Ectopic expression of an AGAMOUS homolog NTAG1 from Chinese narcissus accelerated earlier flowering and senescence in Arabidopsis

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Abstract There are two cultivating varieties of Chinese narcissus, named as Yulinglong (Narcissus tazetta var. chinensis Roem. Florepleno) and Jinzhanyutai (Narcissus tazetta var. chinensis M. Roem.) well known in China. Yulinglong plants exhibit double flower resulted from petaloid stamens. However, the molecular basis of double flower formation is known little and unclear. Based on the flowering ABCDE model, double flower formation is commonly used to link to C functional genes. In this present paper, the isolation and characterization of NTAG1 gene, an AGAMOUS homolog from Chinese narcissus varieties mentioned above are reported. Sequence and expression pattern of NTAG1 gene exhibited the same in both tested varieties. It expresses only in the reproductive organs. Furthermore, functional analysis by using ectopic tests in Arabidopsis showed that NTAG1 might be involved in the carpel identity and floral transition. The effects of ectopic expression of NTAG1 mainly include dwarfing, early flowering, losing inflorescence indeterminacy, branch number increasing, and advancing senescence, whereas some of homeotic phenotypes gradually disappeared in higher generation of transgenic plants. The utilizations of NTAG1 gene in the future gene engineering were also discussed in the paper.

Keywords Chinese narcissus AGAMOUS homologue; NTAG1; Flower development; Senescence

Background Certain flower characters including the flowering time, floral architecture and petal color are usually concerned by ornamental plants breeders. Double flowers resulted from increased number of petals were selected for their showy appearance in many domesticated plant families. Chinese narcissus (Narcissus tazetta var. chinensis) is popular flower with high cultural value in the ornamental market. But the problem of unitary culture variety have become to be the restrict factor for the Chinese Narcissus industry achieving further development. There are only two cultivating varieties, named as Yulinglong (Narcissus tazetta var. chinensis Roem. Florepleno) and Jinzhanyutai (Narcissus tazetta var. chinensis M. Roem.) respectively, in Chinese narcissus. The flower of Yulinglong exhibits double flower resulted from petaloid stamens. However, the molecular basis of the double flower formation is known little and unclear. And there are only a few reports about flower formation of Chines narcissus. The genetic mechanism regulating floral formation is extensively studied in model plants and the ABCDE model has been established (Bowman et al., 1989; Parcy et al., 1998; Pelaz et al., 2001; Pelaz et al., 2000; Roeder and Yanofsky, 2001). In this model, A and E gene classes determine sepal identity, A, B and E determine petal, B, C and E specify stamen, C and E specify carpel and D functional gene is involved in the development of ovule identity. Functional flower organ identity genes including APETALA1 (A class), APETALA3 and PISTILLATA (B class), AGAMOUS (AG) (C class), FLORAL-BINDING PROTEIN (FBP) 7 and FBP11 (D class), and SEPALLATA1/2/3 are (E class) have been isolated (Coen and Meyerowitz, 1991; Colombo et al., 1997; Colombo et al., 1995; Drews et al., 1991; Jofuku et al., 1994; Mandel et al., 1992; Pelaz et al., 2000; Rounsley et al., 1995; Theissen, 2001; Weigel and Meyerowitz, 1993; Weigel et al.,
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1992). Most of these genes (except APETALA2) belong to MADS-box transcription factors gene family which control floral organ identity (Melzer et al., 2009; Saedler et al., 2001; Theissen, 2001). The C-function gene AG plays a central role not only in specifying sexual organ identity but also in determining floral meristem termination (Bowman et al., 1989; Lohmann and Weigel, 2002). In Arabidopsis, AG mutation results in the expansion region of the A gene class into the center of the flower, which makes stamens change into petals and carpels into sepals. In addition, there are additional abnormal flower produced in the center of ag flower. There is increasing evidence that the role of floral organ genes is conserved in different plants, although differences in regulation, redundancy and function of these genes exist between species (Ferrario et al., 2006).

