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Isolation and characterization of the *AGAMOUS* homologous gene *NTAG* in Chinese narcissus (*Narcissus tazetta* var. *chinensis* Roem)

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Abstract Amaryllidaceae, a monocot plant family, consists of many important ornamental bulb flower species. Chinese narcissus (*Narcissus tazetta* var. *chinensis* Roem), its flowers developed at high temperatures and bloomed at lower temperatures during the Chinese Spring Festival, is a traditional Chinese flower with high economic and ornamental value. To study its flower development, a full length cDNA containing MADS box domain from narcissus was isolated by a reverse transcription polymerase chain reaction (RT-PCR) with degenerate oligo-nucleotide primers derived from a conserved MADS- and K-box domain sequence. The 5′ and the 3′ regions of the gene were amplified using the PCR protocol for the rapid amplification of cDNA ends (RACE). The 690 bp open reading frame encodes 230 amino acid residues. A comparison of the deduced amino acid sequence of *NTAG* with the sequence of other MADS box proteins showed 91.3% amino acid identities with *HAG* (*Hyacinthus orientalis*). Sequence analysis and alignment showed significant similarity with other *AG* homologues. RNA blot analysis indicated that the narcissus *NTAG* gene was expressed only in reproductive organs, especially in stamens and carpels. These results indicated that the *NTAG* gene was an *AG* homologue and that the *AG* genes appeared to be structurally and functionally conserved between dicots and monocots.

Key words *NTAG* (*Narcissus tazetta* var. *chinensis* Roem *AGAMOUS*), cDNA amplified fragment length polymorphism, Chinese narcissus

1 Introduction

Despite the existing divergence among flower forms in different plant genera, a typical flower of an eudicotyledonous flowering plant consists of four whorls at the tip of a floral shoot: sepals, petals, stamens and carpels. Extensive molecular and genetic studies in the homeotic mutants in dicots such as *Arabidopsis thaliana* and *Antirrhnum majus* have led to the establishment of a genetic model (the ABC model) that explains how the fates of floral organ primordia are determined (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994, Ng and Yanofsky, 2000). According to the ABC model, flower organ identity genes can be divided into three classes corresponding to three homeotic functions A, B and C in *Arabidopsis thaliana* and *Antirrhium majus*. In this model, the A function genes alone control the sepal identity, the A and B function genes control the petal identity, the B and C function genes determine the stamen identity and the C function genes alone directs carpel identity. In *Arabidopsis*, the known A function genes are *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the B function genes are *APETALA3* (*AP3*) and *PISTILIA* (*PI*) and the only known C functional gene is *AGAMOUS* (*AG*). Only mutation in the *AGAMOUS* (*AG*) gene causes a complete loss of carpel identity and *ag* mutant flowers produce an indeterminate reiteration of the pattern: sepals, petals (Yanofsky et al., 1990). Petals replaced stamens in the third whorl. Carpels (in the fourth whorl) were replaced by a new flower that repeated the same pattern. It was proposed that A and C function genes are antigonistic such that A function genes prevent the action of C in sepal and petal, whereas C function genes prevent the action of A in stamens and carpels (Bowman et al., 1991). The evidence that A and C function genes act antigonistically has been obtained from the ectopic expression of AG genes under control of the CaMV 35S promoter. *AG* RNA accumulated in stamens and carpels throughout their development, consistent with its mutant phenotype. Transgenic *AGAMOUS* antisense gene plants of *A. thaliana*, *B. napus*, petunia, tobacco and tomato resulted in homeotic conversion of sepal to carpel and petal to stamen (Mandel et al., 1992; Kempin et al., 1993; Tsuchimoto et al., 1993; Pnueli et al., 1994; Mizukami and Ma, 1995).

