Floral organ MADS-box genes in *Cercidiphyllum japonicum* (Cercidiphyllaceae): Implications for systematic evolution and bracts definition

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Abstract

The dioecious relic *Cercidiphyllum japonicum* is one of two species of the sole genus *Cercidiphyllum*, with a tight inflorescence lacking an apparent perianth structure. In addition, its systematic place has been much debated and, so far researches have mainly focused on its morphology and chloroplast genes. In our investigation, we identified 10 floral organ identity genes, including four A-class, three B-class, two C-class and one D-class. Phylogenetic analyses showed that all ten genes are grouped with *Saxifragales* plants, which confirmed the phylogenetic place of *C. japonicum*. Expression patterns of those genes were examined by quantitative reverse transcriptase PCR, with some variations that did not completely coincide with the ABCDE model, suggesting some subfunctionalization. As well, our research supported the idea that the bract actually is perianth according to our morphological and molecular analyses in *Cercidiphyllum japonicum*.

Introduction

*Cercidiphyllum japonicum* Sieb. Et Zucc. is a tertiary relic plant and only occurs as a species of east Asian flora. Paleontology research shows that it was once widely distributed in the northern hemisphere. Due to quaternary glaciations, it is now only sporadically found in China and Japan [1,2]. As a cretaceous relic, *C. japonicum* has considerable presence as a tree with colorful leaves. The tree displays typically colored leaves showing amaranthine in the spring, emerald in the summer, golden in the fall and carmine in the winter. As well, it has great economic value given that its fruits and leaves can be used as medicines and the bark is used for tannic extracts. Furthermore, its dioecious, achlamydeous and extreme simplification inflorescence makes it an ideal material for the study of sexual differentiation and regulation of floral development.

Since it was established by Siebold and Zuccarini in 1846 [3], the systematic position of *C. japonicum* has always been in dispute. In the early years, researchers classified it according to its morphology and it was once placed in the *Magnoliaceae* [4]. Baillon [5] proposed that...
*Cercidiphyllum* may be closely related with *Hamamelidaceae* plants, which was approved later and *Cercidiphyllum* was taken into the *Hamamelidaceae* [6]. On the other hand, Van Tieghem put forward that *Cercidiphyllum* should be its own family, a proposal generally accepted [7]. Much later, *Cercidiphyllum* was placed in *Trochodendrales* [8], *Hamamelidales* [9] or *Cercidiphyllales* [10] and *Cercidiphyllaceae* was regarded as the bond connecting *Hamamelidaceae*, *Trochodendrales* and *Magnoliales*. With sequence analysis, the molecular phylogeny of *rbcL* showed that *Cercidiphyllaceae* is close to *Daphniphyllaceae*, *Hamamelidaceae* and *Saxifragaceae*. Combining their morphological characteristics, both *Cercidiphyllaceae* and *Daphniphyllaceae* should be classified with *Hamamelidales* [11]. Analysis of matK sequences declared that *Cercidiphyllaceae* has a distant relationship with *Tetracentraceae* [12]. The APG II [13] and APG III [14] classification systems put *Cercidiphyllum* as an independent family in *Saxifragales*. Combining the floral morphogenesis, the type of vascular perforated plate and anatomical characteristics of *Cercidiphyllaceae*, Yan et al. [15] considered it was suitable to place *Cercidiphyllum* into *Saxifragales*. But the floral morphology and developmental processes were quite distinct from other *Saxifragales* plants. Since flowers are the most conserved organs for angiosperms, it is of great importance to investigate the systematic process according to the floral identity genes.

The ABCDE-model is the most acceptable model explaining flora development. In this model, A- and E-class genes determine sepal formation. A-, B- and E-class genes are responsible for petals. Stamens are determined by B-, C- and E-class genes, while C- and E-class genes determine the identity of carpels. D-class genes are involved in ovule development [16–18]. Almost all genes execute A, B, C, D and E functions, *APETALA1* (*AP1*), *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), *AGOUMOUS* (*AG*), *AGOUMOUS-Like* (*AGL11*) and *SEPALLATA* (*SEP*) lineages belong to the MIKC-type MADS-box family, except for *APETALA2* (*AP2*). Studies showed that these genes have the similar structure consisting of M, I, K and C domains with high conservation. B-/C-class genes were relatively conserved in function of controlling pistilate and staminal development [19]. A-class genes were diversified; for example, *API* mutation resulted in the absence of petals in *Arabidopsis*, but a recent study, about the spiral flowers of *Nigella damascena*, claimed that the *AGL6*-lineage, rather than the *API*-lineage, is an A-class gene, which is the key regulator of sepal and petal development [20]. Modified models have been discussed in many species for clarifying special flower structures.

The flowers of *C. japonicum* were considered to be very special and hence some arguments were cropped up over its flora structures. Solereder [6] and Harms [21] believed that its outward ventral suture characteristics showed that the flowers were inflorescence, thinking that the orientation may be resulted from the absence of an opposite carpel. However, Swamy and Bailey [22] tried to draw arguments for the loss of a second carpel. Both Van Heel [23] and Endress [24] observed early developmental stages of *C. japonicum*. Their descriptions suggested that the flowers develop in a decussate way and that the bracts outside the first couple were not opposite while the second couple were. Moreover, they agreed that the perianth and nectar of *C. japonicum* were missing. Yan et al. [15] observed the morphogenesis of *C. japonicum* and concluded that the bracts were lanceolate, membranous, not phyllose and associated with carpel development and hence the so-called bracts should be tepals. By this token, the floral structure of *C. japonicum* still remains a controversial issue.

