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Overexpression of the NAC transcription factor family gene *ANAC036* results in a dwarf phenotype in *Arabidopsis thaliana*

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SUMMARY

NAC proteins comprise one of the largest families of transcription factors in the plant genome. They are known to be involved in various aspects of plant development, but the functions of most of them have not yet been determined. ANAC036, a member of the *Arabidopsis* NAC transcription factor family, contains unique sequences that are conserved among various NAC proteins found in other plant species. Expression analysis of the *ANAC036* gene indicated that this gene was strongly expressed in leaves. Transgenic plants overexpressing the *ANAC036* gene showed a semidwarf phenotype. The lengths of leaf blades, petioles and stems of these plants were smaller than those in wild-type plants. Microscopy revealed that cell sizes in leaves and stems of these plants were smaller than those in wild-type plants. These findings suggested that ANAC036 and its orthologues are involved in the growth of leaf cells.

Introduction

Transcription factors are key proteins involved in regulation of gene expression at the transcriptional level by specifically binding to the regulatory regions of their target genes. Computational predictions have indicated that the *Arabidopsis* genome contains more than 1500 transcription factor-coding genes, which are classified into families on the basis of their conserved domain structures (Qu and Zhu, 2006). NAC (NAM, ATAF1/2 and CUC2) proteins are plant-specific transcription factors that constitute a large family with more than 100 predicted members in *Arabidopsis* (Olsen et al., 2005). The first discovered NAC gene is *NO APICAL MERISTEM* (*NAM*) in petunia (Souer et al., 1996). Mutation of the *NAM* gene results in a lack of shoot apical meristem formation during embryogenesis. In *Arabidopsis*, *CUP-SHAPED COTYLEDON 1* (*CUC1*), *CUC2* and *CUC3* genes are functionally redundant in the developing shoot apical meristem (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). It has been reported that some members of the NAC protein family play different roles in lateral root formation, auxin signaling, defense and abiotic stress (Olsen et al., 2005). Recent studies have revealed that VASCULAR-RELATED NAC-DOMAIN 6 (VND6) and VND7 act as regulators of xylem formation (Kubo et al., 2005) and that NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1) and NST2 regulate secondary wall thickening (Mitsuda et al., 2005). Membrane-associated NAC transcription factors that are involved in stress response and plant development have also been found (Chen et al., 2008). NAC WITH TRANSMEMBRANE MOTIF 1 (NTM1) mediates cytokinin signaling during cell division (Kim et al., 2006) and NTM1-LIKE 8 regulates salt-responsive flowering (Kim et al., 2007) and gibberellic acid-mediated salt signaling in seed germination (Kim et al., 2008). However, the roles of many NAC transcription factors in plants are still not known. It is important to determine the biological functions of novel NAC proteins required for plant development.

NAC proteins commonly have highly conserved sequences named NAC domains in their N-terminal regions (Aida et al., 1997). NAC domains consist of five subdomains, subdomains A–E (Kikuchi et al., 2000). NAC subdomains D and E are required for DNA-binding ability, while the C-terminal region can function as a transcriptional activation domain (Xie et al., 2000; Duval et al., 2002). Ooka et al. (2003) classified 105 and 75 predicted NAC proteins in *Arabidopsis* and rice, respectively, into 18 subgroups by their amino acid sequence similarities of NAC domains. They found two novel subgroups, ONAC022 and ANAC011, that did not include any NAC proteins of which functions have been

Abbreviations: CUC, cup-shaped cotyledon; GUS, β-glucuronidase; LOV, long vegetative phase; NAC, (NAM, ATAF1/2 and CUC2); NAM, no apical meristem; NST, NAC secondary wall thickening promoting factor; NTM, NAC with transmembrane motif; RNAi, RNA interference; RT-PCR, reverse transcriptase polymerase chain reaction; VND, vascular-related NAC domain; XND, xylem NAC domain

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characterized. Six members of the family of *Arabidopsis* NAC proteins belonged to the ONAC022 subgroup.

We found that ANAC036 (locus name, At2g17040), a member of the *Arabidopsis* NAC protein family in the ONAC022 subgroup, had amino acid sequence uniqueness in its C-terminal region when it was compared to other NAC proteins of *Arabidopsis*. On the other hand, these unique amino acid sequences were partially conserved among various NAC proteins found in other plants. RNA gel blot analysis showed that the *ANAC036* gene was strongly expressed in leaves. Transgenic plants overexpressing the *ANAC036* gene showed a semidwarf phenotype. The cell size was reduced in the dwarf tissues of these plants. These results suggested that NAC proteins homologous to ANAC036 exist in a wide range of plant species and are involved in cell growth.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used as a wild-type plant in this study. Plants were grown on rock-wool and vermiculite or on Murashige–Skoog medium containing 1% sucrose and 0.8% agar. They were grown at 22 °C under continuous light.