Floral organ identity genes *AP1*, *AP3*, *PI* and *AG* encode transcription factors with a highly conserved DNA binding domain, the MADS-box and expressed only in the regions of the developing flower that require their activities (Riechmann and Meyerowitz,

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1997). The isolation of angiosperm and gymnosperm MADS box genes that exhibit strong sequence similarities to the ABC genes provides powerful tools to test evolutionary relationships of not only regulatory gene functions but also developmental structures. It is likely that the ABC MADS box genes have their origins in plants long before the appearance of angiosperms. This conclusion is based on molecular clock estimates and a phylogenetic analysis (Purugganan, 1997). In spite of this conservation, the precise functions of many MADS box genes remain unclear and it appears that, in addition to their essential roles during floral organ determination, they also act as regulators for various other aspects of plant development (Rounsley et al., 1995).

The monocot plant family Amaryllidaceae consists of many important ornamental bulb flower species. Chinese narcissus (*Narcissus tazetta* var. *chinensis*), a traditional Chinese flower with high economic value, grows in low temperature, dies in high temperature and blooms during the Chinese Spring Festival. In narcissus, instead of sepals and petals, flowers have two whorls of tepals which resemble petals. To understand the molecular mechanism of narcissus flower development, in this study, we isolated a narcissus *AGAMOUS* homologue, *NTAG*, with 690 bp an open reading frame and 230 deduced amino acid residues and investigated its expression profiles in different organs in Chinese narcissus.

2 Materials and methods

2.1 Plant material

Three-year-old bulbs of narcissus (*N. tazetta* var. *chinensis* Roem) were obtained from a commercial market. The bulbs were stored under natural conditions. After the flowers developed, some bulbs were grown in the field. Flower buds in bulb, blooming flowers, leaves and stems were harvested to isolate total RNA.

2.2 Total RNA isolation

Various organs such as capel, stamen, tapel, leaf and stem of narcissus were prepared by a guanidium thiocyanate extraction method, employing phenol/chloroform followed by precipitation of RNA with LiCl (Hu et al., 2001).

2.3 Reverse transcription (RT)-PCR

Two μg of total RNA were used in a 20 μL reverse transcription reaction as described by Mou et al. (2000) with some modifications. Briefly, the 2 μg of total RNA were denatured at 70°C for 10 min and quickly ice-quenched. Reverse transcription was performed in a 4 μL of 5× buffer, 1 μL of 10 $\mu mol \cdot L^{-1}$ dNTP, 1 μL of RNA inhibitor, 1 μL of 5 $\mu mol \cdot L^{-1}$ T_{16} adapter primer P3′-A (5′-ATTCCAGACCGGAATTCCGGCG ACAtG$(T)_{16}$(A/G/C)N-3′) and 5′RACE primer P5′-A (5′-AAGCAGTGGTATCAACGCAGAGTCCGGAAT TCCGGCGGG-3′) with 20 units of Supersript II RNA H⁻ reverse transcriptase (Gibco). The reverse transcription reaction was performed at 42°C for 1 h, then stopped by 10 min at 70°C heating followed by immediate incubation on ice.

The degenerate primers were synthesized according to the consensus sequences in the MADS- and K-box domain of different *AGAMOUS* homologues as follows:

AGUMF: 5′-GA(G/A) GT(T/C) GCN CT(C/T/G) (A/G)TN GT(C/T) TTC TC-3′

AGMF: 5′-GGA(A/G)G(A/G)GG(A/G)AA(G/A)A (T/C)(T/C) GA(T/C)GA -3′

AGMR: 5′-(T/G)TNTGCA(A/G)NTC(A/C)A(T/C) (T/C)TCCCT-3′

100-μL PCR was performed with 2.5 μL of the first-strand cDNA, 10 μL of 10× PCR buffer, 8.0 μL of 2.5 $mmol \cdot L^{-1}$ dNTPs (promega), 5 μL of 100 $\mu mol \cdot L^{-1}$ degenerate primer and 4.0 units of Taq polymerase on PE-9600. The reaction was carried out under the following conditions: 94°C for 3 min and then 35 cycles of 94°C for 40 s, 56°C for 2 min, 72°C for 1 min 30 s with a final step of 72°C for 10 min.