In other words, *C. japonicum* is the ideal material to investigate its sex differentiation and floral developmental mechanism. Our research based on the ABCDE model further confirms the systematic evolution of *C. japonicum* by analyzing MADS-box homologs. We discuss its floral structure on the basis of morphologic observations and relative genes expression patterns.
Material and methods

Plant materials

Flower buds were collected from *C. japonicum* growing under natural conditions in Beijing with the cooperation of Dr. Guoke Chen from Institute of Botany, the Chinese Academy of Sciences. One part of the buds were immersed in glutaraldehyde. The others buds for cloning were separated into seven parts: outer scale (OS), middle scale (MS), inner scale (IS), stamens (ST) or carpels (CA), juvenile leaves (LE), stipule (STI) and bracts (BR) and immediately frozen in liquid nitrogen and stored at -80°C until used.

Isolation and identification of genes

Total RNA was extracted from floral buds using the EASYspin plant RNA Extraction Kit (Aidlab, China) following instructions from the manufacturer. First-strand cDNA was synthesized from 1 μg of the DNase I-treated RNA, using adaptor primers and M-MLV Reverse Transcriptase (TaKaRa, Japan). Initial amplification for core sequences were based on homologous cloning. The PCR reagents were composed of 1 μL cDNA, 0.5 μL of each primer (10 mM each), 2.5 μL Ex Taq buffer, 2 μL dNTP (2.5 mM each), 0.3 μL Ex Taq polymerase (TaKaRa, Japan) and adjusted with water to a final volume of 25 μL. PCR was performed with a 3 min 95°C denaturation step, followed by 35 cycles of 30 s at 95°C, 30 s annealing at 52–57°C, a 30–60 s extension at 72°C and a final extension period of 10 min. The PCR products were purified with the gel extraction kit (TaKaRa) and cloned into pMD18®-T vector (TaKaRa). Ligation products were transformed into Escherichia coli Top10 cells (Aidlab China) following instructions by the manufacturer. Then we used 3’ RACE and 5’ RACE system kits (TaKaRa) to obtain the 3’- and 5’-end sequences of each gene. Full-length cDNA of each gene was obtained by PCR-based cloning with gene-specific forward and reverse primers designed according to the corresponding 3’- and 5’-end sequences. Names and sequences of the primers used in this study are presented in Tables 1 and 2.

Sequence alignments and phylogenetic analysis

Selected sequences were downloaded from the National Center for Biotechnology Information GenBank. The taxa were selected on the basis of aligning results and the representative angiosperm classification according to the APGIII system (APGIII, 2009). Only one taxon provided relatively complete cds and was chosen per order. Alignments were conducted by Clustal X 2.0 using protein sequences and phylogenetic trees were formed by software MEGA7.0 using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) Method. *Gnetum gnemon* and *Picea abies* were chosen as outgroups. Support for the branches was assessed using bootstrap analysis with 1000 replicates.

Gene expression analysis

For our semi-quantitative RT-PCR analysis, total RNA was extracted from seven parts described earlier. Each first-strand cDNA was synthesized using an oligo (dT)15 primer and the M-MLV reverse transcriptase kit. To precisely analyze the tissue-specific expression patterns of each lineage genes, real-time quantitative PCRs are conducted. The experiment was accomplished with SYBR premix Ex Taq (Takara, Japan) using the following program: 95°C for 30 s; 40 cycles of 95°C 5 s, and 60°C for 30 s. The beta-actin gene of *C. japonicum Cejaactin* is referred as internal reference.
Table 1. A list of all primers used for gene cloning and qRT-PCR in this study.

| Primer | gene     | core sequences | 3'RACE | 5'RACE | qRT-PCR |
|--------|----------|----------------|--------|--------|---------|
|        |          |                | F      | R      | first   | second  | F      | R      | first   | second  | F      | R      | first   | second  | F      | R      |
| CejaAP1 | CejaAP1  | AP1-F          | AP1-R  |         | 3'AP1-2 |         | qAP1-R | AP1-R  |         | q-AP1-F | q-AP1-R |
| CejaFUL | CejaFUL  | FUL-F          | FUL-R  |         | 3'AF    |         | q-FUL-F| q-FUL-R |
| CejaFUL-like | CejaFUL-like |            | 3'AF    |         | 5'FUL-like-2 | q-FUL-like-F | q-FUL-like-R |
| CejaAGL6 | CejaAGL6 | AGL6-F         | AGL6-R |         | 3'AGL6-2 |         | q-AGL6-F| q-AGL6-R |
| CejaAP3_1 | CejaAP3_1 | AP3-F          | AP3-R  |         | 3'AP3-2 |         | q-AP31-F| q-AP31-R |
| CejaAP3_2 | CejaAP3_2 | PI-F           | PI-R   |         | 3'PI-2  |         | q-PI-F  | q-PI-R  |
| CejaAG1 | CejaAG1  | AG-F           | AG-R   |         | 3'AG1-2 |         | q-AG1-F | q-AG1-R |
| CejaAG2 | CejaAG2  | AGL11-F        | AGL11-R|         | 3'AGL11-2| q-AGL11-R| AGL11-R |
| CejaActin | CejaActin | actin-F        | actin-R|         |         |         |         |         |

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Table 2. Sequence information of the primers listed in Table 1.

