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BRIEF COMMUNICATION

Isolation and characterization of two MADS-box genes from *Lycium barbarum*

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Abstract

To broaden our knowledge of flower development, two floral homeotic genes, *LbAG* and *LbSEP3*, were isolated from the flower of *Lycium barbarum* L. The open reading frame length of *LbAG* and *LbSEP3* were 1090 and 992 bp encoding 249 and 242 amino acids, respectively. Sequence alignment and phylogenetic analysis indicated that *LbAG* belonged to C-type MADS-box gene and that *LbSEP3* was E-type MADS-box gene. Compared with other floral homeotic proteins, LbAG held the conserved AG motif I, II and LbSEP3 conserved SEP3 motif I, II. Expression profile showed that *LbAG* transcripts were abundant in inner two whorls and fruit but not in root, leaf, sepal, and petal, and that *LbSEP3* constitutively expressed in root, leaf, fruit, and all the four floral whorls.

Additional key words: expression profile, floral whorls, open reading frame, PCR, RACE.

A typical eudicot flower comprised of four parts, namely sepals, petals, stamens, and carpels positioning in a concentric-whorl manner from outside to inside. Based on the extensive genetic and molecular studies on the flower development of *Arabidopsis*, *Antirrhinum*, and other non-model plants, a classic ABCE model (Coen and Meyerowitz 1991, Soltis *et al*. 2007) was proposed that four classes of floral homeotic genes were responsible individually or jointly for determining the identity of floral organs.

So far, C-type genes involved in stamen and carpel development have been isolated from lots of species. Typical C-type genes, which were confined to express in the stamen and carpel, were identified in *Arabidopsis* (*AGAMOUS*; Yanofsky *et al*. 1990), tomato (*TAG1*; Pnueli *et al*. 1994), tobacco (*NAG1*; Kempin *et al*. 1993), petunia (*pMADS3*; Kapoor *et al*. 2002), and *Crocus sativus* (*CsAG1*; Tsaftaris *et al*. 2005). Recently, atypical C-type genes, expressing in the vegetative tissues and/or outer two whorls from some species, were also characterized (Van der Linden *et al*. 2002, Endo *et al*. 2006, Tani *et al*. 2009). Although previous studies discovered expression of *SEP3*-like genes restricted to the inner three whorls (Jang *et al*. 1999, Pelaz *et al*. 2001, Ferrario *et al*. 2003), expression of more and more *SEP3*-like genes have been found also in sepal, leaf, seed and fruit (Endo *et al*. 2006, Tani *et al*. 2009). In summary, the divergent expression profiles of homeotic MADS-box genes hinted that MADS-box homologs (*AG*-like and *SEP3*-like) derived from different plants diversified

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Abbreviations: *AG* - *AGAMOUS*; *SEP3* - *SEPALLATA3*; ORF - open reading frame; PCR - polymerase chain reaction; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription polymerase chain reaction.
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functionally. Furthermore, *AG*-like and *SEP3*-like transcripts mentioned above accumulating in non-target tissues indicated that these MADS-box genes were possibly involved in certain biological processes and possessed, so far, unknown function. Therefore, possible new functions of flower developmental genes can be discovered after characterizing the expression profiles of homologous genes in less-studied plant species.

To broaden our knowledge of genes controlling flower development in non-model species, we isolated and characterized two MADS-box genes, *LbAG* and *LbSEP3*, from *Lycium barbarum*, which is one of the important crops belonging to *Solanaceae* family.

