J. Agric. Sci. Technol. (2023) Vol. 25(4): 975-988

Role of *AGAMOUS* Gene in Increasing Tepals of Amaryllis

S. Dastmalchi[1], N. Moshtaghi[1]*, and A. Sharifi[2]

ABSTRACT

MADS-box genes play important roles in the regulation of floral organ development. In this gene family, *AGAMOUS* genes are responsible for stamen and carpel development. In the double-flowered form of Amaryllis, compared to its wild type, the stamen number is reduced to three, there is no pistil, and, in contrast, tepal numbers have increased. In this investigation, we examined the *AGAMOUS* (AG) gene function in these alterations. Therefore, we isolated one *AGAMOUS* coding sequence named *AmAG*. Then, the expression level of this gene in the wild form and double-flowered Amaryllis was evaluated using quantitative real-time PCR. The phylogenetic results showed that the partial *AmAG* gene has high homology with the sequences of *AGAMOUS* ortholog genes in the Amaryllidaceae family and plants close to this family. Also, there were no differences in the sequence of partial *AmAG* genes in wild and double-flowered forms. Real-time PCR revealed that, in wild form, *AmAG* gene expression was low in the first to third whorl and high only in the fourth whorl. While in double flowered form, *AmAG* gene expression in four whorls was low. The lower expression of *AmAG* in the fourth whorl of double-flowered form had caused such morphological alterations, the reasons for which should be determined in other experiments.

Keywords: Double flowered, *Hippeastrum*, *MADS*, Mutant gene.

INTRODUCTION

One of the leading aesthetic indicators in ornamental plants is supernumerary petals, such that double flower production has been considered as one of the breeding goals (Gattolin *et al*., 2018) because double flowers may increase the horticultural value and market appeal. Among double flowers, Amaryllis is one of the popular ones (Liu and Yeh, 2015), which is commercially important. Amaryllis (*Hippeastrum spp.*) is a perennial bulbous plant in Amaryllidaceae family. It has attracted worldwide attention due to flowers with attractive shapes and colors, long-lasting, and glossy strap-like foliage (Ye and Shi, 2008; Y. Wang *et al*., 2018). Its floral architecture is similar to other members in Liliaceae, with two whorls of petaloid organs. The initial studies on the breeding of double-flowered forms in this plant refer to a report presented by McCann (1937).

Molecular genetic analysis of several model plants has provided an overview perception of mechanisms regulating the transition from vegetative to reproductive phase. The general mechanism of this switching emphasizes that flower induction and development require the activation of floral meristem identity genes, which controls the formation of flower organ primordia, then, develop four whorls, i.e., sepals, petals, stamens and carpels (Srikanth and Schmid, 2011; (Matsoukas *et al.*, 2012). *MADS-box* genes encoding transcription factors are the primitive regulators in the orientation of plant development, especially flower morphology (Heijmans *et al.*, 2012; Ng and Yanofsky, 2001; Theißen *et al.*, 2016).

[1] Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.
[2] Academic Center for Education, Culture and Research, Khorasan Razavi Branch, Mashhad, Islamic Republic of Iran.
*Corresponding author; e-mail: moshtaghi@um.ac.ir

[DOI: 10.22034/jast.25.4.15]

Functional analyses on single, double and triple mutants, including studies on homeotic mutants of *Arabidopsis thaliana* and *Antirrhinum majus*, led to the ABCDE model for floral organ determination (Weigel and Meyerowitz, 1994; Pelaz *et al.*, 2000; Theißen, 2001; Zahn *et al.* , 2005; Soltis *et al.*, 2007; Kanno *et al*. 2007; Irish, 2017).

The critical genes in flower organ development were identified in homeotic mutants of *Arabidopsis thaliana* and *Antirrhinum majus,* which are mentioned in different researches (Fornara *et al.*, 2010).

The *AGAMOUS* (*AG*) C/D class gene in *A. thaliana*, belonging to the *MADS-box* gene family, is the main regulator in stamen and carpel development. *AG* gene orthologs exhibited the same expression pattern in monocots and dicots (Akita *et al.*, 2011; Wang *et al*., 2011; Ó'Maoiléidigh *et al*., 2013). In *ag* mutants of *Arabidopsis*, the stamens are converted to the petals, and carpels are changed to another *ag* flower. So, *AG* gene is responsible for the identity of stamens and carpels in flower development and suppression of the activity of the *A-class* gene in these whorls and causes determinacy of floral meristem (Wang *et al*., 2011); Galimba *et al*., 2012).

The essential genes involved in the switching from vegetative to reproductive have been identified in other species. There is much information about the relationship between these genes and how they act. Dubois *et al*. (2010) presented that double-flower phenotype in rose hybrids derived from a shift in *RhAG* expression domain boundary such that its expression was restricted toward the center of the flower in double flowers, while this expression was wider in a single one. In *Prunus lannesiana*, unusual splicing of *PrseAG* gene causes the C-terminal deletion and double flower formation (Liu *et al.*, 2013). In *Thalictrum thalictroides,* retrotransposon insertion in *ThtAG1* gene causes the expression of truncated protein with K-domain deletion and unusual flower type (Galimba *et al*., 2012). Analysis expression of *GLOBOSA* (*GLO*)-like genes in double-flowered lily cultivar, in which stamens were transformed to petal-like organs, indicate that *C-class* genes were limited in whorl 4 and absent in whorl 3 (Akita *et al.*, 2008). *AG* expression in other double-flowered lily showed a relationship between variation in stamen structure and the level of *AG*-like gene expression (Akita *et al*., 2011). Also, in *Tricyrtis. macranthopsis* double-flowered, *TrimAG* gene expression decreased in whorl 3 and 4 compared to the wild type (Sharifi *et al*., 2015).

Previous research demonstrated that despite the generality of the ABCDE model in plants, the genes of each class have different functions in different species, as described above. A study on *AG* gene function in double flowering of Amaryllis has not been done, but what is clear is that in double-flowered Amaryllis, additional tepal-like organs result from anther and pistil transformation (Bell, 1977).

Therefore, for the first time, we aimed to study the partial isolation and quantitative expression of *AGAMOUS* gene in wild-type and double-flowered cultivars to determine whether this new phenotype is related to the *AG* gene or other regulatory genes in the flowering network.

MATERIALS AND METHODS

Plant Material

Two forms of Amaryllis used in this experiment were "double record" as double-flowered and "*Hippeastrum johnsonii*" as wild type. Both were provided from a garden shop and kept in a greenhouse under natural environmental conditions at ACECR greenhouse in Mashhad, Iran. The buds of these plants were used for RNA extraction and cDNA synthesis.