Based on the flowering ABCDE model, double flower formation is commonly used to link to C functional genes. NTAG has been reported being a putative AG ortholog cloned from Chinese narcissus only by the sequence similarity and the expression pattern analysis (Wang et al., 2006). However, no further functional analysis was performed to indicate its involvement in carpel development. In this presmt paper the isolation and characterization of NATG1 gene, an AG homolog from both Chinese narcissus varieties mentioned above are reported. Sequence and expression pattern of NTAG1 gene exhibited the same in both tested varieties. Furthermore, functional analysis by using ectopic tests in Arabidopsis showed that NATG1 might be involved in the carpel identity and floral transition. The utilizations of NATG1 gene in biotechnology are discussed.

1 Results

1.1 Isolation and characterization analysis of NTAG1 genes from two narcissus varieties plants

The flowers of Jinzhanyutai consist of five whors of organs including three sepals, three petals, a golden cup-shaped corona, six stamens, and three fused carpels (Figure 1A). The sepals and petals are white, extremely similar and known as tepals (Figure 1A). Yulinglong plants produce metamorphotic flowers in which stamens are petal-like structure (Figure 1B, Figure 1B C). In order to study the mechanism of such double flower development, we isolated the NTAG1 gene, a putative C-function gene with the information reported by Wang (Wang et al., 2006) from both narcissus plants respectively. Sequence analysis showed that NTAG1 genes from both varieties plants were exactly same. Comparison of the sequence with that presented by Wang (Wang et al., 2006) showed that there were 5 bases pair variation between them. However, there was only one residue changed in the K-box when the deduced amino acid sequence was compared (data not shown). The reason of the sequence difference may be caused by PCR or resulted from the plant materials got from different places.

The NTAG expression has been shown only in the third and forth whorl of the flower of Jinzhanyutai by northern blot (Wang et al., 2006). To further explore whether its expression changed in Yulinglong, semi-quantitative RT-PCR was performed and the results revealed that the expression pattern was nearly similar in both varieties plants, it was only expressed in flowers, but not detectable in leaves, stems and roots (Figure 2A).

1.2 Ectopic expression of NTAG1 caused early flowering and affected the floral organ identity in transgenic Arabidopsis plants

To further investigate whether the sequence and structure similarity (Wang et al., 2006) is coupled to the functional similarity between NTAG1 and C functional genes, We performed functional analysis through transgenic plants. NTAG1 cDNA driven by cauliflower mosaic virus 35S promoter was therefore transformed into Arabidopsis plants. Twenty-one independent transgenic Arabidopsis T1 plants in Col and nine lines in Ler ecotype were obtained through kanamycine screening (results not shown) and PCR (Figure 2B). Most transformed plants produced nearly similar and severe transformation in all development (Figure 1D-J). NTAG1 overexpressing plants in Ler showed reduced size (Figure 1E, compared with 1D), early flowering, and small and curled leaves with yellow tips (Figure 1F). Wild type and transgenic phenotype plants were segregated in T1 generation. All transgenic plants flowered significantly early. Some
lines of transgenic plants showed significantly small structure (showed by arrows in Figure 1G). Some lines showed similar tall as Wt but with obvious curled rosette leaves (Figure 1H). The structure of most flowers of 35S::NTAG1 in Col and Ler in the T₁ generation was similar as that of wild type (Wt),