| Primer | Primer sequences (5' to 3') | Primer | Primer sequences (5' to 3') |
|--------|-----------------------------|--------|-----------------------------|
| AP1-F  | GAGGTTGGCTTTGATGCTCCTCC     | 5'FUL-like-2 | AGAGAAGGAAGTGATTGGTGGAGG |
| AP1-R  | TGGAGTCGAGCTGCTCTCTCT       | 3'AP3-1 | CCTCGCCTCTGAGTGCACCC |
| FUL-F  | GATCAATAGGCAAGTGACGTTTTC    | 5'PI-2 | GGCTTTGATCCCTCCTCCGCCAACAT |
| FUL-R  | CATAGTAGTCTTCTTGAGGCC       | 5'AG1-2 | TCCCGAGATCGTAAGGAGGAGA |
| AGL6-F | GAGAGAGAATGGGGAGAGGAAG      | q-actinF | AAGATCGATCACTACACCTTCA |
| AGL6-R | CCGAGGCTCTCCATTTGCTCT      | q-actinR | ATAAAATGGAACGTAGGCTCACCC |
| AP3-F  | GGCCTTCCTCACAAGAGCCAAATG    | q-AP1-F | GCATACCTCTCCATATACCACA |
| AP3-R  | CTTGCAAGATTTCCTACTCTGATTAG | q-AP1-R | AATACATAATTCTCATACCAAGCT |
| PI-F   | ATGGGAGAGGGAGAGATTGAGTAT    | q-FUL-F | ACCACAGAGAGATGTAGGAGAGA |
| PI-R   | GCTAACATTTGCGATGGCTGCCAC   | q-FUL-R | ATGTCGACGAAATATATAGAGA |
| AG-F   | CAGACTACCTCTCTTGAGCC        | q-FUL-like-F | CTCAACCACCTACCTTCCCTCCT |
| AG-R   | CTCAATTGCCAGACTCTTTGCTCG    | q-FUL-like-R | GGTGTTGGAAGATTCTCCATCC |
| AGL11-F | GATGCTGAAGTTGCTCCTCAT     | q-AGL6-F | CATCCTCATTCACTCCACCAT |
| AGL11-R | CCAATGTCTGCTGTGAGACCTCCTC  | q-AGL6-R | GATTATAAAGGACCATCCCTCGGA |
| actin-F | AAGATCTGCCATACACTTCTTCAAG   | q-AP31-F | ATTAGCCAGAGATGGTCCAGAG |
| actin-R | GACCAGCTCATACATACACTT       | q-AP31-R | AGGAGACCTGTCTGATTTGTTC |
| 3'AP1-2 | AGCATGGGAGAAAATCCTTGAGACG  | q-AP32-F | ATGCAATTGATGCAGGAGAGAC |
| 3'AF   | GAGGCTGGGTGTTGCTGCTCTCAC   | q-AP32-R | CCTACTTTTGATACACGAGAAGCAG |
| 3'AF   | TGAAGTCCTCTGAAAGGACCTAAGG  | q-PI-F  | GGCTATGGGAGATATATGGAGG |
| 3'AGL6-2 | GCTTTCCTGCTGTGATGATCTG     | q-PI-R  | CCTACTTACAAACCCGCAAAAGCA |
| 3'AP3-2 | GAGGTGATTTACAAACACTGCGGC   | q-AG1-F | TCTCGCCCATACGCTGCGAA |
| 3'PI-1 | GAGAATCTCAACTAACTCAGGGCTGTA | q-AG1-R | GGTTCCTCCACAGAGAAGTCCAA |
| 3'PI-2 | TGAGGAAGATTTGTGGAATCTAGA   | q-AG2-F | TGGTAGACAGTACCTGATGAGGAGC |
| 3'AG1-2 | CAAAGTGCCAAAGTGACCTGTTGG   | q-AG2-R | GAGGGAGCGAGATAGAATCCCAAGAT |
| 3'AG2-2 | GAAACAAATCCGAGTAACAAAAAAG | q-AGL11-F | CAAGTACAGAGATACCGGAGAG |
| 3'AGL11-2 | CTGGGAGAAATAGAATTAACGGG   | q-AGL11-R | ATGCAAGATGATACATAGGGGC |

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Table 3. All the MADS-box proteins in protein sequence comparisons and phylogenetic analysis.

