers and
3 the determination of adaxial identity of floral organs in the model developmental
4 system of *Antirrhinum majus*. However, whether *CYC* homologue genes also control
5 floral zygomorphy in monocotyledon *Alstroemeria* plants is yet unknown. In this
6 study, we investigated *CYC*-like genes in the monocotyledons *Alstroemeria aurea*, *A.*
7 *magenta*, and *A. pelegrina* var. *rosea*, all of which have zygomorphic flowers. Since
8 the *CYC* gene belongs to the T-complex protein (TCP) gene family of transcription
9 factors, cloning of *CYC*-like sequences was performed using rapid amplification of
10 cDNA ends (RACE)-polymerase chain reaction (PCR) by using degenerate primers
11 designed for the TCP domain. We cloned 1 *CYC*-like sequence each from *A. aurea*
12 (*AaTCP1*, accession number AB714967 in the GenBank/EMBL/DDBJ databases) and
13 *A. magenta* (*AmTCP1*, AB714970), and 2 *CYC*-like sequences from *A. pelegrina* var.
14 *rosea* (*ApTCP1*, AB714968; and *ApTCP2*, AB714969). The deduced amino acid
15 sequences of *AaTCP1*, *AmTCP1*, *ApTCP1*, and *ApTCP2* shared 67.7%, 67.7%, 71.0%,
16 and 64.5% identities, respectively, with the TCP domain in *CYC*. Molecular
17 phylogenetic analysis indicated that 3 *CYC*-like genes from *Alstroemeria* belonged to
18 the *ZinTBL1b* clade in the *CYC/tb1*-like subfamily. Reverse transcription (RT)-PCR
19 and *in situ* hybridization analyses showed that *AaTCP1* transcripts were specifically
20 detected in flower buds and localized in the base of adaxial inner perianth of *A. aurea*.
21 These results suggest that *CYC*-like genes are also involved in the development of
22 floral asymmetry and the determination of adaxial identity of floral organs in the
23 monocotyledon *Alstroemeria*.

1 **Keywords:** Alstroemeriaceae; *CYCLOIDEA*-like genes; floral asymmetry;
2 monocotyledon; zygomorphy
3
4 **Abbreviations:** *CYC*, *CYCLOIDEA*; *DICH*, *DICHOTOMA*; RACE-PCR, Rapid
5 amplification of cDNA ends-polymerase chain reaction; *Tb1*, *Teosinte branched 1*;
6 TE, Tris-EDTA
7

1. Introduction

Zygomorphic flowers are thought to have evolved from radially symmetric flowers in response to the evolution of specialized pollinators, since their corollas (petal whorls) encourage an approach from one particular direction (Stebbins 1974; Endress, 1999). Thus far, the genetic mechanisms of floral zygomorphy have been studied in Ranunculales, Fabales, Brassicales, Lamiales, and Dipsacales (Jabbour et al., 2009). Endress (1999) and Stebbins (1974) indicated that zygomorphic flowers independently evolved in several clades in order to adapt to diverse pollination methods associated with specialized pollinators. This suggests that the occurrence of floral zygomorphy plays an important role in the diversification of flowering plants (Cubas, 2004; Citerne et al., 2010).

The genetic machinery for the occurrence of zygomorphic flowers was first identified in *Antirrhinum majus*, where it was found that the *CYCLOIDEA* (*CYC*) gene is required for the development of zygomorphic flowers and the determination of adaxial identity of floral organs (Luo et al., 1996; 1999). Both *CYC* and *DICHOTOMA* (*DICH*) are expressed in the adaxial region of the floral meristem of *A. majus*. The expression of the *CYC* and *DICH* genes is restricted in the adaxial region during petal and stamen formation (reviewed in Preston and Hileman, 2009). Coordination of expression patterns of these genes contributes to the determination of floral asymmetry.

The *CYC* gene belongs to the T-cell protein (TCP) gene family of transcription factors, which are characterized by a highly conserved DNA-binding region (Kosugi and Ohashi, 1997; 2002). This conserved region was named the TCP domain, which originated from *Teosinte branched 1* (*Tb1*) from *Zea mays* (Doebley et al., 1997),

1 *CYC* from *A. majus* (Luo et al., 1996), and *PROLIFERATING CELL FACTORS 1* and
2 *2* (*PCF1* and *PCF2*) from *Oryza sativa* (Kosugi and Ohashi, 1997).

3 TCP gene family is divided to 2 major clades: Class I (PCF-like genes) and
4 Class II (Kosugi and Ohashi, 1997; 2002; Cubas et al., 1999; Howarth and Donoghue,
5 2006; Martín-Trillo and Cubas, 2010). Class II comprises of *CYC-tb1*- and *CIN*-like
6 clades. By using degenerate primers designed for the TCP domain, researchers have
7 cloned the *CYC* homologues from a wide range of eudicotyledonous plants belonging
8 to the families Gesneriaceae (Citerne et al., 2000), Fabaceae (Fukuda et al., 2003;
9 Citerne et al., 2006; Feng et al., 2006; Wang et al., 2008), Caprifoliaceae (Howarth et
10 al., 2011), Fumariaceae (Kölsch and Gleissberg, 2006), Papaveraceae (Kölsch and
11 Gleissberg, 2006), Solanaceae (Reeves and Olmstead, 2003), and Plantaginaceae
12 (Baldwin et al., 2011), as well as from the model plant *Arabidopsis thaliana* (Cubas et
13 al., 1999). Howarth and Donoghue (2006) analysed *CYC-tb1* clade in eudicots and
14 revealed the duplications during the evolutionary process. Two duplication events in
15 *CYC-tb1*-like genes were proposed to have led to the development of 3 subgroups,
16 *CYC1*, *CYC2*, and *CYC3* (Howarth and Donoghue, 2006; Chapman et al., 2008).

17 Unlike the extensive studies in eudicots, the role of *CYC* genes for the
18 establishment of floral zygomorphy in monocots has not been widely investigated
19 (Mondragón-Palomino and Trontin, 2011). *CYC* research in monocotyledonous plants
20 has been reported in graminaceous plants such as *Oryza sativa* (Kosugi and Ohashi,
21 1997; Yuan et al., 2009) and *Zea mays* (Doebley et al., 1997). Rudall and Bateman
22 (2004) summarized patterns and processes that induced floral zygomorphy in
23 monocots. Recently, Bartlett and Specht (2011) analysed *CYC* genes in Zingiberales
24 (Costaceae and Heliconiaceae) and suggested that changes of expression pattern of
25 *TB1*-like genes provide a mechanism for evolutionary shifts in floral zygomorphy.

1 Preston and Hileman (2012) also characterized *CYC* genes in *Commelina* and
2 *Tradescantia* (Commelinaceae). Monocot *TCP*-like sequences were found to be
3 associated in 20 major groups with an average identity of $\geq 64\%$ and corresponded to
4 well-supported clades of the phylogeny. In order to resolve the detailed common
5 genetic machinery of floral zygomorphy in eudicotyledonous and monocotyledonous
6 plants, further cloning and analysis of *CYC* homologue genes in monocotyledonous
7 plants is required.