2.4 Isolation full length gene with RACE-PCR

In order to obtain the full-length gene, RACE (rapid amplified cDNA end)-PCR was performed as described by Mou et al. (2002) with some modifications. Briefly, the nest primers were designed according to the partial cDNA sequence. The 3′-RACE-PCR was performed with 5 μL of 2 $\mu mol \cdot L^{-1}$ Enf (5′-ATTCCA GACCGGAATTCCGGC-3′) and 2 $\mu mol \cdot L^{-1}$ NTAGF (5′-CTCGTGGTTTTCTCTACCCGT-3′), then 5 μL of 2 $\mu mol \cdot L^{-1}$ Enf and 5 μL of 2 $\mu mol \cdot L^{-1}$ NTAGF (5′-AGAAGCTTCCAAGT TGCGCCA-3′).

The 5′RACE-PCR was performed with 5 μL of 2 $\mu mol \cdot L^{-1}$ P5′-B (5′-AGCAGTGGTATCAACGCAGA GT-3′) and 5 μL of 2 $\mu mol \cdot L^{-1}$ NTAG1R (5′-TGG CGCAACTTG GAAGCTTCT-3′), then 5 μL of P5′-B NTAG2R (5′-TGCTGATGCCTTTCTCTAGCC-3′).

RACE-PCR reaction was performed as above under the following conditions: 94°C for 3 min, then five cycles of 94°C for 10 s, 72°C for 3 min, followed by five cycles of 94°C for 10 s, 70°C for 20 s, 72°C for 3 min, followed by 30 cycles of 94°C for 10 s, 68°C for 20 s, 72°C for 3 min, then with a final step of 72°C for 10 min.

All PCR products were separated on 1.2% agarose gel, subcloned into pGEM-T easy (Promega) vector

and sequenced.

2.5 Northern hybridization

Twenty μg total RNA of each lane from different tissue leaves and flowers of narcissus were separated on agarose gel containing 10% formaldehyde and transferred onto a hybond-plus membrane (Amersham). RNA gel blot was performed as described previously (Hu et al., 2001) using NTAG cDNA as probe.

Microscopy: a narcissus bulb was dissected and flower development observed with a stereomicroscope (model SZX-12, Olympus, Tokyo, Japan).

3 Results

3.1 Morphology of narcissus flowers

To provide narcissus flower development, the morphology of flower development was investigated. Figures 1A and 1B showed the inflorescence and the mature flower of narcissus. The mature flower was composed of four whorls of floral organs. Both first and second whorls consisted of three tepals. The third whorls had six stamens and the forth whorl has a gyneocium. Early flower development was observed by stereomicroscopy. Figures 1C and 1D showed the florescence development. The second flower on the top of florescence was fully developed; however, the third and the first flowers were then in the development phase. The sixth flower was not formed at that stage.

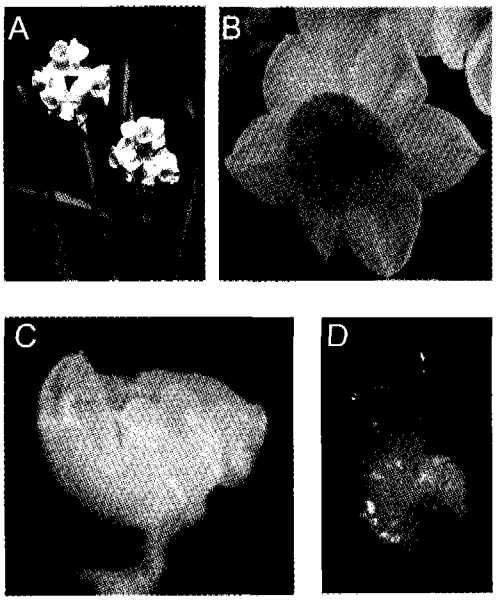

Fig. 1 Inflorescence and flowers of *Narcissus tazetta* var. *chinesis*. A: an inflorenscence; B: front view of a flower; C: early development of flower on the florescence; D: different flower developmental stage.