Plasmid construction and plant transformation

DNA fragments to construct plasmids were amplified by polymerase chain reaction (PCR) using genomic DNA as a template. Primer sequences are listed in Table 1. The PCR was carried out with PfuUltra High-Fidelity DNA polymerase (Strategene, USA). The amplification profile was an initial denaturation at 94 °C for 2 min, followed 24 cycles of denaturation at 96 °C for 20 s, annealing at 64 °C (primer MA14-OE5 and primer MA14 –OE3) or 56 °C (other primers) for 30 s and extension at 68 °C for 3 min, and final extension at 72°C for 10 min. PCR products were separated by agarose gel electrophoresis and extracted from the gel piece containing the amplified DNA. After adding A residues at their 3′ sites using rTaq polymerase (Takara, Japan) and dATP, these fragments were ligated to the *Xcm*I site in p3T vector (Molecular Biotechnology, Germany). The resultant plasmids were used to construct plasmids for transformation of plants, after the nucleotide accuracies had been confirmed by nucleotide sequencing.

Table 1
Primer sequences for plasmid constructs, probe preparation and RT-PCR.

Primer name	Sequence
MA14-OE5	gtctAGAGATGGGAAAAGATATTGAACTCCCG
MA14-OE3	ggagctCATATCCTACCAATATATGTTAACTATTG
MA14-KO5	ggaattcaagcTTGTAGGATGTTGTGCTTTGTAAG
MA14-KO3	ggtcgacggatccATCGTCAAATATGACTATAAATAG
MA41-KO54	ggaattcGAGTAAGAGCTTTTTTTCATATAG
MA41-KO34	gaagcttACTCTATTACATGTAAAACATATG
MA14-Pro5	gggatcCTCAGAGTGTTGAAAATTATACTG
MA14-Pro3	CTCAGTCGGGTGGAACCTA
MA14-5	ATGGGAAAAGATATTGAGCTCC
MA14-3	CTACCAATATATGTTAACTATTGG
EF1a-51	CTCCTTTCAGATTCTTACTTGC
EF1a-52	GGTGACCTTGGCTCCAGTTG

Small letters showed the non-corresponding sequences to the template for adding restriction enzyme sites.
A with underline in MA14-OE5 was different from G in the template sequence to delete the *Sac*I site. Although the codon was changed from GAG to GAA, the amino acid was not changed.

The coding region including introns of the *ANAC036* gene was amplified using MA14-OE5 and MA14-OE3 primers. After cloning the amplified fragment in p3T vector, the inserted DNA was excised by *Xba*I and *Sac*I digestion, and it replaced the *β-glucuronidase* (*GUS*) gene on *Xba*I and *Sac*I sites in pBI121 vector (Clontech, USA). The resultant plasmid was used as *35S::ANAC036* construct.

The 3′ portion of the coding region (corresponding to amino acids from 143 to 276) and 149-bp 3′-untranslated sequence of the *ANAC036* gene were amplified using MA14-KO5 and MA14-KO3 primers. The plasmid containing the amplified fragment in p3T vector was named pM14-3. The second intron sequence of At2g32140 was amplified using MA41-KO54 and MA41-KO34 primers. The plasmid containing the amplified fragment in p3T vector was named pM41-Int. The inserted DNA fragment of pM14-3 was excised by *Hin*dIII and *Sal*I digestion and it was ligated to *Hin*dIII and *Sal*I sites in pM41-Int. We named the resultant plasmid pM14-3Int. The inserted DNA of pM14-3 was excised by *Bam*HI and *Eco*RI digestion and it was ligated to *Bam*HI and *Eco*RI sites in pBluescript vector. We named the resultant plasmid pM14-3PBS. The inserted DNA of pM14-3Int was excised by *Sal*I and *Eco*RI digestion and it was ligated to *Sal*I and *Eco*RI sites in pM14-3PBS. We named the resultant plasmid pM14-3Int3. The *GUS* gene in pBI121 vector was excised by digestion with *Bam*HI and *Sac*I and replaced by the *Bam*HI–*Sac*I adaptor sequence. The inserted DNA of pM14-3Int3 plasmid was excised by *Bam*HI digestion and it was ligated to the *Bam*HI site in pBI121 vector with deletion of the *GUS* gene. This final plasmid was used as *ANAC036* RNAi construct.

The upstream sequence of the *ANAC036* gene and a part of the coding region were amplified using MA14-Pro5 and MA14-Pro3 primers. After cloning the amplified fragment in p3T vector, the inserted DNA was excised by *Bam*HI and *Sma*I digestion, and it was ligated to *Bam*HI and *Sma*I sites in pBI101.1 vector (Clontech, USA). The *Sma*I site exists at the 23-bp downstream of the 5′ end of the coding region, although the MA14-Pro3 primer has no *Sma*I site. The resultant plasmid containing a 1993-bp upstream sequence and a 26-bp sequence of the 5′-coding sequence was used as *ANAC036-promoter::GUS* construct.

The plasmids were introduced into *Agrobacterium tumefaciens* strain C58 by the freeze–thaw method (Nishiguchi et al., 1987) and used to transform the wild-type plant by vacuum infiltration (Bechtold et al., 1993). T1 transformants were selected by resistance to kanamycin. We checked the introduced DNA fragments in transformants by the PCR analysis of genomic DNA.

Microscopic observation

All microscopic samples were fixed overnight in FAA solution (50% ethanol, 5% acetic acid and 5% formaldehyde). For observation of leaves, fixed samples were cleared by incubation overnight in chloral hydrate solution (chloral hydrate:glycerol:water=8:2:1) and directly mounted on slides. The cell areas were measured by ImageJ software (National Institutes of Health, USA). For observation of stems, fixed samples were dehydrated by ethanol, transferred into Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), and sealed with Technovit 3040. The blocks were sectioned and stained with 0.1% toluidine blue in 0.1 M phosphate buffer, pH 7.0.