AGAMOUS Ortholog Gene Isolation

In order to isolate the *AGAMOUS* ortholog gene, total RNA was extracted from 10 mg of the stamen and pistil of the wild type and double flowered of Amaryllis flower bud, which had the highest amount of

[DOI: 10.22034/jast.25.4.15]

AGAMOUS mRNA. RNA extraction was performed using the total RNA Extraction Kit (Parstous, Iran) according to the manufacturer's protocol. The quantity and quality of RNA were evaluated by Nanodrop and loading on 1.8% agarose gel. DNase1 (Fermentas, Canada) was used to eliminate residual DNA. First-strand cDNA was synthesized in a 20 µL reaction base on a Parstous cDNA synthesis kit (Parstous, Iran). Reaction components included 1 µg of total RNA, 1X buffer mix, and 1X enzyme mix. Subsequently, cDNA synthesis was done at 25°C for 10 minutes, then 60 minutes at 47°C, and the reaction was stopped by heating at 85°C for 5 minutes. Synthesized Cdna was chilled on ice.

For *AGAMOUS* ortholog gene isolation, we needed to do cDNA library screening with suitable primers. The nucleotide sequence of *AGAMOUS* ortholog gene in Amaryllis was not identified. Therefore, in the first step, the nucleotide sequence of homologous *AGAMOUS* genes in other plants of the same family and families close to Amaryllis were extracted from the NCBI database. These plants and their accession number in NCBI databases were *Narcissus tazetta* (EF421828.1), *Asparagus virgatus* (AB125347.1), *Hosta plantaginea* (EU429307.1), and *Agave tequilana* (JF699273.1). After aligning the sequences with the multiple sequence alignment program ClustalW in BioEdit7.2 software, highly similar regions of the sequences were selected to design the degenerate primers. Two forward degenerate primers and three reverse degenerate primers were designed (Table 1)

Then, 2 µl of synthesized cDNA was used as a template for PCR amplification in a mixture of 0.1 mM forward primer (F1 or F2), 0.1 mM Oligo(dt)18 primer, 0.2 mM dNTPs, 1X Ex Taq buffer (Mg^{2+}plus), and 1 U of ExTaq DNA polymerase (Takara Bio, Japan) in a 20 µL total volume of PCR reaction. The thermocycler (Bio-Rad Laboratories, USA) program was 5 minutes at 95°C, 40 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds, and a final amplification at 72°C for 2 minutes. Next, the PCR product was diluted (1:1000) and used as a template for the second PCR reaction. The PCR technique was employed as described before, only with different primers. In this step, degenerate primers F1R1, F2R2, and F2R3 with annealing temperatures of 50, 50, and 50°C and expected amplified fragments of 300, 140, and 160 bp, respectively, were assayed. The quality of the final product was evaluated by loading on 1.8% agarose gel. The amplified fragments were purified by PCR clean-up kit (Denazist, Iran) and cloned into a pTG19-T vector (Vivantis, Malaysia). Vectors were transferred to the competent cells of *Escherichia coli* (DH5α) by heat shock method (Chang *et al.*, 2017). Transformed *E. coli* containing considered fragments were selected on Luria-Bertani medium supplemented with 50 mg L^{-1} ampicillin, 40 µg mL^{-1} X-Gal and 0.5 mM IPTG. Cases visualizing white appearance were used for PCR reaction with specific vector primers (M13 and T7). Selected clones were sent for sequencing to the

Table 1. Properties of degenerate primers designed for amplification of *AGAMOUS* gene in Amaryllis.[a]

Primer name	Forward sequence (5	Reverse sequence	Annealing temperature (°C)
F1,R1	GGTSGCCCTYATCGTCTTCT	TTCTTATTTTGYTGATGCCT	50
F2, R2	TACAAGAAAGCWTGYACTGATACA	TCVCCCAWCAAAKTCCTGTT	50
F3, R3	CARGTSACYTTYTGYAAGCG	AGRCRCATTGWRCTVAGAGA	50

[a] (S: C, G; Y: T, C; W: A, T; K: G, T; R: G, A; V: G, C, A).

[DOI: 10.22034/jast.25.4.15]

Macrogen Company (South Korea). PCR conditions were similar to the amplification step of *AGAMOUS* ortholog gene in Amaryllis but different in annealing temperature (58°C) because the used primers were also different. The cDNA from double-flowered and wild forms were used as templates for PCR. PCR products were cloned in *E. coli* (DH5α), then, three colonies with 335 bp insertion fragments were randomly selected and sent for sequencing.

After identifying the sequence of 335 bp gene fragment, the upstream region of the *AGAMOUS* ortholog gene was isolated using degenerate forward primer (5′*GCTGGAKCCCAAGGAGAAG* 3') and specific reverse primer of *AGAMOUS* ortholog gene (5′*GCCAGCAAATAACCAACTTACAG* 3'). Then, three colonies were randomly selected and sent for sequencing

Phylogenetic Analyses of *AGAMOUS orthologs*

Blast analyses were done on the amino acid sequences of the *AmAG* gene with highly similar sequences of *AGAMOUS* ortholog genes recorded in NCBI and other plants in Poaceae and model plants. The following sequences were used: *AGAMOUS* from *Arabidopsis thaliana* (X53579), *AtqMADS4* from *Agave tequilana* (JF699273), *AVAG1* from *Asparagus virgatus* (AB125347), *CeMADS1* and *CeMADS2* from *Cymbidium ensifolium* (GU123626 and GU123627), *CsAG1a* from *Crocus sativus* (KF916013), *DcOAG1* from *Dendrobium crumenatum* (DQ119840), *FARINELLI* from *Antirrhinum majus* (AJ239057), *FBP6* and *pMADS3* from *Petunia x hybrid* (X68675 and X72912), *HplaAG* from *Hosta plantaginea* (EU429307), *HvAG1* and *HvAG2* from *Hordeum vulgare* (AF486648 and AF486649), *NtazAG* from *Narcissus tazetta* (EF421828), *OitaAG* from *Orchis italica* (JX205496), *OsMADS3* and *OsMADS58* from *Oryza sativa* (L37528 and AB232157), *PeMADS1* from *Phalaenopsis equestris* (AF234617), and *PhalAG1* from *Phalaenopsis hybrid* (AB232952). ClustalW multiple alignment was conducted by BioEdit7.2.5 to align the amino acid sequences of each case. A Phylogenetic tree was constructed by the Neighbor-Joining method with 10,000 bootstrap replicates using MEGA X software. We selected JTT+G to construct the Phylogenetic tree.

Quantitative Real-Time PCR of *AmAG* Gene

Total RNA was extracted from the sepal, petal, stamen and pistil whorls of a flower bud in wild and double-flowered forms using a total RNA Extraction Kit. Then cDNAs were synthesized based on the description mentioned above. Specific primers used for Real-time PCR were: 5′ ACCAACACTGCCACTGTCTC 3′ as forward and 5′ *GCCAGCAAATAACCAACTTACAGA* 3′ as reverse for *AmAG* gene with product size 88bp. and 5′ *TGAGAAACGGCTACCACATC* 3′ as forward and 5′ *AGACTCATAGAGCCCGGTATT 3′* as reverse for *18SrRNA* gene with amplicon size of 103 bp, used as the normalization control. The cDNA of the pistil in serial dilutions (6 steps) was used to evaluate the efficiency of primers in amplification using the standard curve slope.

