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Morphogenesis of Stamens and Petaloid Stamens in *Lilium hybrid* 'Red Twin' under Different Temperatures and the Expression Characteristics of Two *AGAMOUS*-like Genes Linked to These Processes

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Abstract: The double-flowered lily 'Red Twin', in which stamens are transformed into petaloid organs, sometimes exhibits normal stamens owing to an unknown mechanism and thus greatly affecting its commercial quality. In this study, the morphogenesis of stamens and petaloid stamens in this cultivar grown under different temperature treatments were investigated. Two *AGAMOUS*-like genes were isolated and their expression levels were analyzed. The results showed that relatively high temperatures induced the morphogenesis of stamens, while relatively low temperatures promoted petaloidy in 'Red Twin'. The stage with 1–6 mm flower buds was identified as the critical development period for stamen morphogenesis; furthermore, keeping the flower buds under relatively low temperatures in the 1–3 or 3–6 mm stages would be sufficient for the formation of petaloid stamens to a high degree. In addition, *LrtAG1* and *LrtAG2* showed the highest expression level in whorls 3 and 4 of 3–6 mm flower buds, respectively. *LrtAG1* showed a higher reduction ratio than *LrtAG2* when the flower buds were transferred to low temperature, implying that the decreased *LrtAG1* expression level is associated with low temperature and might be correlated with the petaloidy of the stamens.

Keywords: *Lilium*; double flower; petaloid stamens; morphogenesis; temperature; *AGAMOUS*-like genes

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1. Introduction

The genus *Lilium* is a perennial bulbous plant belonging to the family Liliaceae, a popular flower in the international flower market because of its large flowers, varied colors, graceful appearance, and varied uses (pot plants, cut flowers, and landscape plants).

Wild-type lily flowers are single flowers bearing four whorls of floral organs, with three outer tepals in the first whorl, three inner tepals in the second whorl, six stamens in the third whorl, and three carpels in the fourth whorl. Double flowers in some varieties show petaloid stamens and even petaloid carpels followed by increasing number of floral organs. These are popular in the international flower market because of their attractive appearance and pollenless feature, avoiding potential cloth staining and allergies. Most lily varieties have consistent single- or double-flower traits. However, owing to an unknown reason and molecular mechanism, some varieties such as *Lilium hybrid* 'Red Twin', 'Gold Twin', and 'Elodie' sold as double-flower varieties sometimes exhibit single-flowers with normal stamens, greatly affecting the performance of these cultivars.

The classical ABC model of floral development in *Arabidopsis* has elucidated the specification of floral organ identity [1,2]: sepal identity (whorl 1) is specified by A-class genes, petal identity (whorl 2) is specified by A- and B-class genes, stamen identity (whorl 3) is determined by B- and C-class genes, and carpel identity (whorl 4) is conferred by C-class genes. As a C-class gene, *AGAMOUS* (*AG*) is a core regulator to control the formation of reproductive organs in *Arabidopsis* [3]. In addition, it also controls the determinacy of

floral meristems [4,5]. The *ag-1* mutation exhibits the conversion of stamens to petals in whorl 3 and carpels to another *ag* mutant flower in whorl 4, which develops constantly repeating structures of 'sepal-petal-petal' from the outside to the inside in flowers [6,7]. The phenomenon of petaloid stamens caused by the mutation, down-regulation, or expressional boundary shift of the *AG* homologs has been reported in other species, such as *Thalictrum thalictroides* [8], *Prunus lannesiana* [9], *Rose hybrida* [10,11], *Petunia hybrida* [12], *Camellia japonica* [13], *Cyclamen persicum* [14], *Matthiola incana* [15], *Chrysanthemum morifolium* [16], and *Hippeastrum hybridum* [17].

In *Lilium* species, down-regulation of the *AG*-like genes, such as *LaphAG1* in 'Aphrodite' [18], *LelAG1* in 'Elodie' [19], and *LiAG* in 'Annemarie's Dream' [20], is considered one of the major factors for stamen petaloidy in these cultivars. In addition, the 'Elodie' cultivar exhibits different strengths of petaloidy of stamens (weak, intermediate, and strong), which is correlated with the expression level of *LelAG1*. However, little is known about the key factors which induce the variation in the degree of petaloidy and the expression level of *LelAG1* in this cultivar.

The effect of temperature or phytohormones on floral morphology has been investigated in many species or varieties of flowering plants. A higher temperature regime of 80 °F (about 26.6 °C, day)/70 °F (about 21.1 °C, night) was reported to induce more petaloid stamens than 70 °F (day)/60 °F (about 15.5 °C, night) and 60 °F (day)/50 °F (10 °C, night) in *Daucus carota*, while neither gibberellic acid, maleic hydrazide, nor kinetin increased or decreased petaloidy [21]. In tomato (*Lycopersicon esculentum*), the flowers contained greater numbers of petals, stamens, carpels, and locules under low temperatures than those under high temperatures; moreover, gibberellic acid (GA_3) could also induce this increase of floral organs under high temperatures [22]. In carnations (*Dianthus caryophyllus*), low temperatures promoted the formation of secondary growing centers in flowers and more petals, which was also promoted by applying GA_3 or indole-3-acetic acid (IAA) to the shoot tip during flower initiation [23]. Furthermore, the carnation 'Cherry' *mlf* mutant produced several malformed floral phenotypes, except for secondary flower formation, under a low temperature of 17/5 °C (day/night), such as proliferated petaloids, proliferated pistillodes with or without petals, phyllody-like sepaloids, and a flattened receptacle [24]. Phyllody-expressing flowers and increasing petal numbers were also observed in *Rosa hybrida* cv. Motrea, but the proportion of the flowers exhibiting this phenotype at a high temperature of 26/21 °C (day/night) was higher than at 21/15 °C (day/night) [25]. However, it was also reported that a low temperature of 15/5 °C (day/night) would result in more irregular flower centers and more petal numbers than a normal temperature of 25/15 °C (day/night) in *Rose hybrida* cv. Vendela [11]. Furthermore, attenuated *RhAG* expression level by the hypermethylation of its promoter induced by low temperatures might be responsible for the increase in petal numbers. This contradictory phenomenon under different temperatures might imply two distinct mechanisms controlling the petal numbers in different varieties of rose. In addition, male fertility is also affected by low or high temperatures. In *Capsicum annuum*, stamens produced abnormal non-viable pollen and were thus functionally male-sterile under low temperatures (18 °C day/15 °C night) [26]. In *Glycine max* cv. Toyomusume, tetrad-shaped abnormal pollen grains were observed when flowers were subjected to low temperatures at the sensitive stage [27]. An Asiatic hybrid lily cultivar 'Akita Petit White' is a variety with antherless stamens. The antherless phenotype was stable under low temperatures. However, it was restored to contain intact anthers under high temperatures, and its pollens produced viable seeds almost equal to those of other Asiatic hybrid lilies after pollination [28]. In general, higher or lower temperatures might induce various floral phenotypes by regulating the expression of key genes, and these changes differ among varieties.