| Protein   | Species                          | Accession number         |
|-----------|----------------------------------|--------------------------|
| CejaAP1   | *Cercidiphyllum japonicum* (this paper) | KY285019                 |
| CejaFUL   | *Cercidiphyllum japonicum* (this paper) | KY285024                 |
| CejaFUL-like | *Cercidiphyllum japonicum* (this paper) | KY285022                 |
| DAL1      | *Picea abies*                     | CAAS6864                 |
| GGM1      | *Gnetum gemon*                    | CABA4447                 |
| FL2       | *Dicentra eximia*                | AGX01574                 |
| MpMADS15  | *Magnolia praecocissima*          | BAB70749                 |
| CsAP1     | *Chloranthus spicatus*            | AAQ83693                 |
| BUseFL2   | *Buxus sempervirens*             | ABG94514                 |
| PAteFL1   | *Pachysandra terminalis*          | ABG49521                 |
| PAteFL2   | *Pachysandra terminalis*          | AAP83389                 |
| TraFUL1   | *Trochodendron aralioides*        | ABQ85944                 |
| TraFUL2   | *Trochodendron aralioides*        | ABQ85945                 |
| FL1       | *Dicentra eximia*                | AGX01534                 |
| GumaFUL-like | *Gunnera manicata*             | AFO68793                 |
| VFUL-L    | *Vitis vinifera*                  | NP_001268211 XP_002281526 XP_002281532 |
| VFUL      | *Vitis vinifera*                  | ACZ26529                 |
| HeaFL     | *Heuchera americana*              | AAP83373                 |
| AcFUL-like | *Actinidia chinensis*             | ADU15471                 |
| RhFUL     | *Rosa hybrid cultivar*            | ACS74808                 |
| MADS4     | *Betula pendula*                  | CAAS67968                |
| HeaFUL    | *Heuchera americana*              | AAP83374                 |
| CsFUL     | *Corylopsis sinensis*             | AAP83371                 |
| GimAP1a   | *Glycine max*                     | ABZ80361                 |
| FUL       | *Arabidopsis thaliana*            | OAO94650                 |
| LcAP1     | *Litchi chinensis*                | AEU55406                 |
| PpAP1-2   | *Pyrus pyrifolia*                 | AJW29022                 |
| MADS5     | *Betula pendula*                  | CAAS67969                |
| CcAP1     | *Carya cathayensis*               | AHI85952                 |
| CoarFUL   | *Coffea arabica*                  | AHW58040                 |
| AcFUL     | *Actinidia chinensis*             | ADU15472                 |
| SpFUL     | *Spinacia oleracea*               | ACE75945                 |
| SpAP1-1   | *Spinacia oleracea*               | ACE75943                 |
| GsAP1     | *Gentiana scabra*                 | BAS0447                  |
| CoarAP1   | *Coffea arabica*                  | AHW58038                 |
| SiAP1     | *Sesamum indicum*                 | AIS82596                 |
| CokoAP1   | *Cornus kousa*                    | AQA61753                 |
| PalaAP1   | *Paeonia lactiflora*              | AGH61290                 |
| VvAP1     | *Vitis vinifera*                  | NP_001268210 XP_002263170 |
| HeaAP1    | *Heuchera americana*              | AAP83372                 |
| CsAP1     | *Corylopsis sinensis*             | AAP83370                 |
| CasiAP1   | *Camellia sinensis*               | AIC75372                 |
| CpAP1     | *Cyclamen persicum*               | BAK09614                 |
| MnAP1     | *Morus notabilis*                 | EXB04487                 |
| ZjAP1     | *Ziziphus jujuba*                 | AG709964                 |
| MADS3     | *Betula pendula*                  | CAAS67967                |
| CcAP1     | *Carya cathayensis*               | AHI85952                 |

(Continued)
| Protein   | Species | Accession number |
|-----------|---------|-----------------|
| CisiAP1   | Citrus sinensis | AAR01228 |
| AP1       | Arabidopsis thaliana | CA7A890 |
| VuAP1     | Vigna unguiculata | BAJ2385  |
| PpAP1-3   | Pyrus pyrifolia | AJW29025  |
| FaAP1     | Fragaria x ananassa | AFA42327 |
| PsAP1-1   | Populus simonii x Populus nigra | AGR88912 |
| PeAP1     | Passiflora edulis | AER30447  |
| CejaAP1   | Cercidiphyllum japonicum (this paper) | KY285023 |
| CejaAP3_1 | Cercidiphyllum japonicum (this paper) | KY285020 |
| CejaAP3_2 | Cercidiphyllum japonicum (this paper) | KY285021 |
| PrDGL     | Pinus radiata | AAF28863  |
| GGM2      | Gnetum gnemon | CAB44448  |
| AmPI      | Amborella trichopoda | BAD42443 |
| Nyod.PI   | Nymphaea odorata | ADD25210 |
| NymPI     | Nymphaea sp. | AAR87705  |
| IiiPI     | Illicium floridanum | AAY25570 |
| MpMADS8   | Magnolia praecocissima | BAB70743 |
| PeamPI    | Persea americana | AAR06672 |
| EgGLO     | Elaeis guineensis | XP_01091271 |
| CsPI      | Chloranthus spicatus | AAF7939 |
| PIPI      | Phalaenopsis japonica | AJG4730 |
| TraPI1    | Trochodendron aralioides | ABQ85946 |
| TraPI2    | Trochodendron aralioides | ABQ85947 |
| PsPI      | Paeonia suffruticosa | AEE98378 |
| RbFP11    | Ribes diacanthum | AHY19022 |
| DiIPI1    | Dillenia indica | ABR68541  |
| PrpsPI    | Prunus pseudocerasus | AIU94284 |
| PMADS2    | Jatropha curcas | XP_01207322 |
| PdPI      | Populus deltoides | ABS71831 |
| AcPI      | Actinidia chinensis | ADU15475 |
| GLO       | Camellia oleifera | AJN0602 |
| NymAP3    | Nymphaea sp. | AAR87701  |
| AmAP3_1   | Amborella trichopoda | BAD42444 |
| MaspAP3   | Magnolia sprengeri | AFN68915 |
| MAprAP3   | Magnolia praecocissima | BAB70742 |
| CsAP3     | Chloranthus spicatus | AAR06664 |
| PAteAP3_1 | Pachysandra terminalis | ADC79700 |
| RbMAP3    | Ribes diacanthum | AHY19023 |
| MCAP3     | Micranthes careyana | ABF56142 |
| CopAP3    | Corylopsis pauciflora | ABF56128 |
| TroAP3    | Trochodendron aralioides | ABE11601 |
| PaLaAP3_1 | Paeonia lactiflora | AGH61291 |
| MadMdTM6  | Malus domestica | NP_001315678 XP_008344258 |
| PTD       | Populus trichocarpa | AAC13695 |
| HmTM6     | Hydrangea macrophylla | BAG68950 |
| GIAP3_1   | Gunnera tinctoria | AAR06687 |
| GmAP3     | Gunnera manicata | AFO68771 |