The real-time PCR reaction was carried out in 20 µL containing 10 µL of Parstous Real-time PCR 2x Master Mix (SYBR Green), 1 µL of each primer (0.1 mM), 1 µL of the cDNA, and 7 µL double distilled water. Real-time PCR was performed on a CFX96 Bio-Rad thermocycler. The program was: 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 20 seconds. Data were analyzed using the Relative Expression Software Tool by Delta Delta CT method (REST 2009). The expression fold was assayed based on 3

[DOI: 10.22034/jast.25.4.15]

replications of real-time PCR reaction for each sample.

RESULTS

Flower Morphology of Amaryllis in Double Flowered and Wild Type

Comparing the flower morphology of double-flowered and wild type of Amaryllis revealed a remarkable difference between these two forms. The wild-type flower possesses three petaloid tepals in the first whorl, three petaloid tepals in the second, six stamens in the third, and three carpels in the fourth whorl (Figure 1). In the double-flowered form, three stamens converted completely to tepals, and the carpels converted to a carpel-like tepal (Figure 1-A). stamens conversion to petal in the studied double-flowered phenotype is consistent

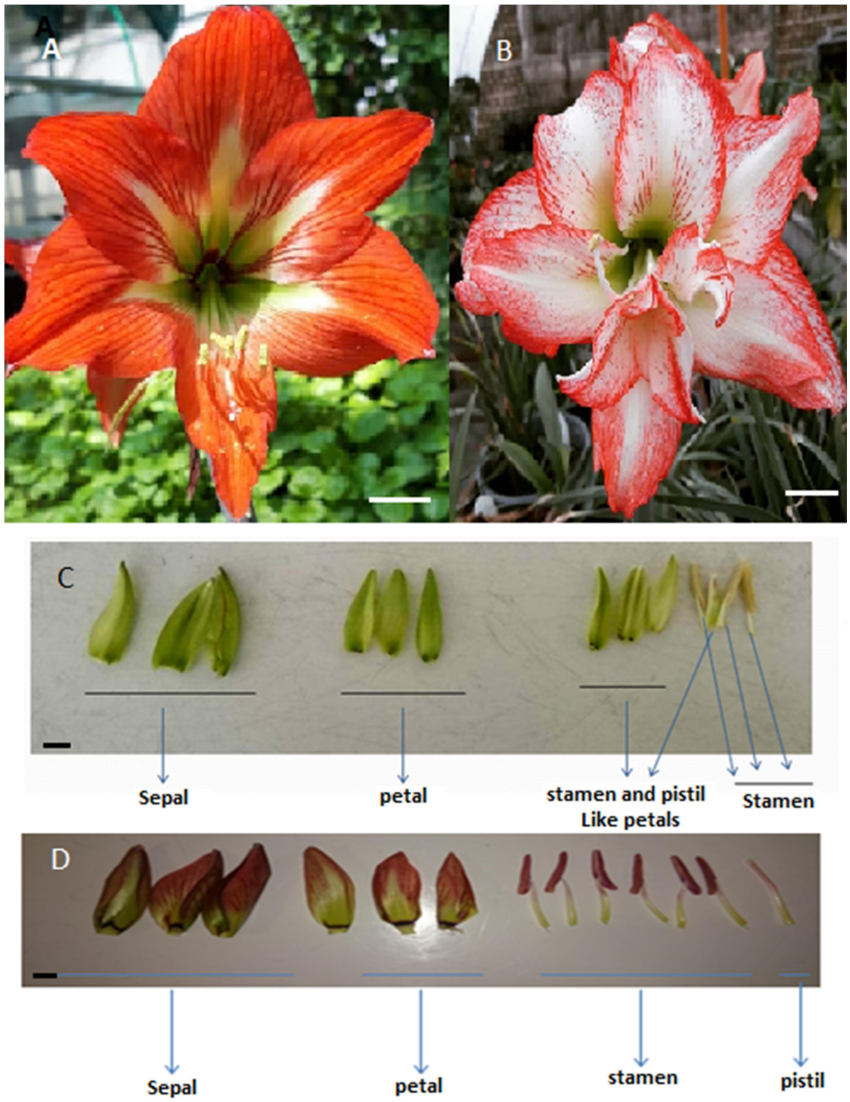

Figure 1. Flower and floral organ morphology in Amaryllis double-flowered (B and C, three stamens converted completely to tepals, and the carpels converted to a carpel-like tepal) and wild type (A and D, three petaloid tepals in the first whorl, three petaloid tepals in the second, six stamens in the third, and three carpels in the fourth whorl). Bars indicate 2 cm.

[DOI: 10.22034/jast.25.4.15]

with previous reports on *A. thaliana* ag mutant (Bowman *et al.*, 1989, 1991). There is a difference in *AGAMOUS* ortholog gene of Amaryllis double-flowered form, as seen in the *A. thaliana ag* mutant. Therefore, we analyzed the sequences of *AGAMOUS* ortholog gene and its expression in wild type and double-flowered forms.

Isolation of *AGAMOUS* Ortholog Gene

Based on the reverse transcription PCR technique using degenerate primers, we identified that the partial length cDNA *AGAMOUS* ortholog gene, named *AmAG* in Amaryllis, is 335bp (Figure 2).

The putative protein encoded by *AmAG* is 105 amino acid with a 20 bp upstream of the gene. Multiple sequence alignment program ClustalW of amino acid sequences of *AmAG* revealed conserved domains of *AGAMOUS* orthologs (Figure 3). The presence of MADS domain and partial K-domain in the *AmAG* protein confirmed that this gene belongs to the *AG* ortholog (Yun *et al.*, 2004; Xu *et al.*, 2006; Chen *et al*., 2012; Sandoval *et al.*, 2012; Waters *et al*., 2013).

The phylogenetic results showed that *AmAG* gene was closer to *NtazAG* gene. Both of these genes belong to the same family, namely, Amaryllidaceae. Considering the output of this phylogenetic analysis, they were placed into the same group, as depicted in Figure 3. Furthermore, this family with Asparagaceae are arranged in the higher Asparagales (Nyffeler and Eggli, 2010). The phylogenetic tree showed considerable results about different species (Figure 4).

The partial *AmAG* gene is 97-98% homologous with *AGAMOUS* orthologs of Asparagaceae plant family, 84-89% homologous with *AGAMOUS* orthologs of Orchidaceae plant family and 86-87% identical with *AGAMOUS* orthologs of Poaceae plant family. In the model plants such as *A. thaliana*, *A. majus*, and *P. hybrida*, the identity was 94% for *AGAMOUS*, 84.7% for *FARINELLI*, and 85-90% for *FBP/PMADS3*, respectively. This high homology of *AmAG* gene with other *AGAMOUS* orthologs in monocots and dicots suggests that *AmAG* gene belongs to the *C-class* genes. Also, sequencing results of partial *AmAG* genes in wild type and double-flowered forms showed no differences between them.