L. hybrid 'Red Twin' has normal outer tepals, inner tepals, carpels, and fertile ovaries inside but petaloid stamens in most cases; however, it sometimes also exhibits normal stamens with fertile pollen. The underlying mechanism of this phenomenon is unknown. In this study, different temperature treatments and a temperature-changing experiment

were performed during the entire floral development process or special stages of 'Red Twin' to determine the critical development period of stamens or petaloid stamens and whether the petaloidy of stamens is induced by low or high temperatures. Two *AGAMOUS*-like genes, *LrtAG1* and *LrtAG2*, were isolated and their expression levels were analyzed in flower buds containing normal or petaloid stamens and different floral organs at the critical development stages. To the best of our knowledge, this is the first study to identify the critical development stage of stamens in the *Lilium* species. These results could be provided to commercial producers of 'Red Twin' flowers to help them avoid the formation of normal stamens and maintain the double-flower trait in this cultivar. In addition, the reduced expressional level of *LrtAG1* in stamens was speculated to be the key reason for the petaloidy of stamens, which will provide a candidate gene for double-flower breeding in lilies.

2. Materials and Methods

2.1. Plant Material and Temperature Treatment

Lilium hybrid 'Red Twin' bulbs of 16 to 18 cm in circumference were kept at 3 °C in the dark for 2 months to break dormancy. Thereafter, they were planted in six growth chambers under different temperature treatments (T1–T6) with a long-day regime (16 h light at 300 μmol·m^{-2}·s^{-1}/8 h dark) (Figure 1a). The bulbs planted in the 28 °C and 22 °C growth chambers were used to conduct a temperature-changing treatment (S1–S10) by transferring the bulbs between the two growth chambers when the first emerged flower buds in the bulbs (FFs) reached a certain size (0.7-mm, 1-mm, 3-mm, 6-mm, and 1-cm) (Figure 1b).

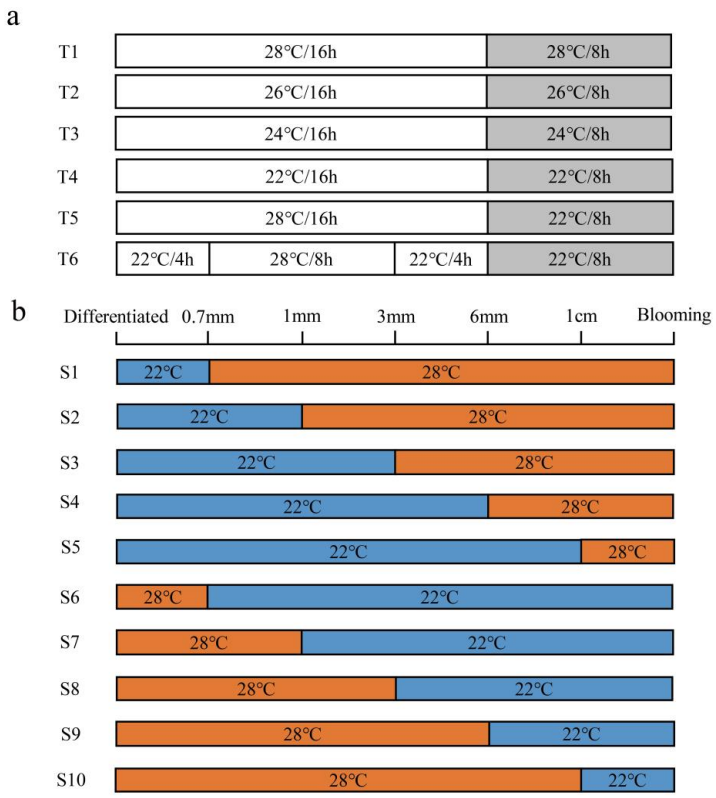

Figure 1. Different temperature treatments of 'Red Twin' every day from planting to blooming (**a**) and temperature-changing treatment of flower buds at different sizes (**b**). In T1-T6, the white box represents the light and the gray box represents the dark period, the different treatment temperatures and durations are shown in the box. S1–S5 represent flower buds that were transferred to 28 °C from 22 °C at 0.7 mm, 1 mm, 3 mm, 6 mm, and 1 cm, respectively. S6–S10 represent flower buds that were transferred to 22 °C from 28 °C at 0.7 mm, 1 mm, 3 mm, 6 mm, and 1 cm, respectively.

Three biological replicates of floral organs in the four whorls of FFs at 3–6 mm and contemporaneous leaves that grew under treatments T1 and S7 were collected, immediately frozen in liquid nitrogen, and kept at −80 °C for RNA extraction.

2.2. Morphological Observation and Statistics of Petaloid Value

Floral organs in whorl 3 (stamens and petaloid stamens) of all flowers under different temperature treatments (T1–T6) and the FFs under different temperature-changing treatments (S1–S10) were observed at their blooming stage. Based on the morphological change, the petaloid degree of stamens was divided into six grades (petaloid value of 0–5). The petaloid value of each treatment was calculated based on the petaloid degree of 30 stamens in 5 FFs from five bulbs.

Flower buds at 0.7 mm, 1 mm, 3 mm, 6 mm, 1 cm, and 4 cm that grew at 28 °C and 22 °C were used to observe the morphology of stamens and petaloid stamens with a dissecting microscope or the naked eye. Flower buds at 1 mm, 3 mm, and 6 mm were also embedded in paraffin blocks for routine longitudinal sectioning and staining with safranin O and fast green.

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit (Takara Bio Inc., Kusatsu, Japan). The RNA concentration, purity, and integrity were analyzed using an ND-1000 spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Qualified RNA was chosen to synthesize cDNA using the Primer-Script^{TM} RT Reagent Kit (Takara Bio Inc.) according to the manufacturer's instructions.

2.4. Isolation of the AGAMOUS-like Genes in 'Red Twin' and Sequence Analysis

Based on the nucleotide sequences of *AGAMOUS*-like genes in *Lilium* spp., including *LLAG1* (AY500376), *LMADS10* (KJ819937) from *L. longiflorum*, *LFMADS4* (Kj489393) from *L. formosanum*, *LelAG1* (AB596844) from *L. hybrid* 'Elodie', *LLAG* (HM030993) from *L. hybrid* 'Siberia', and *LaphAG1* (AB359183) from *L. hybrid* 'Aphrodite', a pair of degenerate primers (forward primer, 5′-ATGGGKAGRGGWAAGATWGAG-3′, and reverse primer, 5′-TTAWCCWAGTTGGAGKGCAGT-3′) were designed to amply the cDNA fragment of their homologs *LrtAG1* and *LrtAG2* in *L. hybrid* 'Red Twin'. The 5′ and 3′ terminal cDNA sequences were obtained by using the 5′ and 3′ rapid amplification of cDNA ends (RACE) system (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The primers used are listed in Table 1. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced by an automated sequencer (Model 310, Applied Biosystems, Waltham, MA, USA).

Amino acid sequences of *LrtAG1*, *LrtAG2*, and 25 C-class genes from 12 monocots and dicots were aligned by Clustal W in MEGA5 [29] with default parameters and displayed with the GeneDoc program [30]. The phylogenetic tree was constructed with the neighbor-joining method using the JTT + G model in MEGA5, and the bootstrap analysis was based on 1000 replicates.

