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A membrane-tethered transcription factor ANAC089 negatively regulates floral initiation in *Arabidopsis thaliana*

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The plant-specific NAC (NAM, ATAF1/2, and CUC2) transcription factors have a regulatory function in developmental processes and stress responses. Notably a group of NAC members named NTLs (NTM1-Like) are membrane-tethered, ensuring plants rapidly respond to developmental changes and environmental stimuli. Our results indicated that ANAC089 was a membrane-tethered transcription factor and its truncated form was responsible for the physiological function in flowering time control.

***Arabidopsis thaliana*, ANAC089, floral initiation, membrane-tethered transcription factor**

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Transcription factors regulate many vital cellular pathways by controlling the expression of downstream genes. There are more than 110 NAC (NAM, ATAF1/2, and CUC2) members comprising one of the largest transcription factor families in *Arabidopsis* [1]. NAC proteins have a highly conserved NAC DNA-binding domain in their N-terminal regions and quite diverse C-terminal regions with transcriptional activities. Several NAC proteins have been functionally described in a variety of plant growth and developmental processes, such as apical meristem formation [2,3], cell cycle control [4], flower development [5], lateral root formation and development [6,7], secondary wall thickenings [8,9], and leaf senescence [10,11]. Additionally, A few NAC members have been reported to contribute to various stress responses [12–14].

A group of NAC members named NTLs (NTM1-Like) are membrane-tethered transcription factors (MTTFs) which ensure that plants rapidly respond to developmental or environmental stimuli [15–17]. MTTFs differ from cytosolic transcription factors in that they are innately inserted into a membrane and exist in a dormant state [18]. The structures of MTTFs are very similar, including a transmembrane domain (TMD) as the membrane anchor and a cytosolic domain containing the transcription factor (TF) motif. MTTFs are activated by proteolytic cleavage, which liberates the cytosolic domain from the TMD and enables the MTTFs to travel to the nucleus. MTTFs identified in *Arabidopsis* include four members of the bZIP family (AtbZIP17, AtbZIP28, AtbZIP49 and AtbZIP60) [19–21] and four of the NAC family (NTM1, NTL6, NTL8 and NTL9) [4,11,15].

Genome-scale analysis shows that over 10% of the NAC transcription factors are membrane-tethered transcription factors (MTTFs) in *Arabidopsis* [15]. Only a few plant MTTFs have been identified and functionally studied. Therefore, characterization of MTTFs is vital not only because these proteins are involved in development and stress response but also because doing so provides a model for studying the mechanism for selective proteolysis and the mo-

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lecular schemes that guide activated proteins to the nucleus.

Floral initiation at an appropriate time is necessary for plants to facilitate reproduction. There are four flowering pathways, including photoperiod, autonomous, GA pathway, and vernalization, which regulate the transition from vegetative phase to reproductive phase in *Arabidopsis*. It is known that a small set of key regulatory genes integrate the interaction of these multiple flowering pathways and such a signal convergence allows plants to control floral initiation in the face of environmental stimuli and internal cues [22–24]. One key *Arabidopsis* transcriptional regulator of floral initiation in the photoperiod pathway is CO (constans) [25], a B-box zinc-finger transcription factor controlling the time of flowering by positively regulating two floral integrators, *FT* (*flowering locus t*) and *SOC1* (*suppressor of overexpression of co 1*) [26,27]. FT is expressed in leaves and translocated to the shoot apex where it regulates the expression of such floral meristem identity genes as *LFY* [27–29]. Another significant regulator is FLC (flowering locus c), a MADS-box transcription factor, which acts as a repressor in both the autonomous and vernalization flowering pathways and also acts upstream of *FT* and *SOC1* [30,31]. In the GA signaling pathway DELLA proteins as key regulators modulate all aspects of GA responses. For example, RGA (repressor of *ga1-3*) and GAI (gibberellin insensitive) repress GA-induced vegetative growth and floral initiation [32]. Recent studies have revealed the timing of floral transition was controlled by chromatin modifications [33–36].

