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ORIGINAL RESEARCH

Arabidopsis MiR396 Mediates the Development of Leaves and Flowers in Transgenic Tobacco

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Abstract MicroRNAs (miRNAs) are single-stranded, non-coding small RNAs that usually function as post-transcriptional negative regulators by base pairing to target genes. They are pivotal to plant development. MiR396 is conserved among plant species and is predicted to target *GRF* (growth-regulating factor) genes in *Arabidopsis*. Here, overexpression of ath-miR396 in tobacco reduced the levels of three *NtGRF*-like genes containing an miR396 match site. Furthermore, its elevated expression resulted in a small, narrow leaf phenotype similar to that found with the *Arabidopsis grf1grf2grf3* triple mutant. We also demonstrated that *35S:MIR396a* transgenic plants were defective in the four whorls of floral organs. These results provide a link between the miR396-mediated regulatory pathway of *NtGRF*-like gene expression and the developmental processes for leaves and flowers in tobacco.

Keywords GRF · miR396 · Narrow leaf · Tobacco

MicroRNAs are a class of about 20- to 24-nucleotide, single-stranded RNAs processed from typical stem loop precursors by the Dicer-like (DCL) family of plant enzymes (Ambros et al. 2003; Lu and Fedoroff 2000; Park et al. 2002; Bartel 2004; Vaucheret et al. 2004). They function as regulators in plants by targeting mRNAs for cleavage or transcriptional repression (Llave et al. 2002; Chen 2004; Juarez et al. 2004; Oka et al. 2008). Some are involved in floral organ identity, leaf morphogenesis, lateral root initiation, and vascular development (Palatnik et al. 2003; Achard et al. 2004; Mallory et al. 2004; Baker et al. 2005; Guo et al. 2005; Kim et al. 2005; Cho et al. 2007; Reyes and Chua 2007). Other miRNAs contribute to disease and nutrient-stress resistance (Sunkar and Zhu 2004; Fujii et al. 2005; Navarro et al. 2006; Dugas and Bartel 2008; Kawashima et al. 2009).

MiR396, a single-stranded RNA of 21-nt microRNA, has been found in 12 plant species (Griffiths-Jones et al. 2006; Zhang et al. 2006). Highly conserved in terms of both primary and mature miRNAs, it is predicted to target *AtGRF* (*Arabidopsis thaliana* growth-regulating factor) family members (At*GRF1*, *AtGRF2*, *AtGRF3*, *AtGRF4*, *AtGRF7*, *AtGRF8*, and *AtGRF9*; Jones-Rhoades and Bartel 2004; Jones-Rhoades et al. 2006). GRFs contain conserved QLQ and WRC domains in their N-terminal region, which define the GRF protein family (van der Knaap et al. 2000). Their QLQ domain may function in protein–protein interactions, as evidenced by its similarity to the N-terminal part of the yeast SWI2–SNF2 protein, which mediates the interaction with another component of the functional complex (Treich et al. 1995; van der Knaap et al. 2000). The WRC domain contains a nuclear localization signal and a DNA-binding motif that comprises the conserved spacing of three Cys and one His residue. Finally, the C-terminal regions of GRF proteins have features common to transcription factors without regard to variations in their length and amino acid sequences (van der Knaap et al. 2000; Kim et al. 2003).

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Growth-regulating factors are present in all seed plants examined thus far and have been well investigated in *Arabidopsis* (van der Knaap et al. 2000; Kim et al. 2003; Choi et al. 2004). Of the nine members found in that genus, AtGRF1, 2, and 3 are involved in mediating leaf shape. For example, transgenic plants overexpressing *AtGRF1* or *AtGRF2* develop larger leaves than do wild-type plants, whereas the triple null mutant *grf1grf2grf3* has smaller, more narrow leaves (Kim et al. 2003; Kim and Kende 2004; Horiguchi et al. 2005).

Although numerous microRNAs function in *Arabidopsis* growth and metabolism, little is known about their role in tobacco (*Nicotiana tabacum* L.). Here, we generated transgenic lines overexpressing ath-miR396 in tobacco. Our objective was to examine how it affects leaf and flower development in that crop.

