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ORIGINAL ARTICLE

AGL6-like MADS-box Genes are Sister to *AGL2*-like MADS-box Genes

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Abstract *AGL6*-like genes form one of the major subfamilies of MADS-box genes and are closely related to the *AGL2* (E-class) and *SQUA* (A-class) subfamilies. In *Arabidopsis*, *AGL6* and *AGL13* have been reported from the *AGL6* subfamily, and *AGL6* controls lateral organ development and flowering time. However, little is known about homologs of these genes in basal angiosperms. We identified new *AGL6*-like genes from several taxa from gymnosperms, basal angiosperms, monocots, and eudicots. These genes were analyzed together with previously reported *AGL6*-like genes. Structural analyses showed 1) a one-aa (amino acid) gap in the I-domain in all *AGL6*-like genes relative to *AGL2*-like and *SQUA*-like genes, 2) a seven-aa insertion in the C-domain of genes from asterids, and 3) a one-aa insertion in the C-domain of genes from gymnosperms. Broad phylogenetic analyses strongly showed that *AGL6*-like genes are sister to *AGL2*-like genes, and *SQUA*-like genes are sister to these two groups. The phylogenetic tree of *AGL6*-like genes generally tracks organismal phylogeny as inferred from multigene data sets; several gene duplications were detected in angiosperms (e.g., within Magnoliales), and one duplication was detected in gymnosperms. We hypothesize that the split between *AGL6*-like and *AGL2*-like genes occurred at least 290-309.2 mya based on our phylogenetic tree and the fossil record.

Key words: *AGL2*, *AGL6*, Gene phylogeny, MADS-box, *SQUA*

Introduction

MADS-box genes encode transcription factors which play important roles in developmental control in plants, animals, and fungi (for reviews, see Shore and Sharrocks 1995; Theissen et al. 1996; Riechmann and Meyerowitz 1997; Theissen et al. 2000; Ng 2001; Theissen 2001; De Bodt et al. 2003). These genes encode a highly conserved domain (MADS domain), of approximately 55 amino acids, that is involved in recognition and binding to a specific DNA region (CArG boxes) (West and Sharrocks 1999). Functions of MADS-box genes vary. In plants, some MADS-box genes are involved in the regulation of floral organ identity, predicted by the ABCDE model in *Arabidopsis* (*AP1*, *AP3*, *PI*, *AG*, *SHP1*, *SHP2*, *STK*, *SEP1, SEP2, SEP3,* and *SEP4*) (Coen and Meyerowitz 1991; Colombo et al. 1995; Pelaz et al. 2000; Theissen 2001; Ditta et al. 2004). Examples of additional known functions of plant MADS-box genes are the regulation of floral meristem identity (*AP1*) (Gustafsonbrown et al. 1994), the timing of flower initiation (*FLC*) (Michaels and Amasino 1999; Alvarez-Buylla et al. 2000), and fruit, leaf, and root development (*PkMADS1*, *FUL*, and *ANR1*) (Gu et al. 1998; Zhang and Forde 2000; Prakash and Kumar 2002).

The MADS-box gene family can be subdivided into two major classes termed Type I and Type II genes (Alvarez-Buylla et al. 2000). Both types of genes have been found in animals, fungi, and plants. Plant type II genes are also called MIKC-type genes (Munster et al. 1997) because these genes share a conserved structural organization: **M**ADS, **I**ntervening, **K**eratin-like, and **C**-terminal domains. Two different types of MIKC-type genes, MIKC^C-type and MIKC*-type, are recognized via the characterization of MADS-box genes from a club moss (*Lycopodium*) and a moss (*Physcomitrella*), respectively (Henschel et al. 2002; Kwantes et al. 2012). MIKC*-type MADS proteins have a longer I-domain region than those of MIKC^C. The presence of MIKC^C-type as well as MIKC*-type genes in both lycophytes (the sister group to all other vascular plants) and mosses suggests that a gene duplication generated these two gene groups before the divergence of mosses (and perhaps all bryophytes) and

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vascular plants (Henschel et al. 2002). Phylogenetic studies of MIKC^C-type genes recognized 14 major clades (Becker and Theissen 2003; Nam et al. 2003; Pařenicová et al. 2003; Kim et al. 2005a). Becker and Theissen (2003) suggested names for these clades (subfamilies) based on the first clade member that was identified: *STMADS11, AGL17, GGM13, DEF, GLO, AG, AGL12, SQUA, AGL2, AGL6, TM8, TM3, AGL15*, and *FLC*. In most studies, these major clades are relatively well supported whereas the relationships among the clades are poorly supported (e.g., Becker and Theissen 2003; Nam et al. 2003; Pařenicová et al. 2003; Kim et al. 2005a; Gramzow et al. 2012). In most cases, members of each clade share similar expression patterns and have closely related functions. For example, members of the *DEF/GLO* clade are responsible for B-function in floral organ identity, and those of the *AGL2* (also referred to as *SEPALLATA*) clade confer E-function. Therefore, the establishment of each clade by gene duplication, diversification, and fixation probably was an important step toward the establishment of floral homeotic functions (Theissen et al. 1996).