3.2 Isolation of *NTAG* gene

In order to understand the mechanism of narcissus flower development, we isolated the C-function genes with limited information about Chinese narcissus. The first strand cDNA was prepared from mRNA isolated from unopened flowers of *N. tazetta* var. *chinensis*. RT-PCR products of 350, 500 and 750 bp were obtained with highly degenerated primers AGUMF and AGMR. These three RT-PCR products were sequenced and blasted against the GenBank and found that the 350 bp RT-PCR product has a high similarity to the AGAMOUS gene of *Arabidopsis thaliana*. Nest primers were designed according to the 350 bp RT-PCR product. All 3′RACE-PCR and 5′RACE-PCR products were separated, isolated, sequenced and blasted against the GenBank and we found that the 750 bp 5′RACE-PCR product and 500 bp 3′RACE-PCR product had great similarity with the *AGAMOUS* homologues with 200 bp overlap. The full length of the gene sequence is 1,418 bp encoding 230 amino acid (Fig. 2).

The isolated cDNA sequence is 1,304 bp excluding the poly (A^+) tail. It has an open reading frame of 690 bp. The deduced amino acid sequence contains the conserved MADS box domain between amino acid residues 2 and 57 and the K box domain between amino acid residues 92 and 158 (underline). This gene was nominated *NTAG* (*Narcissus tazetta* var. *chinensis AGAMOUS* homologue).

Several *AGAMOUS* homologues have now been isolated from dicotyledons and monocotyledons. The hypothetical amino acid sequence of this *AGAMOUS* homologue exhibits significant similarity to the members of the MADS box gene family. From data base sequence analysis we deduced that the NTAG protein shares 67% identity amino acid residues with *AGAMOUS* (*Arabidopsis thaliana*). The *NTAG* amino acid sequence is most homologous to *HAG* (*Hyacinthus orientalis*) with 91.3% identity and in the MADS domain, there is 96% identity (100% similarity) in the amino acids (Fig. 3).

3.3 Phylogeny of MADS-box genes

To explore the evolutionary relationships among the isolated narcissus MADS box genes, a phylogenetic gene tree was constructed based on a comparison of the amino acid sequence of *NTAG* and a selection of well characterized plant MADS-box genes (Fig. 4). The result shows that the *NTAG* belongs to the C function gene class *AG* rather than to another seed plant MADS genes group. In the *AG* group, *NTAG* is more closely related to *HAG* and other *AGAMOUS* homologues of monocotyledons than that from dicotyledons and gymnosperm plants.