Materials and Methods

Plant Materials and Growing Conditions

Seeds from tobacco (*N. tabacum* cv. Xanthi NC), rice (*Oryza sativa* cv. Nipponbare), and *A. thaliana* ("Columbia") were surface-sterilized, cultured for two weeks in a solidified MS medium (0.65% agar, *w*/*v*), then transferred to soil under a 16-h photoperiod at 24°C to 28°C. Roots, stems, and leaves of 4-week-old tobacco seedlings, as well as their floral organs at the flowering stage, were harvested for miR396 expression analysis. Likewise, leaves, roots, stems, and floral tissues of *Arabidopsis* were harvested from 4-week-old plants, and young leaves and panicles were sampled from 14-day-old rice seedlings.

Plasmid Construction and Tobacco Transformation

A 544-bp fragment (containing the 151-bp *miR396*a precursor) was PCR-amplified from *Arabidopsis* genomic DNA, using specific primers for miR396a (5′-TGC TGT AAA AGA ATG ACC CTT-3′ and 5′-AAA CTC ATA GAC AGA AGT TAG GGT T-3′). The amplified fragment was verified by sequencing, then inserted into transformation vector pOCA30 downstream of the constitutive 35S promoter. This construct was introduced into *Agrobacterium tumefaciens* strain GV3101 for transformation of tobacco by the leaf-disk method, as described by Curtis et al. (1995). Transgenic plants were screened on an MS medium containing 300 μM kanamycin, and two independent lines of T_2 plants were analyzed in detail.

RNA Gel Blotting

Total RNA (10 μg) from tobacco (*N. tabacum* cv. Xanthi NC), rice (*O. sativa* cv. Nipponbare), and *A. thaliana* ("Columbia") were extracted with Trizol reagent (Invitrogen). Samples were resolved on a 15% denaturing polyacrylamide/1× TBE/7 M urea gel and subjected to blot hybridization with [^{32}P]-ATP-labeled single-stranded DNA complementary to an miR396a probe (5′-CAGTTCAAGA AAGCTGTGGAA-3′), as described by Akbergenov et al. (2006).

RT-PCR

RNA was prepared from 4-week-old tobacco seedlings for reverse-transcription with 1 μg of total RNA and a kit (Fermentas, Vilnius, Lithuania). PCR reactions (25 μl) were performed with 1 μl of cDNA, 200 nM start-stop target primers, and the following gene-specific primers:

FG165999-A, 5′-ATCTAACACATCGGGTTTGGAT-3′;
FG165999-B, 5′-TGTCGCTCACAATATTTCTGATC-3′;
FG165999-C, 5′-AATTGCCTCGTTACAGGGAAGA-3′;
FG165999-D, 5′-AAGCCACTGTGACCTCAAACCT-3′;
FG137771-A, 5′-TGAATCTGCTGGCTTCACAACT-3′;
FG137771-B, 5′-TCATGTGCCGCTCACAATACTT-3′;
FG137771-C, 5′-ACTTCAAGGGCTGAATTTGGAT-3′;
FG137771-D, 5′-TCTGGCCAAGAAACTGTGGAT-3′;
FG167390-A, 5′-TACCAAGACTTGTTCTTGATCTT GA-3′;
FG167390-B, 5′-TGCACCAGCTAACATATGTCTG TA-3′;
FG167390-C, 5′-ACGGCGGTGAAGTCCTTAAAGT-3′;
FG167390-D, 5′-TCATGGGCCTTTGCTCATATG-3′;
FG194569-A, 5′-TTTGTTGCGAGGTTCAGAGGT-3′;
FG194569-B, 5′-GCCAGTGAAACCAAGATGGAA-3′;
FG194569-C, 5′-CTGGATCCACCAATTCAAAGGT-3′;
FG194569-D, 5′-TCT TTCCATCTGTTCGACGAC-3′

Measurement of Leaf Dimensions

Lengths and widths were measured with a Vernier caliper for leaves detached from 12 4-week-old WT and transgenic plants. Their blade-surface areas were determined with a LAI-3000 C (Li-Cor). All data were analyzed by the SPSS program.