Expression analysis

Total RNA was prepared from various tissues by a method using guanidium thiocyanate (Chirgwin et al., 1979). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed

using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara, Japan). The cDNA was amplified using Ex-Taq (Takara, Japan) with MA14-5 and MA14-3 primers for the *ANAC 036* gene, and with EF1a-51 and EF1a-31 primers for the *EF1α* gene (Table 1). The amplification profile was an initial denaturation at 94 °C for 2 min, followed 21, 24 or 27 cycles of denaturation at 96 °C for 20 s, annealing at 56 °C for 30 s and extension at 68 °C for 2 min, and final extension at 72 °C for 10 min. RT-PCR products were examined by agarose gel electrophoresis.

For RNA gel blots, 20 μg of total RNA was separated on 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond-N+ membrane, and fixed by UV crosslinking. A DNA fragment for a probe was prepared by purification of a RT-PCR product using agarose gel electrophoresis. Probe labeling, hybridization and detection were performed using a Gene Images Random-Prime Labelling and Detection System (GE Healthcare Bio-Sciences, USA). Histochemical staining for GUS activity in transformants was performed as described by Jefferson et al. (1987).

Results

Sequence characteristics of ANAC036

The *ANAC036* gene encodes a protein of 276 amino acids, which belongs to the *Arabidopsis* NAC transcription factor family. ANAC036 is a member of the ONAC022 subfamily, which includes in total six *Arabidopsis* NAC proteins (Ooka et al., 2003). The amino acid sequence of the NAM domain containing NAC subdomains A, B, C and D was found to be conserved between ANAC036 and other NAC proteins of *Arabidopsis*. However, amino acid sequence homology between ANAC036 and other NAC proteins in *Arabidopsis* was not detected in the C-terminal region. When the amino acid sequences of the C-terminal region in ANAC036 were compared with those of various NAC proteins reported in other plants, two conserved sequences were found. We named these conserved sequences C1 and C2 domains and their consensus sequences were estimated (Fig. 1). The C1 domain consisted from 27 amino acids and almost all of the amino acids in its domain were well conserved. This domain contained the putative NAC subdomain E and its immediately downstream region. The C2 domain consisted of approximately 40 amino acids and 22 amino acids in its domain were highly conserved. This domain was located near the C-terminal end.

NAC proteins containing C1 and C2 domains were found in poplar, soybean, castor bean, chickpea, grape, Himalayan rhubarb, daisy, rice, sorghum and maize. The amino acid identities to the consensus sequences in C1 and C2 domains of these NAC proteins were 74–100% and 55–95%, respectively (Table 2). Two NAC proteins similar to ANAC036 were found in poplar, soybean, grape, rice and sorghum. In these plants except sorghum, the amino acid sequence identities to the consensus sequences in C1 and C2 domains of one NAC protein were higher than those of the other. When the more similar NAC proteins to ANAC036 in each species were examined, the amino acid identities to the consensus sequences in C1 and C2 domains were 85–100% and 64–95%, respectively. ANAC034, ANAC042 and ANAC094, which belong to the ONAC022 subfamily, had less than 20% amino acid sequence