8 Plants belonging to the monocotyledonous genus *Alstroemeria*, family
9 Alstroemeriaceae, have recently become popular as ornamentals, and thus are prized
10 for their floral morphology. Furthermore, molecular mechanisms controlling flower
11 development in this plant species have been studied by analysing class B genes (Hirai
12 et al., 2007) and *LEAFY*-like gene (Hirai et al., 2012). Additionally, genetic
13 transformation of *Alstroemeria* has been established by using *Agrobacterium*
14 *tumefaciens* (Kim et al., 2007; Hoshino et al., 2008). These research findings will be
15 useful for further analyses of flower development in this plant species. Therefore, in
16 this study, we investigated *CYC* homologue genes in controlling floral symmetry in
17 *Alstroemeria aurea*, *A. magenta*, and *A. pelegriana* var. *rosea*, all of which have
18 zygomorphic flowers. The genus *Alstroemeria* contains 75 species (Hofreiter and
19 Rodriguez, 2006), of which, these 3 species retain horticultural importance because of
20 their high ornamental values (Fig. 1) and are considered to be the origins of present
21 cultivars. Furthermore, flower characteristics of these species have been extensively
22 evaluated (Kashihara et al., 2011). Therefore, these 3 species were selected for this
23 study in order to establish a model for analysing floral zygomorphy in *Alstroemeria*.
24 We cloned *CYC*-like genes from these plants and analysed their expression pattern by
25 using reverse transcription polymerase chain reaction (RT-PCR). We then used *in situ*

hybridization to investigate the localization of *CYC*-like gene expression in the floral organs. Finally, we investigated how floral zygomorphy is related to *CYC*-like gene expression in *Alstroemeria*.

2. Materials and methods

2.1. Plant materials

Three species of *Alstroemeria* (*A. aurea*, *A. magenta*, and *A. pelegrina* var. *rosea*; Fig. 1) were used in the present study. Potted plants of each species were cultivated in a greenhouse at Experiment Farms, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Plants were grown under natural light conditions, and the greenhouse was maintained at a minimum temperature of 15°C by heating during winter.

2.2. Extraction of total RNA

Total RNA was extracted from ca. 3–5-mm flower buds using the Concert Plant RNA Reagent (Invitrogen, San Francisco, CA, USA), following the manufacturer's instructions. Approximately 5 g fresh weight of flower buds was ground in liquid nitrogen, following which, 2 mL of Concert Plant RNA Reagent was added to the samples. Total RNA was then extracted according to the manufacturer's instructions, and purified RNA was dissolved in 100 µL of Tris-EDTA (TE) buffer. The concentration of extracted total RNA was measured using a spectrophotometer (ND-1000; Nano-Drop Technologies, Wilmington, DE, USA).

2.3. Cloning of *CYC* homologues

1 The total RNA extracted from flower buds was used as a template for PCR at a
2 concentration of $5\ \mu\text{g}\cdot\text{mL}^{-1}$. Next, $1\ \mu\text{L}$ of an adapter primer (10 mM) (5'-GGC CAC
3 GCG TCG ACT AGT ACT₁₇) was mixed with $10\ \mu\text{L}$ distilled water (DW). The
4 samples were denatured by incubating for 10 min at 70°C and then quickly transferred
5 onto ice. For the reverse transcriptase reaction, the reaction solution ($2\ \mu\text{L}$ 10× PCR
6 buffer, $2\ \mu\text{L}$ 25 mM MgCl_2 , $1\ \mu\text{L}$ 10 mM dNTP mix, $2\ \mu\text{L}$ 0.1 M dithiothreitol
7 (DTT); total $7\ \mu\text{L}$) was added to the template RNA, and the mixture was then
8 incubated for 5 min at 42°C . Subsequently, $1\ \mu\text{L}$ of the SuperScriptII reverse
9 transcriptase (Gibco BRL; $200\ \text{U}\cdot\mu\text{L}^{-1}$) was added to the mixture, which was then
10 incubated at 42°C for 50 min and at 70°C for 15 min. The first-strand cDNA obtained
11 from this procedure was treated with $1\ \mu\text{L}$ RNase H ($2\ \text{U}\cdot\mu\text{L}^{-1}$) for 20 min at 37°C .

12 Three degenerate primers for the *CYC* homologue genes were constructed
13 using previously published data [see Additional Information–Table 1] and used for
14 PCR: TCP1 (5'-AAA GAY CGV CAC AGC AAR RTA), TCP2 (5'-CAC AGC AAR
15 ATA TAC ACV BCM CAA), and R primer (5'-CTT CTC TTD GTT CKY TCC CT).
16 PCR products were purified using the Wizard SV Gel and PCR Clean-Up System
17 (Promega).

18 The PCR products were ligated with the pGEM-T Easy Vector (Promega) and
19 used to transform competent cells of *Escherichia coli* XL1-Blue. Samples were
20 incubated overnight, white colonies were selected, and PCR was performed using
21 M13 primer M4 (5'-GTT TTC CCA GTC ACG AC) and M13 primer RV (5'-CAG
22 GAA ACA GCT ATG AC).

23 The sequences obtained in this way from *A. aurea* were used for the
24 construction of the specific primers 3'TCP-1 (5'-CGA TTT CTT CAA GCT CCA A)
25 and 5'TCP-1 (5'-ACC TTT GCT CTA CAC TCC CTA). *ApTCP1* and *AmTCP1* were

1 then cloned from these primers. The PCR products were treated with a Dye
2 Terminator Cycle Sequencing Kit (DTCS; Beckman Coulter) and analysed using an
3 automatic sequencer (CEQ 8000; Beckman Coulter).

5 **2.4. Phylogenetic analysis**

6 Phylogenetic analysis of amino acid sequences of *CYC* homologue genes was
7 performed using the programme SEAVIEW (Galtier et al., 1996). The amino acid
8 sequences were aligned using TCP domain with the SEAVIEW, and a phylogenetic
9 tree was constructed using the maximum likelihood (ML) method by using PhyML
10 3.0 program. Bootstrap analysis (100 replicates) was performed on the data set.

12 **2.5. RT-PCR analysis for *CYC* homologue gene expression**

13 Total RNA was extracted from *A. aurea* flower buds of different lengths (0.5–1, 1–2,
14 and 2–3 mm longitudinal diameter), leaves, and stems. cDNA was synthesized from
15 the total RNA using RT-PCR. The specific primers for *AaTCP1*, 3'TCP-1 (5'-CGA
16 TTT CTT CAA GCT CCA A) and 5'TCP-1 (5'-ACC TTT GCT CTA CAC TCC
17 CTA), were prepared. Pprimers specific to the actin gene of *A. aurea* [ACTIN-1 (5'-
18 GTA TTG TGT TGG ACT CTG GTG ATG GTG T) and ACTIN-2 (5'-GAT GGA
19 TCC TCC AAT CCA GAC ACT GTA)] were used as a control, on the basis of the
20 assumption that different genes with the same transcript numbers will have equal PCR
21 threshold cycle values. Amplification was carried out using an iCycler thermocycler
22 (Bio-Rad, Hercules, CA), with 1 cycle at 94°C for 5 min, and either 20, 25, or 35
23 cycles for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C in a 50 µL PCR solution,
24 including 1 µL first-strand cDNA, 0.5 µL Ex Taq DNA polymerase (5 U·µL⁻¹)
25 (Takara), 5 µL 10× PCR buffer with MgCl₂, 4 µL 2.5 mM dNTP mix, 5 µL of each of

the primers, and 29.5 μ L DW. Subsequently, gel electrophoresis of the PCR products was performed.

2.6. Histological *in situ* hybridization

Flower buds of *A. aurea* were fixed in FAA (50% formaldehyde, 5% acetic acid, 4% formaldehyde) and immediately aspirated for 40 min. The samples were then dehydrated in an ethanol series, and maintained in 99.5% ethanol overnight at 4°C. The samples were soaked several times in absolute ethanol and dehydrated completely in absolute ethanol for 20 min at room temperature. They were then passed through a graded tertiary butyl alcohol (TBA) series and embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis, USA).