Results

MiR396 is Evolutionarily Conserved Among Plant Species

We used Northern blots to investigate the expression patterns of miR396 in *Arabidopsis*, tobacco, and rice (Fig. 1a). For *Arabidopsis*, miR396 accumulated predominantly in the flowers and was apparently detected in leaves, stems, and siliques, but was less abundant in the roots

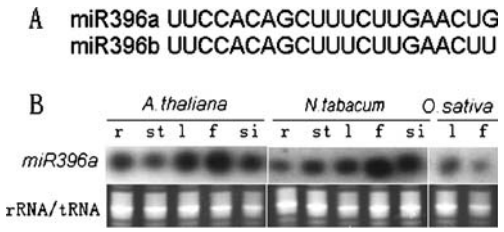

Fig. 1 Expression patterns of miR396 in different tissues and plant species. **a** Sequence similarity between *Arabidopsis* miR396 isoforms a and b. **b** RNA gel blots of total RNA isolated from *Arabidopsis*, tobacco, and rice were probed with labeled anti-miR396. Samples are root (*r*), stem (*st*), leaf (*l*), flower (*f*), and silique (*si*). rRNA and tRNA staining is shown as loading control

(Fig. 1b, left). In tobacco, it accumulated mostly in flowers and leaves and less so in the stems (Fig. 1b, middle). It was also clearly detected in young rice leaves and panicles (Fig. 1b, right). This demonstrated that miR396 is expressed in all plant tissues, as a conserved sequence from both monocot and dicot species. Expression of homologous miR396 suggested the existence of a tobacco ortholog and the possibility that these sequences are almost identical.

Generation of *35S:MIR396a* Transgenic Tobacco

Given that the miR396 is conserved in distantly related plant species (Zhang et al. 2006) and that miR396a differed from miR396b by only one nucleotide in *Arabidopsis* (Fig. 1a), we generated transgenic tobacco overexpressing *ath-miR396a*. DNA fragments corresponding to the precursor fold-back structure of miR396 (pre-miR396a) genes were amplified from *Arabidopsis* genomic DNA. After verification by sequencing, we inserted the pre-miR396a fragment into pOCA30 downstream of the constitutive 35S promoter (Fig. 2a). This construct was then introduced into tobacco by *Agrobacterium*-mediated transformation.

A total of 31 independent transgenic lines were obtained and a subset was examined for the presence of pre-miR396a. T-DNA insertions containing *35S:MIR396a* were detected in all of our transgenic plants (Fig. 2b). Among these, two lines displayed severe phenotypes with leaves that were smaller and narrower than from the wild type. The remainder had less severe phenotypes, with leaves that also were more narrow than those of WT.

We next analyzed *miR396* expression, using Northern blotting of five T_1 plants with distinctly smaller and more narrow leaves. All had higher levels compared with the wild type (Fig. 2c). Lines 2 and 5 were selected for subsequent experiments; their T_3 generation also had much more *miR396* transcript, indicating that this overexpression was heritable.

Transgenic Plants Have Reduced Levels of *NtGRF*-Like Genes

The miR396 targets *GRF* genes in *Arabidopsis* (Jones-Rhoades et al. 2006; Liu et al. 2009), and the latter regulates leaf development in *A. thaliana* (Kim et al. 2003). Because our results suggested that miR396 is conserved in tobacco, we hypothesized that some *NtGRF*-like genes might be targeted by miR396 in that crop.

We searched the tobacco EST sequence database with the conserved sequences of *A. thaliana GRF* (*AtGRF*) as query. Four putative partial cDNA sequences (GenBank accessions FG137771, FG165999, FG167390, and FG194560) contained match sites with ath-miR396 (Fig. 3a). These sequences were aligned with *AtGRF* gene sequences via ClustalX and were translated into inferred amino acid sequences by Premier Primer 5 and aligned with AtGRF proteins by ClustalX (Fig. 3b). All four were highly homologous in both their nucleotide and amino sequences. Additionally, those deduced amino sequences contained a specific GRF DNA-binding sequence that was defined as the WRC domain. This implied that these four partial cDNAs are from four different *NtGRF*-like genes.