Among the MIKC^C-type MADS-box genes, *AGL6* homologs represent one of the major subfamilies and form a monophyletic group in recent phylogenetic analyses (e.g., Becker and Theissen 2003; Nam et al. 2003; Pařenicová et al. 2003; Kim et al. 2005a; Rijpkema et al. 2009; Li et al. 2010; Gramzow et al. 2012). These genes are closely related to the *AGL2* (E-class) and *SQUA* (A-class) subfamilies of MADS-box genes. Conflicting relationships among these three groups have been reported in previous phylogenetic analyses: in some cases *AGL6* is sister to *AGL2* (e.g., Becker and Theissen 2003; Nam et al. 2003; Gramzow et al. 2012; Fig. 1), and in other analyses *AGL6* is sister to *SQUA* and *AGL2* (e.g., Pařenicová et al. 2003; Kim et al. 2005a; Fig. 1). However, in most cases these conflicting relationships were not strongly supported, and in all previous studies these three groups form a clade regardless of their interrelationships (e.g., Winter et al. 1999; Becker and Theissen 2003; Nam et al. 2003; Kim et al. 2005a; Kim et al. 2005b). In the *AGL6*-like genes, recent phylogenetic analyses showed the duplication history of the *AGL6*-like genes: one at the base of the core eudicots resulting in *euAGL6* and *AGL6*-like gene clades, one during basal angiosperm diversification, and two in monocot evolution (Viaene et al. 2010).

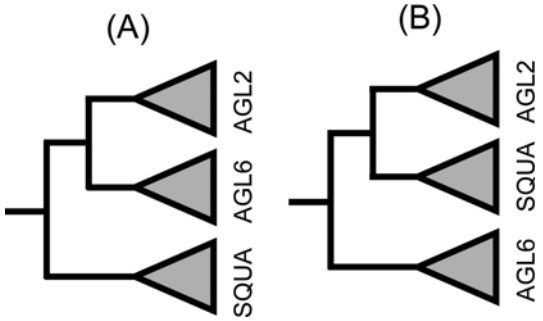

Fig. 1. Two different relationships among *AGL2*, *AGL6*, and *SQUA* subfamilies of MADS-box genes as suggested in previous studies. (A) *AGL6* is sister to *AGL2* (e.g., Becker and Theissen 2003; Nam et al. 2003; Gramzow et al. 2012). (B) *AGL6* is sister to *SQUA* and *AGL2* (e.g., Pařenicová et al. 2003; Kim et al. 2005). For names of subfamilies, we followed Becker and Theissen (Becker and Theissen 2003).

Unlike other major subgroups of MADS-box genes, *AGL6* homologs have not been investigated extensively, and their functions are not completely characterized. In *Arabidopsis*, *AGL6* (At2g45650) and *AGL13* (At3g61120) have been reported from the *AGL6* subfamily (Ma et al. 1991; Rounsley et al. 1995). *AGL6* has recently been shown to control 1) lateral organ development and flowering time (Koo et al. 2010), 2) circadian clock (Yoo et al. 2010), and 3) negative regulation of the *FLC/MAF* clade genes and positive regulation of *FT* (Yoo et al. 2011). For *AGL13,* broad expression including all floral organs and leaves (especially strong expression in ovules) has been reported (Rounsley et al. 1995). In *Petunia*, *SEPALLATA*-like function of petunia *AGL6* (*PhAGL6*) has been reported (Rijpkema et al. 2009). However, *AGL6* and *AGL13* homologs in basal angiosperms have rarely been reported and their functions remain obscure.

Studies of MADS-box gene protein-protein interactions in *Arabidopsis* (de Folter et al. 2005) showed that the AGL6 protein has an interaction pattern closely resembling that of *APETALA1*, suggesting that this protein plays a role in the flowering program. This hypothesis is strengthened by the fact that over-expression of *OMADS1* and *HoAGL6* (from *Oncidium* and *Hyacinthus*, respectively), which are included in the *AGL6* subfamily, resulted in early flowering and loss of inflorescence indeterminacy in *Arabidopsis* (Hsu et al. 2003; Fan et al. 2007). *OsMADS6*, an *AGL6*-like gene from rice, also plays a role in the flowering program: it was strongly expressed in the floral meristem at early stages, and *osmads6* mutants displayed altered palea identity, extra glume-like or mosaic organs, abnormal carpel development, and loss of floral meristem determinacy (Ohmori et al. 2009; Reinheimer and Kellogg 2009; Li et al. 2010). In core eudicots, gene expression studies showed that *AGL6*-like genes acquired expression in vegetative tissues, while expression of its paralog, *euAGL6*, remains predominantly confined to reproductive tissues (Viaene et al. 2010).

In this study, we report 13 new *AGL6*-like genes from phylogenetically pivotal taxa in angiosperm evolution, examine their structure, analyze these new sequences together with previously reported *AGL6*-like genes, and compare the results with previously reported phylogenetic trees. We consider the evolution and diversification of *AGL6*-like genes in a phylogenetic context.

Results

Sequence Structure

Thirteen new *AGL6*-like sequences were identified in this study from *Zamia vazquezii* (*Za.va.AGL6.1* and *Za.va. AGL6.2*), *Welwitschia mirabilis* (*We.mi.AGL6*), *Nuphar advena* (*Nu.ad.AGL6*), *Illicium parviflorum* (*Il.pa.AGL6*), *Magnolia grandiflora* (*Ma.gr.AGL6.2*; previously reported *Ma.gr.AGL6* in Kim et al. (2005a) was treated as *Ma.gr.AGL6.1*), *Liriodendron tulipifera* (*Li.tu.AGL6*), *Eupomatia bennettii* (*Eu.be.AGL6-1* and *Eu.be.AGL6-2*), *Acorus americanus*

Table 1. Sequences of *AGL6*-like genes used in this study. Angiosperm species are arranged by families and higher groups according to the AGP III system (APGIII 2009). Newly identified genes in this study are indicated in bold.