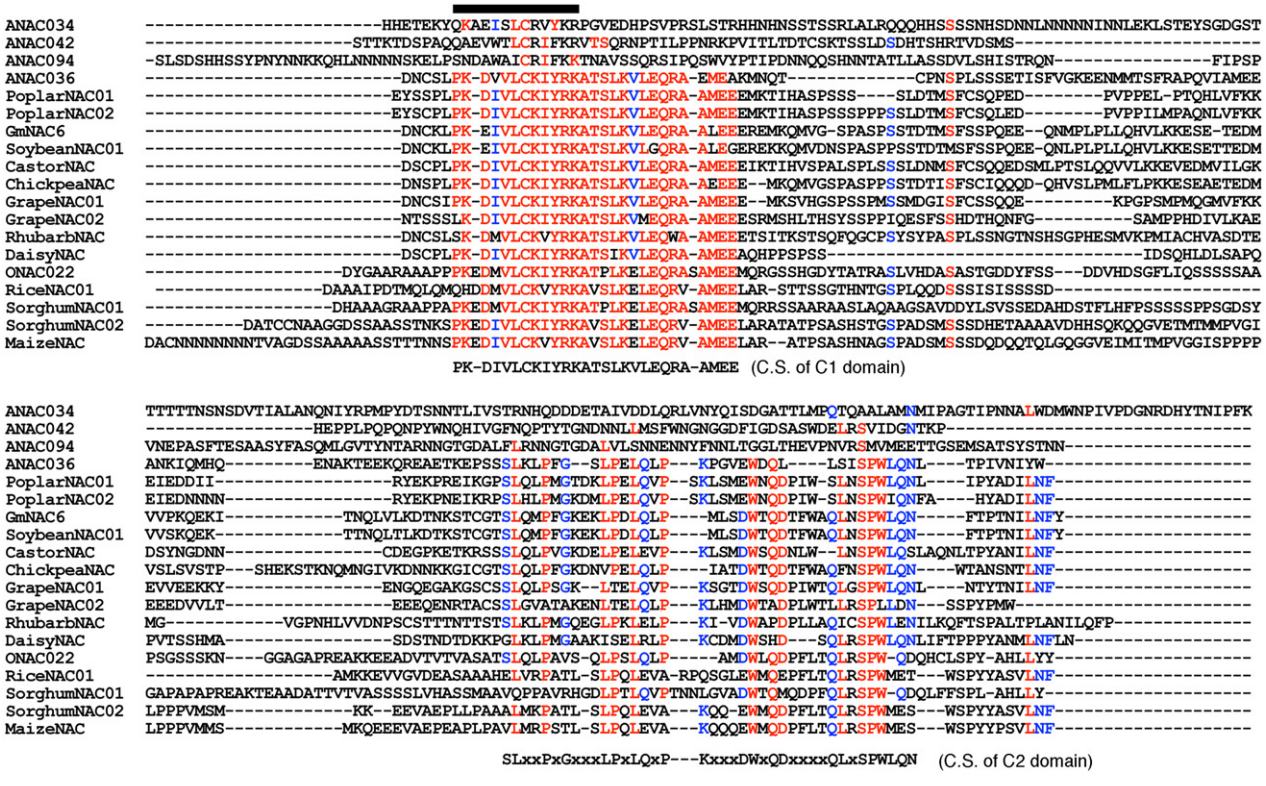

Fig. 1. Alignment of amino acid sequences of the C-terminal region in various NAC proteins. Deletions needed for the alignment are indicated by -. Protein names correspond to those in Table 2. Conserved residues among 16 proteins other than ANAC042, ANAC094 and ANAC034 are shown in red (for more than 13) and blue (for more than 10). Consensus sequences (CS) of C1 and C2 domains are also shown below the alignment. x means any amino acids. In ANAC042, ANAC094 and ANAC034, residues identical to the conserved residues among other proteins are shown in red and blue. A bar above the alignment indicates the region of the putative NAC subdomain E.

Table 2
Sequence characteristics of various NAC proteins. ANAC009, ANAC034, ANAC035, ANAC036, ANAC042 and ANAC094 are members of the ONAC022 family. ANAC043, ANAC054, ANAC081 and ANAC101 are members of other families. Other NAC proteins are homologues to ANAC036, which were found in UniPlot database.

Accession no.	Species	Protein name	Whole length	NAM domain	C1 domain	C2 domain
Q84WP6	*Arabidopsis thaliana*	ANAC043 (NST1)	365 aa	16–145 (48%)	ND	ND
Q9FRV4	*Arabidopsis thaliana*	ANAC054 (CUC1)	310 aa	20–148 (52%)	ND	ND
Q9C598	*Arabidopsis thaliana*	ANAC081 (ATAF2)	283 aa	7–132 (52%)	ND	ND
Q9LVA1	*Arabidopsis thaliana*	ANAC101 (VND6)	348 aa	7–136 (50%)	ND	ND
Q9ZVH0	*Arabidopsis thaliana*	ANAC009	425 aa	23–151 (60%)	ND	ND
Q9ZVP8	*Arabidopsis thaliana*	ANAC035	414 aa	51–178 (63%)	ND	ND
Q8H131	*Arabidopsis thaliana*	ANAC034	379 aa	51–178 (63%)	186–214 (19%)	305–346 (9%)
Q9SK55	*Arabidopsis thaliana*	ANAC042	275 aa	18–144 (63%)	155–183 (19%)	231–272 (18%)
Q9FIW5	*Arabidopsis thaliana*	ANAC094	334 aa	20–145 (58%)	176–274 (11%)	278–319 (14%)
Q7XJT9	*Arabidopsis thaliana*	ANAC036	276 aa	6–135	141–168 (89%)	235–267 (82%)
B9H1P6	*Populus trichocarpa*	PoplarNAC01	278 aa	9–138 (79%)	145–172 (100%)	230–268 (91%)
B9HQZ3	*Populus trichocarpa*	PoplarNAC02	284 aa	9–138 (79%)	145–172 (100%)	236–276 (86%)
Q52QR0	*Glycine max*	GmNAC6	294 aa	12–141 (79%)	147–174 (93%)	247–284 (95%)
C6T864	*Glycine max*	SoybeanNAC01	302 aa	18–147 (79%)	153–180 (85%)	255–292 (95%)
B9SIC1	*Ricinus communis*	CastorNAC	294 aa	9–138 (77%)	144–171 (100%)	244–280 (86%)
C7AFG3	*Cicer arietinum*	ChickpeaNAC	307 aa	15–144 (77%)	150–177 (96%)	260–297 (86%)
A7P8L4	*Vitis vinifera*	GrapeNAC01	276 aa	6–135 (77%)	141–168 (100%)	230–266 (95%)
A7R0R9	*Vitis vinifera*	GrapeNAC02	278 aa	9–138 (74%)	144–171 (93%)	233–271 (68%)
B5M1 × 9	*Rheum australe*	RhubarbNAC	317 aa	21–150 (77%)	156–183 (85%)	260–297 (82%)
A5Z0S7	*Chrysanthemum lavandulifolium*	DaisyNAC	262 aa	9–138 (76%)	144–171 (96%)	211–247 (82%)
Q10S65	*Oryza sativa*	ONAC022	316 aa	17–146 (77%)	158–186 (89%)	269–303 (82%)
Q5Z7Q4	*Oryza sativa*	RiceNAC01	292 aa	19–152 (69%)	166–194 (74%)	242–281 (55%)
C5WZU6	*Sorghum bicolor*	SorghumNAC01	327 aa	15–144 (74%)	157–185 (89%)	275–315 (64%)
C5Z2I7	*Sorghum bicolor*	SorghumNAC02	325 aa	13–154 (66%)	176–204 (89%)	278–314 (64%)
C4J546	*Zea mays*	MaizeNAC	326 aa	6–143 (65%)	175–203 (85%)	278–315 (64%)