Figure 1 A-C: The flower structure of Chinese narcissus; Bar=4 mm; A: The flower of Jinchanyutai; B: The flower of Yulinglong; C: The innerest two wholes of Yulinglong; The arrow shows the carpel; D-H: Phenotypes of transgenic Arabidopsis plants expressing 35S::NTAG1 in the T₁ generation; D,A: 14-d-old Wt Arabidopsis (Ler), Bar=3 mm; E-F, A: 14-d-old (E) and a 20-d-old (F) NTAG1 overexpressing plant in Ler showed reduced size, early flowering, and small and curled leaves; Arrow shows thin and curled leaf with yellow tips, Bar=3 mm; G-H: Eighteen-d-old transgenic plants expressing 35S::NTAG1 in Col in the T₁ generation; Wild type and transgenic phenotype plants segregated in this generation; All transgenic plants flowered significantly early; Some lines of transgenic plants showed significantly small structure (showed by arrows in G) with only 1 cm tall during this stage. Some lines showed similar tall as Wt but with obvious curled rosette leaves (H), Bar=3 mm; I: Most flowers of 35S::NTAG1 in Col in the T₁ generation are smaller, Bar=250 μm; J: First-whorl organs were transformed into carpelloid sepals with stigmatic papillae (showed by arrows), Bar=2 mm; K: Compared with Wt, the 35S::NTAG1 transgenic plants flowered early (left) by producing only six-seven small, curled rosette leaves (right), Bar=5 mm; L: The 35S::NTAG1 transgenic plants (right) began to senescence when Wt plants (left) bolted, Bar=10 mm; M-N: Scanning electron micrographs of sepals observed in Wt (M) and 35S::NTAG1 transgenic plants (N), Bar = 20 μm
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Figure 2 A: The expression of NTAG in Chinese narcissus; B: The 35S::NTAG1 transgenic plants were verified by PCR using genomic DNA as templates; C: The 35S::NTAG1 transgenic plants were verified by RT-PCR; D: The expression SAG12 gene was enhanced in the 35S::NTAG1 transgenic plants; E: The MDA content was higher in the 35S::NTAG1 transgenic plants however the size of flowers was smaller (Figure 1J). Three lines of transgenic plants produced transformed flowers, first-whorl organs were transformed into carpelloid sepals containing stigmatic papillae (showed by arrows in Figure 1J) and second whorl organs absent sometimes, the typical homeotic conversion of sepals and petals similar to that observed in ap2 mutants or AG ortholog overexpressed transgenic Arabidopsis plants (Mizukami and Ma, 1992; Mizukami and Ma, 1997; Tzeng et al., 2002).

Because the phenotypes varied with the expression of the transgenic gene, the expression of NTAG1 gene was analyzed in five lines of the homozygous kanamycin-resistant T3 generation plants by RT-PCR. The results showed that NTAG1 gene were ectopic expressed in these plants (Figure 2C). Compared with Wt, the 35S::NTAG1 transgenic plants flowered early (Figure 1K, left) by producing only six-seven small, curled rosette leaves (Figure 1K, right, Table 1), loss of inflorescence indeterminacy. The branch number and the number of inflorescence buds of the transgenic plants were increased (Table 1). All transgenic plants produced terminal flowers (Figure 1K) similar to those observed in Arabidopsis plants ectopically expressing the AG or AG orthologs (Mizukami and Ma, 1992; Rutledge et al., 1998). However, there was no obvious homeotic conversion of flower organs in the transgenic plants. When the epidermal cells of the flower organs were examined by scanning electron micrographs, the inner three whorls were morphologically similar to the Wt organs epidermis (data not shown). The surface of irregularly shaped cells in Wt sepals are cuticular thickening, whereas the surface of irregularly shaped cells along with the interspersed stomata in Wt carpels are smooth (Tzeng et al., 2002). A 35S::NTAG1 flower produced sepals nearly similar to wild type except that surface cells containing less cuticular thickenings (Figure 1M, N).

Additionally, we also investigated, the morphological features of transgenic plants (Table 1). The height of 35S::NTAG1 plants was 5.6 cm, which was shorter than that of Wt (11.5 cm). The leaf was smaller, and the siliques was shorter (the average siliques length of 35S::NTAG1 plants was 7.5 mm versus 10.5 mm of Wt), and the number of branch per plant increased significantly (the average branch number of 35S::NTAG1 plants was 8.1 versus 4.0 of Wt).

1.3 Ectopic expression of NTAG1 accelerated senescence in transgenic Arabidopsis plants

Even in the vegetative stage, the leaves produced by

| Plants   | Leaf number (pc) | Leaf length (mm) | Plant height (cm) | Branch number (pc) | Siliques length (mm) |
|----------|-----------------|------------------|-------------------|--------------------|----------------------|
| 35S::NTAG1 | 7.3 ± 1.1       | 8.9 ± 1.1        | 5.6 ± 2.1         | 8.1 ± 2.6          | 7.5 ± 1.0            |
| Wt       | 9.6 ± 0.8       | 12.9 ± 1.0       | 11.5 ± 3.6        | 4.0 ± 2.6          | 10.5 ± 1.3           |
the 35S::NTAG1 transgenic plants were often yellow which is characteristic of senescence. Most leaves of the transgenic plants began to lose green when Wt plant bolted (Figure 1L). To analyze the effect of the ectopic expression of NTAG1 on senescence, the MDA content, a stress or senescence indicator, was assayed in the 14-d-old plants. It showed that the transgenic plants had much higher MDA level than Wild type plants (Figure 2E). In addition, the expression of SENESCENCE ASSOCIATE GENE 12 (SAG 12), a senescence marker (Gan and Amasino, 1995) was advanced by ectopic expression of NTAG1 (Figure 2D).