Expression of AmAG Gene in Double Flowered and Wild Type

The mRNA levels in different floral organs (i.e., sepal, petal, stamen, and carpel) of Amaryllis double-flowered and wild type were assayed by real-time PCR to determine the *AmAG* expression. The results indicated *AGAMOUS* expression was different in various tissues of the wild type. The highest *AGAMOUS* expression was recorded in carpels, almost 6.5-fold higher than others. In contrast, the other parts of the flower displayed a low-level expression of this gene (Figure 4). Such expression pattern for the *AGAMOUS* gene in Amaryllis is similar to the previous reports for its other orthologs in other plants (Akita *et al*., 2011; Sandoval *et al*., 2012; Waters *et al*., 2013). The expression of *TrimAG* gene in petaloid petals of *Tricyrtis macranthopsis* was low (Sharifi *et al*., 2015). Real-time PCR analysis of *Agamous* gene in the double-flowered of *Tricyrtis macranthopsis* from

GCTGGAGCCCAAGGAGAAGATGGGTAGGGGGAAGATAGAGATCAAAAGGATCGAA
AATACGACTAACAGGCAAGTCACCTTTTGCAAACGTCGAAATGGGTTGCTCAAAAA
GGCCTATGAATTGTCTGTGCTTTGCGATGCGGAGGTCGCTCTTATTGTCTTCTCCAGC
CGCGGTCGCCTCTACGAGTATGCAAACAATAGTGTGAAAGCGACAATTGAGAGGTA
CAAGAAAGCATGCAGTGATACAACCAACACTGCCACTGTCTCAGAGGCCAATTCTC
AGTACTACCAACAAGAAGCTTCCAAGTTGCGCCAGCAAATAACCAACTTACAGA

Figure 2. Sequence of *AmAG* in Amaryllis.

[DOI: 10.22034/jast.25.4.15]

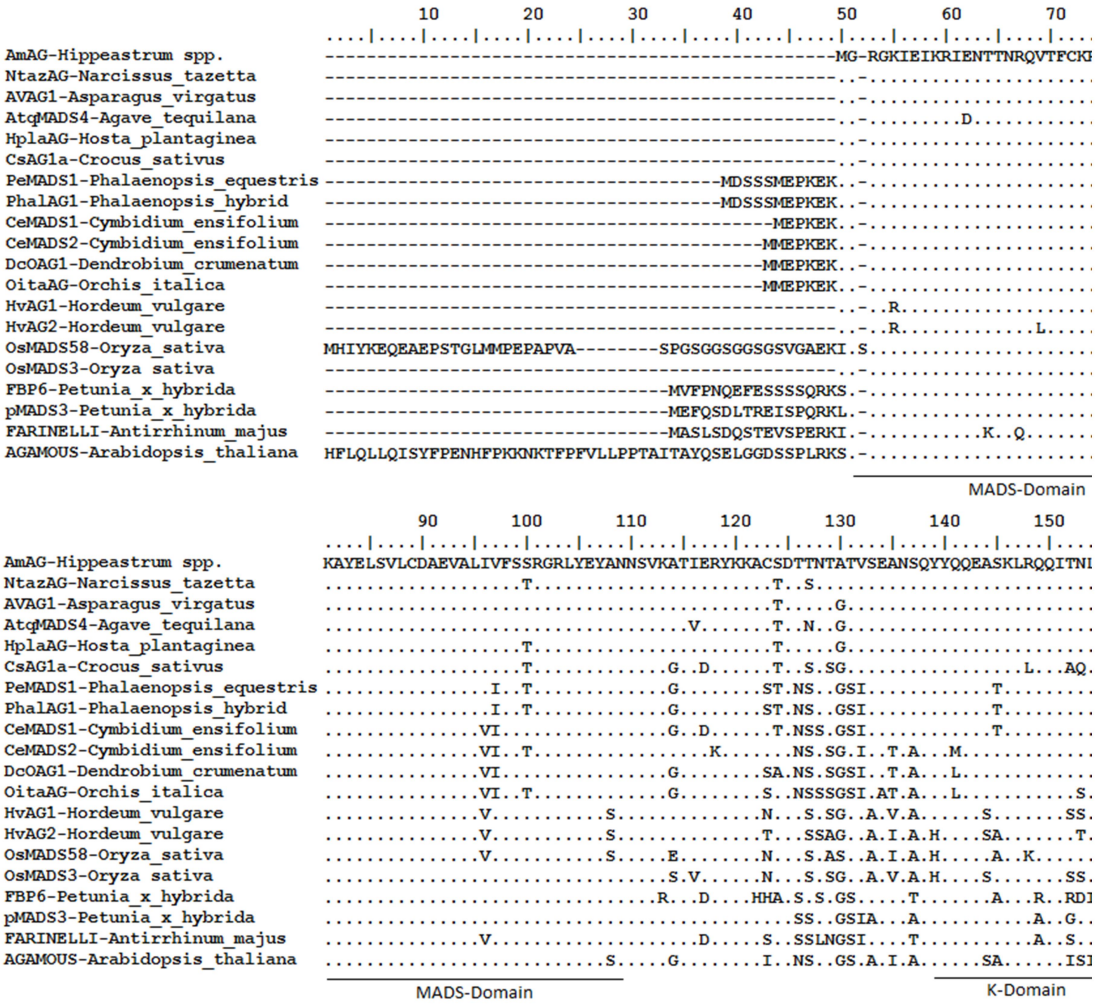

Figure 3. ClustalW multiple alignment of amino acid sequences of *AmAG* and *AGAMOUS* orthologs. The first line is the isolated gene of *AmAG* from Amaryllis. Similar amino acids to *AmAG* are defined by dot (.), while space (-) was used for maximizing the alignment. MADS and K domains are underlined.

the liliaceae family showed that its expression in the two inner flower whorls was dramatically reduced in comparison with that of the wild type, resulting in changed floral structure, and the two inner whorls resembled the two outer flower whorls. Decreased expression of TrimAG in *T. macranthopsis* double-flowered form caused stamens and carpels to be replaced with tepals (Sharifi *et al*., 2015).

In double flower form, gene expression in all studied tissues decreased at least six times compared to the wild-type carpel tissue. This reduction of expression in the double-flower carpel was 10-fold. These results indicate a down-regulation of the gene in the double-flower form. Therefore, this gene can be considered involved in the double-flowering process in Amaryllis (Figure 5).