Classification	Taxa	Gene name	GenBank accession Number	Reference
GYMNOSPERMS				
Cycadales				
Zamiaceae	*Zamia vazquezii*	***Za.va.AGL6.1***	KC899695	This study.
		Za.va.AGL6.2	KC899696	This study.
Ginkgoales				
Ginkgoaceae	*Ginkgo biloba*	*GbMADS1*	AB029463	Unpublished.
		GbMADS8	AB029470	Unpublished.
Gnetales				
Gnetaceae	*Gnetum gnemon*	*GGM9*	AJ132215	Winter et al. (1999)
		GGM11	AJ132217	Winter et al. (1999)
		GpMADS3	AB022665	Shindo et al. (1999)
Welwitschiaceae	*Welwitschia mirabilis*	***We.mi.AGL6***	KC899694	This study.
Coniferales				
Pinaceae	*Picea abies*	*DAL1*	X80902	Tandre, Albert et al. (1995)
	Pinus resinosa	*MAD1*	Y09611	Liu et al. (1997), Electronic Plant Gene Register PGR97-032
	Pinus radiata	*PrMADS2*	U42400	Mouradov et al. (1996), Electronic Plant Gene Register PGR96-124
		PrMADS3	U76726	Mouradov et al. (1997), Electronic Plant Gene Register PGR97-032
ANGIOSPERMS				
Amborellales				
Amborellaceae	*Amborella trichopoda*	*Am.tr.AGL6*	AY936234	Kim et al. (2005)
Nymphaeales				
Nymphaeaceae	*Nuphar advena*	***Nu.ad.AGL6***	KC899697	This study.
Austrobaileyales				
Schisandraceae	*Illicium parviflorum*	***Il.pa.AGL6***	KC899698	This study.
MAGNOLIIDS				
Laurales				
Lauraceae	*Persea americana*	*Pe.am.AGL6.1*	DQ660395	Chanderbali et al. (2006)
		Pe.am.AGL6.2	DQ660396	Chanderbali et al. (2006)
Magnoliales				
Magnoliaceae	*Magnolia grandiflora*	*Ma.gr.AGL6.1*	AY936233	Kim et al. (2005)
		Ma.gr.AGL6.2	KC899699	This study.
	Magnolia kobus (=*M. praecocossima*)	*MpMADS3*	AB050645	Unpublished.
		MpMADS4	AB050646	Unpublished.
	Michelia figo	*MfAGL6A*	AY306157	Litt and Irish (2003)
		MfAGL6B	AY306158	Litt and Irish (2003)
	Liriodendron tulipifera	***Li.tu.AGL6***	KC899700	This study.
Eupomatiaceae	*Eupomatia bennettii*	***Eu.be.AGL6-1***	KC899701	This study.
		Eu.be.AGL6-2	KC899702	This study.
Piperales				
Saururaceae	*Houttuynia cordata*	*HcAGL6*	AB089160	Unpublished.
MONOCOTS				
Acorales				
Acoraceae	*Acorus americanus*	***Ac.am.AGL6***	KC899703	This study.

Table 1. Continued

Classification	Taxa	Gene name	GenBank accession Number	Reference
Asparagales				
Agapanthaceae	*Agapanthus praecox*	*ApMADS3*	AB079261	Unpublished.
Asparagaceae	*Asparagus officinalis*	*AOM3*	AY383559	Unpublished.
Hyacinthaceae	*Hyacinthus orientalis*	*HoAGL6*	AY591333	Unpublished.
Iridaceae	*Crocus sativus*	*AGL6a*	EF041505	Unpublished.
		AGL6b	EF041506	Unpublished.
COMMELINIDS				
Arecales				
Arecaceae	*Elaeis guineensis*	*AGL6-1*	AY739701	Unpublished.
		mads4	AJ581469	Unpublished.
Poales				
Poaceae	*Dendrocalamus latiflorus*	*MADS17*	AY599754	Unpublished.
		MADS18	AY599755	Unpublished.
	Hordeum vulgare	*HvAGL6*	AY541067	Unpublished.
	Lolium perenne	*LpMADS4*	AY198329	Petersen et al. (2004)
	Oryza sativa	*OsMADS6*	U78782	Unpublished.
			(=AP004178)	Unpublished.
		OsMADS17	AF109153	Moon et al. (1999)
			(=AF095646)	Unpublished.
			(=AL606688)	Feng et al. (2002)
			(=AY551918)	Unpublished.
	Poa annua	*PaMADS1*	AF372840	Unpublished.
	Triticum aestivum	*TaMADS12*	AB007505	Murai et al. (1998), Electronic Plant Gene Register PGR98-159
	Zea mays	*ZAG3*	L46397	Mena et al. (1995)
		ZAG5	L46398	Mena et al. (1995)
Zingiberales				
Musaceae	*Musa acuminata*	*MuaMADS2*	AY941799	Unpublished.
EUDICOTS				
Ranunculales				
Papaveraceae	*Eschscholzia californica*	***Es.ca.AGL6***	KC899704	This study.
Ranunculaceae	*Ranunculus bulbosus*	*RbAGL6*	AY306184	Litt and Irish (2003)
Core eudicots				
Saxifragales				
Saxifragaceae	*Ribes sanguineum*	***Ri.sa.AGL6***	KC899705	This study.
ROSIDS				
Vitales				
Vitiaceae	*Vitis vinifera*	*VvMADS3*	AF373602	Boss et al. (2002)
FABIDS				
Cucurbitales				
Cucurbitaceae	*Cucumis sativus*	***Cu.sa.AGL6***	KC899706	This study.
Fabales				
Fabaceae	*Pisum sativum*	*PEAM5*	AY884289	Hecht et al. (2005)
Rosales				
Rosaceae	*Malus domestica*	*MdMADS11*	AJ000763	Yao et al. (1999)
MALVIDS				
Brassicales				
Brassicaceae	*Arabidopsis thaliana*	*AGL6*	M55554	Ma, Yanofsky et al. (1991)
		AGL13	U20183	Rounsley et al. (1995)
	Brassica oleracea	*BoAGL6a*	AJ508055	Unpublished.
		BoAGL6b	AJ508409	Unpublished.
ASTERIDS				
LAMIIDS				
Lamiales				
Oleaceae	*Syringa vulgaris*	*SvAGL6*	AY306188	Litt and Irish (2003)
Solanales				
Solanaceae	*Solanum lycopersicon*	*SlMBP6*	TC146409*	Hileman et al. (2006)

