

Original Article

## Isolation of an *AGAMOUS* homolog from *Zamia muricata*

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### Abstract

*AGAMOUS* (*AG*) is a class C MADS-box gene that plays an important role in the development of reproductive organs in angiosperms and gymnosperms. Here, an *AGAMOUS* homolog was isolated from male and female *Zamia muricata*. Lengths of nucleotide sequences were 1,262 and 1,537 bp, respectively, and both represented 672 bp of open reading frame corresponding to 224 amino acid residues of predicted protein. The amino acid sequences showed high similarity to *AG* protein from *Cycas* (*C. elephantipes*, *C. nongnoochiae*, *C. taitungensis*, *C. pranburiensis* and *C. edentata*) and revealed a conserved region at MADS-box (M) and Keratin-like box (K). A phylogenetic tree based on the amino acid sequences of *AG* protein of angiosperms and gymnosperms indicated that *Zamia* and gymnosperms were grouped together with high bootstrap support.

**Keywords:** *AGAMOUS*, cycad, gymnosperms, phylogenetic tree, *Zamia*

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### 1. Introduction

Cycads are the oldest living seed plants with a fossil record stretching back to at least the early Paleozoic period, approximately 250 million years ago (MYA) (Schwendemann, Taylor, & Taylor, 2009; Zhifeng & Thomas, 1989). They form a monophyletic group classified as a single order, Cycadales, which has been divided into two suborders with three families and approximately 331 species (Osborne, Calonje, Hill, Stanberg, & Stevenson, 2012). The family Cycadaceae contains only a single genus *Cycas*; the family Stangeriaceae consists of two genera, *Bowenia* and *Stangeria*; while the family Zamiaceae is the most diverse and includes the genera *Ceratozamia*, *Chigua*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas* and *Zamia* (Stevenson, 1992).

Several previous studies of cycad phylogenetic relationships were based on molecular markers (Chaw, Walters, Chang, Hu, & Chen, 2005; Crisp & Cook, 2011; Zgurski *et al.*, 2008). All these phylogenetic analyses indicated the positions of *Bowenia*, *Stangeria* and *Dioon* as incongruent with earlier classifications based on morphology (Stevenson, 1992). Recently, a new classification represented the order Cycadales as consisting of two families: Cycadaceae with the single genus *Cycas* and Zamiaceae with 9 genera as *Bowenia*, *Ceratozamia*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas*, *Stangeria* and *Zamia* (Christenhusz *et al.*, 2011). In all genera of cycads (except *Cycas*) males and females produce compact strobili (cones) composed of either megasporophylls or microsporophylls on separate plants. *Zamia* is the second largest and most diverse genus of the order Cycadales consisting of more than 70 species (Osborne *et al.*, 2012). Although male and female cones are generally dissimilar in shape and size, the sex of cycads cannot be distinguished at the juvenile stage. Most cycads have a very long life cycle.

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The time from germination until the seedling grows into a reproductive plant is unknown for most species but would appear to be a very long period (Stevenson, 1990). The molecular mechanism of cycad sex determination has so far remained elusive.

In higher plants, genetic control of reproductive organs is explained by the ABCDE model where the class C genes specify the formation of stamens and carpels in the third and fourth whorls of the flowering plant (Theissen, 2001). The C-class gene of *Arabidopsis thaliana* is the *AGAMOUS* (*AG*) gene which encodes a MADS-box transcription factor. *Arabidopsis ag* mutants reveal that the stamens and carpels are replaced by petals in a new flower (Bowman, Drews, & Meyerowitz, 1991). *AG* orthologous genes have been studied in many plants species that revealed a conserved functional role in flower development. The *AGAMOUS* gene is also present in gymnosperms and controls the development of megasporophylls and microsporophylls. Therefore, this gene was present before the divergence of gymnosperms and angiosperms lineages (Lovisetto, Baldan, Pavanello, & Casadoro, 2015; Zhang, Tan, Pwee, & Kumar, 2004) indicating that the genetic pathways controlling flower and cone development are probably homologous (Rutledge *et al.*, 1998). In this study, we investigated DNA sequences of the *AGAMOUS* gene from male and female *Zamia muricata* that may be related to sex determination.