We tentatively named protein names other than *Arabidopsis* NAC proteins, one of *Oryza sativa* NAC proteins and one of *Glycine max* NAC proteins.
Q10S65 (Os03g0133000) corresponded to ONAC022 in Ooka et al. (2003) and Fang et al. (2008) and Q52QR0 corresponded to GmNAC6 in Meng et al. (2007).
The region of the NAM domain was determined by a MOTIF search using the Pfam database in the WWW site of GenomeNet (http://motif.genome.jp/).
Columns of the NAM domain, C1 domain and C2 domain show the amino acid sequence positions and identities of their domains. Amino acid identities of the NAM domain and C1 and C2 domains were calculated by comparison with ANAC036 and their consensus sequences, respectively. ND, not determined.

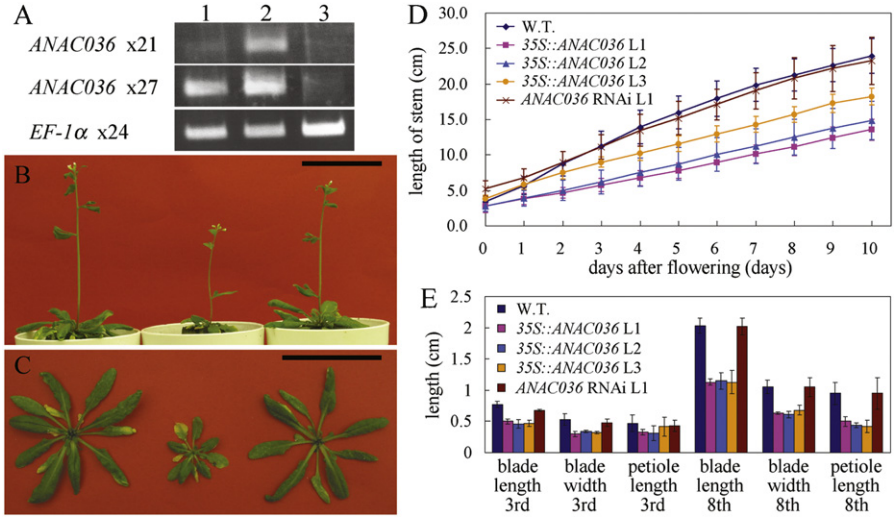

Fig. 2. Morphological phenotype of transgenic plants. (A) Detection of *ANAC036* mRNA. RT-PCR was performed using total RNA from leaves of wild-type (lane 1), *35S::ANAC036*L1 (lane 2) and *ANAC036* RNAi L1 (lane 3) plants. The cycle numbers of PCR of each gene are shown at the left. *EF-1α* gene was used as a control. (B) Wild-type (left), *35S::ANAC036* L1 (center) and *ANAC036* RNAi L1 (right) plants. Plants are three days old after anthesis of the first flower. Scale bar=5 cm. (C) Rosette leaves of wild-type (left), *35S::ANAC036* L1 (center) and *ANAC036* RNAi L1 (right) plants. Plants are three days old after anthesis of the first flower. Scale bar=5 cm. (D) Measurements of stem length of primary inflorescence of wild-type (n=8), *35S::ANAC036* L1 (n=8), L2 (n=8), L3 (n=8) and *ANAC036* RNAi L1 (n=8) plants. Data were obtained by measuring stem length of primary inflorescence each day after anthesis of the first flower. Every point represents the average with standard deviation. (E) Measurements of leaf sizes of wild-type (n=4), *35S::ANAC036* L1 (n=4), L2 (n=4), L3 (n=4) and *ANAC036* RNAi L1 (n=4) plants. Blade length, blade width and petiole length of the 3rd and 8th leaves were measured. The leaves were harvested 3 days after anthesis of the first flower. Color bars indicate the average with standard deviation.

identities to the consensus sequences of C1 and C2 domains (Fig. 1, Table 2).

The amino acid sequence of the NAM domain in the NAC proteins containing C1 and C2 domains was similar to that of ANAC036 (Table 2). The amino acid identities to ANAC036 in the NAM domain of these NAC proteins were 65–79%, which were higher than those of other *Arabidopsis* NAC proteins belonging to the ONAC022 subfamily (58–63%) and other subfamilies (48–52%).