Table 1. Continued

Classification	Taxa	Gene name	GenBank accession Number	Reference
	Petunia × hybrida	*pMADS4*	AB031035	Tsuchimoto et al. (2000)
CAMPANULIDS				
Asterales				
Asteraceae	*Chrysanthemum × morifolium*	*CDM104*	AY173062	Unpublished.
	Gerbera sp.	*grcd3*	AJ784157	Unpublished.

*tomato EST library number (http://tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato).

(*Ac.am.AGL6*), *Eschscholzia californica* (*Es.ca.AGL6*), *Ribes sanguineum* (*Ri.sa.AGL6*), and *Cucumis sativus* (*Cu.sa.AGL6*) (Table 1). Numbers following gene names indicate multiple homologs in each taxon. Because the two sequences of *E. bennettii* differ by one nucleotide, we treated them as different alleles (multiple clones were identified for each allele). Two pairs of sequences in *Z. vazquezii* and *M. grandiflora* were treated as different genes because they showed relatively high sequence differences, respectively (25% and 29% divergence in amino acid and DNA sequences between *Zamia* homologs and 15% and 17% sequence divergence between *Magnolia* homologs). Furthermore, each number of a pair was placed in a different clade in the phylogenetic tree (see phylogenetic analyses).

Aligned sequences of *AGL6*-, *AGL2*-, and *SQUA*-like genes showed that the first 20 amino acids of the I-domain were well aligned, as was the MADS-domain. In the I domain, a one-aa gap was detected in all *AGL6* sequences except a sequence from *Ranunculus* (AY306184) (Fig. 2A). In the 5' end of the K-domain region, a seven-aa insertion was found only in the *AGL6* from *Petunia*, *Lycopersicon*, *Syringa*, *Chrysanthemum*, and *Gerbera*, all asterids (APG III 2009) (Fig. 2B). In the same region, a gymnosperm-specific one-aa insertion was also detected (Fig. 2B).

Phylogenetic Analyses

In all phylogenetic analyses, *AGL2*-like genes were sister to *AGL6*-like genes, and *SQUA*-like sequences were sister to the clade of AGL2 + AGL6 (Fig. S1, summarized in Fig. 3). When we used *TM3*-like genes as the outgroup, the sister-group relationship between *AGL6*-like and *AGL2*-like genes was supported with 71% ML bootstrap and a posterior probability of 0.94 (Fig. 3A). When we used *AG*/*AGL12* genes as the outgroup, this relationship was weakly supported by ML bootstrap analysis, but the Bayesian posterior probability was 0.95 (Fig. 4B). In both analyses, the AGL6 + AGL2 + SQUA clade is highly supported, indicating a close relationship among these three gene groups (Fig. 3).

For intensive analyses of the *AGL6* group, various phylogenetic analyses were performed using selected *AGL2*-like genes as the outgroup (Fig. 4). Our phylogenetic tree of *AGL6* genes generally tracks organismal phylogeny as inferred from multigene molecular data sets (e.g., Soltis et al. 1999, 2000, 2011). Gymnosperm genes were sister to angiosperm genes, and these two clades were highly supported (posterior probabilities of 0.99 and 1.0, respectively). In the gymnosperm clade, two major lineages were clearly recognized, and these two clades had high posterior probability (1.0 and 0.96, respectively; Fig. 4). Each sublineage in the gymnosperm clade contains sequences from the four major gymnosperm lineages (Soltis et al. 2005): *Ginkgo*, cycads (*Zamia*), conifers (*Pinus* and *Picea*), and Gnetales (*Gnetum* and *Welwitschia*) (Soltis et al. 2005; Fig. 4), representing an ancient gene duplication in the common ancestor of gymnosperm *AGL6*. Although a duplication at the base of the eudicots was detected in a recent phylogenetic analysis of *AGL6*-like genes focused on core eudicots (Viaene et al. 2010), it is not shown in our tree because some core eudicot genes detected from recent genome studies (e.g., genes from *Vitis, Populus*, and *Citrus*) were not included in our study. Instead, localized gene duplications were detected in our tree: Magnoliales, Brassicaceae, and potentially Lauraceae (Fig. 4, large stars), respectively. Many recent duplications were also detected at the genus or species levels (Fig. 4, small stars). Our tree agrees well with that from a recent study focused on monocot MADS-box genes (Li et al. 2010). However, supporting values (posterior probabilities) for the eudicot and monocot clades in our tree are much higher: 1.0 rather than >0.5 for eudicots and 0.96 rather than >0.5 for monocots. Gaps and insertions recognized in the sequence matrix were synapomorphic characters in relevant clades (Fig. 4, gray bars on the nodes).