2 Discussion
In Arabidopsis, AG is required for both floral meristem determinacy and reproductive organ identity (Bowman et al., 1989; Lohmann and Weigel, 2002). In Arabidopsis, AG mutation results in the expansion region of the A gene class into the center of the flower, which makes stamens change into petals and carpels into sepals. In addition, there are additional abnormal flower produced in the center of ag flowers suggesting that the floral meristem of this mutant is indeterminate. To explore the possible molecular mechanisms involved in double Narcissus flower, we isolated and characterized NTAG1 gene, an AG homolog from both Chinese narcissus varieties. Both the results that the sequence and expression pattern exhibited same in both varieties, and evidence of functional NTAG1 gene existing in double flowers showed by ectopic tests in Arabidopsis, indicate that the increase of petal number in narcissus flower is not caused by the sequence mutation or a deregulation of expression of NTAG1 gene. Gao etc. al (Gao et al., 2008) isolated a MADS-box gene NTMADS1, an AG ortholog, from Chinese narcissus double flower plants and speculated that the sequence variation from that of NTAG is the reason of double flower formation in narcissus. According to our data, such sequence difference may be caused by PCR or resulted from the plant materials got from different places. Based on the flowering ABCE model, the double flower might be caused by the restriction of the AG ortholog expression, or by the expansion of A-function genes expression domains (Dubois et al., 2010). So further analysis of NTAG1 expression domains, the isolation and characterization of other C-function genes and A-function genes in narcissus, will be necessary to draw a clear conclusion.

Not similar to the most AG homolog transgenic plants (Mizukami and Ma, 1992; Mizukami and Ma, 1997; Tzeng et al., 2002), ectopic expression of NTAG1 in Arabidopsis is not often accompanying with the homeotic transformation flower (Less than 10% lines in T1 generation). Especially with the generation increased, the transgenic plants developed normal flowers. There are three explanations for such data. Firstly, the expression of NTAG1 gene in most transgenic plants, especially in higher generations, was not enough for the flower transformation. Secondly, NTAG1 gene might not be the only one C-function genes in narcissus. Indeed, the C-function is shared by two partially redundant genes in many plant species (Dubois et al., 2010). For instance, AG performs the sexual organ identity and floral meristem termination, whereas, SHATTERPROOF (SHP) is involved in later carpel development stages in Arabidopsis (Causier et al., 2005). Conversely, in Antirrhinum majus L., the ortholog of SHP, PLENA is essential for sexual organ identity (Davies et al., 1999). It is therefore of interest to identify SHP lineage in narcissus. Additionally, in line with the quartet model (Theissen, 2001), another reason might be the different NTAG1 interaction factors existed in narcissus from that in Arabidopsis.

The novel phenotypes of the NTAG1 transgenic plants include early flowering, losing inflorescence indeterminacy and increasing branch number, which are usually candidate characters of ornamental plants selected by people. So it will be an ideal candidate gene for the genetic modification in narcissus or other plants. However, ectopic expression of NTAG1 accelerated earlier senescence. This might be a consequence of the competition between sink and source, the poor source caused by fewer and smaller leaves, the competitive sink characterized by early reproductive development. So in order to obtain more novel phenotypes, adjust the NTAG1 expression driven by certain promoter such as inducible promoter await for future performance.
3 Materials and methods

3.1 Plant materials and growth conditions

Plants of narcissus Yulinglong and Jinzhanyutai used for this study were grown in the field in Chongming, Shanghai. *Arabidopsis thaliana* of ecotype Columbia-0 (Col) and Lersberg (Ler) plants were grown in the green house under constant illumination (−80 μmol·m−2·s−1) at 22±2°C. For transgenic plants to be screened, seeds were surface sterilized, stratified at 4°C for 3 d, plated on 1/2 Murashige and Skoog (Murashige and Skoog, 1962) medium (MS) containing 50 μg·mL−1 kanamycin, and grown under continuous fluorescent light at 22°C.

3.2 Scanning electron microscopy (SEM)

Plants were fixed in FAA (70% ethanol 89%, formaldehyde 5%, acetic acid 6%) at 4°C overnight, dehydrated in an ethanol series, critical-point dried in liquid CO₂, sputter-coated with gold palladium, analyzed and photographed with a Philips XL 30 FEG SEM.