The ath-miR396 sequence was complementary to the four *NtGRF*-like mRNAs that encoded a portion of the conserved N-terminal WRC domain of the proteins. The exceptions were one mismatch and one bulge for the *NtGRF*-like pairing regions among the 21 nts. To further determine whether the increased level of ath-miR396 transcript could cause degradation of the four *NtGRF*-like genes, we detected their mRNA in wild-type and transgenic tobacco through semiquantitative RT-PCR. By amplifying the cleavage site-spanning fragment, we determined that, compared with WT, levels of three *NtGRF*-like genes were

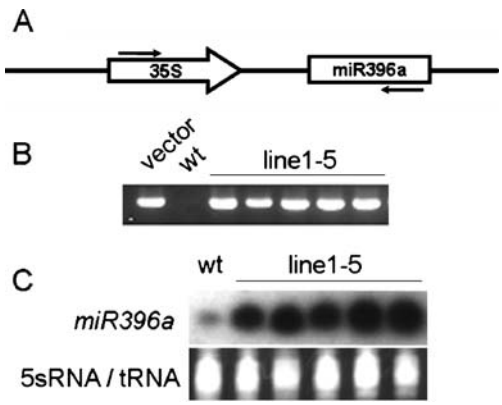

Fig. 2 Characterization of *35S:MIR396a* transgenic plants. **a** Structure of *35S:MIR396a* fusion. *Arrows* indicate positions of primers used to detect transgene. **b** Identification of *35S:MIR396a* plants by PCR. Products were from DNAs of six independent T_1 transgenic plants. **c** MicroRNA blot hybridization using miR396a antisense probe

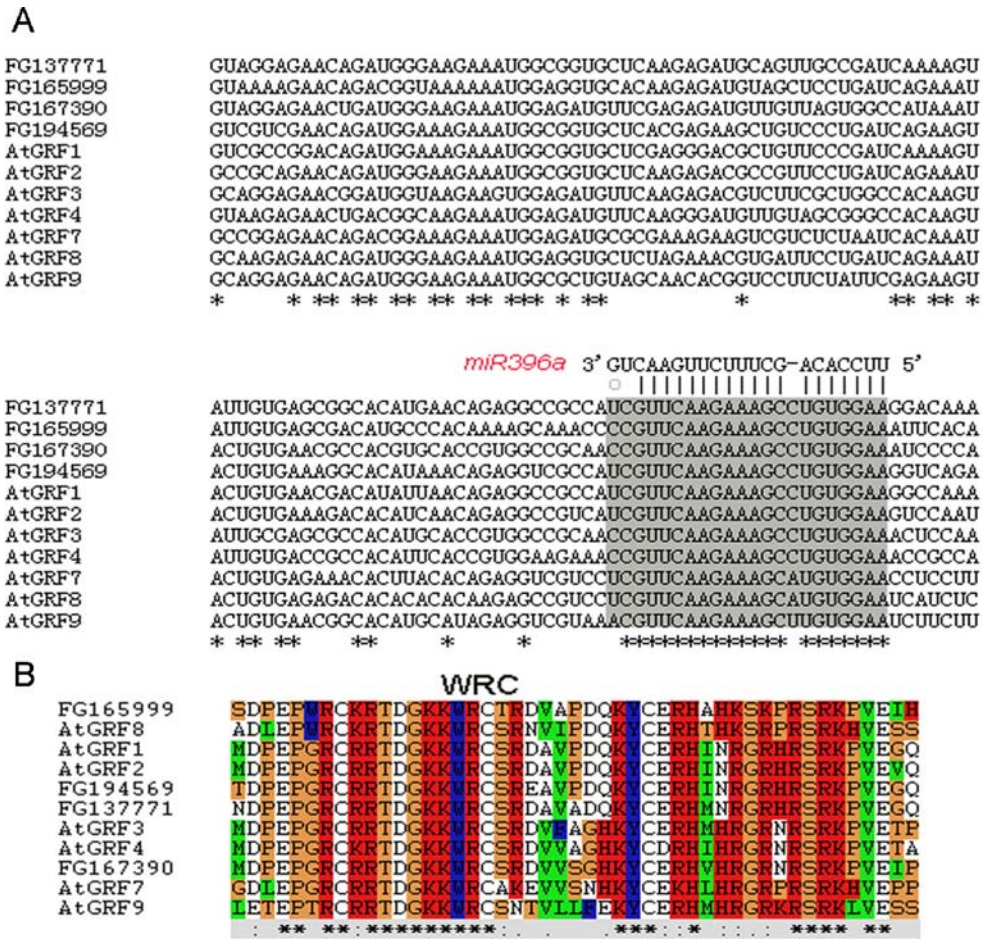

Fig. 3 Comparison of nucleotide and amino sequences of GRFs. **a** Alignment of *AtGRFs* and *NtGRF*-like partial nucleotide acid sequences. *Black box* indicates miR396 match site. **b** Alignment of AtGRF and NtGRF-like partial amino acid sequences. *WRC* indicates domain of GRF proteins. NtGRF-like sequences were acquired from NCBI (http://www.ncbi.nlm.nih.gov) and aligned by ClustalX

significantly decreased in the transgenics while that of FG167390 was nearly unchanged (Fig. 4b).