Since the gene expression in the third whorl of the double-flower was not significantly different from the gene expression in the same whorl in the wild type, it can be conclude that the conversion of the three of six stamens to the tepals

[DOI: 10.22034/jast.25.4.15]

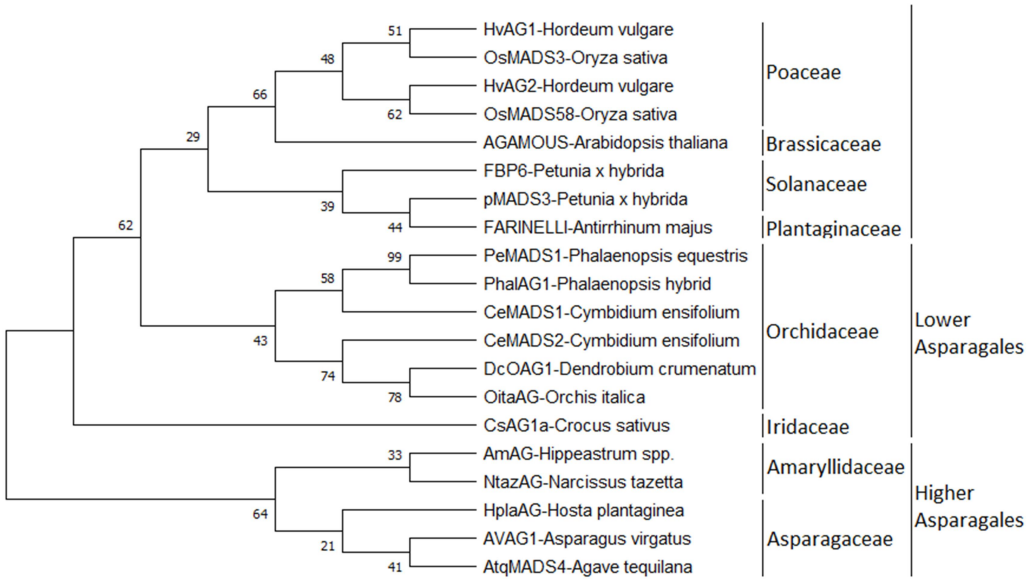

Figure 4. Phylogenetic tree of *AmAG* and *AGAMOUS* orthologs based on the neighbor-joining method. The numbers in each branch give bootstrap values from 10,000 replicates.

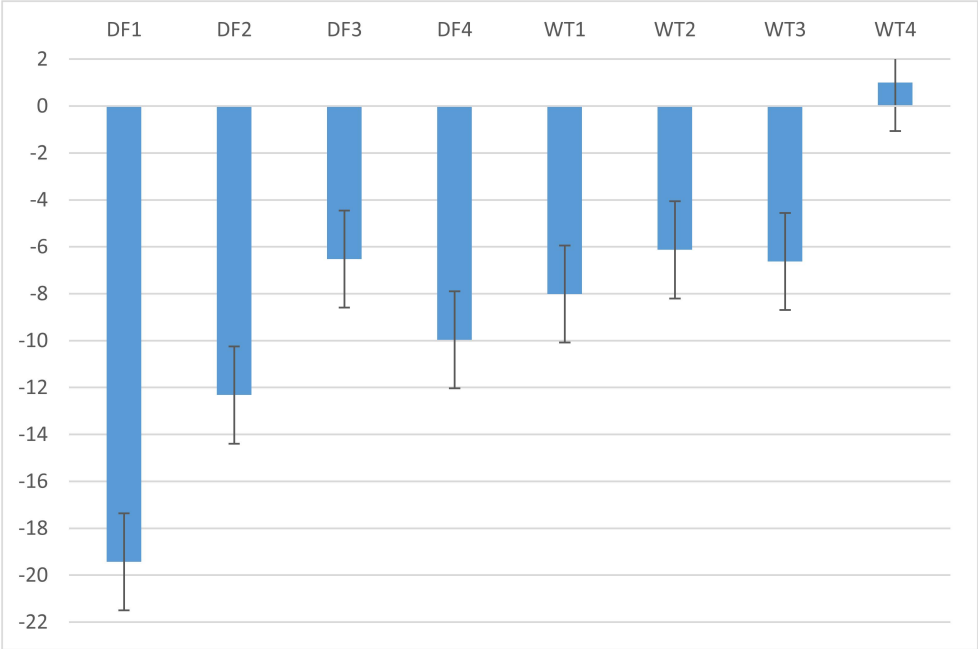

Figure 5. *AmAG* gene expression assay in *Amaryllis* double-flowered and wild type by Real-time PCR. Bars represent fold expression means ±SE (n= 3). DF1, DF2, DF3 and DF4: Double Flowered (whorl 1, 2, 3, 4), WT1, WT2, WT3, WT4: Wild Type (whorl 1, 2, 3, 4).

[DOI: 10.22034/jast.25.4.15]

results from reduced gene expression in the fourth whorl, i.e., pistil. It may suggest the interaction of the genes involved in classes C and D in Amaryllis.

This phenotype was similar to the phenotype observed in Arabidopsis *ag* mutant, in which the reproductive organs became sepals and petals (Bowman *et al*., 1989, 1991). In Arabidopsis, the *AGAMOUS* gene is widely expressed in stamen and pistil primordia during the early stages of development. It controls stamen and pistil development and inhibits the activity of *A-class* genes in the third and fourth whorls. In the absence of the *AGAMOUS* gene, the activity of the A-class genes extends to the third and fourth whorls. Therefore, in *ag* mutant, the third and fourth whorls also produce petals (sepals, petals, petals, petals) (Mizukami and Ma, 1995; Sieburth *et al.*, 1995).

DISCUSSION

During flower development in *A. thaliana*, the *AGAMOUS* gene is a key regulator in stamen and carpel organogenesis. Evidence for replacement of stamens with petals and carpels with a reiteration of the sepals and petals (Bowman *et al*., 1989), 1991) was observed either in the phenotype of *ag* mutants such as *A. majus* (Davies *et al*., 1999), *T. thalictroides* (Galimba *et al*., 2012), *T. macranthopsis* (Sharifi *et al*., 2015), or with silencing of *AGAMOUS* ortholog in cyclamen (Tanaka *et al*., 2013) and petunia (Noor *et al*., 2014).

We chose an *ag* mutant phenotype of Amaryllis to study the role of the C-class MADS-box gene in flower development in wild type and double-flowered forms. We isolated a partial *AGAMOUS* ortholog with MADS domain and K-domain regions. It was confirmed that *AmAG* is an *AGAMOUS* ortholog through the presence of these conserved motifs in *AmAG* protein and high homology with *AGAMOUS* ortholog genes in other plants of the same family and families close to Amaryllis (Yun *et al*., 2004; Xu *et al*., 2006; Chen *et al*., 2012; Sandoval *et al*., 2012; Waters *et al*., 2013).