In this study we report a membrane-tethered transcription factor *ANAC089* negatively regulating floral initiation in *Arabidopsis*.

1 Materials and methods

1.1 Plant materials, growth conditions, and *Arabidopsis* transformation

Arabidopsis thaliana (ecotype Col-0) was used in the present study. Seeds were surface-sterilized and sown on solid agar plates containing 4.3 g L^{-1} Murashige and Skoog (MS) Basal Salt Mixture Substratum (Sigma, St. Louis, MO, USA) with 3% (w/v) sucrose and 0.8% (w/v) agar, adjusted to pH 5.8. The plates were kept in the dark at 4°C for 3 d before being placed in a growth chamber for germination. Seven-day-old seedlings were transferred to soil and grew to maturity in a controlled culture room set at 23°C with a relative humidity of 65% under fluorescent illumination (100 μmol m^{-2} s^{-1}, 16 h of light and 8 h of darkness).

Agrobacterium-mediated transformation of *Arabidopsis* plants was carried out by a modified floral dip method [37]. To generate transgenic *Arabidopsis* plants, the gene constructs were subcloned into the modified binary pCAMBIA1300 vector under the control of the CaMV 35S promoter.

1.2 Protein sequence analysis

Amino acid sequences of ANAC089 (At5g22290) and NTL8 (At2g27300) were compared using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) and the clustalW server (http://www.ebi.ac.uk/clustalw). TMs (Transmembrane Motifs) were predicted using the ARAMEMNON membrane protein database (http://www.aramemnon.botanik.uni-koeln.de).

1.3 Subcellular localization analysis of ANAC089 proteins

Localization of the ANAC089 protein was assessed by transient expression of ANAC089F (full coding region, 1–340 aa) or ANAC089 Δ C (1–310 aa) and green fluorescence protein (GFP) fusion protein in *Arabidopsis* protoplasts and in *Nicotiana Benthamiana* leaves. The ANAC089F and ANAC089 Δ C coding regions were obtained by RT-PCR amplification. The PCR-amplified fragments were respectively inserted into the modified pUC-EGFP vector and pGPTVII-GFP vector. Protoplast preparation and transient transformation were performed as previously described [38]. For transient analysis in *Nicotiana benthamiana* leaf cells, *Agrobacterium tumefaciens* containing the pGPTVII-GFP vector with fusion genes were directly injected into the leaves. The endoplasmic reticulum marker (ER-mCherry) was used as a control (ER-rk: *Arabidopsis* Information Resource stock No. CD3-959) [39]. Images were obtained using a confocal laser microscope (LSM 510 CLSM, Zeiss). The GFP was excited at 488 nm and the emission was collected at 515–530 nm; mCherry was excited at 543 nm and the emission was collected at 585–615 nm. Adobe Photoshop 8.0.1 was used for further processing of all of the images.

1.4 Transcription activity analysis in yeast

The yeast strain YRG-2 harboring the *LacZ* and *HIS3* reporter genes was used as an assay system. The fragments of *ANAC089-N* (1–167 aa), *ANAC089-C* (168–340 aa), and *ANAC089ΔC-C* (168–310 aa) were respectively obtained by PCR. According to the protocol provided by the manufacturer (Stratagene), pBD-ANAC089-N, pBD-ANAC089-C, pBD-ANAC089ΔC-C, the positive control pGAL4 and the negative control pBD vector were all transformed into the yeast strain YRG-2. The transformed strains were confirmed by PCR and were selected onto an SD/Trp^- media plate. The transcription activation activities were evaluated according to their growth status on SD/His^- media plates.

1.5 Construction of the *ANAC089* promoter::GUS fusion vector and β-glucuronidase analysis

A 1778-bp-long DNA promoter fragment upstream from the ATG start codon of the *ANAC089* was amplified by PCR. The amplified DNA product was inserted into the binary

vector pCAMBIA1391 (CAMBIA, Canberra, ACT, Australia). The *pANAC089-GUS* construct was transformed into *Arabidopsis* plants, and homozygotic plants were subjected to GUS staining as described by Jefferson [40].