Semidwarf phenotype induced by overexpression of the ANAC036 gene

In order to characterize the function of the ANAC036 protein, we generated transgenic plants expressing the *ANAC036* gene under control of the cauliflower mosaic virus 35S promoter (*35S::ANAC036* plants). We also generated transgenic plants that produce double-stranded *ANAC036* RNAs to induce RNA interference (*ANAC036* RNAi plants).

Eighteen lines of *35S::ANAC036* plants and 11 lines of *ANAC036* RNAi plants were obtained. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that expression of the *ANAC036* gene was increased in *35S::ANAC036* plants and decreased in *ANAC036* RNAi plants (Fig. 2A). It was remarkable that 16 lines of the *35S::ANAC036* plants showed a semidwarf phenotype, although two lines did not show a clear phenotype. On the other hand, obvious morphological changes were not observed in any line of *ANAC036* RNAi plants (Fig. 2B and C). We used three independent *35S::ANAC036* lines, named L1, L2 and L3, and one *ANAC036* RNAi line, named L1, for detailed analysis.

Successive measurements of stem length of primary inflorescence of these transgenic lines after anthesis of the first flower showed that shortening of the primary inflorescence stem occurred uniformly in each stage of bolting in *35S::ANAC036* plants (Fig. 2D). Ten days after anthesis of the first flower, the stem lengths of primary inflorescence of *35S::ANAC036* L1, L2 and L3 plants were approximately 55%, 60% and 75% of that of wild-type plants, respectively. Measurements of the sizes of rosette leaves of the transgenic plants showed reduction in the sizes of leaf blades and petioles of the *35S::ANAC036* plants (Fig. 2E). For example, blade length, blade width and petiole length of the 8th leaves of *35S::ANAC036* L1, L2 and L3 plants were approximately 55%, 60–65% and 45–55% of those of wild-type plants, respectively. Morphological alterations of other organs, such as flowers and roots, were not found in *35S::ANAC036* plants (data not shown).

Anatomical analysis of 35S::ANAC036 plants

We also observed cell sizes of the *35S::ANAC036* plants. In the 3rd and 8th leaves of *35S::ANAC036* plants, the sizes of epidermal and mesophyll cells were smaller than those in wild-type plants (Fig. 3A–F, Table 3). The cell area of mesophyll cells in *35S::ANAC036* plants was approximately 40–60% of that in wild-type plants. Observation of longitudinal sections of the bottoms of primary inflorescence stems revealed that the length of pith cells in *35S::ANAC036* plants in the longitudinal direction was 65–80% of that in wild-type plants (Fig. 3G and H, Table 3). Observation of transverse sections of stems showed no other morphological alterations in *35S::ANAC036* plants (Fig. 3I–L).

Analysis of ANAC036 gene expression

To determine the organs in which the *ANAC036* gene is expressed, RNA gel blot analysis and histochemical analysis of GUS activity in transgenic plants containing chimeric genes constructed by fusion of the *GUS* gene and the upstream sequence of the *ANAC036* gene (*ANAC036-promoter::GUS* plants) were performed. For RNA blot analysis, the coding region (corresponding to amino acids from 1 to 276) of the *ANAC036* gene was used as a probe, and the probe specificity was confirmed by genomic DNA blot analysis (data not shown). Fig. 4A shows that the *ANAC036* gene is highly expressed in rosette leaves and slightly expressed in seedlings and inflorescences.