The total RNA from *Lycium barbarum* L. flowers was isolated according to the instruction of *TRIzol* kit (*Invitrogen*, USA) and were reverse-transcribed by *Powerscript*^{TM} *II* (*Takara*, Japan) with PCR primers SMART IV (AAGCAGTGGTATCAACGCAGAGT GGCCATTACGGCCGGG) and CDS III (ATTCTA GAGGCCGAGGCGGCCGACATG-d(T)30$_{N-1}$N). Long distance (LD) PCR for double strand cDNA amplification was carried out following the *SMART*^{TM} cDNA library construction kit user manual. A degenerate primer, pMADS (GTKCTHTGYGAYGCYGARRTTGC), combined with CDSIII was used for 3'RACE. Consequently, PCR fragment about 750 bp was obtained and sequenced. Based on the known sequences, two gene-specific primers, LbAG3-5 (TTCCTGTTCTGATTCTGCGG) and LbSEP3-5 (TTGATAGGAAAACCATTGAGCATATT), were designed and used for 5' RACE together with the SMART IV primer. After cloning, sequencing, and assembling, two primer pairs, 5LbSEP3 (GAGAGA AAAAAAGAAAAAGAAA) and 3LbSEP3 (TGATAG GAAAACCATTGAGCAT) for *LbSEP3*, as well as 5LbAG (TCTCTATTTTCATCTTCCAACCCTTT) and 3LbAG (CACAGCAGAAAGAAGACCATCAACCA) for *LbAG*, were designed to confirm the assembled full-length cDNA sequences. Finally, two MADS-box genes referred to *LbSEP3* and *LbAG* were isolated and deposited in *NCBI* with the accession number of GU144015 and GU144014, respectively. The PCR programs for isolating MADS-box genes were performed as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, annealing at 68 °C for 2.5 min, and final extension at 72 °C for 10 min.

To reveal the expression pattern of *LbAG* and *LbSEP3*, root, young leaf, sepal, petal, stamen, carpel, and fruit samples were separately collected for isolating RNA and 10 μg of the total RNA was utilized for the first strand cDNA synthesis. The RT-PCR primers were designed spanning the C domain and 3'UTR in order to guarantee the primers in a gene-specific manner. Gene specific primers were: LbAG-RT-F (ATAAAAAAC CACAAGAGAAG) and LbAG-RT-R (TATGAACAA AAGGAACAGTA) for *LbAG,* and LbSEP3-RT-F (CTT GAATCACTTGAGAGGCA) and LbSEP3-RT-R (TAG GAAAACCATTGAGCATA) for *LbSEP3*. PCR products were then cloned and sequenced to confirm the transcripts of *LbAG* and *LbSEP3*. The actin gene was used as an internal control gene, and primers were *Actin-F* (GATATGGCACCATACGTTTTATAACG) and *Actin-R* (GTGGTTTCGTGGATACCCGCGGCTT). The expres-

Table 1. Proteins homologous to LbAG and LbSEP3.

Gene name	Accession	Species	Identities [%]	E-value
NtAG	AAA17033	*Nicotiana tabacum*	93	3.00E-129
SlAG	AAA34197	*Solanum lycopersicum*	89	9.00E-128
PhAG	CAA51417	*Petunia × hybrida*	89	1.00E-126
InAG	BAF36711	*Ipomoea nil*	80	5.00E-110
AmAG	CAB42988	*Antirrhinum majus*	78	6.00E-104
TcAG	ABA39727	*Theobroma cacao*	76	3.00E-101
CsAG	AAC08528	*Cucumis sativus*	74	6.00E-95
CuAG	BAF34911	*Citrus unshiu*	73	1.00E-100
CmAG	BAG06959	*Chrysanthemum morifolium*	72	1.00E-97
GhAG	ACF93432	*Gossypium hirsutum*	72	7.00E-93
AtAG	NP_567569	*Arabidopsis thaliana*	68	2.00E-90
NsSEP3	AAD39034	*N. sylvestris*	98	7.00E-136
SlSEP3	AAP83377	*S. lycopersicum*	96	1.00E-115
PhSEP3	AAP83395	*P. hybrida*	95	1.00E-114
GhSEP3	AAN15182	*G. hirsutum*	83	5.00E-106
AmSEP3	AAP83364	*A. majus*	85	1.00E-103
LjSEP3	AAX13298	*Lotus japonicus*	84	1.00E-115
TrSEP3	ABD19719	*Taihangia rupestris*	81	3.00E-110
CmSEP3	AAO22982	*C. morifolium*	79	5.00E-107
AtSEP3	NP_564214	*A. thaliana*	75	3.00E-97
CsSEP3	ACB69509	*Crocus sativus*	74	8.00E-95
MdSEP3	CAA04919	*Malus × domestica*	65	4.00E-81