## 2. Materials and Methods

### 2.1 Plant materials

Megaspores and microspores of *Z. muricata* were collected from plants cultivated in Nong Nooch Botanical Tropical Garden, Chonburi, Thailand.

### 2.2 Isolation of RNA and cDNA synthesis

Total RNA was isolated from megaspores and microspores using RNeasy Plant Mini Kit (QIAGEN, Germany). First strand cDNA was synthesized from total RNA using adapter-(dT)<sub>17</sub> primer (P18C1, Table 1) by ReverTra-Ace- $\alpha$ -kit (Toyobo, Japan) according to the manufacturer's instruction. The oligo dT primer was added as an adapter at 5' end to facilitate RACE or high throughput sequencing.

Table 1. Primers used in this study

Name	Nucleotide sequence (5'-3')
AG-F	ATTGAGATAAAGAAGATCGAGAA
AG-R	TTTCATCCAAGCTGAAGGGC
P19C1	GGCTGCAGTCGACATTGATTTTTTTTTT TTTTTTT
P18C1	GGCTGCAGTCGACATTGA
SP1 (5'-RACE)	CTGCCTGAGTTTTCTGCCTCC
SP2 (5'-RACE)	GCGTGTGTCAGCGCAAGTCT
SP3 (5'-RACE)	GCCACTTCTGCATCACATAGCAC
Intron1-F	AAGGCTGTATGTTTGCTAATA
Intron1-R	CTCTCGATTGTTCTTCACG
Intron2-F	GGAGCCATTTCAGAGTCCAAT
Intron2-R	CTGCCTCTGTTGCCAGTA
Intron3-F	GGCAGCAGATTGACATTCTAC
Intron3-R	TTCAGGTCCCGGATTAGGA

### 2.3 RT-PCR

cDNA was used as a template for PCR amplification with specific primers designed from the *AGAMOUS* genes of *Cycas edentata* (AY295079) and *Ginkgo biloba* (AY114304). PCR amplification was performed in 50  $\mu$ l of reaction mixture consisting of 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, 1.25 units of *Taq* DNA polymerase, 10  $\mu$ M of each primer (AG-R and AG-F, Table 1) and cDNA template. PCR was carried out at an initial denaturation of 3 min at 94 °C, followed by 35 cycles each with 30 s at 94°C, 30 s at 58°C, 1 min at 72°C and a final extension of 7 min at 72 °C. PCR products were fractionated in 1% agarose gel and DNA bands were visualized using ethidium bromide staining. The amplified cDNA was cloned into pGEM-T easy vector (Promega, USA) and sequenced.

### 2.4 3'-rapid amplification of cDNA ends (3'-RACE)

The 3'-end of *AGAMOUS* gene was amplified using gene-specific primer (AG-F) and adapter primer (p18C1) (Table 1). PCR was carried out at an initial denaturation of 3 min at 94 °C, followed by 35 cycles each with 30 s at 94 °C, 45 s at 58 °C, 90 s at 72 °C and a final extension of 7 min at 72 °C. PCR products were fractionated in 1% agarose gel and DNA bands were visualized using ethidium bromide staining. The PCR product was cloned into pGEM-T easy vector (Promega, USA) and sequenced.

### 2.5 5'-rapid amplification of cDNA ends (5'-RACE)

Three gene-specific primers (SP1, SP2 and SP3) were designed from the 3'-RACE *AGAMOUS* gene sequence. The 5'-RACE was performed according to the method described by Frohman, Dush, & Martin (1988) with minor modification. First-strand cDNAs for 5'-RACE were synthesized using a 5'-RACE kit (Roche) with SP1 primer. cDNA was amplified by gene-specific primer (SP2) and oligo dT-anchor primer. A PCR product was used as a template to perform a nested PCR with gene-specific primer (SP3) and anchor primer. The PCR product was cloned into pGEM-T easy vector (Promega, USA) and sequenced.

### 2.6 PCR amplification of intron of the *AGAMOUS* gene

Genomic DNA was extracted from megaspores and microspores of *Z. muricata*. Specific primers were designed from cDNA sequences in this study and the *AGAMOUS* gene of *Cycas edentata* (Table 1). PCR was carried out at 94 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were extracted and purified from agarose gel bands, cloned into pGEM-T easy vector (Promega, USA) and sequenced.