(Continued)
Table 3. (Continued)

| Protein | Species | Accession number |
|---------|---------|------------------|
| SxcTM6  | Saxifraga careyana | ABF56143 |
| DiiTM6  | Dilenia indica | ABR68544 |
| CejaAG1 | Cercidiphyllum japonicum (this paper) | KY285015 |
| CejaAG2 | Cercidiphyllum japonicum (this paper) | KY285016 |
| CejaAGL11 | Cercidiphyllum japonicum (this paper) | KY285018 |
| DAL2 | Picea abies | CAA55867 |
| GGM3 | Gnetum gnemon | CAB44449 |
| AmAG | Amborella trichopoda | AAY25577 |
| MAvuAG | Magnolia wufengensis | AEO52692 |
| MisiAG | Magnolia sirindhorniae | AGZ63865 |
| LoAG | Lilium hybrid cultivar | AEk94071 |
| EgAG1 | Elaeis guineensis | AAW66881 |
| AoAG | Alpinia oblongifolia | ABB92624 |
| NualAG | Nuphar advena | AAY25576 |
| NymAG1 | Nymphaea sp. | AAS45692 |
| HiCAG | Houttuynia cordata | AAS45684 |
| TraAG1 | Trochodendron aralioides | ABQ85948 |
| TraAG2 | Trochodendron aralioides | ABQ85949 |
| PasuAG | Paeonia suffruticosa | AGS12611 |
| SxcAG1 | Saxifraga careyana | AAS45705 |
| VvAG | Vitis vinifera | NP_001268097 XP_002263066 |
| JacuAG | Jatropha curcas | NP_001292936 XP_012091857 |
| MAG | Mangifera indica | ACN97631 |
| CmMADS2 | Castanea mollissima | AAZ77747 |
| KejaAG | Kerria japonica | AGZ01978 |
| PMAG | Prunus mume | ABU41518 |
| CoAG | Cornus kousa | AGA61751 |
| CoarAG | Coffea arabica | AHWS8037 |
| SiAG | Sesamum indicum | AIS82595 |
| DiAG | Dillenia indica | ABR68545 |
| PLENA | Gunnera manicata | AFO68768 |
| LAG | Liquidambar styraciflua | AAD38119 |
| Mople | Misopates orontium | CAJ44134 |
| plena | Antirrhinum majus | BAI8391 |
| GsAG1 | Gentiana scabra | BAS04480 |
| CoarPLE | Coffea arabica | AHWS8047 |
| GsAG2 | Gentiana scabra | BAS04484 |
| NyodAG3 | Nymphaeas odorata | ADD25206 |
| SxcAG2 | Saxifraga careyana | AAS45704 |
| MADS10 | Malus domestica | NP_001280931 |
| PpAGL11_1 | Pyrus pyrifolia | AJW29026 |
| MADS5 | Vitis vinifera | AAM23145 |
| JacuAGL11 | Jatropha curcas | XP_012073508 |
| GrAGL11 | Gossypium raimondii | XP_012447416 |
| CisiAGL11 | Citrus sinensis | XP_006478235 |
| GmAGL11 | Glycine max | NP_001236130 |
| AGL11 | Arabidopsis thaliana | AAC49080 |
| LjAGL11 | Lotus japonicus | AAX13306 |

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Morphological observations

Mature floral buds from pistillate and staminate flower of *C. japonicum* were dissected with a needle and photographed under a stereoscopic microscope. All parts were separately fixed overnight in glutaraldehyde (2.5% glutaraldehyde in a 25 mM sodium phosphate buffer, pH 6.8) at 4˚C. After dehydration in a graded ethanol series, the specimens were introduced at a critical point into liquid CO₂. The dried material was mounted and coated with gold-palladium using a Hitachi E-1010 sputter Coater. Specimens were examined using a FEI-Quanta 200F scanning electron microscope with an accelerating voltage of 15 kV.

Results

Morphological observations

The flowers of *C. japonicum* are small and inconspicuous, with similar flowering buds and leaf buds. The inflorescence has a juvenile leaf and a stipule which are embedded in three scales. The outer scales are russety, thick and sclerotic. The middle and inner scales are membranous, stretching out from the outer ones as they develop. When young, the middle and inner scales are peak green with a rose-red margin and turn yellowish with a red margin when mature. Juvenile leaves and stipules are found at the bottom of the pedicel. Juvenile leaves with transparent scrotiform glands in the margin are involute when they are wrapped in scales. The stipules are lanceolate, subtranslucent and membranous. The inflorescence of *C. japonicum* is highly simplified, with their pistillate inflorescence formed by four subtranslucent peak green bracts and 2–6 carpels, whose flat and upturned stigma is yellowish-green when young and turn scarlet when mature (Fig 1A). From our observations, we conclude that there are only two membranous bracts and several stamens whose heads are a bit sharp. The anthers are greenish when young and turn crimson when mature, with filaments almost did not elongate until when they are nearly mature (Fig 1B).