Serial sections were cut at 10 μ m by using a microtome (HM315; Carl Zeiss, Oberkochen, Germany) and placed on glass slides coated with APS (S8111, Matsunami Glass Ind., Ltd., Osaka, Japan). The slides were treated twice in xylene for 10 min, and passed through a graded ethanol series (2 $\times$ absolute ethanol for 30 min, followed by 95%, 85%, 70%, 50%, and 30% ethanol for 30 s each). The slides were immersed in sterilized DW twice for 30 s and placed in 0.2 N HCl for 20 min. The slides were then immersed in a protease buffer containing 100 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0) and treated with RNase-free proteinase K (1 mg $\cdot$ mL⁻¹ in protease buffer) for 30 min at 37°C. Next, the slides were immersed in sterilized DW for 5 min and passed through 0.1 M triethanolamine for 5 min (pH 8.0) twice, acetic anhydride (0.25% in 0.1 M triethanolamine) for 10 min, 2 $\times$ SSC for 5 min, sterilized DW for 5 min, a graded ethanol series (30%, 50%, 75%, and 95% ethanol for 5 min each), and 99.5% ethanol for 5 min twice. The slides were dried under reduced pressure with an aspirator for 1 h.

1 For hybridization, 2 mg·mL⁻¹ (final concentration) of a DIG-labelled probe
2 was added to hybridization buffer consisting of 50% (v/v) formaldehyde, 300 mM
3 NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1× Denhardt (0.02% (w/v)
4 Ficoll 400, 0.02% (w/v) polyvinylpyrrolidone, 0.02 (w/v) BSA, 0.25% SDS, 125
5 mg·mL⁻¹ denatured DNA, 125 mg·mL⁻¹ yeast RNA, and 10% (w/v) dextran sulphate.
6 The probes were synthesized by PCR from the sequences for *AaTCP1* (348 bp). The
7 probes were used as an antisense probe. Sense probe was also prepared and used as a
8 control experiment. Each hybridization solution was added to each of the tissue
9 sections, and the sections were incubated in a humidified box at 48°C for 12–16 h.

10 Following hybridization, sections were washed by electro-washing, according
11 to the methods of Kobayashi et al. (1994). The hybridization signals were detected
12 using anti-DIG conjugated with alkaline phosphatase and visualized with reaction
13 buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂, 0.2 mM nitroblue
14 tetrazolium chloride, and 0.2 mM 5-bromo-4-chloro-3-indolyl-phosphate). Following
15 visualization (2–3 h incubation), the sections were treated twice with a solution of 10
16 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 5 min and covered in 70% glycerol with
17 a coverslip. The slides were observed using a stereomicroscope (SMZ800, Nikon) and
18 imaged using a digital camera (Digital Sight DS-L, Nikon).

19

20 **3. Results**

21 **3.1. *CYC-like genes from Alstroemeria***

22 The deduced amino acid sequences of the cloned cDNAs *AaTCP1*, *AmTCP1*, *ApTCP1*,
23 and *ApTCP2* were aligned with those of *CYC*, *DICHOTOMA (DICH)*, *TB1*, *OsTB1*,
24 and *TCP1* (Fig. 2). The deduced amino acid sequences shared 67.7%, 67.7%, 71.0%,
25 and 64.5% identities, respectively, with the TCP domain in *CYC*, indicating that *CYC*-

1 like derived from *Alstroemeria* have high sequence similarity to other *CYC*-like genes
2 (Fig. 2). *ApTCP1* and *AmTCP1* had very high similarity (approximately 93%) to the
3 entire sequence of *CYC*, whereas *AaTCP1* and *ApTCP2* had considerably lower
4 similarities (68.3% and 49.0%, respectively).

6 **3.2. Phylogenetic analysis**

7 TCP family genes have previously been separated into 2 subfamilies: Class I (*PCF*-
8 like) and Class II (Kosugi and Ohashi, 1997, 2002; Cubas et al., 1999). Furthermore,
9 Bartlett and Specht (2011) classified 8 clades of *ZinTBL1a*, *ZinTBL1b*, *ZinTBL2*,
10 *PoaTBL1*, *PoaTBL2*, *CYC1*, *CYC2*, and *CYC3*. In the present study, the deduced
11 amino acid sequences from the TCP domain of *AaTCP1*, *ApTCP1*, *AmTCP1*, and
12 *ApTCP2* were aligned and analysed using the ML method (Fig. 3). This suggested that
13 *AaTCP1*, *ApTCP*, and *AmTCP1* were located in *ZinTBL1b*. Amongst the *Alstroemeria*
14 sequences in the present study, *ApTCP2* was separated from *AaTCP1*, *ApTCP1*, and
15 *AmTCP1*. This indicated that *A. pelegrina* might have 2 types of TCP genes.

17 **3.3. Expression of *AaTCP1* in *Alstroemeria aurea***

18 In *A. aurea*, the specific band showing RNA expression of *AaTCP1* was observed in
19 flower buds but not in leaves or stems (Fig. 4). When 3 developmental stages of
20 flower buds (0.5–1, 1–2, and 2–3 mm) were analysed, the number of PCR cycles was
21 found to affect the detection of *AaTCP1*: when 25 PCR cycles were run, *AaTCP1* was
22 only detected in 2–3 mm flower buds, whereas 35 PCR cycles detected *AaTCP1*
23 bands in all sizes of flower buds (Fig. 5). In contrast, *ACTIN*, which was used as the
24 control, was detected in all samples under all conditions (Fig. 5). This indicates that
25 *AaTCP1* is developmentally expressed in flower buds.

3.4. Localization of AaTCP1 mRNA during flower development

To identify the localization pattern of *AaTCP1* mRNA, transverse sections of flower buds of *A. aurea* were hybridized *in situ* with DIG-labelled sense or antisense *AaTCP1*. For samples that were hybridized with antisense probes, signals were detected in the adaxial inner perianth, gynoecium, and filaments of basal flower buds (Fig. 6). No signal was obtained from the samples that were hybridized with sense probes. Localization pattern of *AaTCP1* mRNA was examined in longitudinal sections (Fig. 7). The signals were observed in central portion of flower buds. Strong signals were localized in base of inner and outer perianth, filaments, anthers, and gynoecium. The localization patterns were similar to those of transverse sections shown in Fig. 6. These findings suggest that *AaTCP1* expression might be involved in flower formation, resulting in floral zygomorphy.

4. Discussion

Previous studies have investigated floral zygomorphy in *Antirrhinum majus* and the role of the *CYC* gene in controlling floral zygomorphy (Luo et al., 1996; Cubas et al., 1999). In the present study, we analysed floral zygomorphy in 3 species of the monocotyledon *Alstroemeria*. Conserved amino acid sequences from these genes were used to design degenerate primers, which were used to clone 4 *CYC*-like sequences (*AaTCP1*, *ApTCP1*, *ApTCP2*, and *AmTCP1*) using 5'RACE. Phylogenetic analysis was then performed on the deduced amino acid sequences of *AaTCP1*, *ApTCP1*, *ApTCP2*, and *AmTCP1* by using the ML method, which indicated that *AaTCP1*, *ApTCP1*, and *AmTCP1* were located in *ZinTBL1b* clade of the *CYC*-/*tb1*-

1 like subfamily, suggesting that these sequences have a high degree of similarity with
2 other *CYC*-like genes.

3 Two clones (*ApTCP1* and *ApTCP2*) were isolated from *A. pelegrina* var. *rosea*
4 in this study, indicating that gene duplication might have occurred at some time in the
5 past. *ApTCP1* was more similar to *AaTCP1* and *AmTCP1* than to *ApTCP2*. *CYC* and
6 *DICH* have been reported to have arisen from gene duplication in *A. majus* (Luo et al.,
7 1999), and species closely related to *A. majus* have also been found to have *CYC*
8 genes with gene duplication (Vieira et al. 1999). Gene duplication of *CYC* might be
9 responsible for the development of the unique flower shape (papilionoid flowers) in
10 plants belonging to Fabaceae (Fukuda et al., 2003). Gene duplication of *CYC* might
11 also be related to floral evolution in monocotyledons such as *Alstroemeria*.