There were no differences between double-flowered and wild type in partial *AmAG* gene sequences. However, the pattern of the *AmAG* expression in double-flowered and wild type was different. Thus, its expression in wild-type was restricted to the fourth whorl, and in double-flowered, it was low in all whorls. Such sharp changes may be related to other factors such as variation in upstream regulatory genes, *cis* regulatory elements in promoter or introns which should be examined in later experiments. Such pattern was observed in *AGAMOUS* orthologs in other plants with high expression in two inner whorls and low expression or absence in sepal and petal (Yusuke Akita *et al*., 2011; Sandoval *et al*., 2012; Waters *et al*., 2013; Tanaka *et al*., 2013). Declining or eliminating *C-class* gene expression in double-flowered cultivars, replaces petaloid tepals with stamens, carpels and floral meristem indeterminacy. Such mutant phenotype was observed in *Arabidopsis* (Bowman *et al*., 1989, 1991) , *Ipomoea nil* (Nitasaka, 2003) and *A. majus* (Davies *et al*., 1999). Alternative splicing of *MastAG*, an *AGAMOUS* homolog, caused double flower formation in *Magnolia stellata* (Zhang *et al.*, 2015). Furthermore, restriction in the *AGAMOUS* ortholog gene expression showed the same phenotype, for example, in Rose, the *RhAG* expression in double-flowered hybrids was limited to the center and led to replacing stamens with petals (Dubois *et al*., 2010). It was reported in *Rosa hybrid* that DNA hypermethylation induced by low temperature affected the *RhAG* expression and increased petal number (Ma *et al*., 2015). Recently, a study on *Rosa sp*. suggests the relation between the regulation of *RcAGAMOUS* expression and miR172 resistant *RcAP2L* (*APETALA2-like* gene) in double flower formation (François *et al*., 2018). Virus-based silencing of *ThtAG1* in *T. thalictroides* caused the stamens and carpels transformation to sepaloid organs and flower

[DOI: 10.22034/jast.25.4.15]

indeterminacy (Galimba *et al*., 2012). Akita *et al*. (2008, 2011) reported a strong relation between organ development in third whorl and *AGAMOUS* ortholog expression in lily. In wild type, this expression was high in third and fourth whorls, but it was low in third whorls in double flowered (Akita *et al*., 2008), 2011). In *T. macranthopsis* double flowered, the *TrimAG* gene expression decreased in third and fourth whorls compared to the wild type. *TrimAG* promoter sequences analyses indicate no significant differences concerning cis-regulatory elements in wild type and double-flowered plants (Sharifi *et al*., 2015). In *TrimAG* intron II sequence, only in a double flowered cultivar, two repetitive 'CT' and 'AG' sequences might cause the formation of a stem-loop structure and lead to the silencing of *TrimAG* gene (Sharifi *et al*., 2015). The observation of C-class functional mutant phenotypes in the silencing of *AGAMOUS* ortholog in *petunia* (Noor *et al*., 2014), *gerbera* (Yu *et al*., 1999) and *cyclamen* (Tanaka *et al*., 2013) was the witness of *AGAMOUS* gene as a controller of organ identity in whorls 3 and 4. Silencing *AGAMOUS* genes in apples by RNA interference (RNAi) leads to double flower formation (Klocko *et al*., 2016). *Medicago truncatula* double mutant of *mtaga mtagb* leads the conversion of stamens and carpels to numerous vexillum-like petals (Zhu *et al*., 2018). In contrast, Salamah and Rostina (2019) reported that crested peach and double flower phenotypes in *Hibiscus rosa-sinensis* were not related to the loss of *AGAMOUS* gene expression (Salamah and Rostina, 2019).

The absence of *AG* function leads to the extension of a class genes activity in whorls 3 and 4. Therefore, this caused the development of petals in whorl 3, and carpels changed to new flower that reiterates this pattern, (sepal, petal, petal) Two nearly related genes in *Antirrhinum*, *PLENA* (*PLE*) and *FARINELLI* (*FAR*) as *C-class* genes, have, to some extent, overlap functions. The *ple* mutants develop petals in whorl 3 and organs similar to sepaloid/carpeloid/petaloid in whorl 4, while *far* mutants have slight changes. In contrast, *ple far* double mutants show petals in whorls 2 to 4, a phenotype more like the *ag* mutants (Bradley *et al.*, 1993); Davies *et al*., 1999).

Examination of the sequence of *AmAG* in miRBase Database (https://www.mirbase.org) did not find a sequence with E value lower than 0.61. The lowest E value with 0.61 belonged to gma-miR1516c microRNA of *Glycine max,* which act in plant NB-LRR defense gene family (Zhai *et al*. 2011). Moreover, search in the partial protein sequence of *AmAG in www.genome.jp/tools/motif/ tool found* 3 motifs in Pfam, namely, SRF-TF, DUF6146, and K-box with E values of (1.5e-27), (0.032), and (0.062), respectively. The first one is a SRF-type transcription factor (DNA-binding and dimerization domain), the second one is family of Unknown Function (DUF6146), and the last one is K-box region.

CONCLUSIONS

This experiment analyzed a double flowered Amaryllis in which three stamens and carpel converted to petaloid organs. To determine the molecular mechanism of the double flowering phenotype, we isolated the partial *AmAG* as *AGAMOUS* ortholog and analyzed its expression. There were no differences in sequences of partial *AmAG* in wild type and double-flowered. The expression pattern of *AmAG* gene in wild type like other *AGAMOUS* orthologs was restricted to the inner whorl, but in double-flowered, *AmAG* gene expression, like other C-class functional mutants, declined dramatically. Such sharp changes may be related to other factors such as variation in upstream regulatory genes, cis regulatory elements in promoter, or introns, etc.

ACKNOWLEDGEMENTS

This research was conducted with the research grant in Ferdowsi University of Mashhad, Iran. We thank Jahad Daneshgahi

[DOI: 10.22034/jast.25.4.15]

of Mashhad, ACECR for their recommendations and facilities.