To further confirm that the *NtGRF*-like genes were cleaved by ath-miR396, we conducted RT-PCR for 5′(AB) and 3′ (CD) cleavage fragments (Fig. 4a). In contrast to a decrease in the AD fragment from transgenic plants, their AB or CD fragments for the four *NtGRF*-like genes were at least as numerous as those in the wild type (Fig. 4b). This indicated that the *NtGRF*-like genes were likely cleaved by ath-miR396.

Transgenic Plants Show a Narrow-Leaf Phenotype

To examine the functions of miR396 in tobacco, we analyzed its phenotypic characteristics. Compared with WT plants, the transgenics were shorter and had smaller, more narrow leaves (Fig. 5a). Surface areas of the first two leaves were reduced by nearly 50% or 90% for lines 2 and 5, respectively, because both length (by 35% to 48%) and width (by 47% to 75%) of the blade were reduced for these most mature leaves (Fig. 5b–d). As a result, the leaf index (leaf length/leaf width) was higher and leaves were more narrow than for WT. Similar decreases were noted in the widths and lengths of leaves at other positions on those transgenic plants. These phenotypes resembled those of

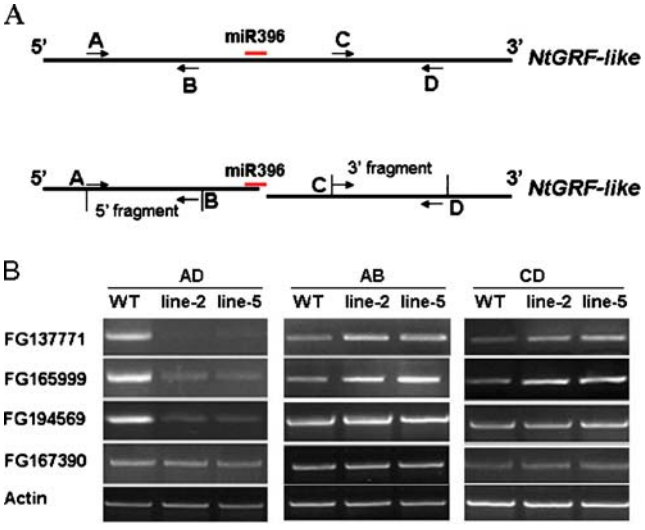

Fig. 4 RT-PCR analyses of *NtGRF*-like gene expression. **a** Positions of primers. *Red line*, miR396 and position of target; *arrow*, primer orientation. **b** Semiquantitative RT-PCR analysis for *NtGRF*-like gene. *AD* full-length; *AB* 5′ cleavage fragment, *CD* 3′ cleavage fragment