12 In *A. majus*, *CYC* is involved in petal and gynoecium development in the
13 abaxial region of flower buds (Luo et al., 1996; 1999). To determine the temporal and
14 spatial expression patterns of *CYC* homologue genes in *Alstroemeria*, we used RT-
15 PCR and *in situ* hybridization to analyse the expression of *AaTCP1*. RT-PCR
16 indicated that *AaTCP1* is expressed in flower buds but not in leaves or stems, and that
17 during floral development, *AaTCP1* expression is enhanced and maintained in the late
18 stage of flower bud formation. Previous studies have reported that *TCP1* expression
19 disappears in late flower buds in *Arabidopsis* having symmetrical flowers (Cubas et
20 al., 2001), while *CYC* expression is maintained throughout floral development in *A.*
21 *majus* (Luo et al., 1996; 1999). This difference in expression patterns between
22 symmetrical and actinomorphic flowers matches the pattern observed in this study for
23 *Alstroemeria* plants with floral zygomorphy. The expression regions of *CYC*
24 homologue genes in *Alstroemeria* were identified by hybridizing flower buds *in situ*
25 using an antisense probe. Signals were observed at the inner perianth of the adaxial

1 region (Figs. 6 and 7). In *Lupinus nanus* (Fabaceae), *LegCYC1A* and *LegCYC1B* were
2 also detected in similar floral tissue (Citerne et al., 2006). Hence, the *CYC* homologue
3 gene expression in *A. aurea* might be involved in floral zygomorphy, as is involved in
4 *A. majus* and *L. nanus*. This result suggests that the expression pattern of *CYC*
5 homologue genes has been conserved between eudicot and monocot plants.

6 *Alstroemeria* flowers that possess specific spots on the perianth (Fig. 1e) and
7 distinguishable perianth shapes are considered to be useful for the analysis of floral
8 zygomorphy. Furthermore, an *Agrobacterium*-mediated transformation system has
9 now been established in *Alstroemeria* (Kim et al., 2007; Hoshino et al., 2008). Thus,
10 *Alstroemeria* will continue to be used in future studies to investigate the role of *CYC*
11 in the expression of floral zygomorphy.

12

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6

1 **Figure legends**

2 **Figure 1. *Alstroemeria* spp. used in the present study and floral zygomorphy in**
3 ***Alstroemeria*.** (a) Plants of *A. pelegrina* var. *rosea* at the flowering stage (scale = 10
4 cm); (b) flower of *A. pelegrina* var. *rosea* (scale = 3 cm); (c) flower of *A. aurea* (scale
5 = 3 cm); (d) flower of *A. magenta* (scale = 3 cm). (e) A representative *Alstroemeria*
6 flower showing floral zygomorphy. Black straight line indicates a symmetrical axis.
7 Specific spots are observed on 2 perianths enclosed with curve lines.

8

9 **Figure 2. Conserved amino acid sequences of the TCP and R domains in *CYC***
10 **homologue genes of *Alstroemeria* spp. and reference species.** Deduced amino acid
11 sequences of *AaTCP1*, *ApTCP2*, *ApTCP1*, and *AmTCP1* were aligned with
12 *CYCLOIDEA* (accession number Y16313), *DICHOTOMA* (AF199465), *TB1*
13 (U94494), *OsTB1* (AB088343), and *TCP1* (AC002130).

14

15 **Figure 3. Molecular phylogenetic analysis of amino acid sequences of TCP**
16 **domain in *CYC*-like genes using the maximum likelihood (ML) method.**

17 Numerals indicate bootstrap values from 100 replicates. The abbreviations of gene
18 names and GenBank/EMBL/DDBJ database accession numbers are as follows:

19 *OsTCP6* (*Oryza sativa* *TCP6*; GQ229483), *AtTCP8* (*Arabidopsis thaliana* *TCP8*;
20 NM_001084270), *SlTCP11* (*Solanum lycopersicum* *TCP11*; NM_001247902),
21 *AtTCP6* (*Arabidopsis thaliana* *TCP6*; NM_123468), *ZmTCP-domain* (*Zea mays*
22 *TCP-domain protein*; NM_001158303), *AtTCP9* (*Arabidopsis thaliana* *TCP9*;
23 NM_130131), *AtTCP7* (*Arabidopsis thaliana* *TCP7*; NM_122234), *AtTCP3*
24 (*Arabidopsis thaliana* *TCP3*; AF072134), *AtTCP4* (*Arabidopsis thaliana* *TCP4*;
25 NM_180258), *SlCycloidea* (*Solanum lycopersicum* *Cycloidea*; NM_001247406),

1 SITCP3-1 (*Solanum lycopersicum* TCP3; NM_001247438), SITCP10 (*Solanum*
 2 *lycopersicum* TCP10; NM_001247647), SITCP1-2 (*Solanum lycopersicum* TCP1;
 3 NM_001246854), ZmTCPfam (*Zea mays* TCP family transcription factor;
 4 NM_001157836), OsTCP11 (*Oryza sativa* TCP11; GQ229484), SITCP5 (*Solanum*
 5 *lycopersicum* TCP5; NM_001246863), AtTCP5 (*Arabidopsis thaliana* TCP5;
 6 NM_125490), SITCP4 (*Solanum lycopersicum* TCP4; NM_001247635), SITCP6
 7 (*Solanum lycopersicum* TCP6; NM_001247639), AtTCP2 (*Arabidopsis thaliana*
 8 TCP2; AF072691), PaTB1-TCP (*Plagiostachys albiflora* TB1-TCP; HM775146.1),
 9 PmTB1-TCP (*Plagiostachys mucida* TB1-TCP; HM775147.1), PmTCP1 (*Plantago*
 10 *major* TCP1; AY168138), AmTCP4 (*Antirrhinum majus* TCP4; AY168143),
 11 AmTCP3 (*Antirrhinum majus* TCP3; AY168142), AmDICHOTOMA (*Antirrhinum*
 12 *majus* DICHOTOMA; AF199465), AtTCP1 (*Arabidopsis thaliana* TCP1;
 13 NM_001160982), AmTCP2 (*Antirrhinum majus* TCP2; AY168141), AmCycloidea
 14 (*Antirrhinum majus* Cycloidea; Y16313), LoTCP1 (*Ligustrum ovalifolium* TCP1;
 15 AY168156), SITCP2 (*Solanum lycopersicum* TCP2; AY168166), SITCP7 (*Solanum*
 16 *lycopersicum* TCP7; NM_001246868), SpTCP7 (*Schizanthus pinnatus* TCP7;
 17 AY168174), SpTCP5 (*Schizanthus pinnatus* TCP5; AY168172), SpTCP6
 18 (*Schizanthus pinnatus* TCP6; AY168173), PmTCP2 (*Plantago major* TCP2;
 19 AY168139), CtTCP1 (*Calceolaria tenella* TCP1; AY168152), CtTCP2 (*Calceolaria*
 20 *tenella* TCP2; AY168153), LoTCP3 (*Ligustrum ovalifolium* TCP3; AY168158),
 21 AmTCP1 (*Antirrhinum majus* TCP1; AY168140), SpTCP3 (*Schizanthus pinnatus*
 22 TCP3; AY168170), SpTCP4 (*Schizanthus pinnatus* TCP4; AY168171), EpTCP1
 23 (*Echium plantagineum* TCP1; AY168175), SITCP1 (*Solanum lycopersicum* TCP1;
 24 AY168165), NsTCP1 (*Nicotiana glauca* TCP1; AY168163), AcTBLb (*Acorus*
 25 *calamus* TBLb; HM775145.1), AcTBLa (*Acorus calamus* TBLa; HM775144.1),