For an individual flower, the morphology of epidermal cells among the various parts-three scales, juvenile leaf, stipule, stamen or carpel and bract-are clearly distinct. When comparing the male and female flowers, except for the carpels and stamens, the other corresponding parts of flowers do not show clear differences on epidermal cells. The abaxial epidermal cells on the outer scales are long, fibrous and relatively smooth except for a few short horns (Fig 2A). While the adaxial epidermis can be clearly distinguished, the cells are short, irregular and rough with a raised edge in the middle (Fig 2B). Most epidermal cells on both adaxial and

![Fig 1. Morphology of *Cercidiphyllum japonicum* flowers. (A) Female inflorescence bud and dissections parts. (B) male inflorescence bud and dissections parts. OS = outer scale, MS = middle scale, IS = inner scale, ST = stamens, CA = carpels, LE = juvenile leaves, STI = stipule, and BR = bracts. Male and female inflorescence are showing the same outlook of OS, MS, IS, LE and STI.](https://doi.org/10.1371/journal.pone.0178382.g001)
Fig 2. Epidermal cells of leaves and floral parts of *Cercidiphyllum japonicum*. Since male and female flowers are the same besides floral organs, so just female ones were displayed. (A) Abaxial (bar = 50 μm) and amplified (inset; bar = 10 μm) epidermal cells of a outer scale at mature stage. (B) Adaxial (bar = 30 μm) epidermal cells of a outer a scale at mature stage. (C) Abaxial (bar = 25 μm) and adaxial (inset; bar = 20 μm) epidermal cells of a inner scale at mature stage, showing irregular striation. (D) Abaxial (bar = 30 μm) and adaxial (inset; bar = 10 μm) epidermal cells of a inner scale at mature stage. (E) Carpels from a mature flower (bar = 200 μm). (F) Epidermal cells of a stigma (left; bar = 15 μm) and back (right; bar = 5 μm) of carpe. (G) A stamen from a mature flower (bar = 200 μm). (H) Surface of anther (left; bar = 10 μm) and filament (right; bar = 20 μm). (I) Juvenile leaves (bar = 200 μm) and the abaxial and amplified epidermal cells (inset; bar = 15 μm). (J) Epidermal cells of glands (bar = 30 μm). (K) Surface of a stipule (bar = 500 μm), showing relatively regular sculpturing (insert; bar = 30 μm). (L) Bracts (bar = 300 μm), showing middle slotted or tee or cross grooves (bar = 20 μm).
abaxial sides of the middle scales are short and square, while cells on the edge are longer and with irregular prismatic protuberances (Fig 2C). The inside and outside epidermal cells on the inner scales are basically the same, regular and square in the middle, longer in the margin and straddle parallel grooves (Fig 2D). Epidermal cells on stigma are sunken and irregular in shape; it is hard to distinguish between individual cells. Cells on ventral sutures are square and arranged densely, while the peripheral cells are relative long and smooth (Fig 2F). The epidermal cells on the head of stamens and cells at the stomium of anther are spheroidal or square, but other places of the anthers are irregular, distorted strips, difficult to affirm as single cells (Fig 2G). Elsewhere, the filament cells are smooth and regular and elongated (Fig 2H). Cells of veins are larger and protuberant, while the mesophyll cells are smaller, round or square protuberances (Fig 2I). Epidermal cells of glands on the edge of juvenile leaves are nearly square and smooth (Fig 2J). The epidermal cells on the cusp of stipules are short and round and the margin consists of monolayer cells, while the lower cells are regular strip foundations with parallel contorted folds with spiny protuberances in the margin (Fig 2K). The epidermal cells on bracts are distinct ellipsoid with regular horizontal slender striate bulges and most of them are slotted in the middle or have tee or cross grooves (Fig 2L).

Screening and phylogenetic analysis of homeotic genes

Ten floral organ identity genes were obtained by homologous cloning and RACE methods. Among these, four clones were identical to AP1, FUL, FUL-like and AGL6 genes. These genes were respectively referred as CejaAP1, CejaFUL, CejaFUL-like and CejaAGL6. Three B-class transcripts were identified and referred as CejaPI, CejaAP3_1 and CejaAP3_2. Two C-class gene were called CejaAG1, CejaAG2 and the only D-class homologous gene was named CejaAGL11. We performed phylogenetic analyses and constructed trees of each gene and classified them into four trees.

According to the phylogenetic analysis of A-class genes, CejaAP1, CejaFUL and CejaFUL-like genes are respectively classified with euAP1, euFUL and FUL-like lineages in the basal core eudicots. CejaAP1 and CsAP1 of Corylopsis sinensis (Saxifragales) are sister groups, given bootstrap support under ML (94%) and form a clade with other euAP1 homologues of Saxifragales. CejaFUL and CsFUL of Corylopsis sinensis (Saxifragales) are sister groups and form a clade with HeaFUL of Heuchera americana (Saxifragales) with bootstrap support under ML (95%). CejaFUL-like also forms sister groups with HeaFUL-like of Heuchera americana (Saxifragales) (Fig 3). Since AGL6 lineage was not a typical A-class gene, the phylogenetic tree of CejaAGL6 was constructed only with its own lineage genes. The analysis shows that CejaAGL6 groups with RsAGL6 of Ribes sanguineum (Saxifragales) in the basal core eudicots (bootstrap 82%) (Fig 4).

CejaPI, the homologue of PI in C. japonicum, forms a sister group with PsPI of Paeonia suffruticosa (Saxifragales) and RbFPI of Ribes diacanthum (Saxifragales) even with low bootstrap support. CejaAP3_1 and CejaAP3_2 are grouped with RbMAP3 of Ribes diacanthum (Saxifragales) and CopAP3 of Corylopsis pauciflora (Saxifragales) with bootstrap support under ML (98%). This clade clearly branches off TM6 lineages (Fig 5).