1.6 Flowering time measurements

Plants were grown under LD (16 h of light and 8 h of darkness) conditions for flowering time measurements. The total number of rosette leaves and the days to the inflorescence stem height at 1 cm were used to measure flowering times. At least 30 plants were measured and averaged for each measurement.

1.7 Analysis of flowering time genes expression by reverse-transcriptase quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, California, USA) from 100 mg of 20-day-old seedlings under LD conditions. The cDNAs were synthesized using M-MLV reverse transcriptase with oligo-d (T) 15 primers according to the manufacturer's instructions (Promega, Madison, WI, USA). Reverse-transcriptase quantitative PCR (RT-qPCR) was performed with SYBR-Green I intercalating dye (Bio-Rad, Hercules, USA) using a MyiQ Single Color Real Time PCR Detection System (Bio-Rad). The following PCR program was used: an initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. *ACTIN2* gene was used as an internal control. The RT-qPCR data were analyzed using the MyiQ software (Bio-Rad). All experiments were independently repeated three times.

1.8 Primers

The primers used in this paper are listed in Table 1.

Table 1 Primer sequences used for cloning and PCR

Primer name	Sequences (5′–3′)	Applications
ANAC089ΔC F	TTCTGCAGATGGACACGAAGGCGGTTG	*ANAC089ΔC::35S* cloning
ANAC089ΔC R	TCGGTACCTTACACGAAGCAGCTCGACAAT	*ANAC089ΔC::35S* cloning
ANAC089gfp F	AAGGATCCATGGACACGAAGGCGGTTG	*ANAC089::GFP* cloning
ANAC089gfp R	GCGGTACCTTCTAGATAAAACAACATTG	*ANAC089::GFP* cloning
ANAC089ΔCgfp F	AAGGATCCATGGACACGAAGGCGGTTG	*ANAC089ΔC::GFP* cloning
ANAC089ΔCgfp R	GCGGTACCCACGAAGCAGCTCGACAATG	*ANAC089ΔC::GFP* cloning
ANAC089-N F	CTGAATTCATGGACACGAAGGCGGTTG	*ANAC089-N* cloning for transcription activity analysis
ANAC089-N R	AACTGCAGTTAGTTCCTCCTAACCCGGCAA	*ANAC089-N* cloning for transcription activity analysis
ANAC089-C F	GGGAATTCAAAGAATACAATAGTGGTA	*ANAC089-C* cloning for transcription activity analysis
ANAC089-C R	CCTGCAGTTATTCTAGATAAAACAACATTG	*ANAC089-C* cloning for transcription activity analysis
ANAC089ΔC-C F	GGGAATTCAAAGAATACAATAGTGGTA	*ANAC089ΔC-C* cloning for transcription activity analysis
ANAC089ΔC-C R	AACTGCAGTTACACGAAGCAGCTCGACAAT	*ANAC089ΔC-C* cloning for transcription activity analysis
ANAC089promoter F	TGGATCCTTTGATGGCCGTTGGTCTG	*pANAC089::GUS* cloning
ANAC089promoter R	TGAATTCAAACTCCAACCGCCTTCGT	*pANAC089::GUS* cloning
Actin real F	GGTAACATTGTGCTCAGTGGTGG	Actin for qRT-PCR
Actin real R	AACGACCTTAATCTTCATGCTGC	Actin for qRT-PCR
ANAC089 real F	GGAACACACCAAACGAAGTGCCAA	*ANAC089* for qRT-PCR
ANAC089 real R	TAAACCCACGAAGCAGCTCGACAA	*ANAC089* for qRT-PCR
CO real F	GCCATCAGCGAGTTCCAATTCTAC	*CO* for qRT-PCR
CO real R	CCTTCCTCTTGATCCACCACCAG	*CO* for qRT-PCR
SOC1 real F	TAAGGATCGAGTCAGCACCAAACC	*SOC1* for qRT-PCR
SOC1 real R	AGCTCCTCGATTGAGCATGTTCCT	*SOC1* for qRT-PCR
FT real F	TCCCTGCTACAACTGGAACAACCT	*FT* for qRT-PCR
FT real R	GCCTGCCAAGCTGTCGAAACAATA	*FT* for qRT-PCR
FLC real F	CGTCGCTCTTCTCGTCGTCTC	*FLC* for qRT-PCR
FLC real R	TTCGGTCTTCTTGGCTCTAGTCAC	*FLC* for qRT-PCR
LFY real F	TCCACTGCCTAGACGAAGAAGC	*LFY* for qRT-PCR
LFY real R	TCCCAGCCATGACGACAAGC	*LFY* for qRT-PCR
GAI real F	ACGCCGAGTACGATCTTAAAGC	*GAI* for qRT-PCR
GAI real R	TTTCCACGACGCCGTTTGAG	*GAI* for qRT-PCR
RGA real F	AAGCATACGACGGTGGTGGAG	*RGA* for qRT-PCR
RGA real R	TTCAGTTCGGTTTAGGTCTTGGTC	*RGA* for qRT-PCR