### 2.7 Phylogenetic analysis

*AGAMOUS* sequences were edited and assembled using the program GeneStudio (<http://genestudio.com/>). DNA sequences were deposited in the DDBJ/EMBL/GenBank DNA

database under accession numbers. Nucleotide sequences translation was performed with the ExPASy program (<http://web.expasy.org/translate>). The predicted amino acid sequences and relative *AGAMOUS* gene from GenBank were aligned using ClustalX multiple sequence alignment software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). A phylogenetic tree was reconstructed using the Maximum likelihood method (ML) of MEGA 7 program (Kumar, Stecher, & Tamura, 2016). JTT+G (Jones-Taylor-Thornton with invariable sites) was the best amino acid evolution model and the log likelihood for the analysis = -1682.8914. ML heuristic searches were performed by applying the Neighbor-Joining (NJ) method to a matrix of pairwise distances and all positions containing gaps and missing data were eliminated. Supports for clusters on the tree were assessed with 1,000 replicates of bootstrapping. GenBank accession numbers of amino acid sequences used were as follows: *C. elephantipes* [AKG92785.1], *C. nongnoochiae* [AKG92785.1], *C. pranburiensis* [AKG92785.1], *C. taitungensis* [AKG92785.1], *C. edentata* [AAM74074.1], *Ginkgo biloba* [BAD93166.1], *Pinus radiata* [AAD09342.1], *Magnolia paenetaluma* [AFH74399.1], *Arabidopsis thaliana* [P17839.2], *Nicotiana benthamiana* [AFK13159.1], *Meliosma dilleniifolia* [AAS45686.1] and *Brassica napus* [XP\_013668951.1].

### 3. Results and Discussion

#### 3.1 Isolation of the *AGAMOUS* gene from *Zamia muricata*

Total RNA isolated from megaspores and microspores of *Z. muricata* was used as a template to synthesize complementary DNA (cDNA). Primers for amplification of the *AGAMOUS* gene (AG-F and AG-R) were designed from *C. edentata* and *G. biloba* (Table 1). These primers were used to amplify cDNA from megaspores and microspores of *Z. muricata*. The PCR products were approximately 622 bp in length. Percentage of similarity of this sequence was closer to five *Cycas* species (*C. elephantipes*, *C. nongnoochiae*, *C. taitungensis*, *C. pranburiensis* and *C. edentata*) than to *G. biloba*. The 5'-RACE primers (SP1, SP2 and SP3, Table1) were designed from this sequence. The first strand cDNA was synthesized using P19C1 primer. Subsequently, the P18C1 and AG-F primers were used in PCR reaction to amplify the 3' end fragment. DNA sequences of *Z. muricata* varied in length between sexes; 981 bp (*Z. muricata* male) and 1,202 bp (*Z. muricata* female). Difference in size was in the 3' untranslated region (3' UTR) that showed variation of polyadenylation sites. Accordingly, the *GMB5* gene of *G. biloba* associated with *AGAMOUS* was also reported to have multiple polyadenylation sites (Jager, Hassanin, Manuel, Guyader, & Deutsch, 2003). However, two sequences which represented polyadenylation signal (TATAA) were found in 3' UTR before poly A tail. For the 5' end fragment, the sequences of two samples were different in size. The full-length cDNA was 1,262 bp in male and 1,537 bp in female, and the GenBank accession numbers for the two sequences were KP238767 and KP23866, respectively. These two sequences could be further used to design specific primers for sex differentiation in this plant.

The contig from the assembled sequences revealed the same open reading frame corresponding to 224 deduced