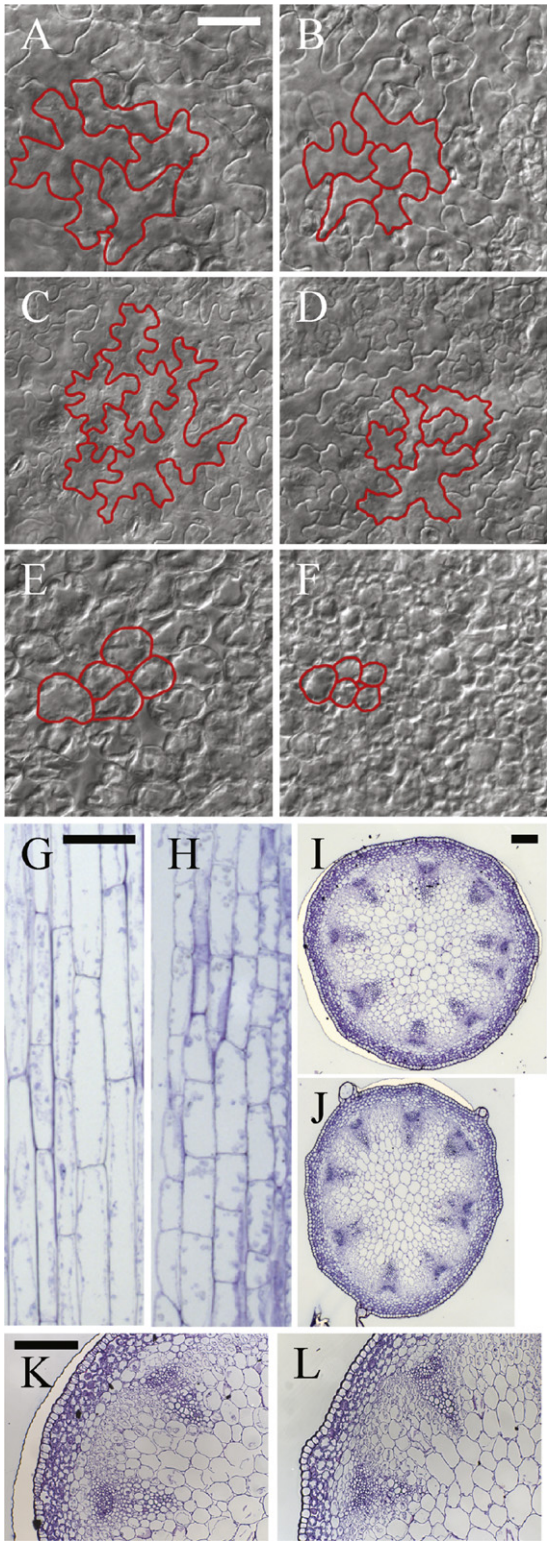

Fig. 3. Anatomical phenotype of *35S::ANAC036* plants. Samples were harvested 3 days after anthesis of the first flower. (A–F) Images of epidermal and mesophyll cells. Adaxial epidermal cells in 8th leaves of wild-type plants (A) and *35S::ANAC036* L1 plants (B), abaxial epidermal cells in 8th leaves of wild-type plants (C) and *35S::ANAC036* L1 plants (D) and mesophyll cells in 8th leaves of wild-type plants (E) and *35S::ANAC036* L1 plants (F). Scale bar=50 μm. (G, H) Longitudinal sections of stems of wild-type plants (G) and *35S::ANAC036* L1 plants (H). Scale bar=50 μm. (I–L) Transverse sections of stems of wild-type plants (I, K) and *35S::ANAC036* L1 plants (J, L). Scale bar=100 μm.

Table 3
Lengths of pith cells (μm) and areas of leaf cells (μm^2) of wild-type and *35S::ANAC036* plants.

Sample	Wild type	*35S::ANAC036*L1	*35S::ANAC036*L3
Pith cells of primary inflorescence stem	161.0 ± 32.4	106.6 ± 20.0 (66.2%)	130.0 ± 26.8 (80.7%)
Adaxial epidermal cells of the 3rd leaf	2550.0 ± 884.8	1212.4 ± 303.9 (47.5%)	1910.6 ± 812.6 (74.9%)
Abaxial epidermal cells of the 3rd leaf	1861.5 ± 598.1	1104.9 ± 248.7 (59.4%)	1609.0 ± 716.2 (86.4%)
Mesophyll cells of the 3rd leaf	820.4 ± 223.6	320.5 ± 93.1 (39.1%)	433.1 ± 121.0 (52.8%)
Adaxial epidermal cells of the 8th leaf	2922.1 ± 837.5	1669.7 ± 384.1 (57.1%)	1915.9 ± 694.2 (65.6%)
Abaxial epidermal cells of the 8th leaf	2195.1 ± 611.8	1649.7 ± 455.5 (75.2%)	1626.5 ± 524.2 (74.1%)
Mesophyll cells of the 8th leaf	813.4 ± 208.1	476.8 ± 97.8 (58.6%)	443.6 ± 96.9 (54.5%)

Samples were harvested and used for measurements 3 days after anthesis of the first flower.
Sixty cells and 40 cells were measured in wild-type and *35S::ANAC036* L1 plants, respectively. In *35S::ANAC036* L3 plants, 40 pith cells and 60 leaf cells were measured. Averages with standard deviation are shown.
Ratios of transgenic plants to wild-type plants are shown in parentheses.

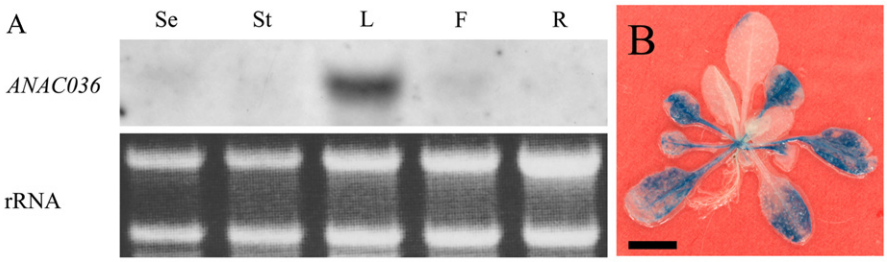

Fig. 4. Expression analysis of the *ANAC036* gene. (A) RNA gel blot analysis of the *ANAC036* gene in different organs. RNA was extracted from 1-week-old seedlings (Se), stems (St), rosette leaves (L), inflorescences (F) and roots (R). Ethidium bromide-stained rRNAs are shown as a loading control. (B) Histochemical GUS staining of a transgenic plant carrying the GUS coding region fused to the *ANAC036* promoter. A 3-week-old plant grown in soil was stained. Scale bar=1 cm.

The sites of GUS expression in *ANAC036-promoter::GUS* plants were examined by histochemical staining. We observed GUS expression in rosette leaves of adult plants. Some leaves showed strong GUS activity and others scarcely showed GUS activity in one individual (Fig. 4B). Although we examined five transgenic lines, the same results of GUS staining were obtained. A difference, e.g., leaf stage and leaf size, between stained leaves and non-stained leaves was not found. GUS activity was not detected in other organs, including stems, flowers and roots.