2.5. Quantitative Real-Time PCR Analysis

Quantitative real-time PCR (qRT-PCR) of *LrtAG1* and *LrtAG2* was carried out with TB Green^{TM} Premix Ex Taq^{TM} II (Takara Bio Inc.) on a Roche LightCycler 480II System (Roche Diagnostics GmbH, Mannheim, Germany). We selected *18S ribosomal RNA* (*18S*, AY684927) as a reference gene to standardize the results, and the primers used are listed in Table 1. The standard curve of a 5-fold dilution series from a pool of cDNAs was made to calculate the gene-specific PCR efficiency ($E = 10^{(-1/slope)} - 1$) and regression coefficient (R^2) to confirm the suitability of these primers for qRT-PCR analysis. Relative expression quantification was calculated by the $2^{-\Delta\Delta CT}$ algorithm based on the expression of *18S*.

Table 1. Primers for rapid amplification of cDNA ends (RACE) and real-time quantitative PCR (qRT-PCR).

Gene	Oligo Name	Sequence (5′-3′)	Remarks
LrtAG1	5GSP1	GTTGCGCCCTTTACTTGCCA	5′-RACE
	5GSP2	GAGCTCATAGGCCTTCTTGAGCAGGCCA	5′-RACE
	5nested-GSP	CTGCCTGTTGGTGGTGTTCTCGATCCTC	5′-RACE
	3GSP	CACATGGACATGGACCGAACGCAGCAGC	3′-RACE
	3nested-GSP	GCGACACTTGGAGATGCTGCCCACCAC	3′-RACE
	RT-F	CCATGCCTACATTCGATTCG	qRT-PCR
	RT-R	GGAGATTGTTCAGAGTCTTCATAA	qRT-PCR
LrtAG2	5GSP1	TCGCTTTGCAATTCCATCTC	5′-RACE
	5GSP2	TCATGTTGCTGAGGGACTCGCCCAACAG	5′-RACE
	5nested-GSP	GGCGGCCGCGAGTGGAGAAGACGATGAG	5′-RACE
	3GSP	AGATCTGTCTCGGAAGCCAATGCACAGT	3′-RACE
	3nested-GSP	AGGAATCTGTTGGGCGAGTCCCTCAGCA	3′-RACE
	RT-F	GCCACACTTCGATTCACGG	qRT-PCR
	RT-R	GATTCAGCATAAAACCACATTG	qRT-PCR
18S	RT-F	CGTTTCGGGCATGATTTGTGG	qRT-PCR
	RT-R	TCGCATTTCGCTACGTTCTTC	qRT-PCR

3. Results

3.1. Petaloidy of Stamens under Different Temperatures

All flowers in 'Red Twin' that were kept at a consistent temperature of 28, 26, 24, and 22 °C from planting to blooming (Figure 1a, T1–T4) had normal outer petals, inner petals, and pistils at blooming stage, but their stamens in whorl 3 exhibited different degrees of petaloidy. Stamens of the plants grown at 28 °C had the normal form with four pollen sacs, more pollen grains, and a filiform filament. As the temperature decreased, the strength of the petaloidy of stamens increased, from reduced to disappeared pollen sacs and grains and filiform to petaloid filaments (Figure 2). Stamens of the plants grown at 22 °C had no pollen sacs but a petaloid filament, similar to those grown at 20 °C or 18 °C (Figure S1). The petaloid value of 0–5 was defined based on the changed morphological characteristics of stamens under different temperatures (Table 2). Statistical analysis showed the petaloid value of stamens of plants kept at 28 °C for 16 h (T5) and 8 h (T6) daily, at a daily mean temperature of 26 °C (T5) and 24 °C (T6), was close to the petaloid value of stamens of plants kept at 26 °C and 24 °C for 24 h (T2 and T3), respectively (Figure 3a). This morphogenesis of stamens under relatively high temperatures and petaloid stamens under relatively low temperatures was dose dependent.

Table 2. Definition of the petaloid value of stamen at the blooming stage.

Petaloid Value	Characterization	Related Pictures
0	Four pollen sacs, more pollen grains, a filiform filament	Figure 2b,c
1	Two pollen sacs, less pollen grains, a filiform filament	Figure 2e,n
2	Abnormal pollen sacs, pollen-free, a filiform filament	Figure 2f,h,o
3	Abnormal pollen sacs, pollen-free, a petaloid filament	Figure 2q
4	No pollen sacs, a filiform filament	Figure 2i,r
5	No pollen sacs, a petaloid filament	Figure 2k,l