amino acid residues in *Z. muricata* (male and female). In addition, both *AGAMOUS* sequences of *Zamia* male and female revealed the TIERYKK motif in I domain as the typical amino acid sequence present in class C protein in the ABC model (Tandre, Svenson, Svensson, & Engström, 1998; Zhang *et al.*, 2004). A protein database search showed that the amino acid sequence of *Zamia* gave 100% and 96% similarity to AG protein from some *Cycas* and *G. biloba*, respectively. Sequences alignment with AG proteins from gymnosperms (*Z. muricata* (male and female), *C. elephantipes*, *C. edentata*, *C. nongnoochiae*, *C. pranburiensis*, *C. taitungensis*, *G. biloba* and *Pinus radiata*) and angiosperms (*Arabidopsis thaliana*, *Magnolia paenetaluma*, *Meliosma dilleniifolia*, *Brassica napus* and *Nicotiana benthamiana*) revealed that *Zamia AGAMOUS (ZmAG)* was MIKCC-type domain (Figure 1). AG homologs had MIKC domain structure that was characteristic of type II or MIKC-type MADS-box proteins (Kramer, Jaramillo, & Di Stilio, 2004). Absence of NH<sub>2</sub>-domain was found in the *ZmAG* and this also did not appear in all *AGAMOUS* protein gymnosperm families (Jager *et al.*, 2003). *ZmAG* possessed the MADS-box conserved regions commonly found in gymnosperms and angiosperms containing 56 amino acid motifs, involved in binding to DNA based on a consensus CC(A/T)GG sequence (Shore & Sharrocks, 1995; Zhang *et al.*, 2017). In addition, conserved AG motifs I and II were found at C domain and were also represented in AG homologs in angiosperms (Kramer *et al.*, 2004) indicating that *ZmAG* in this study should be functional.

#### 3.2 Genomic organization of the *ZmAG* gene

Alignment of cDNA and the *AGAMOUS* gene in the *Cycas* genome showed that the *ZmAG* consisted of nine exons and eight introns, in which intron numbers and positions were conserved among various plants (Hong, Hamaguchi, Busch, & Weige, 2003; Lee & Pijut, 2017; Liu & Liu, 2008). Three pairs of primers (Intron1-F/R, Intron2-F/R and Intron3-F/R) were designed from the exon region and successfully used to amplify the respective intron fragments of *Z. muricata* (male and female). Primers Intron-1F/R and 2F/R could be used to amplify the second and third intron, respectively, while Intron3-F/R primers were used for amplification of the fourth to seventh intron. Two clones of the second intron were isolated from male and female plants appeared to contain fragments of the same length (1,432 bp), whereas the second intron of *A. thaliana* was longer than that of *ZmAG* (about 3 kb) (Liu & Liu, 2008). Alignment of this intron sequence shared 99% identity with the difference between male and female at 3 positions; 214, 610 and 722 (Figure 2). In contrast, DNA sequences of intron 3 (642 bp), intron 4 (102), intron 5 (163), intron 6 (159) and intron 7 (198) were not different in both plants. This result indicated that the second intron might correlate with development of megaspores and microspores similar to the second intron of *Arabidopsis* which was reported to be a promoter enhancer (cis-regulatory sequences) for expression of carpel and stamen (Hong *et al.*, 2003; Liu & Liu, 2008). Reverse orientation of the second intron sequence of *AGAMOUS* orthologue from *Populus trichocarpa* has also been fused with the minimal 35S promoter (*rPTAG2I*) and could be used as a promoter for cytotoxic gene expression in tobacco (Li *et al.*, 2016).