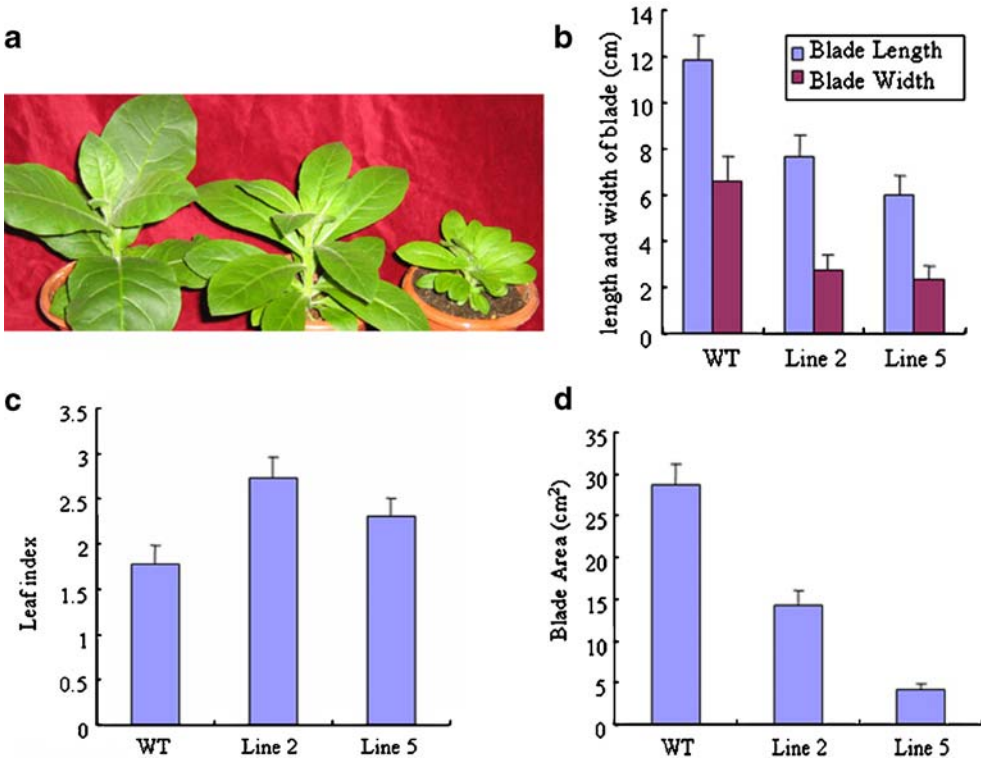

Fig. 5 Phenotypes of leaves from *35S: miR396a* transgenic lines. **a** Height and leaf size for 4-week-old plants. **b**–**d** Dimensions of first two leaves from WT and transgenic plants at day 40: blade area (*b*), blade length and width (*c*), and leaf index (*d*; = leaf length/leaf width)

grf1grf2grf3 triple mutant plants in *Arabidopsis* (Kim and Kende 2004), further demonstrating that elevated levels of ath-miR396 lead to a narrow-leaf phenotype, probably because expression of *NtGRF*-like genes is repressed in tobacco.

Floral Development is Defective in Transgenic Tobacco

In addition to narrow leaves and smaller stature, floral development was affected in transgenic plants. Wild-type