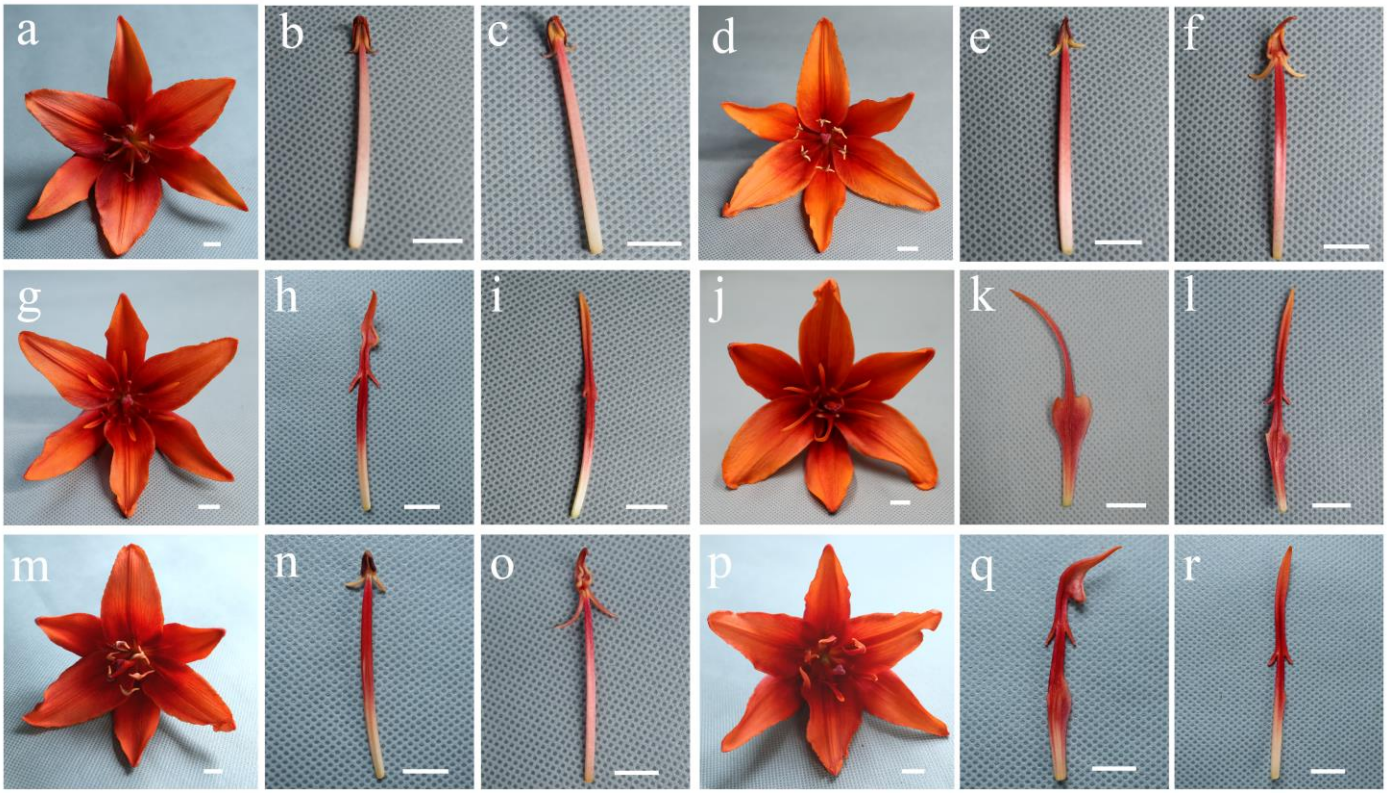

Figure 2. Morphological characteristics of flowers and floral organs in whorl 3 at the blooming stage under different temperature treatments. (**a**–**c**) Treatment T1. (**d**–**f**) Treatment T2. (**g**–**i**) Treatment T3. (**j**–**l**) Treatment T4. (**m**–**o**) Treatment T5. (**p**–**r**) Treatment T6. Treatments T1–T6 are explained in Figure 1a. Bars = 1 cm.

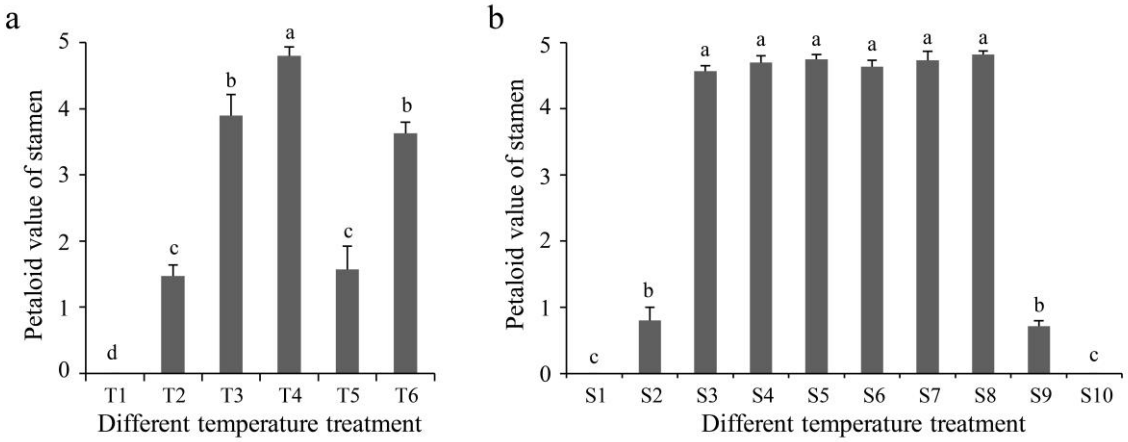

Figure 3. The petaloid value of stamens at the blooming stage under different temperature treatment T1-T6 (**a**) and S1–S10 (**b**). All treatments are explained in Figure 1. The petaloid value of the stamen was calculated according to Table 2. Different letters above bars indicate significant differences at the 0.05 level (Tukey-Krammer test).

3.2. Morphological Courses of Stamens and Petaloid Stamens

Stamens and petaloid stamens were undifferentiated in the 0.7-mm flower buds grown at temperatures of 28 °C and 22 °C (Figure 4a,k), while the dome-shaped primordiums of stamens and petaloid stamens had just emerged in the 1-mm flower buds, with no obvious difference between them (Figure 4b,h,l,r). Stamens and petaloid stamens of the 3-mm flower buds were beginning to show differences: the inside wall of the stamens was full (Figure 4c,i) while petaloid stamens were concave (Figure 4m,s), and the base of

stamens was constricted while the top of petaloid stamens was narrow. More differences were exhibited along with the growth of flower buds at 28 °C and 22 °C. For example, four locules appeared in stamens with developing archesporial cells (Figure 4d,j), but sheet-like structures in petaloid stamens (Figure 4n,t) in the 6-mm flower buds; rod-like filaments of stamens (Figure 4e) but petaloid filaments of petaloid stamens (Figure 4o) in the 1-cm flower buds; colored stamens (Figure 4f) but green petaloid stamens (Figure 4p) in the 4-cm flower buds; and colored pestle-like stamens with dispersed pollen (Figure 4g) but colored sword-like petaloid stamens (Figure 4q) in the blooming flowers. In general, the morphological outline of the stamens was formed in the flower buds at 6 mm, while the petaloid stamens were formed at 1 cm. They began to enlarge, lengthen, change color, and develop interior structures until they matured.