1 LoTCP2 (*Ligustrum ovalifolium* TCP2; AY168157), AmTCP5 (*Antirrhinum majus*
 2 TCP5; AY168144.1), SpTCP2 (*Schizanthus pinnatus* TCP2; AY168169), NsTCP2
 3 (*Nicotiana sylvestris* TCP2; AY168164), SlTCP9 (*Solanum lycopersicum* TCP9;
 4 GQ496327), AtBRANCHED1 (*Arabidopsis thaliana* BRANCHED1; AM408560),
 5 ZmTCPtrans (*Zea mays* TCP transcription factor; NM_001136610), OsDP1 (*Oryza*
 6 *sativa* DP1; EU702407), CcTBL2b (*Calathea crotalifera* TBL2b; HM775133.1),
 7 AvTBL2 (*Alpinia vittata* TBL2; HM775135.1), ZoTBL2b2 (*Zingiber ottensii*
 8 TBL2b.2; HM775138.1), ZoTBL2 (*Zingiber officinale* TBL2; HM775136.1),
 9 ZoTBL2b1 (*Zingiber ottensii* TBL2b.1; HM775137.1), GlTBL2b (*Globba laeta*
 10 TBL2b; HM775134.1), CcTBL2a (*Calathea crotalifera* TBL2a; HM775131.1),
 11 HcTBL2 (*Heliconia chartacea* TBL2; HM775126), HpTBL2 (*Heliconia pendula*
 12 TBL2; HM775128), HsTBL2 (*Heliconia stricta* TBL2; HM775132), CaTBL2 (*Costus*
 13 *amazonicus* TBL2; HM775124.1), SnTBL2 (*Strelitzia nicolai* voucher TBL2;
 14 HM775127.1), GlTBL2a (*Globba laeta* TBL2a; HM775129.1), SlTCP3 (*Solanum*
 15 *lycopersicum* TCP3; AY168167), SlTCP8 (*Solanum lycopersicum* TCP8;
 16 NM_001247643), SpTCP1 (*Schizanthus pinnatus* TCP1; AY168168), AlstpelTCP2
 17 (ApTCP2) (*Alstroemeria pelegrina* TCP2; AB714969), LlTCP1 (*Lilium longiflorum*
 18 TCP1; EF095959.1), OmTBL1a (*Orchidantha maxillarioides* TBL1a; HM775093),
 19 HsTBL1a (*Heliconia stricta* TBL1a; HM775094), AvTBL1a (*Alpinia vittata* TBL1a;
 20 HM775103.1), CcTBL1a (*Calathea crotalifera* TBL1a; HM775096.1), CrTBL1a
 21 (*Curcuma rubrobracteata* TBL1a; HM775098), RiTBL1a (*Riedelia lanata* TBL1a;
 22 HM775099.1), SnTBL1a (*Strelitzia nicolai* voucher TBL1a; HM775102.1),
 23 MuTBL1a (*Monocostus uniflorus* TBL1a; HM775101.1), PhTBL1a (*Pleuranthodium*
 24 *hellwigii* TBL1a; HM775097.1), AlstauTCP1 (AaTCP1) (*Alstroemeria aurea* TCP1;
 25 AB714967), AlstpelTCP1 (ApTCP1) (*Alstroemeria pelegrina* TCP1; AB714968),

1 AlstmagTCP1 (AmTCP1) (*Alstroemeria magenta* TCP1; AB714970), CrTBL1b
 2 (*Curcuma rubrobracteata* TBL1b; HM775107.1), EcTBL1b (*Elettaria cardamomum*
 3 TBL1b; HM775113.1), BnTBL1b (*Burbridgea nitida* TBL1b; HM775110.1),
 4 AvTBL1b (*Alpinia vittata* TBL1b; HM775112.1), EuTBL1b (*Elettariopsis unifolia*
 5 TBL1b; HM775111.1), RITBL1b (*Riedelia lanata* TBL1b; HM775108.1), PhTBL1b
 6 (*Pleuranthodium hellwigii* TBL1b; HM775109.1), CcTBL1b (*Calathea crotalifera*
 7 TBL1b; HM775118.1), CsTBL1b (*Costus spicatus* TBL1b; HM775115.1), CaTBL1b
 8 (*Costus amazonicus* TBL1b; HM775114.1), HsTBL1b (*Heliconia stricta* TBL1b;
 9 HM775117), CspTBL21 (*Canna* sp. TBL2.1; HM775105.1), CspTBL22 (*Canna* sp.
 10 TBL2.2; HM775104.1), MbTBL1b (*Musa basjoo* TBL1b; HM775142.1), SrTBL1b
 11 (*Strelitzia reginae* TBL1b; HM775120.1), SnTBL1b (*Strelitzia nicolai* TBL1b;
 12 HM775121.1), MITBL1b (*Maranta leuconeura* TBL1b; HM775116.1), OsTB1
 13 (*Oryza sativa* TB1; AB088343), PaTB1 (*Pleioblastus amarus* TB1; DQ910764.1),
 14 YnTB1 (*Yushania niitakayamensis* TB1; DQ910763.1), HvTCP (*Hordeum vulgare*
 15 TCP; JF904738.1), ZmTB1 (*Zea mays* TB; U94494), PgTB1 (*Pennisetum glaucum*
 16 Tb1; EF694127.2), PdTB1 (*Phacelurus digitatus* TB1; AF322125), SbTB1 (*Sorghum*
 17 *bicolor* TB1; AF322132), and AgTB1 (*Andropogon gerardii* TB1; AF322119).

18

19 **Figure 4. RT-PCR analysis of *AaTCP1* transcripts in flower buds (FB), leaves (L),**
 20 **and stems (S) of *Alstroemeria aurea*.** The number of PCR cycles was 35. The actin
 21 gene was used as the internal control.

22

23 **Figure 5. RT-PCR analysis of *AaTCP1* transcripts in *Alstroemeria aurea* flower**
 24 **buds of different developmental stages.** Quantitative RT-PCR, conducted by

1 altering the number of PCR cycles for flower buds of 0.5–1, 1–2, and 2–3 mm in
2 length and using the actin gene as the internal control.

3

4 **Figure 6. Localization of *AaTCP1* transcripts detected using *in situ* hybridization**
5 **of flower buds of *Alstroemeria aurea*.** Flower buds of 3 mm longitudinal diameter
6 were used to prepare transverse serial sections of 10 µm thickness. (a) *In situ*
7 hybridization using DIG-labelled antisense *AaTCP1* probe. (b) *In situ* hybridization
8 using DIG-labelled sense *AaTCP1* probe. ip, inner perianth; op, outer perianth; fi,
9 filament; g, gynoecium. Scale = 500 µm.

10

11 **Figure 7. Localization of *AaTCP1* transcripts in longitudinal sections detected**
12 **using *in situ* hybridization of *Alstroemeria aurea*.** Flower buds of 3 mm
13 longitudinal diameter were used to prepare serial sections of 10 µm thickness. (a) *In*
14 *situ* hybridization using DIG-labelled antisense *AaTCP1* probe. (b) *In situ*
15 hybridization using DIG-labelled sense *AaTCP1* probe. an, anther; ip, inner perianth;
16 op, outer perianth; fi, filament. Scale = 500 µm.