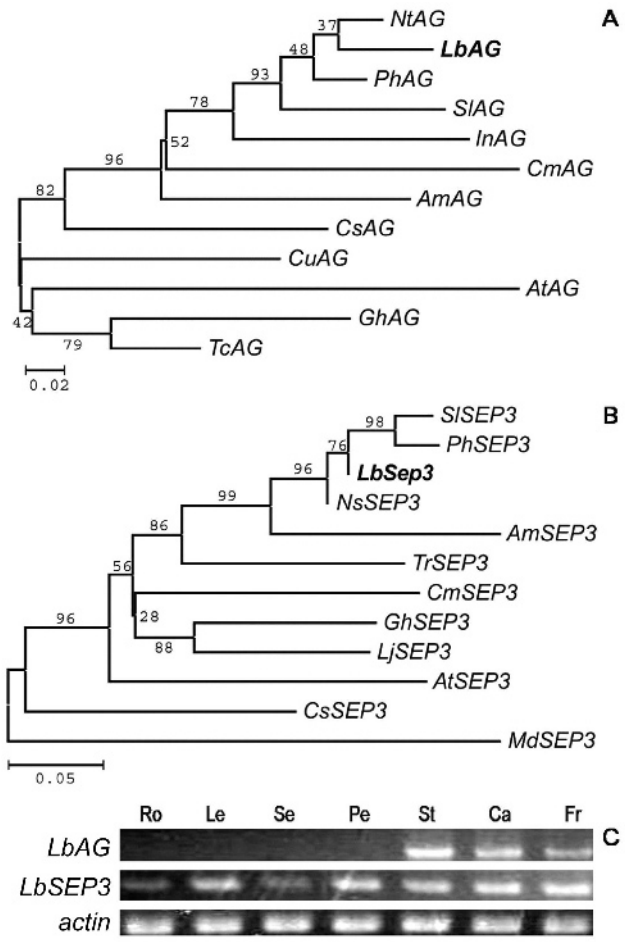

Fig. 1. NJ phylogenetic tree and expression profiles of *LbAG* and *LbSEP3*. *A* - phylogenetic relationship between *LbAG* and *AG*-like homologs from other plants. *B* - phylogenetic relationship between *LbSEP3* and *SEP3*-like homologs from other plants. Bootstrap values (2 000 replications) are shown near the branches and sequence distance is shown in the bottom of the tree. All MADS-box genes selected for phylogeny study are listed in Table 1. *C* - expression profiles of *LbAG* and *LbSEP3*; Ro - root, Le - leaf, Se - sepal, Pe - petal, St - stamen, Ca - carpel, Fr - fruit. *Actin* gene was used as an internal control.

sion profiles of *LbAG* and *LbSEP3* were performed for three replicates following the PCR program with denaturation at 95 °C for 5 min, followed by 33 cycles at 95 °C for 30 s, annealing at 43 °C for 30 s, extension at 72°C for 1 min and final extension for 10 min at 72°C. To detect the phylogenetic relationships, *Lycium* MADS-box genes and their homologs from other plants (Table 1) were aligned using *ClustalX* (Thompson *et al.* 1997) with manual correction. Phylogenetic trees were constructed using *MEGA4.0* software (Tamura *et al*. 2007).