3.3 Determination of malonaldehyde (MDA) levels

MDA determination was followed the method described by Zhang (Zhang et al., 2009). Fresh leaves of 20-d-old plants (0.2 g) were homogenized in 6 mL 10% (w/v) trichloroacetic acid. The homogenates were centrifuged at 5000xg for 10 min. A reaction mixture of the supernatant (2 mL) and 2 mL thiobarbituric acid (0.6%) was incubated in a boiling water bath for 15 min, then cooled immediately before centrifugation. Absorbance of the supernatants was determined at 450, 532 and 600 nm, respectively. Calculation of MDA was based on the following formula: C (μ mol/L) = 6.45(A532−A450)−0.56 A450.

3.4 Total RNA and genomic DNA extraction

Total RNA was isolated using Trizol reagent (Tiangen, Shanghai) plus 5 mol/L NaCl to reduce polysaccharide level in the RNA. For Arabidopsis RNA isolation, total RNA was extracted from the above ground tissues. And RNA was pretreated with RNase-free DnaseI (Takara, Japan) to remove contaminating genomic DNA. Arabidopsis and narcissus genomic DNA was isolated by using the lysis buffer (0.2 mol/L Tris, 0.05 mol/L EDTA, 7 mol/L Urea, 2% Sarkosyl, pH 8.0), employing phenol/chloroform followed by precipitation of DNA with iso-propyl alcohol.

3.5 Isolation and sequence analysis of *NTAG1*

To obtain the main fragment of the C-lineage genes from both narcissus varieties, primers AGAMOUS F (5’-ctc gag ATG GGG AGG GGT AAG ATA GAG ATC AA−3’) and AGAMOUS R (5’-ctc gag TCA TCC CAG TTG AAG GGT AGT−3’) were designed according to the *NAG* gene reported by Wang (Wang et al., 2006). Both the specific 5’ and 3’ primers for *NTAG1* contained the generated KpnI recognition site (5’-ctc gag−3’, a lowercase letter) to facilitate the cloning of this gene. cDNA was synthesized from 500 μg total RNA using a cDNA synthesis kit (TOYOBO, Japan). PCR reactions were performed using the pfu PCR kit (Takara, Japan) and the amplified fragments were purified with the DNA Gel Extraction kit (Tiangen, Shanghai), cloned into the pMD18-T vector (Takara, Japan), and verified by sequencing.

3.6 Plant transformation and transgenic plants analysis

A *KpnI* fragment containing the full-length cDNA for *NTAG1* gene was introduced into the binary T-DNA vector pMon530 (Monsanto, USA), driven by the 35S promoter. The sense orientation construct for this gene was determined using digestion and sequencing. The construct was transferred into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986), and then introduced into Arabidopsis plants using the Floral dip method (Clough and Bent, 1998). Transformants that survived in the medium containing kanamycin (50 μg·mL−1) were further verified by PCR and reverse transcriptase-PCR analyses.

The phenotypic effects of *NTAG1* in transgenic plants were analyzed in the T₁ generation and in the homozygous kanamycin-resistant T₃ generation plants through photographing with a digital camera or SEM. To compare the phenotypes of the transgenic and wild type plants, at least 20 transgenic plants from 5 independent lines respectively and 20 wild-type plants were grown under the same conditions. Plant height, silique length, branch number, leaf number etc. were recorded.
3.7 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Five microgram of total RNA extracted from different plant tissues was used for generating the first strand of cDNA according to the instructions of the Superscript RT (Toyobo, Japan). PCR amplification was performed with gene specific primers. For SAG12 gene, specific forward (5'-CCAATGAGCAATTTGGTGCC-3') and reverse (5'-TGTCGCAATCAACAGCCTTG-3') primers were used. For NTA1 gene, specific AGAMOUS F and AGAMOUS R primers were used. Expression levels of ACTIN were monitored to serve as a quantifying control.

Authors' contributions

DXJ and XLJ carried out the gene cloning and transgenic planting phenotype analysis, and WY helped with the phenotype analysis and took part in the data analysis. SY took part in the technique instruction. LXF performed the experiment designs and drafted the manuscript. All authors read and approved the final manuscript.

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