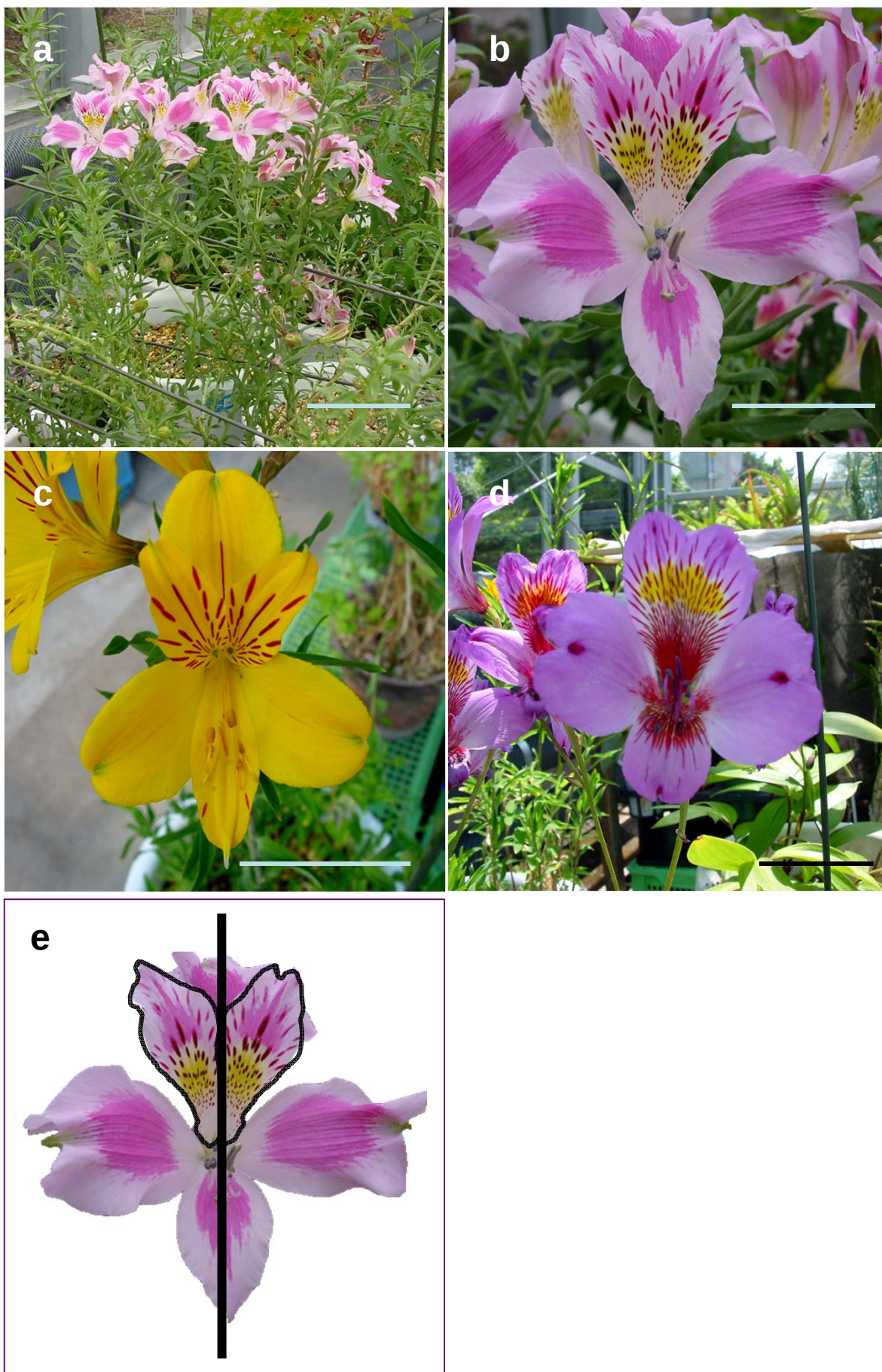


Figure 1

TCP domain

<i>Alstroemeria aurea</i> /AaTCP1	K	I	Y	T	P	Q	G	P	R	D	R	R	M	R	L	S	L	E	V	A	P	D	F	F	K	L	Q	D	M	L	G	F	D	K	A	S	K	I	V	Q	W	L	L	T	M	S	A	S	A	I	Q	E	L	T	P	T	A	R	P	Q	
<i>Alstroemeria magenta</i> /AmTCP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	F	F	K	L	Q	Y	M	L	G	F	D	K	A	S	K	T	V	R	W	L	L	T	M	L	A	S	A	I	Q	E	L	T	P	T	A	R	P	R	
<i>Alstroemeria pelegrina</i> /ApTCP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	F	F	K	L	Q	D	M	L	G	F	D	K	A	S	K	T	V	R	W	L	L	T	M	S	A	S	A	I	Q	E	L	A	P	T	G	R	P	R	
<i>Alstroemeria pelegrina</i> /ApTCP2	K	I	Y	T	A	Q	G	P	R	D	R	R	M	R	L	S	L	I	G	V	A	R	D	F	F	D	L	Q	D	T	L	G	Y	D	K	A	S	Q	T	M	K	W	L	L	T	M	S	A	S	A	I	K	D	L	M	D	G	S	R	Q	H
<i>Antirrhinum majus</i> /CYCLOIDEA	K	I	Y	T	A	Q	G	P	R	D	R	R	M	R	L	S	L	I	G	V	A	R	D	F	F	D	L	Q	E	M	L	G	F	D	K	P	S	K	T	L	D	W	L	L	T	K	S	K	E	A	I	K	E	L	V	Q	S	K	S	T	K
<i>Antirrhinum majus</i> /DICHOTOMA	K	I	N	R	P	Q	G	P	R	D	R	R	V	R	L	S	I	G	I	A	R	K	F	F	D	L	Q	E	M	L	G	F	D	K	P	S	K	T	L	D	W	L	L	T	K	S	K	E	A	I	K	E	L	V	Q	S	K	S	S	K	
<i>Oryza mays</i> /TB1	K	I	C	T	A	G	G	M	R	D	R	R	M	R	L	S	L	D	V	A	R	K	F	F	A	L	Q	D	M	L	G	F	D	K	A	S	K	T	V	Q	W	L	L	N	T	S	K	S	A	I	Q	E	I	M	A	D	D	A	S	S	
<i>Zea sativa</i> /OsTB1	K	I	S	T	A	G	G	M	R	D	R	R	M	R	L	S	L	D	V	A	R	K	F	F	A	L	Q	D	M	L	G	F	D	K	A	S	K	T	V	Q	W	L	L	N	M	S	K	A	A	I	R	E	I	M	S	D	D	A	S	S	
<i>Arabidopsis thaliana</i> /TCP1	K	I	Q	T	A	Q	G	I	R	D	R	R	V	R	L	S	I	G	I	A	R	Q	F	F	D	L	Q	D	M	L	G	F	D	K	A	S	K	T	L	D	W	L	L	K	S	R	K	A	I	K	E	V	V	Q	A	K	N	L	N		

<i>Alstroemeria aurea</i> /AaTCP1	DQLFSESSSTSEECENQS-----TASDHHKGSPPWKKAPNPILPN--RRGWWQHPGPS
<i>Alstroemeria magenta</i> /AmTCP1	DPLFSESSSTSEECENQS-----TASDHHKGGKLPLVESSKNPNPSKP-KRMAASRASRK
<i>Alstroemeria pelegrina</i> /ApTCP1	DQLFSESSSTSEECENLS-----TASDHHKGGKLPLVESSKNPNPSKP-KRMVASRASRK
<i>Alstroemeria pelegrina</i> /ApTCP2	S---VSQSSSTSECNNNS-----ADNKGKSHLEGDTSSAQKF-KREAATAAVVR
<i>Antirrhinum majus</i> /CYCLOIDEA	S---NSSSPDCDEEVVS-----VDSENVTDHSGKSLKANNN--CKEAMDSHQ
<i>Antirrhinum majus</i> /DICHTOMA	S---NISNSPSECDAQEVL-----SADLPYIGSSKGKA AVG LNS-NKCKGGRDVD
<i>Oryza sativa</i> /TB1	ECEVDGSSSSLSDVGKHN-----PAEQLGGGGDHKPKGNCRGEGKKPAKA-SKAAATPKPPR
<i>Azadirachta indica</i> /OBT81	VCEEDGSSSSLSDVGKQQQHSSNPADRGGAGDHKGGAHGHSDBGKAKPR-RRAANPKPPR
<i>Arabidopsis thaliana</i> /TCP1	NDDDEDFGNI GGDVEEQEE-----KEEDDNGDKSFVYGLSPGYGEDEVCEATKAGIR