In the present study, *LbAG* gene with the length of 1090 bp ORF encoding 249 amino acids was isolated. LbAG hit to homologs from other plants with identity ranging from 68 % for *Arabidopsis thaliana* AtAG/AGAMOUS to 93 % for *Nicotiana tabacum* NtAG/NAG1 (Table 1). Sequence alignment showed that LbAG held highly conserved MADS domain, K domain, and the conserved AG motif I and II (Kramer *et al.* 2004) (data not shown). Furthermore, there was an N-terminal polypeptide stretch preceding the MADS domain, which was consistent with the previous results (Yanofsky *et al.* 1990, Pnueli *et al*. 1994). Phylogenetic tree showed that LbAG closely clustered with *Solanaceae* AGAMOUS homologs, namely tobacco NtAG/NAG1 (Kempin *et al*. 1993), tomato SlAG/TAG1 (Pnueli *et al*. 1994), and petunia PhAG/pMADS3 (Kapoor *et al*. 2002), that were identified to share similar functions with *Arabidopsis* AtAG/AGAMOUS (Yanofsky *et al*. 1990) (Fig. 1*A*).

Expression profiles indicated that *LbAG* was restricted to stamen, carpel, and fruit, but not in root, young leaf, sepal, and petal (Fig. 1*C*). Previous studies showed the expression of *Arabidopsis AGAMOUS,* as well as its homologs, occurred in the third whorl (stamen) and the fourth whorl (carpel) (Yanofsky *et al*. 1990, Kempin *et al*. 1993, Tsuchimoto *et al*. 1993, Pnueli *et al.* 1994, Kapoor *et al*. 2002). Therefore, our results were consistent with previous reports, except for expressing in fruit. Recently, *TcAG* from *Theobroma cacao* was

characterized and expressed not only in stamen and carpel but also in fruit at entire developmental stages (Chaidamsari *et al.* 2006). Many researches documented that genes homologous to *Arabidopsis AGAMOUS* expressed not only in the inner two whorls but also in other tissues even in vegetative tissues (Brunner *et al.* 2000, Van der Linden *et al.* 2002, Endo *et al.* 2006, Tani *et al.* 2009). For instance, the transcripts of *MdMADS15* from *Malus domestica* accumulated in shoot and leaf (Van der Linden *et al.* 2002). Analogously, *CsMADS1* from *Citrus unshiu* expressed in flower, seed, peer, juice sac, petal, and whole fruit (Endo *et al.* 2006). Furthermore, *PTAG1* and *PTAG*2 from *Populus trichocarpa* showed a lower expression level in stem and leaf compared with those in flower tissues (Brunner *et al.* 2000). In *Prunus persica*, *PPERAG* was also detectable in a relatively low level in leaf and sepal (Tani *et al.* 2009). These studies suggested that *LbAG* and other *AG*-like genes mentioned above, which were originally regarded to express exclusively in the inner two whorls, expressed at a comparatively low level in vegetative tissues, fruit, seed, and petal. It hinted that *AG*-like genes including *LbAG* were probably involved with unknown functions. Recently, an *AGAMOUS*-related MADS-box gene, *XAL1*, was identified in *Arabidopsis* and regulated the root meristem cell proliferation and flowering transition (Tapia-Lopez *et al.* 2008). Additionally, *AGL8*, which originally functioned in carpel tissue for flower formation, was discovered in vegetative tissues and was proposed to regulate the cellular differentiation during leaf development (Gu *et al.* 1998). Therefore, it was reasonable to speculate that *LbAG* expressing in fruit was possibly involved in processes during fruit development. Although expression in fruit was not examined in other *Solanaceae* species, transcripts of *AG-like* genes were also discovered in citrus fruit (Endo *et al.* 2006). In the past decades, several authors documented functional diversity of genes after duplication and this phenomenon was common in the plant kingdom (Zhou *et al.* 2010). In contrast, functional diversification of orthologs, for instance *LbAG* and *CsMADS1 versus AGAMOUS*, after speciation was rarely reported. Finally, the possible new function of *LbAG* involved for fruit development was needed to be confirmed by further experiments.