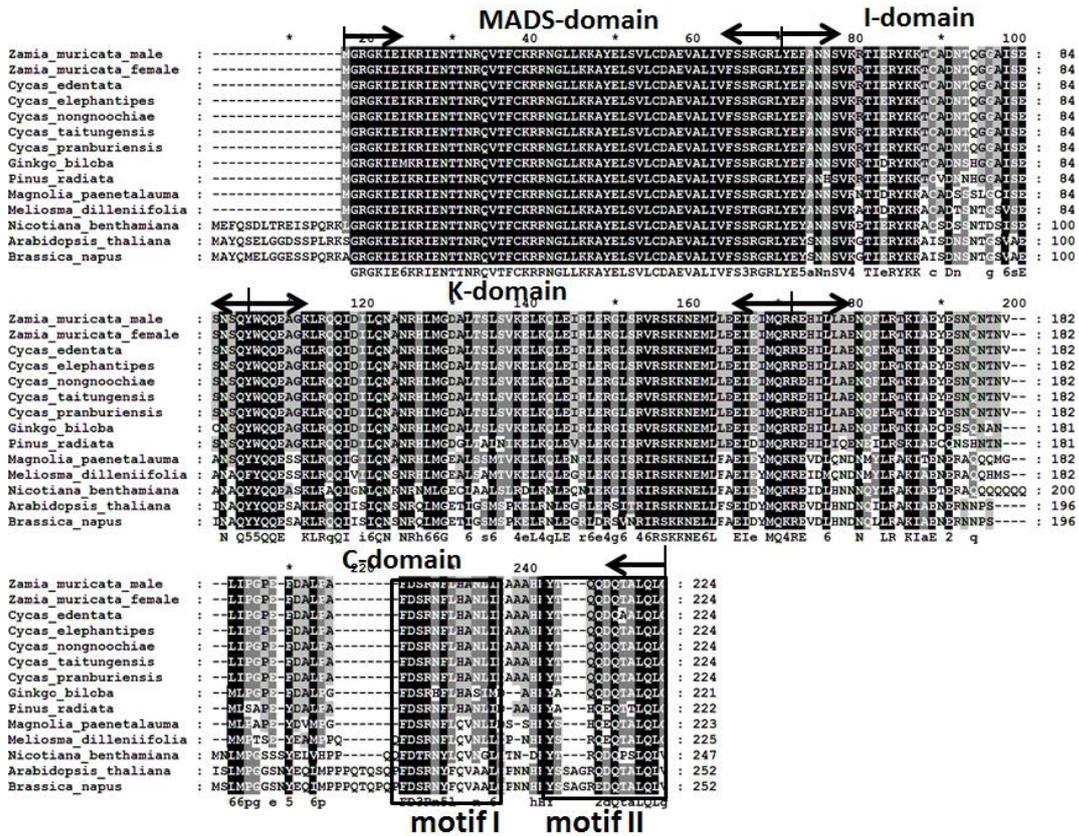


Figure 1. Amino acid sequence alignments of *AGAMOUS* from *Z. muricata* (male), *Z. muricata* (female), *C. edentata*, *C. elephantipes*, *C. nongnoochiae*, *C. taitungensis*, *C. pramburiensis*, *Ginkgo biloba*, *Pinus radiata*, *Meliosma dilleniifolia*, *Magnolia paenetaulauma*, *Nicotiana benthamiana*, *Arabidopsis thaliana* and *Brassica napus*. Identical amino acid residues are black and conserved residues are grey. Dashed lines indicate gaps. A line is drawn above the MADS-box domain, the I-domain, the K-box domain and the C-domain of AG. Boxes are motif I and II.

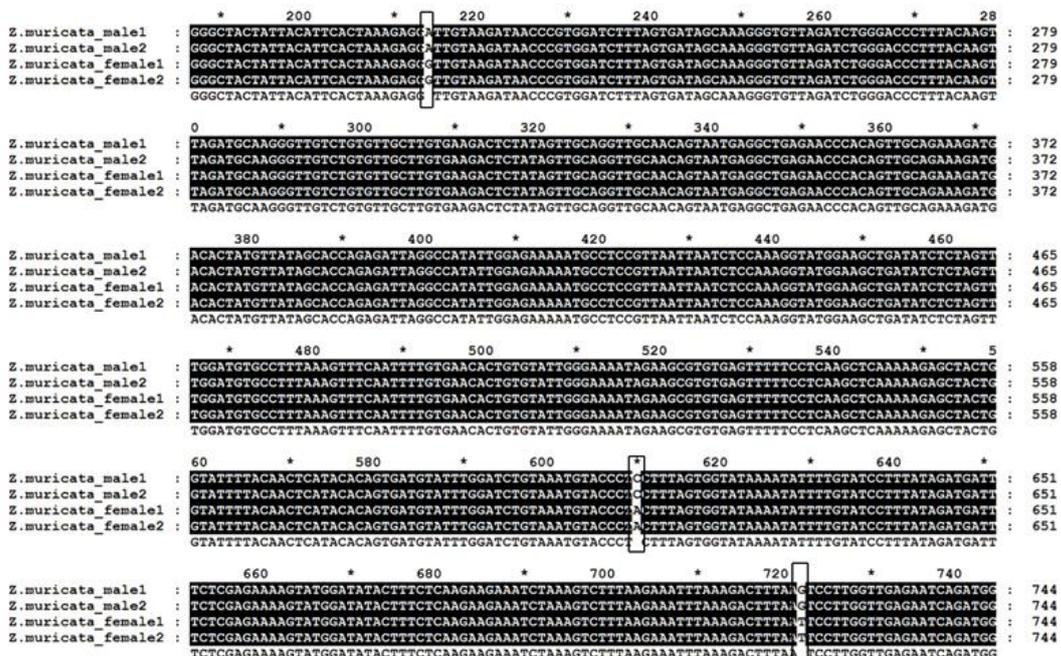


Figure 2. Partial DNA sequence alignments in the second intron of *Z. muricata* (male and female). Boxes represent base substitution sites.