Two C-class genes, CejaAG1 and CejaAG2, were isolated; phylogenetic analysis showed that CejaAG1 belongs to the euAG lineages and CejaAG2 to the PLE lineages. CejaAG1, PasuAG of Paeonia suffruticosa (Saxifragales) and SxcAG1 of Saxifraga careyana (Saxifragales) gather in a group with bootstrap support under ML (61%). CejaAG2 and LAG of Liquidambar styraciflua (Saxifragales) is a sister group in the ML analysis (bootstrap 61% support). The only D-class gene CejaAGL11 forms a clade with SxcAG2 of Saxifraga careyana (Saxifragales) in the ML analysis (bootstrap 71% support) (Fig 6).
Fig 3. Phylogenetic analysis of A-class genes. A phylogenetic tree was built using the maximum-parsimony method through the program MEGA 7.0 based on the protein sequences of different species. GGM1 and DAL1 are used as outgroups. The percentage bootstrap values are indicated by numbers at the branch points.

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Fig 4. Phylogenetic analysis of AGL6 lineages. A phylogenetic tree was built using the maximum-parsimony method through the program MEGA 7.0 based on the protein sequences of different species. DGL14 and GGM11 are used as outgroups. The percentage bootstrap values are indicated by numbers at the branch points.

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Fig 5. Phylogenetic analysis of B-class genes. A phylogenetic tree was built using the maximum-parsimony method through the program MEGA 7.0 based on the protein sequences of different species. PrDGL and GGM2 are used as outgroups. The percentage bootstrap values are indicated by numbers at the branch points.

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Fig 6. Phylogenetic analysis of C/D-class genes. A phylogenetic tree was built using the maximum-parsimony method through the program MEGA 7.0 based on the protein sequences of different species. GGM3 and DAL2 are used as outgroups. The percentage bootstrap values are indicated by numbers at the branch points.

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Expression of ABCD Homologs in *C. japonicum*

The expression patterns of the ABCD Homologs were analyzed by qRT-PCR. The expression patterns of these genes were shown in Fig 7. Except for *CejaPI* which is expressed strongly in male ones and weakly in female ones, the remaining target genes are barely expressed in juvenile leaves.

For A-class genes, *cejaAP1* has similar expression patterns between male and female buds, expressed in inner scales, stipules and bracts. *CejaFUL* is expressed in all scales, stipules and bracts of male and female buds as well as in carpels. *CejaFUL-like* is almost only expressed in bracts. *CejaAGL6* shows different expression patterns between male and female flowers, with relatively strong expressions in the outer scales of males while weakly in those of females, but expressed relatively weak in carpels and stipules. Elsewhere, *CejaAGL6* is detected in female bracts but not in male ones. B-class genes are expressed in almost all male floral organs, especially *CejaPI* which is barely expressed in female buds. *CejaAP3* is expressed most often in both male and female bracts. This *CejaAP3* is expressed most in both male and female bracts, where the expression level of *CejaAP3* is 3–4 times compared with *CejaActin*.

**Fig 7. Expression patterns of floral organ identity genes.** Real time qPCR was performed showing expression in different organs. The *CejaActin* was used as an internal reference. Values represent the means ± standard error of triplicates.

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CejaAP3_2 is expressed higher than CejaAP3_1 in stamens and carpels, but in both male and female bracts, expression level of CejaAP3_2 is much less than CejaAP3_1. Apart from this observation, we found that, CejaAP3_1 displays a similar expression pattern with CejaAP3_2 between other male and female floral parts (low level). For C-class genes, CejaAG1 is mainly expressed in carpels, stamens and both bracts. CejaAG2 is expressed in carpels and both bracts (low level), but less than CejaAG1. The D-class gene CejaAGL11 is expressed quite strongly in carpels.

**Discussion**

Since species identification and classification are based on morphology, an increasing number of studies suggested that sole reliance on this approach may lead to the neglect of a significant number of relevant species [25]. As the development of molecular phylogenetics, DNA and amino acid sequence analyses have been an important method to study systematic evolution and development. As Woese [26] argues, sequential information contains the promise that we will have potentially more evolutionary information than we now possess and allows us to infer a great deal of assurance than we can now.

**MADS-box homologs and systematic place**

We obtained three A-class, three B-class, two C-class homologs and one D-class homolog from Cercidiphyllum japonicum, which has never been reported before. Phylogenetic analyses show that these floral organ identity genes group with the respective classes of the MADS-box genes from other Saxibragales plants, indicating that placing Cercidiphyllum japonicum in Saxibragales in the basal core eudicots is suitable. The C-terminal regions of C. japonicum genes contained conserved characteristic motifs, typical of the genes of each class (Fig 8), therefore indicating their functional similarities with other homologs regulating flower formations in other plants [27,28]. Only the C terminal is shown. Conserved motifs are boxed, as defined by previous studies for the AP1 motif, the PI and AP3 motifs, and the AG motif.