Discussion

Evolution of *AGL6*-like Genes in the MADS-box Gene family

Similar to previous studies (Becker and Theissen 2003; Nam et al. 2003; Pařenicová et al. 2003; Kim et al. 2005a; Gramzow et al. 2012), our phylogenetic analyses revealed a close relationship among *AGL6*-, *AGL2*- (E-class), and *SQUA*- (A-

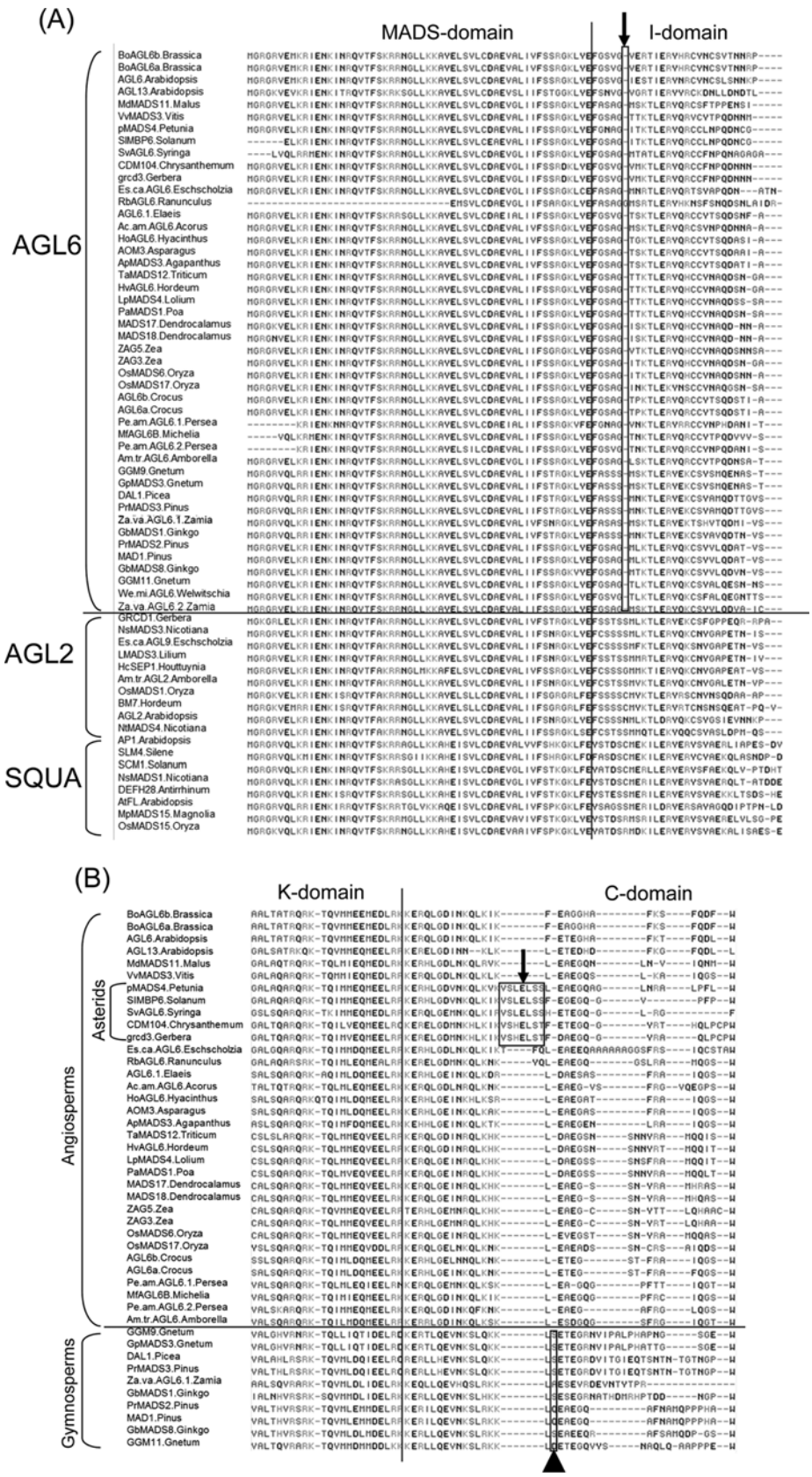

Fig. 2. A part of the alignment of the *AGL6*, *AGL2*, and *SQUA* genes. (A) *AGL6* genes have a one-amino acid deletion in the I-domain (arrow). (B) Gymnosperm-specific one-amino acid insertion (arrowhead) and asterid-specific seven-amino acid insertion were detected in the C-domain (arrow).

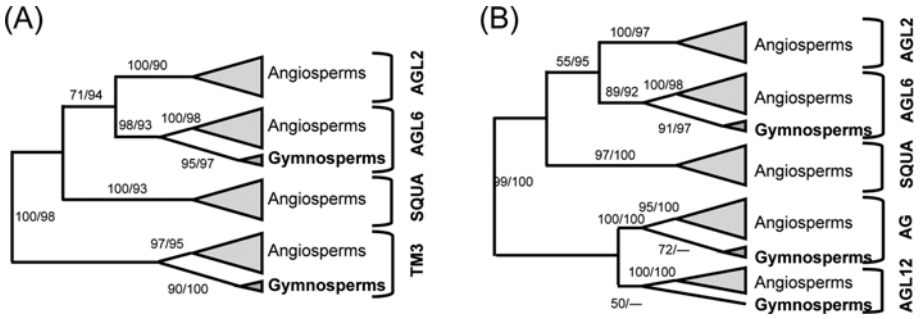

Fig. 3. Summary of phylogenetic analyses of *AGL6*-, *AGL2*-, and *SQUA*-like genes with *TM3*-like genes as outgroup (A) or *AG*-like plus *AGL12*-like genes as outgroup (B). Values above the nodes indicate bootstrap values in maximum likelihood analyses and posterior probability (X 100) in the Bayesian analyses of DNA analyses. "–" indicates value less than 50.