As for *LbSEP3* of 992 bp ORF encoding 242 amino acids was isolated. It shared high level of amino acid sequence identity with homologs ranging from 65 % for *M. domestica* MdSEP3/MdMADS8 to 98 % for *N. sylvestris* NsSEP3/NsMADS3 (Table 1). Sequence alignment showed that LbSEP3 possessed highly conserved MADS domain, K domain, and SEP3 motif I and II (Zahn *et al.* 2005) (data not shown). NJ phylogenetic tree indicated that LbSEP3 tightly clustered with *Solanaceae* SEP3-like homologs and formed a subclade (Fig. 1*B*). Taken together the results of sequence alignment and phylogenetic analysis, it was evident that LbSEP3 was a putative SEP3 homolog (Fig. 1*B*).

According to the expression profiles, *LbSEP3* was found constitutively expressing in all tissues, although the expression levels in root and sepal were relatively lower compared with those in leaf, stamen, carpel, and fruit (Fig. 1*C*). Different from homologs that were confined to express in the inner three whorls, such as *FBP2* (Ferrario *et al.* 2003), *NsMADS3* (Jang *et al.* 1999), and *SEP3* (Pelaz *et al.* 2001), *LbSEP3* transcripts also accumulated in sepal and vegetative tissues other than the inner three whorls. Correspondingly, *SEP3-like* homologs from many other plants were indeed detectable at a considerably low level in vegetative tissues (Endo *et al.* 2006, Tani *et al.* 2009). For example, *PPERSEP3* transcripts were detected in leaf, sepal, fruit and inner three whorls (Tani *et al.* 2009), and *CitMADS3* transcripts accumulated in sepal, fruit, and seed (Endo *et al.* 2006). Similar to the situation of *LbAG*, *LbSEP3* might have evolved new functions after speciation comparing with its *Solanaceae* orthologs.

Taken as a whole, MADS-box genes, *LbAG* and *LbSEP3,* from *L. barbarum* expressed not only in the expected tissues (the inner two and three whorls) but also in fruit and vegetative tissues, which indicated that *LbAG* and *LbSEP3* might have new functions in these tissues. Therefore, further studies are needed to confirm the background expression of *LbAG* and *LbSEP3* without any newly evolved functions, or to elucidate the possible new functions of these two genes evolved after speciation.

References

Brunner, A.M., Rottmann, W.H., Sheppard, L.A., Krutovskii, K., DiFazio, S.P., Leonardi, S., Strauss, S.H.: Structure and expression of duplicate *AGAMOUS* orthologues in poplar. - Plant mol. Biol. **44**: 619-634, 2000.

Chaidamsari, T., Samanhudi, Sugiarti, H., Santoso, D., Angenent, G.C., De Maagd, R.A.: Isolation and characterization of an *AGAMOUS* homologue from cocoa. - Plant Sci. **170**: 968-975, 2006.

Coen, E.S., Meyerowitz, E.M.: The war of the whorls: genetic interactions controlling flower development. - Nature **353**: 31-37, 1991.

Endo, T., Shimada, T., Fujii, H., Omura, M.: Cloning and characterization of 5 MADS-box cDNAs isolated from citrus fruit tissue. - Sci. Hort. **109**: 315-321, 2006.

Ferrario, S., Immink, R.G.H., Shchennikova, A., Busscher-Lange, J., Angenent, G.C.: The MADS box gene *FBP2* is required for SEPALLATA function in petunia. - Plant Cell **15**: 914-925, 2003.

Gu, Q., Ferrandiz, C., Yanofsky, M.F., Martienssen, R.: The *FRUITFULL* MADS-box gene mediates cell differentiation during *Arabidopsis* fruit development. - Development **125**: 1509-1517, 1998.