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tgctgatgcctttctctagccccccacttatccttgaaatcaatggcaatggatgcttgagtcaatagccactctcccaccctctttgtagttctcccactttaattgaattagctggagagaagaacataga 130
gagatttccccctttccttcacccaaaaaaaaaaaaacaattacttttatcagagaggagaaatacgtacatggagagttgattcttagttcataaataagcccctcataaccaaaagcaacvctcattcctc 260
ctattgcacctctctatctcacaagggaaagaaggagaggacatcctccaccttaacaaaccaaccctttttcttcgtttcttcacaacggtcaccacggagtcgagaaggctggatcccaaggagaag 390
ATG GGT AGG GGG AAG ATA GAG ATC AAA AGG ATC GAA AAC ACG ACT AAT AGG CAA GTC ACT TTT TGC AAG CGT CGA AAT 468
M  G  R  G  K  I  E  I  K  R  I  E  N  T  T  N  R  Q  V  T  F  C  K  R  R  N                          26
GGG TTG CTC AAA AAG GCC TAT GAA TTG TCC GTG CTC TGC GAT GCG GAG GTC GCC CTT ATC GTC TTC TCT ACC CGT GGC 546
G  L  L  K  K  A  Y  E  L  S  V  L  C  D  A  E  V  A  L  I  V  F  S  T  R  G                          52
CGC CTC TAT GAA TAT GCA AAC AAC AGT GTG AAA GCG ACC ATT GAG AGA TAC AAG AAA GCA TGC ACT GAT ACA TCC AAC 624
R  L  Y  E  Y  A  N  N  S  V  K  A  T  I  E  R  Y  K  K  A  C  T  D  T  S  N                          78
ACT GCC ACT GTC TCT GAG GCT AAT TCT CAG TAC TAC CAA CAA GAA GCT TCC AAG TTG CGC CAG CAA ATA ACC AAC TTA 702
T  A  T  V  S  E  A  N  S  Q  Y  Y  Q  Q  E  A  S  K  L  R  Q  Q  I  T  N  L                          104
CAG AAT TCT AAC AGG AAT TTG ATG GGG GAG TCT CTG AGC ACA GTG AGC CTT AGG GAC CTG AAG CAG CTT GAG AGC AGG780
Q  N  S  N  R  N  L  M  G  E  S  L  S  T  V  S  L  R  D  L  K  Q  L  E  S  R                          130
CTA GAG AAA GGC ATC AGC AAA ATA AGA ACT AAA AAG AAT GAG TTA TTG TTT GCT GAA ATT GAA TAT ATG CAA AAA AGG 858
L  E  K  G  I  S  K  I  R  T  K  K  N  E  L  L  F  A  E  I  E  Y  M  Q  K  R                          156
GAG ATT GAG TTG CAA AAC GAT AAC ATG TAC CTA CGC AAT AAG ATA ACT GAT AAT GAG AGA GCA CAA CAG CAA ATG AAC 936
E  I  E  L  Q  N  D  N  M  Y  L  R  N  K  I  T  D  N  E  R  A  Q  Q  Q  M  N                          182
ATG CTG CCA TCT GCT GCT ACA ACT TCA ACT CAT GAT CAG TAC GAG GGG ATA CCC CAA TTT GAT TCA AGA AAC TTC CTC 1,014
M  L  P  S  A  A  T  T  S  T  H  D  Q  Y  E  G  I  P  Q  F  D  S  R  N  F  L                          208
CAA GTG AGC TTG ATG GAT CCC GGT CAC CAC TAC TCG CGC CAG CAG CAG ACT ACC CTT CAA CTG GGA TGA acgatgatagatggaat 1,200
Q  V  S  L  M  D  P  G  H  H  Y  S  R  Q  Q  Q  T  T  L  Q  L  G  *                                    230
gactggaggtgccgcttcacgaggcttgggcaagtgaaattttaatgcatgaaggcagtagaagtcttctttctgtgtatgtcacgtataaaagctgcactttgttcgaaaacttaagaactaggagacg 1,328
tctatgctcttatgaatgttaaatgactgtatgacaaatgactggctactatatattgttggngtactactattaaaaaaaaaaaaaaaa                                        1,418
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Fig. 2 DNA sequence and deduced amino acid sequence of NTAG. The MADS-box DNA-binding domain (bolds) and K-box domain are underlined. An asterisk represents the termination coden, 3′ Utr and 5′ Utr is delineated by carets.