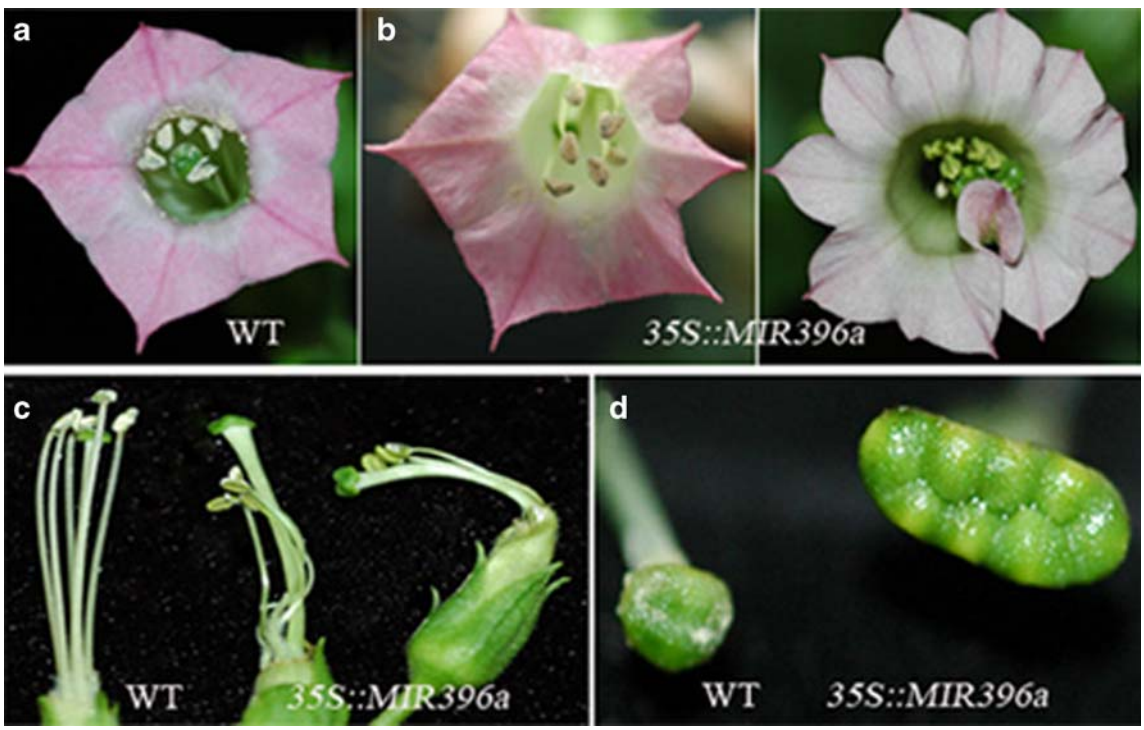

Fig. 6 Phenotypes of flowers from wild-type and *35S: miR396a* transgenic lines. **a** WT. **b** Transgenic plant with slight phenotype (*left*) and severe phenotype (*right*). **c** Comparison of WT and transgenic filaments. **d** Pistils from WT and transgenic plants

tobacco flowers (Fig. 6a) present a first whorl of five sepals that are connately fused. The second whorl is occupied by five pink petals that also are connately fused to form a trumpet-like corolla. The third whorl comprises five stamens capped by pollen-bearing anthers, and the fourth whorl is a two-carpellate gynoecium. In contrast, flowers on our transgenic plants had more than five fused petals; those with the most severe phenotype had up to 11 (Fig. 6b). More than five stamens were shorter and abnormally curved, with their pistils containing more than two carpels (Fig. 6c, d). Some transgenic flowers produced only a few or no seeds. Even if the anthers properly released their pollen, stamens were much shorter than pistils, leading to defective fertility (Fig. 6c).

Discussion

GRF genes are in a novel class of transcriptional regulators. Such families include nine members in *A. thaliana*, 12 in rice, and 14 in maize (Zhang et al. 2006, 2008). Based on homology among GRFs, we obtained four putative NtGRF-like partial sequences with the specific WRC domain of GRF proteins from the tobacco EST database. Their alignment indicated that the four *NtGRF*-like genes are highly similar to *AtGRF* in both nucleotide and protein sequences.

In *Arabidopsis*, the *AtGRF* genes (*AtGRF*1, 2, 3, 4, 6, 7, 8, and 9) are negatively regulated via miR396 at the posttranscriptional level (Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al. 2006). Smaller, more narrow leaves are associated with the *grf1grf2grf3* triple mutant (Kim et al. 2003) whereas *AtGRF5* overexpression leads to larger leaves (Horiguchi et al. 2005), thereby demonstrating that *AtGRF* regulates their development. Here, the reduced expression by *NtGRF*-like genes meant that our *35S:MIR396a* transgenic tobacco developed small and narrow leaves, similar to those of that *Arabidopsis* triple mutant. One explanation is that a decline in *NtGRF*-like transcripts is caused by elevated levels of ath-miR396. Our RT-PCR results suggest that ath-miR396 mediates the cleavage of those *NtGRF*-like transcripts (Fig. 4). Although one of the four *NtGRF*-like genes, FG167390, did not show a clear reduction in mRNAs, it still probably is regulated at the level of translation (Chen 2004; Juarez et al. 2004).

AtGRF1 and AtGIF1 act as a transcription activator and co-activator involved in regulating fertility and the growth and shape of leaves and sepals (Kim and Kende 2004). In *Arabidopsis*, transcript levels of all AtGRFs are the highest in the developing floral buds (Kim et al. 2003). Flowers from our transgenic plants also were aberrant, having many more petals, stamens, and carpels, but lower fertility than WT. Therefore, we propose that the reduced expression of *NtGRF*-like genes, as mediated by ath-miR396, might regulate the interaction between *NtGRF*-like and *NtGIF*, resulting in these floral abnormalities.

Many miRNA families are evolutionarily conserved across all major lineages of plants, based not only on their genes but also their targets, per the EST database (Floyd and Bowman 2004; Zhang et al. 2006). Our spatial expression profiles for miR396 in dicot and monocot species also indicate that miR396 sequences are highly conserved across great phylogenetic distances. Because of this conservation and the occurrence of an miR396 ortholog in tobacco, we conclude that a gene homologous to *miR396* exists in tobacco and that it functions similarly to *Arabidopsis miR396*.

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