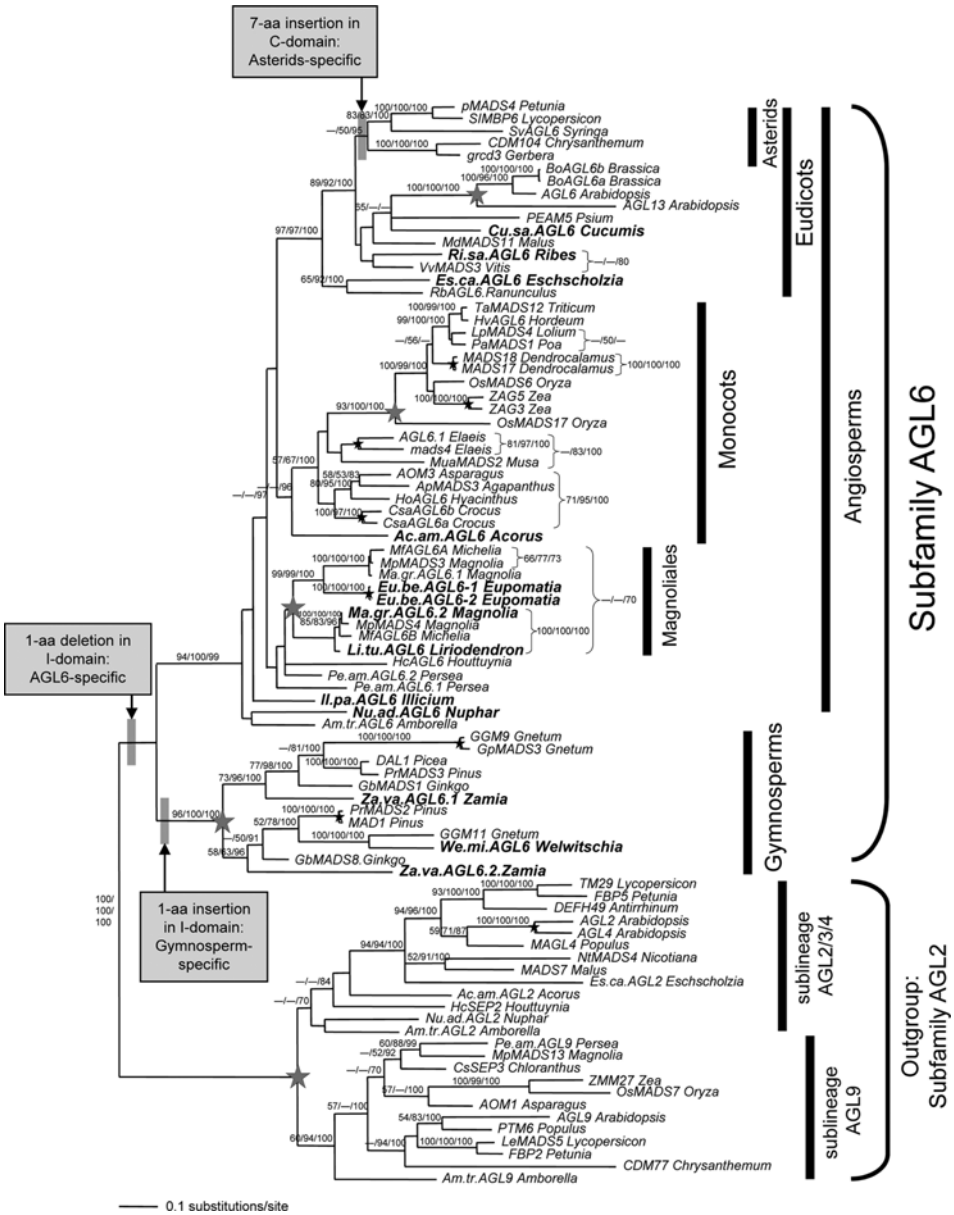

Fig. 4. Maximum likelihood tree of 63 *AGL6* genes and 25 representatives of *AGL2* genes as outgroup. Large stars indicate recent duplications at the genus or species level and small stars indicate duplications at the higher level. Three numbers above each node indicate bootstrap values from parsimony and maximum likelihood analyses and posterior probability from Bayesian analysis (X100). "–" indicates value less than 50.