<i>Alstroemeria aurea</i> /AaTCP1	G N P H S - - I Q R - - - - -
<i>Alstroemeria magenta</i> /AmTCP1	P T L H - - - P A L A R E C R A K V - - - - -
<i>Alstroemeria pelegrina</i> /ApTCP1	P T L H - - - P A L A R E C R A K V - - - - -
<i>Alstroemeria pelegrina</i> /ApTCP2	V A P F - - - A T L A K E S R A K A R G R A R E R T - -
<i>Antirrhinum majus</i> /CYCLOIDEA	A A A K - - - - - E S R A K A R A R A R E R T K E K
<i>Antirrhinum majus</i> /DICHOTOMA	L A K - - - - - E S R A K A R A R A R E R T K E K
<i>Zea mays</i> /TB1	K S A N N - A H Q V P D K E T R A K A R E R A R E R T K E K
<i>Oryza sativa</i> /OsTB1	R L A N - - A H P V P D K E S R A K A R E R A R E R T K E K
<i>Arabidopsis thaliana</i> /TCP1	K K K S E - L R N I S S K G L G A K A R G K A K E R T K E M

R domain

Figure 2

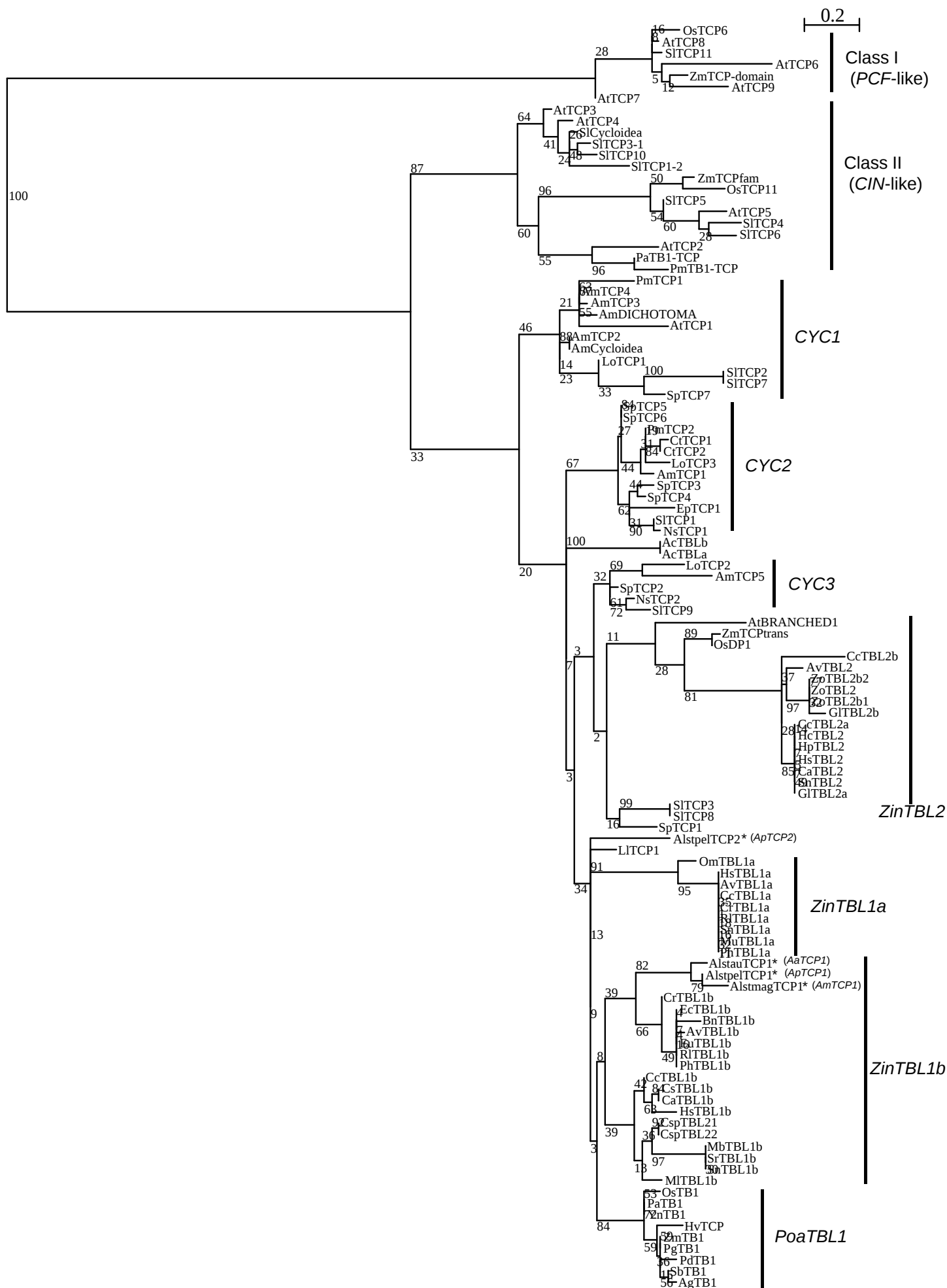


Figure 3

* This study

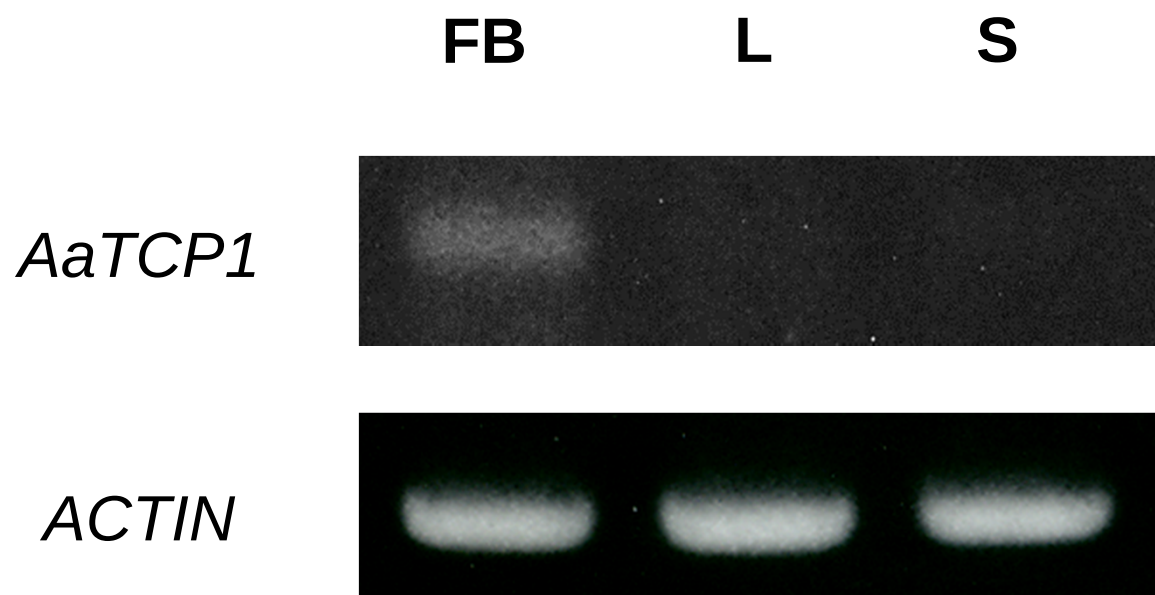


Figure 4

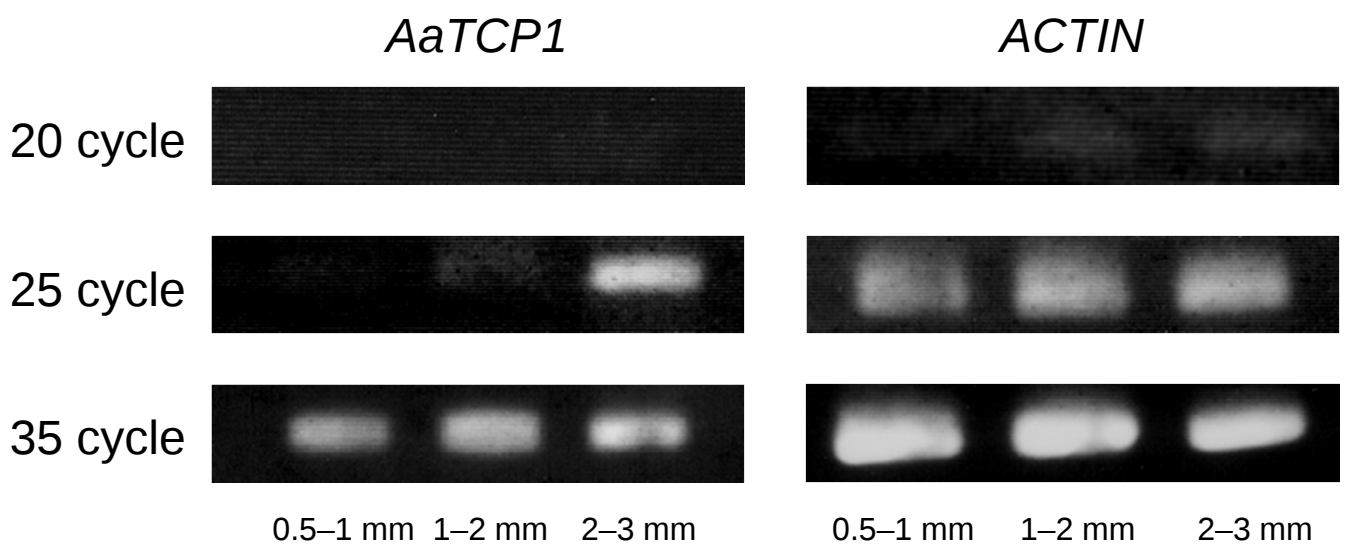


Figure 5

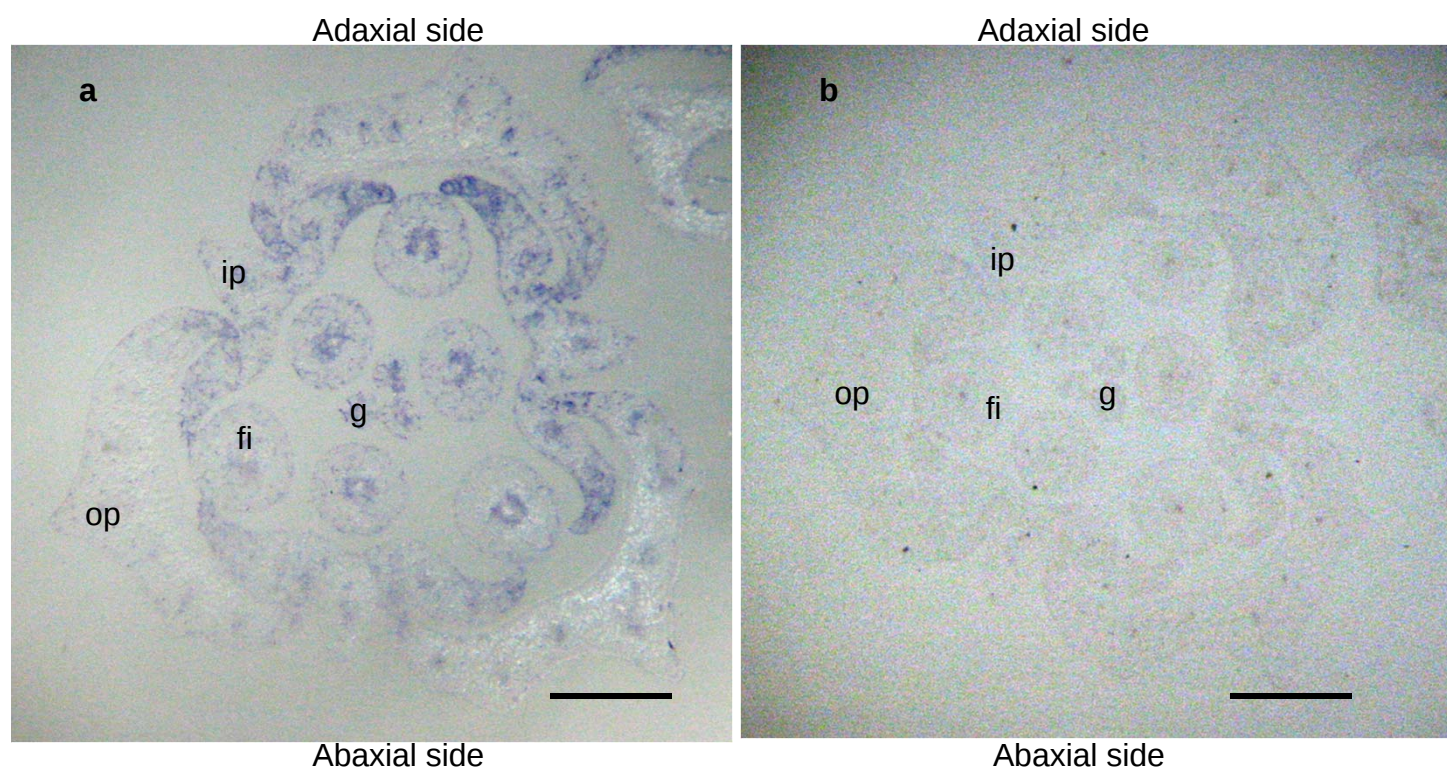


Figure 6

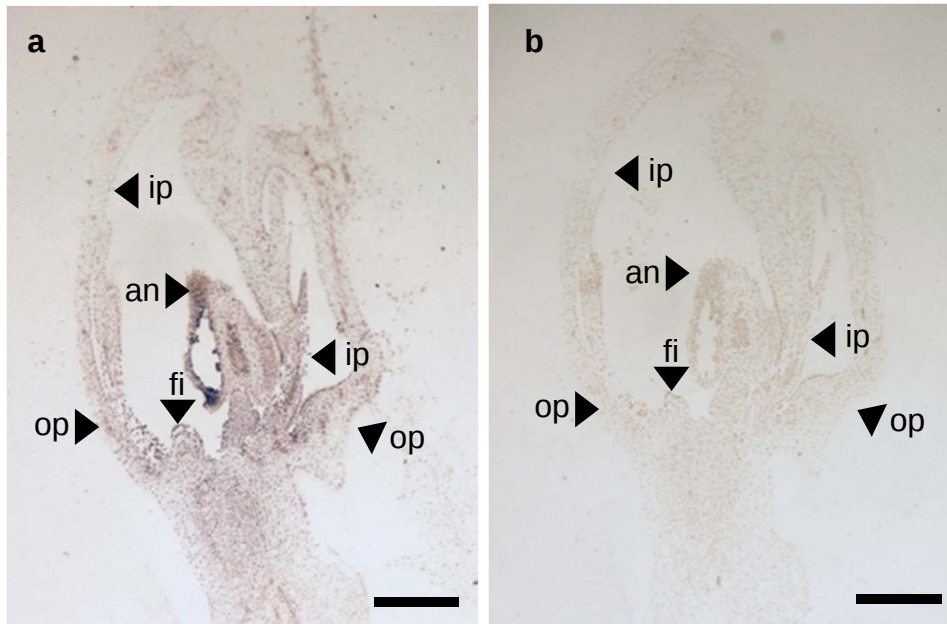


Figure 7