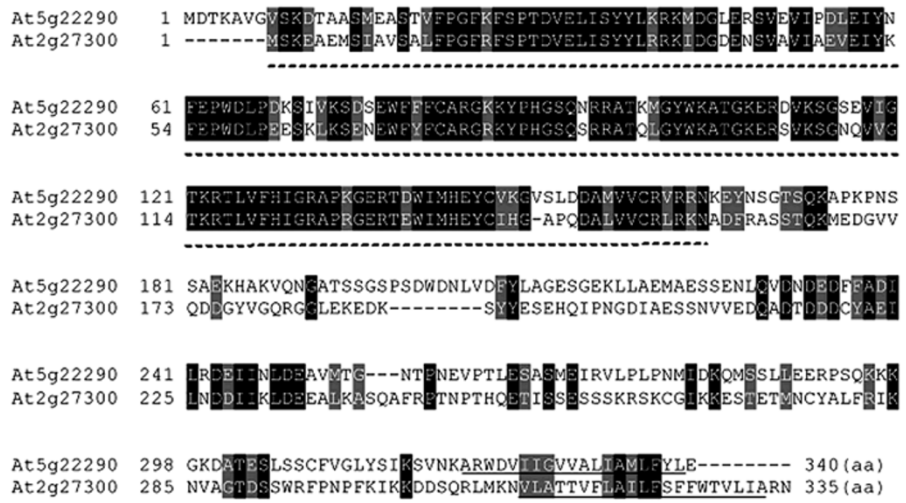

Figure 1 Sequence comparison of ANAC089 (At5g22290) and NTL8 (At2g27300).

2 Results

2.1 Sequence alignment of ANAC089 with NTL8 in *Arabidopsis*

A protein sequence comparison of ANAC089 (At5g22290) with NTL8 (At2g27300) revealed that ANAC089 contained the NAC conserved domain as other NAC transcription factors. A transmembrane domain (TMD) was clearly predicated in the far C-terminal sequence (321–339 aa) of the ANAC089. Although both *ANAC089* and *NTL8* belong to the OsNAC8 subfamily, there is less similarity in their C-terminal regions (Figure 1).

Amino acid sequences were aligned using clustalW. Identical amino acids were shaded in black, and similar amino acids were shaded in gray. The NAC domains were underlined with broken lines and the transmembrane motifs (TMs) were underlined with solid lines.

2.2 Membrane-tethering of the ANAC089 transcription factor

ANAC089 has a potential TMD in the far C-terminal sequence. Therefore we hypothesized that *ANAC089* might be a MTTF. Transient transformation of *Arabidopsis* leaf protoplasts showed that the ANAC089F signal was detected in the cytoplasm while the ANAC089ΔC signal was exclusively localized in the nucleus (Figure 2A). Further research using Agrobacterium coinfiltration of *Nicotiana benthamiana* confirmed that ANAC089F was localized in the endoplasmic reticulum. Figure 2B shows that GFP-tagged ANAC089F was overlapped with the endoplasmic reticulum marker (ER-mCherry).