Recent studies suggested that the major duplication events for floral ABC-class genes occurred at the base of core eudicots [29–32]. For A-class genes, it has been proposed that a major duplication event occurred near the base of their core eudicots, giving rise to euAP1, euFUL and FUL-like lineages [31,33,34]. All the three A-class lineages we obtained from C. japonicum, thus suggesting that it could have originated after this duplication period. For the AP3/PI subfamily, one duplication formed DEF/AP3 (paleoAP3) and GLO/PI lineages. Subsequently, following the duplication in the base of core eudicots, a frame shift mutation occurred in DEF/AP3 copies and formed TM6 and euAP3 lineages [29,35]. Predicted amino acid sequence of CejaAP3_1 contains a paleoAP3 motif, suggesting that C. japonicum may not originate may not have originated later than the base of the core eudicots. In addition, euAG- and PLE-lineage originated on account of a major duplication in the early period of core eudicots and undergone the functional switch between them after rosid and asterid differentiations [30,36,37]. Since both euAG and PLE homologs were found in C. japonicum, it is further demonstrated that C. japonicum may not have originated earlier than the rosid and asterid divergent period. Hence, the summation of molecular evidence limited the systematic place of C. japonicum to the base of core eudicots.

Studies of earlier ABCDE-models were based on the Arabidopsis and Antirrhinum model systems [16,17]. Based on ABCDE-model, we speculated that the sexual differentiation of C. japonicum may be related to the B-/C-class homologs. In the most recent common ancestor of gymnosperms and angiosperms, the primitive function of AG lineage was to differentiate the
reproductive organs from nutritional organs [38,39]. The function of DEF/GLO lineage is to differentiate male and female [40]. The B-class gene SlAP3Y in Silene latifolia is located in the Y chromosome and related to gender decision [41]. The qRT-PCR results show that CejaAG1 is highly expressed in stamens and carpels, while the CejaAG2 is almost only expressed in carpels strongly. Previous studies have indicated that the B-class genes of core eudicots are stably

Fig 8. Representative predicted amino acid sequences of ABCD genes from Cercidiphyllum japonicum and selected taxa.

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expressed in petals and stamens, but this is not always coincident with the B-class genes of basal eudicots and basal angiosperms [42]. For instance, the CejaPI is almost male specific, since it is strongly expressed in all male organs and barely examined in female ones. These results may indicate that CejaAG1 plays an important role in reproductive organ formation. As well, CejaAG2 and CejaPI are crucial to carpels and stamens in floral development of C. japonicum respectively. Since functional verification is difficult to conduct in woody material, evidence for functions of identified ABCDE genes of Cercidiphyllum japonicum should use the corresponding mutant Arabidopsis as medium in future studies.

Confusing structure of C. japonicum
In general, C. japonicum is thought to be missing the perianth. When we observed the male and female inflorescences, we encountered that there were two lamelliform and membranous bracts in male while there were four in female ones. Ding [43] described the ‘bracts’ as four sepals in the Flora of Henan. In another point of view, Yan et al. [15] observed morphogenesis of C. japonicum and considered that bracts should be closer to phyllome, but the so called bracts in C. japonicum developed with their basal stamens or pistils correlatively; hence they proposed that the so called bracts are more closely related to tepals. We found that leaf buds and flower buds are much the same except for their reproductive parts. According to the Agricultural Dictionary, a bract is actually a phyllome. Based on the model, the absence of petals in C. japonicum might be due to the null function of A-class and B-class homologs. The APl/SQUA family, such as AP1 mutant of Arabidopsis and SQUA mutant of Antirrhinum majus, may cause changes of petals and sepals [33,44,45]. Moreover, the petals were converted to sepals and stamens to carpels in the ap3 and def mutants [40]. Unfortunately, definite evidence of A-class homologs has never been demonstrated in woody plants and the expression patterns are not strictly conserved. In most primitive angiosperms, it is the petals not bracts or sepals having high expression levels of both A- and B-class genes, such as Orchid [46,47], Trochodendron [48] and Eucalyptus of Saxifragales [49]. Wróblewska et al. [50] analyzed expression patterns of key flower genes of several Magnoliaceae and found that the B-class genes, AP3 and PI, were restricted to the second and third whorl. In our research, the qRT-PCR results show that both A- and B-class genes, especially CejaAP1 and CejaAP3_1/_2 whose homologous genes are petal decisive in Arabidopsis, had significant expressions in the bracts that are different from other organs of C. japonicum. Recent studies in Arabidopsis and Antirrhinum, as well as several other species, indicate that the function of floral MADS-box genes is largely associated with the expression patterns of these genes, particularly when expression levels are high [51]. What is more, the epidermal cells of the bracts show considerable differences from other phyllomes. In view of this inference, we recommended that the so-called bracts actually should be considered as perianth.

Exon skipping of CejaAP3
Alternative splicing has been found in several MADS-box genes which, to some extent, might have either an important positive or negative impact, typical in Magnolia stellata [52]. During the screening, two CejaAP3_1/_2 spliceosomes were found. After examining the genomic sequence, we found that the two clones may be formed by alternative splicing. In addition, the shorter spliceosome, CejaAP3_2, was confirmed to be missing an exon 4 (Fig 9). What is more, the results of qRT-PCR shows that CejaAP3_2 displays a high expression in stamens and moderate expression in other floral parts, indicating that this abnormal splicing may have a significant impact on the floral development of C. japonicum, especially the perianth. However, the exact nature of this product and its interactions need further study.
We conclude that all floral homeotic gene phylogenies show that *C. japonicum* is closely related to the plants of *Saxifragales*, suggesting that our species should be placed in *Saxifragales* at the base of core eudicots. This result confirms the APGIII system and supports a new train of thought when investigating systematic evolution based on floral organ identity genes. As well, our research supports the conjecture that the so-called bracts of *C. japonicum* actually are perianth, a conclusion based on morphology and expression patterns.

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