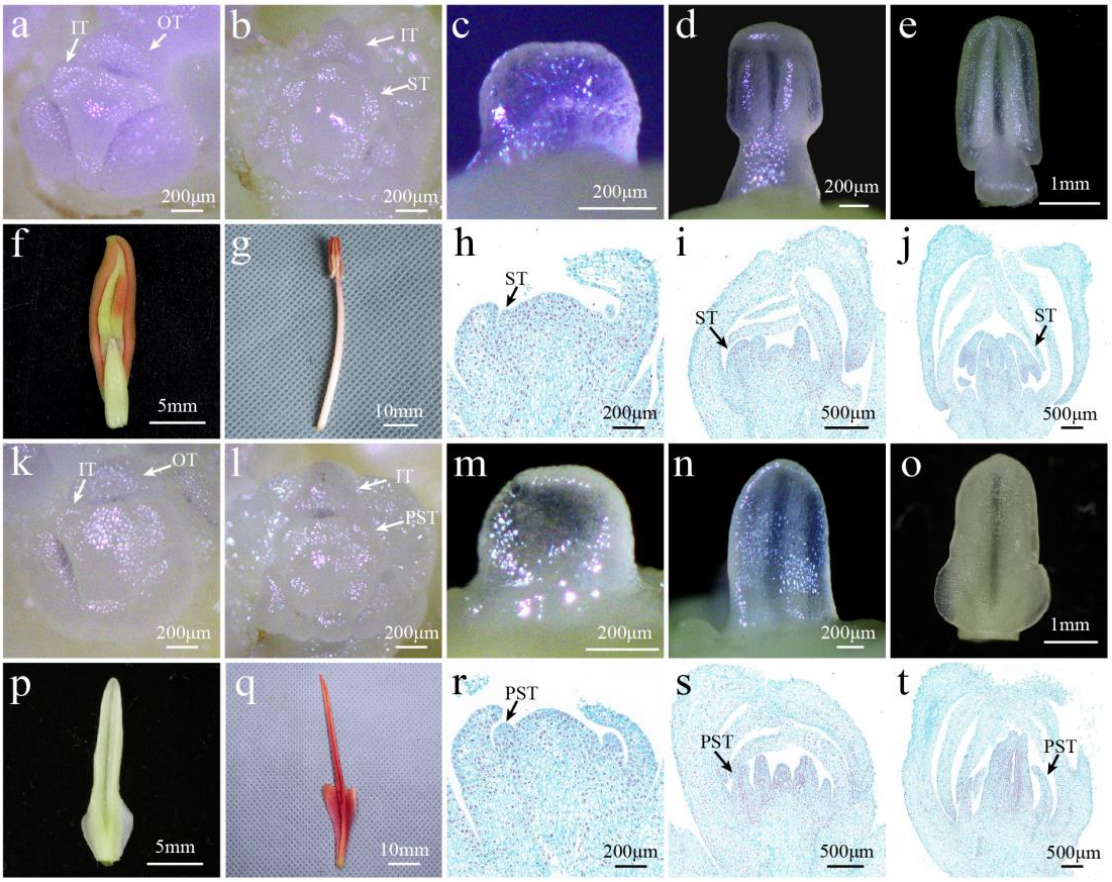

Figure 4. Morphological and cut-section observation of stamens and petaloid stamens in flower buds at different sizes under the temperature treatment of 28 °C and 22 °C, respectively. (**a**–**j**) 28 °C. (**a**) shows the undifferentiated stamens in flower buds at 0.7 mm. (**b**–**g**) show the stamens in flower buds at 1 mm, 3 mm, 6 mm, 1 cm, 4 cm, and in the blooming stage, respectively. (**h**–**j**) show the longitudinal section of stamens in flower buds at 1, 3, and 6 mm, respectively. (**k**–**t**) 22 °C. (**k**) shows undifferentiated petaloid stamens in flower buds at 0.7 mm. (**l**–**q**) show the petaloid stamens in flower buds at 1 mm, 3 mm, 6 mm, 1 cm, 4 cm, and in the blooming stage, respectively. (**r**–**t**) show the longitudinal section of petaloid stamens in flower buds at 1, 3, and 6 mm, respectively. OT, IT, ST, and PST represent outer tepal, inner tepal, stamen, and petaloid stamen, respectively.

3.3. Identification of Critical Developmental Periods of Stamens and Petaloid Stamens

A series of temperature-changing experiments were performed in the growth chambers; the timing to change temperature was decided based on the length of the first emerged flower buds in the bulbs (FFs), and the petaloid value of stamens in FFs were calculated (Figure 3b) to identify the critical developmental periods of stamens and petaloid stamens, which are easily affected by temperature, determining their appearance. The FFs grown

at 22 °C but transferred to 28 °C at 0.7 mm (Figure 1b, S1), and FFs grown at 28 °C but transferred to 22 °C at 1 cm (Figure 1b, S10) all exhibited normal stamens with the petaloid value of 0 at the blooming stage (Figure 3b). This indicated that relatively high temperatures (28 °C) during the 0.7 mm–1 cm development stage induced the morphogenesis of normal stamens, and relatively low temperatures (22 °C) from differentiation to the size of 0.7 mm or from 1 cm to the blooming stage had no effect on the morphogenesis of normal stamens.

The FFs grown at 22 °C but transferred to 28 °C at 1 mm (Figure 1b, S2), and FFs grown at 28 °C but transferred to 22 °C at 6 mm (Figure 1b, S9) exhibited close to normal stamens with fewer pollen grains and filiform filaments at the blooming stage. However, the FFs grown at 22 °C but transferred to 28 °C (Figure 1b, S3), and those grown at 28 °C but transferred to 22 °C at 3 mm (Figure 1b, S8) exhibited petaloidy of stamens close to the highest degree just like FFs under the treatments S4, S5, S6, and S7 (Figure 3b). These results indicated that the development period of 1–6 mm in flower buds was the critical period to regulate the morphogenesis of stamens. Exposure of the flower buds at the period of 1–3 mm or 3–6 mm to relatively low temperatures (22 °C) would lead to the morphogenesis of petaloid stamens at a high degree.

3.4. Isolation and Sequence Analysis of Two AGAMOUS-like Genes from 'Red Twin'

Two *AGAMOUS*-like genes from 'Red Twin' were cloned according to their homologs in other lily cultivars, and the full-length cDNA sequences of these two genes were obtained using 5′ and 3′ RACE technology. Subsequently, these two genes were designated as *LrtAG1* and *LrtAG2* and submitted to the NCBI database with the accession numbers ON756091 and ON756092. *LrtAG1* cDNA has a 5′ leader region of 209 bp, a 3′ untranslated region of 342 bp preceding a poly(A) tail, and encodes a protein with a predicted length of 254 amino acids. *LrtAG2* cDNA has a 5′ leader region of 224 bp, a 3′ untranslated region of 232 bp preceding poly(A) tail, and encodes a protein with a predicted length of 225 amino acids. The 5′ leader region and 3′ untranslated region of *LrtAG1* cDNA have low identities (36.52% and 37.68%) with *LrtAG2* cDNA.

Phylogenetic analysis of 27 C-class gene proteins showed that LrtAG1 was clustered with LLAG1 (*Lilium longiflorum*), LFMADS4 (*Lilium formosanum*), LelAG1 (*Lilium hybrid* '*Elodie*'), LLAG (*Lilium hybrid* 'Siberia'), and LaphAG1 ((*Lilium hybrid* 'Aphrodite'). LrtAG2 was clustered with LMADS10 (*Lilium longiflorum*) and TrimAG (*Tricyrtis macranthopsis*) in the Liliaceae clade, which is closely related to the Asparagales clade in monocot AG orthologues (Figure 5).

Amino acid sequence alignment of these 27 proteins was performed (Figure 6). LrtAG1 shares 100% identity with LelAG1 and LLAG, 95% with LaphAG1, 93% with LFMADS4, 89% with LLAG1, 68% with LMADS10 and LrtAG2 from *Lilium* species, and 60% with AG from *Arabidopsis thaliana*. LrtAG2 shares 98% identity with LMADS10, 70% with LFMADS4 and LaphAG1, 69% with LLAG1, 68% with LelAG1 and LLAG from *Lilium* species, 85% with TrimAG from *Tricyrtis macranthopsis* and HpAG from *Hosta plantaginea*, 82% with CsAG1a and CsAG1b from *Crocus saticus*, 80% with HhAG1 from *Hippeastrum hybridum* and OMADS4 from *Oncidium* Gower Ramsey, 79% with HAG1 from *Hyacinthus orientalis*, and 69% with AG from *Arabidopsis thaliana*. LrtAG1 and LrtAG2 contain the conserved MADS and K domains and AG motifs I and II found in AGAMOUS homologs. No differences were found in the conserved MADS domains between LrtAG1 and LrtAG2, but several different amino acid sequences in the K domain and C-terminal region were found, especially 10 amino acid insertions/deletions in the middle of the C-terminal regions of these two proteins. A comparison of these amino acid sequences showed that the C-terminal region of LrtAG1 clade proteins (LLAG1, LFMADS4, LelAG1, LLAG, LrtAG1, and LaphAG1) are longer than LrtAG2 clade proteins (LrtAG2, LMADS10, and TrimAG) in Liliaceae and the AGAMOUS-like proteins from other families, which could be due to the presence of direct repeat sequences in this region and might influence the differentiation of their function.