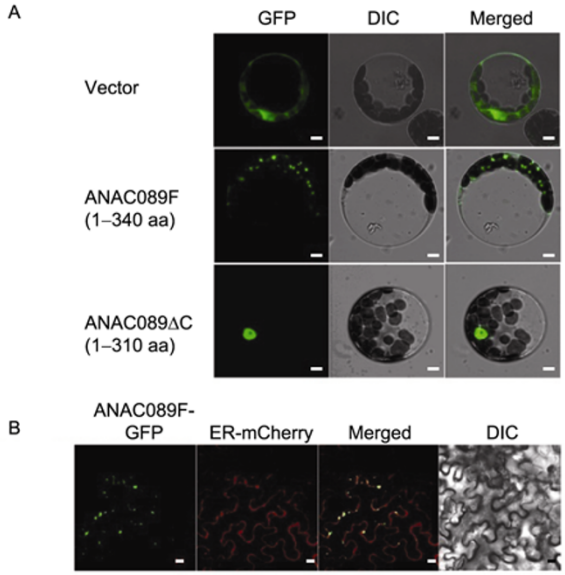

Figure 2 Subcellular localization of *ANAC089* gene products in *Arabidopsis* protoplasts and the *Nicotiana benthamiana* leaf cells. A, *Arabidopsis* protoplasts were transformed with the modified pUC-EGFP vector containing the indicated gene fusions. Protoplasts expressed the black GFP vector as a control. ANAC089 peptide fragments (1–340 aa or 1–310 aa) were fused to the N terminus of GFP for expression in the protoplasts. Cells were examined using a confocal laser scanning microscope. The GFP fusion protein is shown in green. Scale bar=5 μm. B, Subcellular localization of the ANAC089F (1–340 aa) fusion protein by the Agrobacterium coinfiltration assay. Leaves of the *Nicotiana benthamiana* plants were infiltrated with an *A. tumefaciens* strain carrying the modified pGPTVII-ANAC089F-GFP vector together with an *A. tumefaciens* strain carrying the ER-mCherry plasmid. The GFP fusion protein is shown in green; the mCherry fusion protein is shown in red. Scale bar=10 μm.

2.3 Transcription activation activity in the C-terminal region of ANAC089

To investigate whether ANAC089 may function as a tran-

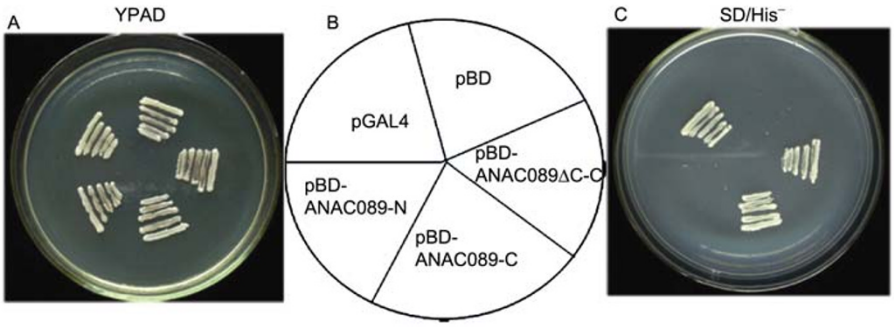

Figure 3 Transcription activity of ANAC089 in yeast. Transcription activity analyses of pBD-ANAC089-N (1–167 aa), pBD-ANAC089-C (168–340 aa), pBD-ANAC089ΔC-C (168–310 aa), pBD and pGAL4 were expressed in yeast strain YRG-2. The transformants were respectively streaked on the YAPD medium (A) and on the SD/His⁻ medium (C) for examination of growth.

scriptional activator like other known NAC transcription factors, the *ANAC089* gene fragments were in-frame fused to the GAL4 DNA binding domain. All the transformants grew well on the YAPD medium (Figure 3A). On an SD medium without histidine, the yeast cells containing pBD-ANAC089-C, pBD-ANAC089ΔC-C and the positive control plasmid pGAL4 all grew well, while the cells containing pBD-ANAC089-N and the negative control plasmid pBD did not grow (Figure 3C). These results indicated that ANAC089 was a transcription factor and its transcription activation activity was determined by the C-terminal domain even without its TMD.