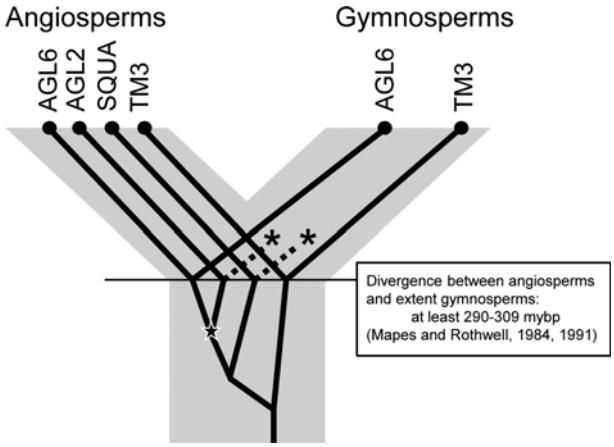

Fig. 5. A hypothesis for the evolutionary history of *AGL6*-, *AGL2*-, and *SQUA*-like genes based on the phylogenetic analyses with *TM3*-like genes as outgroup (Fig. 3A). A duplication between *AGL2*- and *AGL6*-like genes occurred before the divergence of angiosperms and gymnosperms (star). *SQUA* and *AGL2* genes have not been detected in gymnosperms (asterisks).

class) like genes. Furthermore, our phylogenetic analyses strongly demonstrated a sister relationship between *AGL6*-like genes and *AGL2*-like genes. MADS-box genes have diverged into many sublineages via several duplication events (Becker and Theissen 2003; Nam et al. 2003). We can estimate an approximate divergence time of clades by comparison with the organisms from which the genes derive, as long as this group forms a well-supported clade in the phylogenetic tree. For example, it is clear that the AGL6 + AGL2 + SQUA clade (L6L2SQ) diverged from other lineages of MADS-box genes before the split of angiosperms and extant gymnosperms because the L6L2SQ clade contains both angiosperm and gymnosperm genes (Fig. 5). However, of the three subclades found in the L6L2SQ clade, only the *AGL6* clade contains gymnosperm genes. *SQUA*- and *AGL2*-like genes have not been reported in gymnosperms. Although one *AGL2*-like gene from a gymnosperm, *Pinus radiata* (*PrMADS1*; U42399), has been published, this sequence grouped with other angiosperm *AGL2*-like genes in phylogenetic analyses (and was most closely related to a *Eucalyptus* sequence) (Zahn et al. 2005): the identity of this sequence is therefore in question, and it was excluded in the previous phylogenetic study of *AGL2*-like genes (Zahn et al. 2005).

The sister group of the L6L2SQ clade varies among the previous phylogenetic analyses of MADS-box genes (e.g., Winter et al. 1999; Becker and Theissen 2003; Nam et al. 2003). However, previous broad analyses, which contain all *Arabidopsis* and rice MIKC-type MADS-box sequences, suggest that *TM3*-like genes (Nam et al. 2003) or *AG*- plus *AGL12*-like genes (Becker and Theissen 2003) are the best candidate for the sister group of the L6L2SQ clade. Fig. 5 shows the possible evolutionary history of *AGL6*-like genes when the *TM3*-like genes are sister to the L6L2SQ clade. In this case, a duplication to produce *AGL6*-like and *AGL2*-like genes predates the divergence of angiosperms and extant gymnosperms because both the *TM3* and L6L2SQ clades contain gymnosperm and angiosperm sequences. We can explain gymnosperm-less *SQUA* and *AGL2* lineages by the losses of these genes in gymnosperms after the *SQUA* and *AGL2* lineages diverged from the common ancestor of the L6L2SQ clade (Fig. 5 asterisks). Alternatively, it is possible that gymnosperm *SQUA*- and *AGL2*-like genes have not yet been found. However, the former is more likely because these genes were not detected in the intensive screening of expressed genes conducted in reproductive organs of a cycad (ESTs of *Zamia vazquezii* in the Floral Genome Project; see below) or in the genome sequence of a conifer (*Picea abies*; Nystedt et al. 2013).

Extant seed plants originated approximately 290-309.2 mya (Mapes and Rothwell 1984; Mapes and Rothwell 1991), and most evidence indicates a very early split between the living gymnosperms and the line leading to angiosperms (Soltis *et al.* 2002; Donoghue 2010; Magallon 2010; Doyle 2012). Therefore, we hypothesize that the split between *AGL6*-like and *AGL2*-like genes occurred at least 290-309.2 mya based on the above assumptions. This scenario is the same when *AG/AGL12* is the outgroup because gymnosperm genes have also been reported in both *AG* and *AGL12* lineages.

The function of many *AGL2*-like and *SQUA*-like genes is now well characterized and includes the formation of each floral whorl in *Arabidopsis* and *Petunia* (Coen and Meyerowitz 1991; Kotilainen et al. 2000; Ditta et al. 2004). In contrast, the function of *AGL6*-like genes has only been characterized in a few cases (e.g., Rijpkema et al. 2009). Our phylogenetic analyses showed that *AGL6*-like genes are sister to *AGL2*-like genes, and *SQUA*-like genes are sister to these two groups. Recognizing phylogenetic relationships among these orthologous gene groups and their approximate divergence time, when combined with detailed investigation of their expression patterns, will provide a better understanding of the evolutionary history of the genetic control mechanisms of flowers.

Materials and Methods

Data Collection

We collected 62 *AGL6*-like sequences, including 13 newly identified sequences (Table 1). We searched for *AGL6*-like genes using amino acid BLAST (BLASTN) in GenBank with the *Arabidopsis AGL6* protein sequence as the seed and an e^{-5} cut-off level. Some sequences resulting from this search were members of the *AGL2* or *SQUA* subfamilies, but we were easily able to identify members of the *AGL6* subfamily, based on the result of initial phylogenetic analyses and sequence structure analyses (see Results). Through this search, we

identified 48 previously reported angiosperm and gymnosperm *AGL6*-like sequences from GenBank. We also blasted *AGL6* against the expressed sequence tag (EST) collection of the Floral Genome Project (FGP; http://www.floralgenome.org) (cut-off level was e^{-5}). After phylogenetic analysis of these sequences with 108 previously reported *Arabidopsis* MADS-box genes (Pařenicová et al. 2003), we identified 10 *AGL6*-like sequences (phylogenetic analysis not shown) from the FGP EST collections. Genes detected from EST libraries were completed by additional sequencing of identified clones using universal primers included in the library vector. We also added a sequence from the tomato EST library (http://tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato).