Jang, S., Hong, M.Y., Chung, Y.Y., An, G.: Ectopic expression of tobacco MADS genes modulates flowering time and

plant architecture. - Mol. Cell **9**: 576-586, 1999.

Kapoor, M., Tsuda, S., Tanaka, Y., Mayama, T., Okuyama, Y., Tsuchimoto, S., Takatsuji, H.: Role of petunia pMADS3 in determination of floral organ and meristem identity, as revealed by its loss of function. - Plant J. **32**: 115-127, 2002.

Kempin, S.A., Mandel, M.A., Yanofsky, M.F.: Conversion of perianth into reproductive organs by ectopic expression of the tobacco floral homeotic gene *NAG1*. - Plant Physiol. **103**: 1041-1046, 1993.

Kramer, E.M., Jaramillo, M.A., Di Stilio, V.S.: Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. - Genetics **166**: 1011-1023, 2004.

Pelaz, S., Gustafson-Brown, C., Kohalmi, S.E., Crosby, W.L., Yanofsky, M.F.: *APETALA1* and *SEPALLATA3* interact to promote flower development. - Plant J. **26**: 385-394, 2001.

Pnueli, L., Hareven, A.D., Rounsley, S.D., Yanofsky, M.F., Lifschitz, E.: Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. - Plant Cell **6**: 163-173, 1994.

Soltis, D.E., Chanderbali, A.S., Kim, S., Buzgo, M., Soltis, P.S.: The ABC model and its applicability to basal angiosperms. - Ann. Bot. **100**: 155-163, 2007.

Tamura, K., Dudley, J., Nei, M., Kumar, S.: MEGA4: molecular evolutionary genetics analysis (*MEGA*) software version 4.0. - Mol. Biol. Evol. **24**: 1596-1599, 2007.

Tani, E., Polidoros, A.N., Flemetakis, E., Stedel, C., Kalloniati, C., Demetriou, K., Katinakis, P., Tsaftaris, A.S.: Characterization and expression analysis of *AGAMOUS*-like, *SEEDSTICK*-like, and *SEPALLATA*-like MADS-box genes in peach (*Prunus persica*) fruit. - Plant Physiol. Biochem. **47**: 690-700, 2009.

Tapia-Lopez, R., Garcia-Ponce, B., Dubrovsky, J.G., Garay-Arroyo, A., Perez-Ruiz, R.V., Kim, S.-H., Acevedo, F., Pelaz, S., Alvarez-Buylla, E.R.: An *AGAMOUS*-related MADS-box gene, *XAL1* (*AGL12*), regulates root meristem cell proliferation and flowering transition in *Arabidopsis*. - Plant Physiol. **146**: 1182-1192, 2008.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G.: The *CLUSTAL_X* windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. - Nucl. Acid. Res. **25**: 4876-4882, 1997.

Tsaftaris, A.S., Pasentsis, K., Polidoros, A.N.: Isolation of a differentially spliced C-type flower specific *AG*-like MADS-box gene from *Crocus sativus* and characterization of its expression. - Biol. Plant. **49**: 499-504, 2005.

Tsuchimoto, S., Vanderkrol, A.R., Chua, N.H.: Ectopic expression of pMADS3 in transgenic petunia phenocopies the petunia *blind* mutant. - Plant Cell **5**: 843-853, 1993.

Van der Linden, C.G., Vosman, B., Smulders, M.J.M.: Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. - J. exp. Bot. **53**: 1025-1036, 2002.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., Meyerowitz, E.M.: The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. - Nature **346**: 35-39, 1990.

Zahn, L.M., King, H.Z., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., dePamphilis, C.W., Ma, H.: The evolution of the *SEPALLATA* subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. - Genetics **169**: 2209-2223, 2005.

Zhou, L., Zhou, Y., Wang, M., Wang, H., and Zhao, Y.: Expressions and dimerization affinities of three highly identical *APETALA3* genes in *Brassica napus*. - Biol. Plant. **54**: 33-40, 2010.