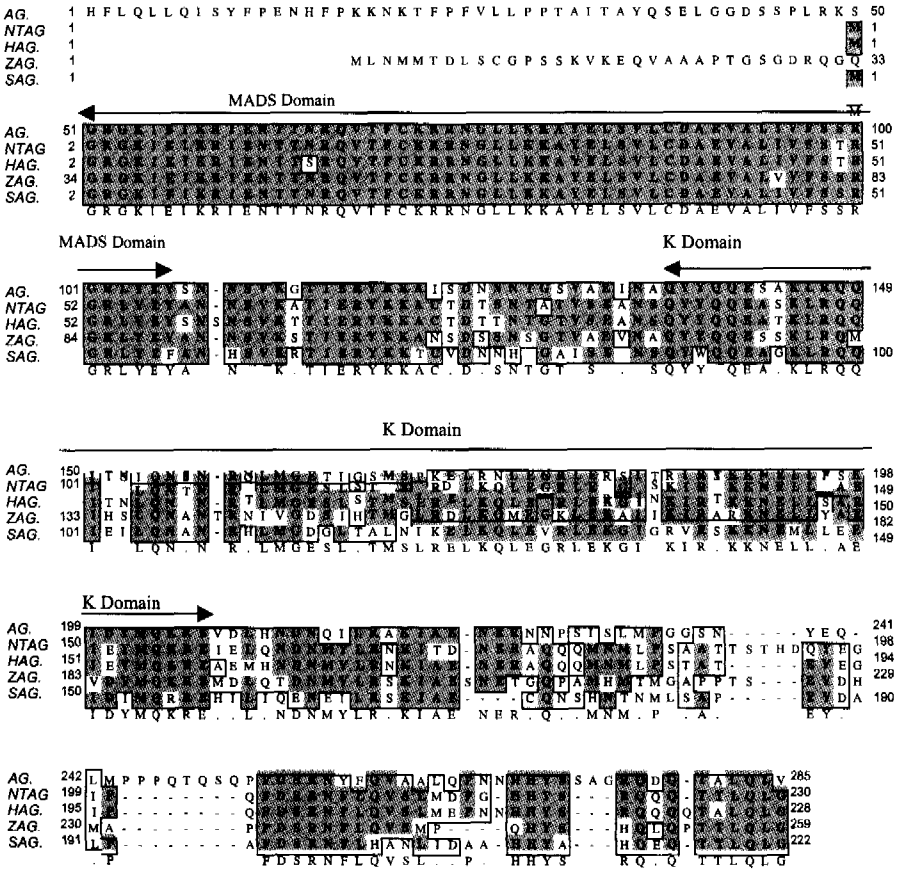

Fig. 3 Alignment of deduced amino acid sequences from selected *AG* homologues in different plants. The MADS-box domain and K domain are indicated by double-ended arrows above the sequences. Amino acids conserved in at least three of the five sequences are shaded and dots indicate gaps inserted into the sequence to optimize the alignment. The alignment was generated using the DNASTAR ALGNMENT program. *AG* homologue selected in different plant as follow: *AG*, *Arabidopsis thiana*; *Zag1*, *Zea mays*; *Hag1*, *Hyacinthus orientalis*; *Sag1*, *Picea mariana*.

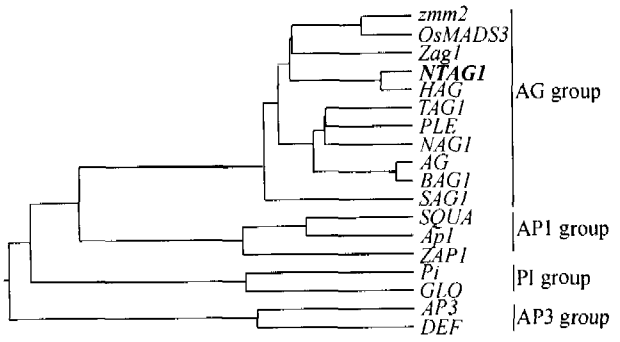

Fig. 4 Phylogenetic tree of plant MADS box proteins to illustrate the degree of relatedness between the AGAMOUS homologues of different plants obtained by the Neighbor Joining method. The sequence encoded by the gene isolated in this study is shown in bold text and the distance along the horizontal axis is indicative of differences between the sequences. The groups of the genes which fall into are indicated by the bar on the right of the figure. The GenBank accession number of all the amino acid sequences used above and abbreviations are as follows: *Tag1*, *Lycopersicon esculentum* (L26295); *Nag1*, *Nicotiana tabacum* (L23915); *Ag*, *Arabidopsis thiana* (X53579); *Bag1*, *Brassica napus* (M99415); *Plena*, *Antirrhinum majus* (S53900); *OsMADS3*, *Oryza sativa* (L37528); *Zag1*, *Zea mays* (L18924); *Zmm2*, *Zea mays* (X81200); *OsMADS3*, *Oryza sativa* (L37528); *Hag1*, *Hyacinthus orientalis* (AF099937); *Sag1*, *Picea mariana* (U69482); *Def*, *Antirrhinum majus* (X52023); *AP3*, *Arabidopsis thaliana* (M86357); *Ap1*, *Arabidopsis thaliana* (Z16421); *ZAP1*, *Zea mays* (l46400); *Squa*, *Antirrhinum majus* (Q38742).

3.4 Expression of *NTAG* gene in different organs of narcissus

In order to analyze the expression of *NTAG*, results of north hybridization using *NTAG* as a probe are shown in Fig. 5. The transcription accumulates at higher levels in flower stamens and carpals. The *NTAG* expression was not detectable in leaves, stems and tepals and only expressed in the third and forth whorl of the flower. The expression pattern of *NTAG* resembles that of *AG* and *TAG*.