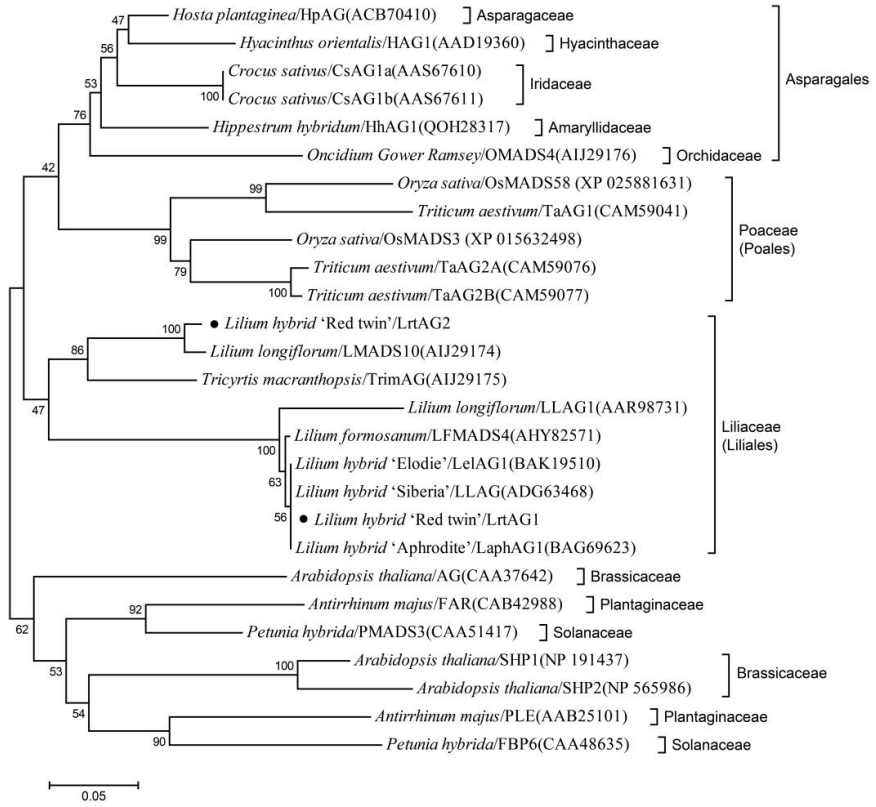

Figure 5. Phylogenetic relationship of LrtAG1 and LrtAG2 from *Lilium hybrid* 'Red Twin' and C-class gene proteins from 12 species. Node values indicate bootstrap support (%) from 1000 replicates. The prefixed black dots indicate LrtAG1 and LrtAG2. The numbers in brackets were the NCBI accession numbers of these proteins.

3.5. Expression Analysis of LrtAG1 and LrtAG2

qRT-PCR of *LrtAG1* and *LrtAG2* was performed on the first emerged flower buds (FFs) at different sizes (1 mm, 3 mm, and 6 mm), the floral organs in four whorls of FFs at 3–6 mm, and contemporaneous leaves under two temperature treatments (T1 and T7) (Figure 7). The amplification efficiency and regression coefficient of the primers for *LrtAG1*, *LrtAG2*, and *18S* calculated by the standard curve method were 92.59%, 92.45%, 93.55%, and 0.9996, 0.9988, and 0.9984, respectively, which confirmed that these primers were suitable for qRT-PCR analysis. Under a temperature of 28 °C, the third whorl floral organs developed four prismatic stamens in FFs at 6 mm, the expression level of *LrtAG1* in FFs at 1–6 mm showed a strong increasing trend, while *LrtAG2* showed a slight increase. *LrtAG1* exhibited the highest expression level in whorl 3 of FFs at 3–6 mm and the second highest in the carpel. However, it had very low to no expression in the leaves and outer and inner tepals. In contrast, *LrtAG2* exhibited the highest expression levels in the carpel (whorl 4), second highest in whorl 3, and very low or no expression in leaves and outer and inner tepals. When the FFs were transferred from 28 °C to 22 °C at 1 mm, the third whorl floral organs developed sheet-like petaloid stamens in FFs at 6 mm. *LrtAG1* was strongly downregulated by approximately 85% and 71% in FFs at 3 mm and 6 mm compared with the FFs grown at 28 °C, while *LrtAG2* was slightly downregulated by approximately 10% and 18%. In the FFs at 3–6 mm under the temperature treatment of 28 to 22 °C, *LrtAG1* showed a reduced expression level by approximately 64% in whorl 3 and 62% in the carpel compared with the same organs grown at 28 °C, while *LrtAG2* was reduced by approximately 41% and 34%. Given the higher expression level of *LrtAG1* than *LrtAG2* in whorl 3 of the FFs and the higher reduction ratio when the FFs were transferred from 28 °C to 22 °C, the downregulated expression level of *LrtAG1* might be responsible for the petaloidy of stamens in 'Red Twin'.