REFERENCES

1. Akita, Y., Horikawa, Y. and Kanno, A. 2008. Comparative Analysis of Floral *MADS-Box* Genes between Wild Type and a Putative Homeotic Mutant in Lily. *J. Hortic. Sci. Biotechnol.,* **83(4):** 453-461.
2. Akita, Y., Nakada, M. and Kanno, A. 2011. Effect of the Expression Level of an *AGAMOUS*-Like Gene on the Petaloidy of Stamens in the Double-Flowered Lily,'Elodie'. *Sci. Hortic.,* **128(1):** 48-53.
3. Bell, W. D. 1977. Double Flowered Amaryllis. *Proc. Fla. State Hort. Soc.* **90**:121–122
4. Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. 1989. Genes Directing Flower Development in Arabidopsis. *Plant Cell,* **1(1)**: 37-52.
5. Bowman, J. L., Smyth, D. R., Meyerowitz, E. M., 1991. Genetic Interactions among Floral Homeotic Genes of Arabidopsis. Development **112**: 1–20.
6. Bradley, D., Carpenter, R., Sommer, H., Hartley, N. and Coen, E. 1993. Complementary Floral Homeotic Phenotypes Result from Opposite Orientations of a Transposon at the Plena Locus of Antirrhinum. *Cell,* **72(1)**: 85-95.
7. Chang, A. Y., Chau, V., Landas, J. A. and Pang, Y. 2017. Preparation of Calcium Competent *Escherichia coli* and Heat-Shock Transformation. *JEMI Methods,* 1, 22-25.
8. Chen, Y. -Y., Lee, P. -F., Hsiao, Y. -Y., Wu, W. -L., Pan, Z. -J., Lee, Y. -I. and Tsai, W. -C. 2012. *C*- and *D-Class MADS-Box* Genes from *Phalaenopsis equestris* (Orchidaceae) Display Functions in Gynostemium and Ovule Development. *Plant Cell Physiol.,* **53(6),** 1053-1067.
9. Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. 1999. PLENA and FARINELLI: Redundancy and Regulatory Interactions between Two Antirrhinum MADS-Box Factors Controlling Flower Development. *EMBO J.,* **18(14):** 4023-4034.
10. Dubois, A., Raymond, O., Maene, M., Baudino, S., Langlade, N. B., Boltz, V. and Bendahmane, M. 2010. Tinkering with the C-Function: A Molecular Frame for the Selection of Double Flowers in Cultivated Roses. *PLoS One,* **5(2):** 1-12.
11. Fornara, F., de Montaigu, A. and Coupland, G. 2010. SnapShot: Control of Flowering in Arabidopsis. *Cell,* **141(3):** 550-550.
12. François, L., Verdenaud, M., Fu, X., Ruleman, D., Dubois, A., Vandenbussche, M., Bendahmane, A., Raymond, O., Just, J. and Bendahmane, M. 2018. A miR172 Target-Deficient *AP2*-Like Gene Correlates with the Double Flower Phenotype in Roses. *Sci. Rep.,* **8(1)**: 1-11.
13. Galimba, K. D., Tolkin, T. R., Sullivan, A. M., Melzer, R., Theißen, G. and Di Stilio, V. S. 2012. Loss of Deeply Conserved C-Class Floral Homeotic Gene Function and C-and E-Class Protein Interaction in a Double-Flowered Ranunculid Mutant. *Proc. Natl. Acad. Sci.,* **109(34):** E2267-E2275.
14. Gattolin, S., Cirilli, M., Pacheco, I., Ciacciulli, A., Da Silva Linge, C., Mauroux, J. B., Lambert, P., Cammarata, E., Bassi, D., Pascal, T. and Rossini, L. 2018. Deletion of the miR172 Target Site in a *TOE*-Type Gene is a Strong Candidate Variant for Dominant Double-Flower Trait in Rosaceae. *Plant J.,* **96(2):** 358-371.
15. Heijmans, K., Morel, P. and Vandenbussche, M. 2012. *MADS-Box* Genes and Floral Development: The Dark Side. *J. Exp. Bot.,* **63(15):** 5397-5404.
16. Irish V., 2017. The ABC Model of Floral Development, *Cur. Biol.* **27(17)**: 887-890.
17. Kanno, A., Nakada, M., Akita, Y., and Hirai, M., 2007. Class B Gene Expression and the Modified ABC Model in Nongrass Monocots. Sci. World J., **7**:268-279.
18. Klocko, A. L., Borejsza-Wysocka, E., Brunner, A. M., Shevchenko, O., Aldwinckle, H. and Strauss, S. H. 2016. Transgenic Suppression of *AGAMOUS* Genes in Apple Reduces Fertility and Increases Floral Attractiveness. *PLoS One,* **11(8):** 1-17.

[DOI: 10.22034/jast.25.4.15]

19. Liu, M. -C. and Yeh, D. -M. 2015. 'TSS No. 1-Pink Pearl': A Double-Flowered and Fragrant Amaryllis Cultivar. *Hort. Sci.,* **50(10)**: 1588-1590.
20. Liu, Z., Zhang, D., Liu, D., Li, F. and Lu, H. 2013. Exon Skipping of AGAMOUS Homolog PrseAG in Developing Double Flowers of *Prunus lannesiana* (Rosaceae). *Plant Cell Rep.,* **32(2)**: 227-237.
21. Ma, N., Chen, W., Fan, T., Tian, Y., Zhang, S., Zeng, D. and Li, Y. 2015. Low Temperature-Induced DNA Hypermethylation Attenuates Expression of RhAG, an AGAMOUS Homolog, and Increases Petal Number in Rose (*Rosa hybrida*). *BMC Plant Biol.,* **15(1):** 1-13.
22. Matsoukas, I. G., Massiah, A. J. and Thomas, B. 2012. Florigenic and Antflorigenic Signaling in Plants. *Plant Cell Physiol.,* **53(11):** 1827-1842.
23. McCann, J. 1937. New Double Hybrid Amaryllis. *Herbertia,* **4:** 185-186.
24. Mizukami, Y. and Ma, H. 1995. Separation of AG Function in Floral Meristem Determinacy from that in Reproductive Organ Identity by Expressing Antisense AG RNA. *Plant Mol. Biol.,* **28(5):** 767-784.
25. Ng, M. and Yanofsky, M. F. 2001. Function and Evolution of the Plant *MADS-Box* Gene Family. *Nat. Rev. Genet.,* **2(3):** 186-195.
26. Nitasaka, E. 2003. Insertion of an En/Spm-Related Transposable Element into a Floral Homeotic Gene DUPLICATED Causes a Double Flower Phenotype in the Japanese Morning Glory. *Plant J.,* **36(4):** 522-531.
27. Noor, S. H., Ushijima, K., Murata, A., Yoshida, K., Tanabe, M., Tanigawa, T., Kubo, Y. and Nakano, R. 2014. Double Flower Formation Induced by Silencing of C-Class *MADS-Box* Genes and Its Variation among Petunia Cultivars. *Sci. Hortic.,* **178:** 1-7.
28. Nyffeler, R. and Eggli, U., 2010. An up-to-Date Familial and Suprafamilial Classification of Succulent Plants. *Bradleya* **28**: 125–144.
29. Ó'Maoiléidigh, D. S., Wuest, S. E., Rae, L., Raganelli, A., Ryan, P. T., Kwaśniewska, K., Das, P., Lohan, A. J., Loftus, B., Graciet, E. and Wellmer, F. 2013. Control of Reproductive Floral Organ Identity Specification in Arabidopsis by the C Function Regulator AGAMOUS. *Plant Cell,* **25(7):** 2482-2503.
30. Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E. and Yanofsky, M. F. 2000. B and C Floral Organ Identity Functions Require SEPALLATA *MADS-Box* Genes. *Nature,* **405(6783)**: 200-203.
31. Salamah, A. and Rostina, I. 2019. Analysis of *AGAMOUS* Gene Expression in *Hibiscus rosasinensis* L. Single Pink, Crested Peach, and Double Orange Flowers. *J. Phys.: Conf. Ser.,* **1245: 1-7.**
32. Sandoval, S. D. C. D., Juárez, M. J. A. and Simpson, J. 2012. *Agave tequilana MADS* Genes Show Novel Expression Patterns in Meristems, Developing Bulbils and Floral Organs. *Sex. Plant Reprod.,* **25(1)**: 11-26.
33. Sharifi, A., Oizumi, K., Kubota, S., Bagheri, A., Shafaroudi, S. M., Nakano, M. and Kanno, A. 2015. Double Flower Formation in *Tricyrtis macranthopsis* Is Related to Low Expression of *AGAMOUS* Ortholog Gene. *Sci. Hortic.* **193**: 337-345.
34. Sieburth, L. E., Running, M. P. and Meyerowitz, E. M. 1995. Genetic Separation of Third and Fourth Whorl Functions of AGAMOUS. *Plant Cell,* **7(8)**: 1249-1258.
35. Soltis, D. E., Chanderbali, A. S., Kim, S., Buzgo, M. and Soltis, P. S. 2007. The ABC Model and Its Applicability to Basal Angiosperms. *Ann. Bot*., **100(2):** 155-163.
36. Srikanth, A. and Schmid, M. 2011. Regulation of Flowering Time: All Roads Lead to Rome. *Cell. Mol. Life Sci*., **68(12):** 2013-2037.
37. Tanaka, Y., Oshima, Y., Yamamura, T., Sugiyama, M., Mitsuda, N., Ohtsubo, N., Ohme-Takagi, M. and Terakawa, T. 2013. Multi-Petal Cyclamen Flowers Produced by AGAMOUS Chimeric Repressor Expression. *Sci,* **3:** 1-6.
38. Theißen, G. 2001. Development of Floral Organ Identity: Stories from the MADS House. *Curr. Opin. Plant Biol.,* **4(1):** 75-85.
39. Theißen, G., Melzer, R. and Rümpler, F. 2016. MADS-Domain Transcription Factors and the Floral Quartet Model of Flower Development: Linking Plant