### 3.3 Phylogenetic analysis

A phylogenetic tree was reconstructed using the maximum likelihood method for determination of the relationships among *AGAMOUS* protein sequences from gymnosperms and angiosperms (Figure 3). The phylogenetic tree could be divided into 2 groups; the first group *ZmAG* was clustered with *AGAMOUS* from gymnosperms. This group comprised *C. elephantipes*, *C. edentata*, *C. pranburiensis*, *C. taitungensis*, *C. nongnoochiae*, *Z. muricata* (male and female) and *AGAMOUS* homologues found in *G. biloba* and *P. radiata*. This result indicated a monophyletic group with high bootstrap support. The second group consisted of *M. paenetaulauma*, *M. dillenifolia*, *N. benthamiana*, *A. thaliana* and *B. napus* which were all in angiosperms and eudicotyledons. This group divided into two sub-groups. *M. paenetaulauma* and *M. dillenifolia* represented a first sub-group that did not have NH<sub>2</sub>-terminal. In contrast, a second sub-group (*N. benthamiana*, *A. thaliana* and *B. napus*) represented an NH<sub>2</sub>-terminal domain that was a fundamental characteristic of the core eudicot members (Kramer *et al.*, 2004). *AGAMOUS* in the basal eudicots are usually related to ovule development while the core eudicots, *AGAMOUS* have been shown to play a role in stamen and carpel development (Carvalho, Schnable, & Almeida, 2018; Jager *et al.*, 2003). Phylogenetic analysis suggested a close relationship of *AGAMOUS* genes from *Cycas* and *Zamia*. The *AGAMOUS* MADS-box gene may have emerged at least 300 million years ago and controlled reproductive organ development in the common ancestor of gymnosperms and angiosperms (Lovisetto *et al.*, 2012; Zhang *et al.*, 2004).

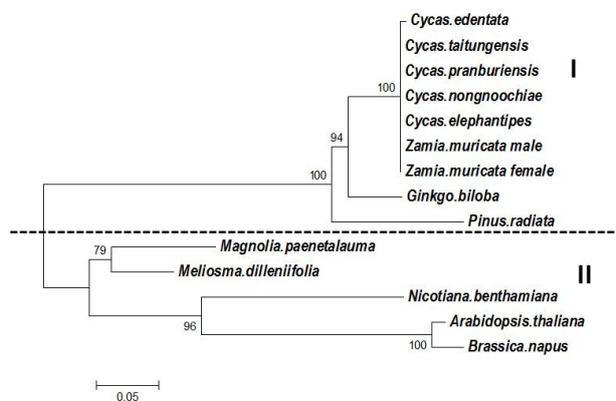


Figure 3. Maximum likelihood analysis of *AGAMOUS* amino acid sequences. Values above branches represent bootstrap values (1000 replicates).

### 4. Conclusions

*AGAMOUS* cDNA sequences were identified from male and female *Z. muricata*. The length of nucleotide sequences varied as 1,262 and 1,537 bp, respectively. Difference in size of female *Z. muricata* occurred in the 3' untranslated region that showed variation of polyadenylation sites. However, both sequences showed 672 bp of open reading frame corresponding to 224 amino acid residues of predicted protein. In addition, the third to seventh introns isolated from male and female had the same sequence and length. However, the

sequence of the second intron was different between male and female at 3 positions; 214, 610 and 722. Protein BLAST showed high similarity to AG protein from *Cycas* and the conserved regions at MADS-box (M) and Keratin-like box (K) as special characters of an *AGAMOUS* protein. Phylogenetic analysis revealed that *ZmAG* was placed in the gymnosperm *AGAMOUS* clade. These findings revealed that *AGAMOUS* genes controlling flower and cone development were homologous.

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