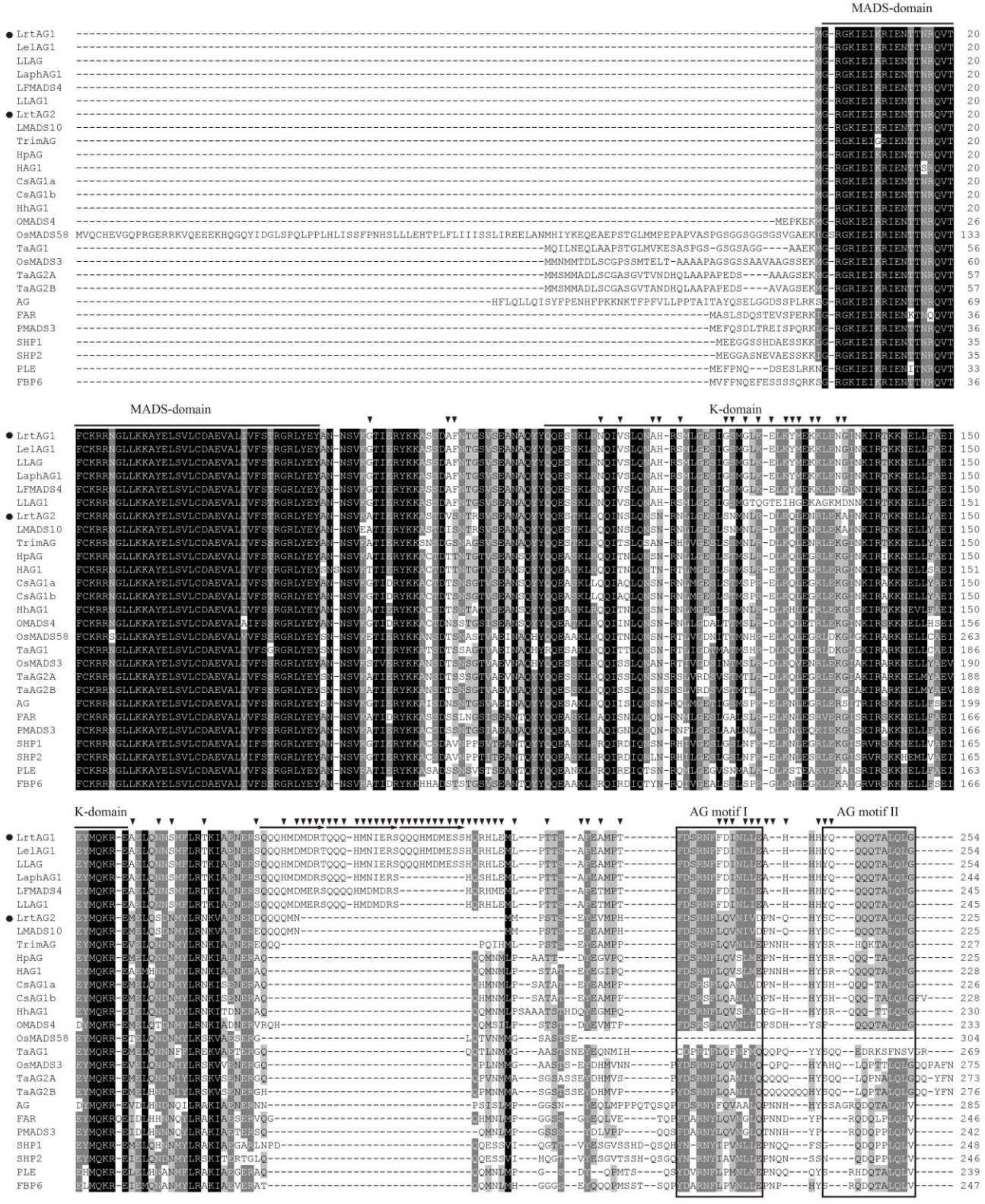

Figure 6. Comparison of amino acid sequences of LrtAG1, LrtAG2, and related C-class gene proteins in monocots and dicots. Identical amino acid residues of these proteins are in black, and conserved residues are in gray. Gaps (-) are introduced to maximize alignment. The amino acid positions are shown on the right. Black lines are drawn above the MADS- and K-domain. AG motif I and AG motif II are indicated by boxes. The repeat sequences are indicated by arrows. The prefixed black dots indicate LrtAG1 and LrtAG2, and the triangles indicate different amino acid sequences between LrtAG1 and LrtAG2.

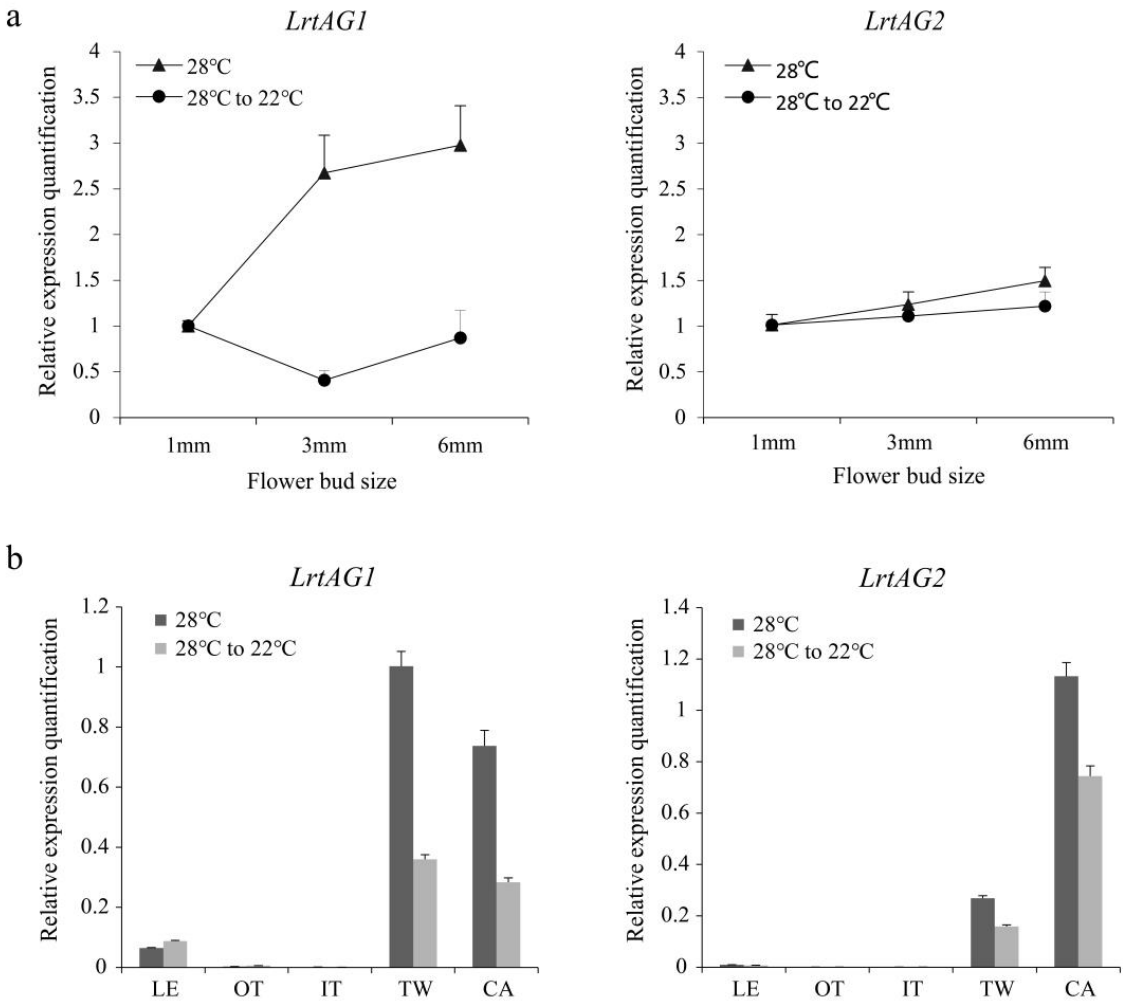

Figure 7. Expression profiles of *LrtAG1* and *LrtAG2* under different temperature treatments. (**a**) qRT-PCR analysis in first emerged flower buds (FFs) at different sizes (1 mm, 3 mm, and 6 mm) under two temperature treatments. (**b**) qRT-PCR analysis of the floral organs in four whorls of FFs at 3–6 mm and contemporaneous leaves under two temperature treatments. 28 °C, flower buds developed at 28 °C from undifferentiated to 6 mm. 28 °C to 22 °C, flower buds developed at 28 °C from undifferentiated to 1 mm but at 22 °C from 1–6 mm. LE, OT, IT, TW, and CA represent leaf, outer tepal, inner tepal, the third whorl floral organ, and carpel, respectively.

4. Discussion

The exposure of 'Red Twin' to constant relatively high temperature (28 °C) from planting to blooming maintained the formation of normal stamens, while exposure to relatively low temperature (22 °C) induced petaloid stamens at a high degree. With a gradual reduction of temperature, the stamens of this cultivar exhibited a strengthening degree of petaloidy (Figure 2), which indicated that the morphogenesis of stamens or petaloid stamens in this cultivar is extremely thermosensitive. This series of changes from stamens to petaloid stamens were similar to that in carrot hybrids. However, the difference in carrots was that accelerated petaloidy of stamens occurred with rising temperature [21,31], which implied the various influence of temperature on the developmental process of stamens and distinct mechanisms in different species. In the lily cultivar 'Akita Petit White', the anthers in stamens also exhibited thermosensitivity; they had intact anthers with functional pollen grains at 32 °C/25 °C, yellowish, swollen, and curved structures at the tips of stamens at 25 °C/18 °C, and no anthers at 18 °C/11 °C. Even though the anthers disappeared, the stamens did not show petaloid structures at low temperatures, indicating that there are some different factors affected by temperature compared with 'Red Twin'.