Discussion

In this study, we characterized a novel member of the NAC protein family in *Arabidopsis*, ANAC036. The amino acid sequence of ANAC036 was unique compared to those of other *Arabidopsis* NAC proteins, suggesting functional originality of ANAC036 in *Arabidopsis*. Two conserved amino acid sequences named C1 and C2 domains were found in ANAC036 and in various NAC proteins reported in other plants. These domains were not found in other *Arabidopsis* NAC proteins. The C1 domain contained the putative NAC subdomain E and its immediately downstream sequence, indicating the possibility that this domain participates in the specificity of DNA binding sequences because NAC subdomain E is required for DNA-binding ability (Duval et al., 2002). These NAC proteins also had high amino acid sequence homology to ANAC036 in the NAM domain. In some plants, two proteins similar to ANAC036 were reported and one of them tended to have a more similar amino acid sequence than the other one. These facts suggest that ANAC036 and its orthologues exist widely in plants and that they have a common role.

We found that transgenic plants overexpressing the *ANAC036* gene showed a semidwarf phenotype. Microscopic observation revealed that cell size of these plants was reduced in leaves and stems, indicating the dwarfing of these plants was primarily due to reduction of cell size. We have not examined the increase of ANAC036 protein in *35S::ANAC036* plants, although the accumulation of its RNA was confirmed by RT-PCR. The possibility remains that other effects, e.g., down-regulation of NAC genes related to ANAC036 by mechanisms such as co-suppression, cause dwarfing of *35S::ANAC036* plants. However, we considered this possibility to be low. The accumulation of ANAC036 RNA in *35S::ANAC036* plants suggests that the small RNAs like siRNAs were not produced from the transcripts of endogenous and exogenous *ANAC036* genes in *35S::ANAC036* plants.

It has been reported that overexpression of the xylem NAC domain 1 (*XND1*) gene (Zhao et al., 2008) and expression of C-terminal truncated VND7 (Yamaguchi et al., 2008), which exhibits a dominant negative effect, cause a dwarf phenotype in *Arabidopsis*. These dwarfing plants have a defect in xylem formation. However, observation of transverse sections of the bottoms of stems showed that no other morphological alterations, e.g., in vascular bundle formation, had occurred in *35S::ANAC036* plants (Fig. 4I–L), indicating that the dwarfing of *35S::ANAC036* plants was induced by different sources. There have been other studies of transgenic plants, in which NAC genes were introduced, showing a dwarf phenotype in *Arabidopsis*. Constitutive expression of active forms of NTM1 causes a dwarf phenotype (Kim et al., 2006) by reduction of cell division, not by reduction of cell size. Constitutive expression of active forms of NTM1-LIKE 8 also results in a dwarf phenotype (Kim et al., 2007). The major functions of NTM1-LIKE 8 are regulation of flowering time and seed germination, which are not related to ANAC036 function (Kim et al., 2007, 2008).

RNA gel blot analysis and histochemical analysis of the *ANAC036-promoter::GUS* plants revealed that the *ANAC036* gene was strongly expressed in rosette leaves. We detected faint hybridization bands by RNA blot analysis using seedling and inflorescence RNAs. Public microarray data in the ATTED-II database indicated that the *ANAC036* gene is expressed in rosette leaves and expressed weakly in cotyledons in 7-d-old seedlings and sepals in flowers. The ATTED-II database also showed that

expression of the *ANAC036* gene was induced by some abiotic stresses, such as osmotic stress, salt stress and UV-B stress, and by cycloheximide treatment. It is possible that overexpression of the *ANAC036* gene leads to excessive stress responses, subsequently compromising cell growth.

In rice, OsNAC6, which is the closest orthologue of *Arabidopsis* ATAF2, has been shown to be involved in abiotic and biotic stress-responsive gene expression. Overexpression of the *OsNAC6* gene caused upregulation of biotic stress-related genes, including *PR* genes, and growth retardation of plants (Nakashima et al., 2007). In *Arabidopsis*, various mutants that have constitutively active salicylic acid or jasmotic acid pathways exhibit a stunted phenotype (Heil and Baldwin, 2002). We examined the expression of *PR-5* and *BGL2* genes, the expression of which is induced by salicylic acid, and that of *PDF1.2* and *THI2.1* genes, the expression of which is induced by jasmotic acid. However, no upregulation of those genes was detected in *35S::ANAC036* plants (data not shown). At present, there is no evidence that the dwarf phenotype caused by overexpression of the *ANAC036* gene resulted from a constitutive defense response. The mechanism by which over-expression of the *ANAC036* gene causes reduction of cell size is not known.

Recently, the functions of two *Arabidopsis* NAC protein members belonging to the ONAC022 subgroup have been reported. ANAC034/035, named LONG VEGETATIVE PHASE 1 (LOV1), is involved in regulation of flowering time and cold stress response (Yoo et al., 2007). ANAC009, named FEZ, controls the cell division in a limited area of root cap stem cells (Willemsen et al., 2008). These findings suggest that there is a functional difference between ANAC036, LOV1 and FEZ despite the fact that they belong to the same subgroup. ANAC036 is thought to have a novel function and to play a common role in various plants. Further works, e.g., identification of target genes of ANAC036, will clarify the role of ANAC036 and its orthologues in plants.

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