4 Discussion

Narcissus flowers were developed under high temperatures. When the three-year-old narcissus bulbs were stored at 4°C after its leaves died in spring, no flowers were developed (data not shown). The flowers were developed at high temperatures in the summer. In order to study the roles of floral organ identity genes in narcissus, partial cDNA fragments were obtained by RT-PCR with high degenerate primers according to the highly conserved MADS box domain. The full length of this gene was obtained by RACE-PCR (Rapid Amplified cDNA End PCR). Comparison of gene sequences shows that *NTAG* and *HAG*, have high identity and share significant similarity. *HAG* is an *AGAMOUS* homologue in *Hyacinthus* (Li et al., 2002), its expression is similar to the *NTAG* expression in narcissus in the inner part of the flower. Both plants are monocotyledons and have a similar flower shape.

Most of the isolated and functional analyzed MADS box genes came from dicotyledons; a limited number of monocotyledon MADS box genes have been studied. This study shows that the *AGAMOUS* subfamily can still be divided into three clusters. *NTAG* and other homologues in the monocots belonged to the same cluster, although the flower shape and size of the narcissus and hyacinth are very different from that of rice and maize. In rice, the *AGAMOUS* homologue OsMADS3 gene determined the third and fourth whorls floral organ identity (Kang et al., 1998; Kyozuka and Shimamoto, 2002). The *ZAG1* gene, an *AGAMOUS* homologue in maize, did not greatly affect the identity of reproductive organs (Mena et al., 1996). A second *AGAMOUS* homolog, *ZMM2*, has a distinct expression pattern of from that of *ZAG1*. C-function organ identity genes in maize have two closely related genes, *ZAG1* and *ZMM2*, with overlapping but non-identical activities. In our study only one C-function gene was isolated, unlike maize. The second cluster includes all the *AGAMOUS* homologues of dicotyledons. The third cluster includes the *AGAMOUS* homologue isolated from fir (Rutledge et al., 1998). The last com-

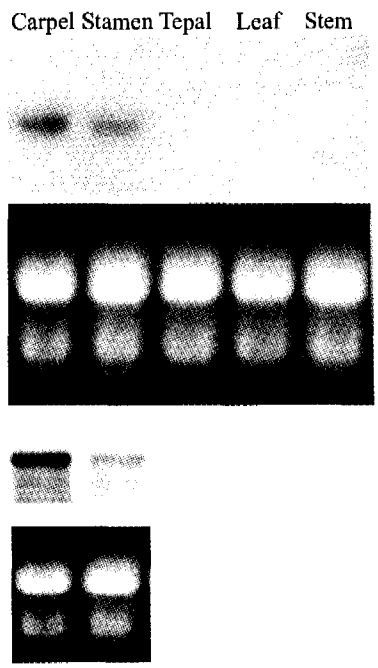

Fig. 5 Expression analysis of narcissus *NTAG* in different tissue: unopened flower bud, leaf, petal. Gel separated total RNA (4 μg) was blotted and hybridized to *NTAG* cDNA. The source of the tissue for each RNA sample is indicated above each lane.

mon ancestor of angiosperms and gymnosperms (a progymnosperm) is believed to have existed 285–350 million years ago (Martin et al., 1993; Munster et al., 1997). Monocotyledons diverged from dicotyledons about 180 million years ago and have evolved flowers that are distinct from those of dicots (Wolfe et al., 1989). Although different plants with different flower morphology, genes with similar functions in different angiosperms and gymnosperms consistently group together. All the *AGAMOUS* homologues of different monocotyledons are in the same subclass.

The *NTAG* expression was only detected in the third and forth whorl of the flower. Its expression could not be detected in tepals and vegetative tissues such as leaf and stem. In summary, *NTAG* cDNA sequence, putative amino acid and its pattern are similar to *AGAMOUS* of *Arabidopsis thaliana* gene suggesting that *NTAG* is the homologue of the *A. thaliana AGAMOUS* from narcissus.

Acknowledgements

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