In the case of sequences of *Eupomatia bennettii* and *Ribes sanguineum*, we screened MADS-box genes from cDNAs of young flowering buds (vouchers are *Endress 5197* and *S. Kim 1143*, respectively) using a MADS-box-specific degenerate primer (5'-GGGGTACCAAYMGI-CARGTIACITAYTCIAAGMGIMG-3'; Kramer et al. 1999). For RNA extraction, reverse-transcription, PCR, cloning, and sequencing, we followed the methods described in Kim et al. (2005b). We distinguished sequences of *AGL6*-like genes from other MADS-box genes using the same phylogenetic analysis approach described above.

Alignment and Phylogenetic Analysis

To clarify the phylogenetic position of *AGL6*-like genes in the MADS-box gene family, we constructed a DNA matrix containing *AGL6*-like genes (62 sequences), along with *AGL2*-like (107 sequences) and *SQUA*-like (91 sequences) genes, which were potential sister groups to *AGL6*-like genes in previous broad phylogenetic analyses (Becker and Theissen 2003; Nam et al. 2003; Pařenicová et al. 2003; Kim et al. 2005a; Gramzow et al. 2012). Then we added either representatives of *TM3*-like genes or *AGAMOUS* (*AG*)-like plus *AGL12*-like genes as the outgroup independently to test the effect of outgroup.

First, we translated these gene sequences to amino acid sequences using Se-Al (http://tree.bio.ed.ac.uk/software/seal/) and aligned the amino acid sequences of *AGL6*-like genes using CLUSTALX (version 1.83) (Thompson et al. 1997) with default options. Then alignments of 107 *AGL2*-like and 91 *SQUA*-like sequences used in the studies of Becker and Theissen (2003) and Zahn et al. (2005) (authors of these studies kindly provided their alignments) were combined with the *AGL6* matrix using the "profile alignment" method in CLUSTALX (file-to-file alignment). Because *TM3*-like genes or *AGAMOUS* (*AG*)-like plus *AGL12*-like genes were sister to the AGL6 + AGL2 + SQUA clade in the previous phylogenetic analyses (Becker and Theissen 2003; Nam et al. 2003), we included these two groups independently in our matrix to test the effect of outgroup. The alignment of amino acid sequences was converted into an alignment of DNA sequences using the program AA2DNA (http://www.mybiosoftware.com/alignment/5250) for DNA analyses.

Phylogenetic analyses were carried out on the nucleotide sequences using maximum-likelihood (ML) (Felsenstein 1981) and Bayesian inference (Huelsenbeck and Ronquist 2001) in PHYML (version 2.4) (Guindon and Gascuel 2003) and MrBayes (version 3.0b4) (Huelsenbeck and Ronquist 2001), respectively. Prior to these analyses, we selected the best model of molecular evolution using MODELTEST (version 3.06) (Posada and Crandall 1998). The GTR + I + Γ model of nucleotide substitution, which assumes general time reversibility (GTR), a certain proportion of invariant sites (I), and a gamma distribution to accommodate rate variation among sites (Ã), was selected for both matrices. For ML analysis, ML parameter values were optimized with a BIONJ tree as a starting point (Gascuel 1997). Support values for nodes on the ML tree were estimated with 250 bootstrap replicates (Felsenstein 1985). For Bayesian analyses, we ran four chains, sampling one tree every 1,000 generations for 10,000,000 generations (starting with a random tree). When we plot log likelihood values to trees from every 1,000 generations, stationarity was reached at approximately 150,000 generations in both analyses; thus, the first 150 trees were considered the "burn in" of the chain, and phylogenetic inferences were based on those trees sampled after 150,000 generations.

For intensive analyses of the *AGL6* group, a reduced data set, which contains *AGL6*-like genes and 25 selected representatives of *AGL2*-like genes as outgroup, was analyzed: for the *AGL2*-like genes, we selected one or two genes from major lineages of angiosperms for the *AGL9* and *AGL2/3/4* sublineages (see Fig 4). In addition to ML and Bayesian analyses (same conditions as full matrix), maximum parsimony (MP) analysis was also conducted for the reduced data set using PAUP* (version 4.0b10) (Swofford 2001): the heuristic search strategy involved 100 random addition replicates with TREE bisection-reconnection (TBR) branch swapping, saving all optimal trees. Bootstrap analyses (Felsenstein 1985) were performed using 500 re-samplings and 10 random addition replicates with TBR branch swapping, saving all optimal trees.

Supporting Information

Additional Supporting Information is in the online version of this article:
Fig. S1. Maximum likelihood tree of *AGL2*-like, *AGL6*-like, and *SQUA*-like genes with outgroup of *TM3*-like genes (A) and *AG*-like/*AGL12*-like genes (B), respectively.

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Author's Contributions

SK carried out sequence analysis, phylogenetic analyses, interpreted the results, and drafted the manuscript. PSS and DES jointly contributed to the conception and coordination of the study, were involved in revising the manuscript. All authors agreed on the contents of the paper and post no conflicting interest.

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