In *Arabidopsis*, the flower development process before anthesis is divided into 12 stages [32]. At stage 3, sepal primordia arise; at stage 5, petal and stamen primordia arise; at stage 6, stamen primordia are dome-shaped and the gynoecium remains invisible; at stage 7, stamens become constricted at the base and the gynoecium begins to form a cylinder; and at stage 8, locules in the stamens are clearly visible. However, this division of developmental stages can also be applied partly to the flower development process of 'Red Twin'. As we observed, stamen primordia were dome-shaped in the 1-mm flower buds of 'Red Twin' (Figure 4b), similar to those in *Arabidopsis* at stage 6; the base of stamen began to constrict in 3-mm flower buds (Figure 4c), similar to that in *Arabidopsis* at stage 7; and 4 locules appeared in 6-mm flower buds (Figure 4d), which occurred at stage 8 in *Arabidopsis*.

The expression of *AG* RNA started in the center of floral primordia at stage 3 in *Arabidopsis* [6], prior to the initiation of petal and stamen primordia formation (stage 5). In *ag-1* 35S:AG-GR flowers of *Arabidopsis*, *AG* expression can be induced by a 10 μM dexamethasone (DEX) solution [33]. Four DEX treatments at 12-h intervals to flower buds from stage 3 or 6 onward result in normal mature stamens. However, treatments from stage 7 onward result in incompletely developed stamens with sterile anthers in whorls 2 and 3. In addition, if less than four DEX treatments at 12-h intervals were applied to flower buds from stage 3 onward, indehiscent anthers, locule-bearing petals, and even normal petals would be observed. This indicated that prolonged AG activity is necessary for the various aspects of stamen morphogenesis. In 'Red Twin', a higher temperature treatment (transferred from 22 °C to 28 °C) was applied to the FFs at 1 mm (stage 6) and lasted until blossoming. This induced nearly normal stamens with fewer pollen grains and filiform filaments at the blooming stage. However, a similar treatment applied to FFs at 3 mm (stage 7) only induced a slightly lower degree of petaloid stamens compared with the FFs with no treatment (grown at a constant temperature of 22 °C). Furthermore, FFs that grew at 28 °C but were transferred to 22 °C at 6 mm (stage 8) also exhibited nearly normal stamens, while those transferred to 22 °C at 3 mm (stage 7) exhibited petaloid stamens at a high degree. These results indicated that the developmental process of stamens from stage 6 to stage 7 was crucial for normal morphogenesis in *Arabidopsis* and *Lilium*, and the period of 1–6 mm (stage 6 to stage 8) of flower buds was identified as the critical development period for stamen morphogenesis in 'Red Twin'. Maintaining the flower buds under relatively low temperatures in the 1–3 mm or 3–6 mm stages would be sufficient for the formation of petaloid stamens to a high degree, thereby improving the double-flower trait in the commercial production of 'Red Twin' flowers. In addition, the stamen morphogenesis of 'Red Twin' under relatively high temperatures may also require a constant and high expression level of *AG*-like genes, similar to *Arabidopsis*.

However, it was observed that the transformation of sepals to carpels or petals to stamens were stronger at higher temperatures in mutants of *AG* repressors, *blr* [34] and *ap2* [35,36], in *Arabidopsis*. This might imply that the inhibition of *AG* expression in whorl 1 and 2 weakened at higher temperatures, and its expression level may be regulated by temperature. In *Rose hybrida* cv. Vendela [11], *RhAG* exhibited thermosensitivity, and its expression level was attenuated under low temperatures, which was considered one of the major factors that led to the increased petals. These additional petals mainly originated from the petaloidy of partial stamens. We also isolated two *AG*-like genes, *LrtAG1* and *LrtAG2,* in the lily cultivar 'Red Twin'. *LrtAG1* exhibited the highest expression level in whorl 3 of 3–6 mm FFs, while *LrtAG2* expression was the highest in whorl 4. *LrtAG1* showed a higher reduction ratio than *LrtAG2* when the FFs were transferred to 22 °C from 28 °C at 1 mm. This indicated that *LrtAG1*, rather than *LrtAG2*, might play a crucial part in stamen morphogenesis, and the decrease of *LrtAG1* expression level induced by relatively low temperatures might be responsible for the petaloidy of stamens. In addition, ectopic expression of the LFMADS4 and LLAG1 protein in *Arabidopsis*, sharing 93% and 89% identity, respectively, with the LrtAG1 protein, both caused the conversion of sepals into carpelloid sepals and petals into stamens [37,38]. In contrast, the LMADS10 protein,

sharing 98% identity with the LrtAG2 protein, only caused carpelloid sepals [39]. These results further implied the crucial function of the LrtAG1 clade rather than the LrtAG2 clade on stamen morphogenesis in *Lilium*. This division of function might be mainly attributed to differences in the C-terminal sequences of those proteins.

Phytohormones such as GA_3 and IAA were reported to promote additional petals in tomatoes and carnations [22,23], and high IAA and ABA concentrations were associated with stamen sterility in the *stamenless-2* mutant of tomato [40,41]. In *Lilium*, numerous phytohormone-related genes are involved in the biosynthesis or signaling of GA_3, IAA, jasmonic acid, abscisic acid (ABA), cytokinin, or ethylene identified by transcriptome analysis and were considered to participate in the regulation of stamen petaloidy [20,42]. Therefore, it is a strong possibility that variations of phytohormones were involved in the petaloidy of stamens induced by relatively low temperature in 'Red Twin'. Future research should focus on this aspect.

5. Conclusions

In this study, we found that relatively high temperatures induced the morphogenesis of stamens, while relatively low temperatures promoted their petaloidy in the *Lilium* cultivar 'Red Twin'. The period of 1–6 mm of flower buds was identified to be the critical development period for stamen morphogenesis based on the temperature-changing treatment. Maintaining the flower buds at relatively low temperatures during the 1–3 mm or 3–6 mm periods would be sufficient for the formation of petaloid stamens to a high degree in 'Red Twin'. Two *AG*-like genes, *LrtAG1* and *LrtAG2,* were isolated in 'Red Twin' and exhibited differences in the C-terminal sequences. *LrtAG1* showed the highest expression levels in whorl 3 of 3–6 mm flower buds, while *LrtAG2* expression was the highest in whorl 4. *LrtAG1* showed a higher reduction ratio than *LrtAG2* when the flower buds were transferred to 22 °C from 28 °C at 1 mm, which implied that the decreased expression level of *LrtAG1* associated with low temperature might be correlated with the petaloidy of the stamens. These results can provide guidance to promote the formation of petaloid stamens in the commercial production of 'Red Twin' flowers and provide a candidate gene for double-flower breeding in lilies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8121184/s1, Figure S1: Morphological characteristics of flowers in 'Red Twin' at a consistent temperature of 20 and 18 °C from planting to blooming. (a) 20 °C. (b) 18 °C. Bars = 1 cm.

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