[DOI: 10.22034/jast.25.4.15]

Development and Evolution. *Dev.,* **143(18):** 3259-3271.

40. Wang, S. -Y., Lee, P. -F., Lee, Y. -I., Hsiao, Y. -Y., Chen, Y. -Y., Pan, Z. -J., Liu, Z. -J. and Tsai, W. -C. 2011. Duplicated *C-Class MADS-Box* Genes Reveal Distinct Roles in Gynostemium Development in *Cymbidium ensifolium* (Orchidaceae). *Plant Cell Physiol.,* **52(3):** 563-577.
41. Wang, Y., Chen, D., He, X., Shen, J., Xiong, M., Wang, X., Zou, D. and Wei, Z. 2018. Revealing the Complex Genetic Structure of Cultivated Amaryllis (*Hippeastrum hybridum*) Using Transcriptome-Derived Microsatellite Markers. *Sci. Rep.,* **8(1)**: 1-12.
42. Waters, M. T., Tiley, A. M., Kramer, E. M., Meerow, A. W., Langdale, J. A. and Scotland, R. W. 2013. The Corona of the Daffodil *Narcissus bulbocodium* Shares Stamen-Like Identity and Is Distinct from the Orthodox Floral Whorls. *Plant J*. **74(4):** 615-625.
43. Weigel, D. and Meyerowitz, E. M. 1994. The ABCs of Floral Homeotic Genes. *Cell,* **78(2)**: 203-209.
44. Xu, Y., Teo, L. L., Zhou, J., Kumar, P. P. and Yu, H. 2006. Floral Organ Identity Genes in the Orchid *Dendrobium crumenatum. Plant J*., **46(1):** 54-68.
45. Ye, L. and Shi, Y. 2008. Research on Pollen Germination and Pollen Preservation Characteristic of Hippeastrum. *J. Shanghai Jiaotong Univ. (Agric. Sci.),* **1**: 3.
46. Yu, D., Kotilainen, M., Pöllänen, E., Mehto, M., Elomaa, P., Helariutta, Y., Albert, V. A. and Teeri, T. H. 1999. Organ Identity Genes and Modified Patterns of Flower Development in *Gerbera hybrida* (Asteraceae). *Plant J.,* **17(1)**: 51-62.
47. Yun, P.-Y., Ito, T., Kim, S. -Y., Kanno, A. and Kameya, T. 2004. The *AVAG1* Gene Is Involved in Development of Reproductive Organs in the Ornamental Asparagus, *Asparagus virgatus*. Sex. *Plant Reprod.,* **17(1)**: 1-8.
48. Zahn, L. M., Leebens-Mack, J., DePamphilis, C., Ma, H. and Theissen, G. 2005. To B or Not to B a Flower: The Role of DEFICIENS and GLOBOSA Orthologs in the Evolution of the Angiosperms. *J. Hered.,* **96(3):** 225-240.
49. Zhai, J., Jeong, D. H., De Paoli, E., Park, S., Rosen, B. D., Li, Y., González, A. J., Yan, Z., Kitto, S.L., Grusak, M. A., Jackson, S. A., Stacey, G., Cook, D. R., Green, P. J., Sherrier, D. J. and Meyers, B. C. 2011. MicroRNAs as Master Regulators of the Plant NB-LRR Defense Gene Family Via the Production of Phased, Trans-acting siRNAs. *Genes Dev.* **25(23)**:2540-53.
50. Zhang, B., Liu, Z. -X., Ma, J., Song, Y. and Chen, F. -J. 2015. Alternative Splicing of the *AGAMOUS* Orthologous Gene in Double Flower of *Magnolia stellata* (Magnoliaceae). *Plant Sci.,* **241**: 277-285.
51. Zhu, B., Li, H., Wen, J., Mysore, K. S., Wang, X., Pei, Y., Niu, L. and Lin, H. 2018. Functional Specialization of Duplicated AGAMOUS Homologs in Regulating Floral Organ Development of *Medicago truncatula*. *Front. Plant Sci.,* **9:** 1-14.

[DOI: 10.22034/jast.25.4.15]

نقش ژن AGAMOUS در افزایش تعداد گلبرگ در گیاه آماریلیس

س. دستمالچی، ن. مشتاقی، و ا. شریفی

چکیده

ژن‌های MADS-box نقش مهمی در تنظیم رشد اندام‌های گل دارند. در این خانواده ژنی، ژن های AGAMOUSمسئول رشد برچه و پرچم هستند. در شکل دابل فلاور آماریلیس نسبت به نوع وحشی آن، تعداد برچه به سه کاهش یافته است و مادگی وجود ندارد. در مقابل، تعداد گلبرگها افزایش یافته است. در این تحقیق، عملکرد ژن AGAMOUS (AG) در این تغییرات بررسی شده است. در تحقیق حاضر، بخشی از توالی کد کننده ژن AGAMOUS به نام AmAG تکثیر شد و توالی حاضر تحت بررسی های فیلوژنتیک قرار گرفت. سپس میزان بیان این ژن در فرم وحشی و آماریلیس دابل فلاور با استفاده از روش Real-Time PCR بررسی شد. نتایج بررسی های فیلوژنتیک نشان داد که بخش جداسازی شده ژن یا AmAG همسانی بالایی با توالی ژن‌های ارتولوگ AGAMOUS در خانواده Amaryllidaceae و گیاهان نزدیک به این خانواده دارد. همچنین، تفاوتی در توالی ژن AmAG در فرمهای وحشی و دابل فلاور وجود نداشت. نتایج Real-time PCRنشان داد که در شکل وحشی، بیان ژن AmAG در حلقه اول تا سوم کم و فقط در حلقه چهارم زیاد بود. در حالی که در فرم دابل فلاور، بیان ژن AmAG در چهار حلقه پایین بود. بیان کمتر AmAG در حلقه چهارم فرم دابل فلاور باعث افزایش تعداد گلبرگها و مشاهده تغییرات مورفولوژیکی در فرم گل شده است.

[DOI: 10.22034/jast.25.4.15]