2.4 Tissue-specific expression of the *ANAC089*

To examine the tissue expression pattern of *ANAC089*, we generated and analyzed the *ANAC089 promoter::GUS* transgenic plants. 20 independent transgenic lines were analyzed by histochemical detection of GUS activity. The expression profiles were similar for all tested lines. The GUS signal was first observed in cotyledons of the germinating seeds (Figure 4A). Strong GUS staining was primarily localized in the vascular tissues of hypocotyls, cotyledons, roots (except root tips), rosette leaves and cauline leaves (Figures 4B–E). Among the floral organs, the vascular tissues of the corolla, filaments and chapiters also exhibited a distinct GUS signal (Figure 4F). However, no expression was observed in mature siliques, stems and seeds (Figures 4E and G). These results revealed that *ANAC089* was primarily expressed in the vascular tissues.

2.5 Delayed flowering in the *35S::ANAC089ΔC* transgenic plants under the LD condition

To obtain insights into the physiological role of *ANAC089*, the truncated *ANAC089* form (*ANAC089ΔC*) was overexpressed in transgenic *Arabidopsis* plants under the control of the CaMV 35S promoter. More than 30 independent transgenic lines were obtained and many of them exhibited the distinct phenotypes: Dwarfed growth with small, dark

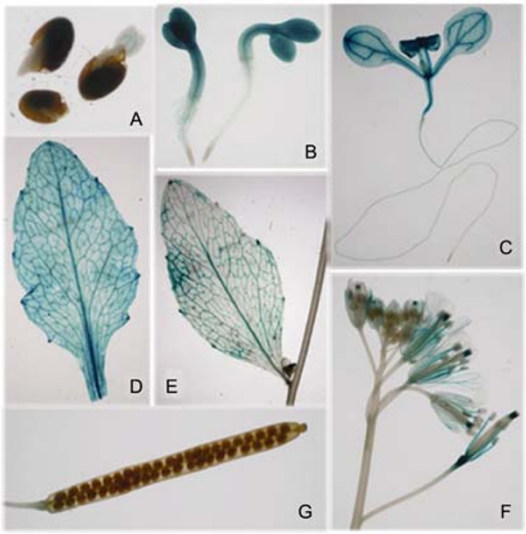

Figure 4 *ANAC089 promoter-GUS* expression pattern in transgenic *Arabidopsis* plants. A, Seeds germinated for 24 h. B, three-day-old seedlings. C, Ten-day-old seedlings. D, A rosette leaf from a three-week-old plant. E, Stem and cauline leaf from a five-week-old pant. F, Inflorescence from a five-week-old plant. G, Mature siliques.

green leaves and late flowering as compared to wild-type plants (Figure 5A). Three of the transgenic lines (OE-1, OE-2 and OE-3) were selected for detailed investigation. Under the LD condition, the wild type, OE-1, OE-2 and OE-3 began respectively flowering with 12.10, 16.52, 20.13 and 22.87 rosette leaves. The number of days of wild-type plants at the time of flowering was 27.73, but that of OE-1, OE-2 and OE-3 were respectively 33.17, 38.85 and 42.54 (Figure 5C). Our results showed that *35S::ANAC089ΔC* plants exhibited a delayed flowering phenotype compared to wild-type plants under the LD condition. The phenotypic variations were related to the transcript level of *ANAC089ΔC* among transgenic lines. A higher transcript level of *ANAC089ΔC* corresponded to a more severe phenotypic change (Figures 5A and B), suggesting that the phenotypic differences of the transgenic lines might be caused by *ANAC089ΔC* overexpression with a gene dosage effect.

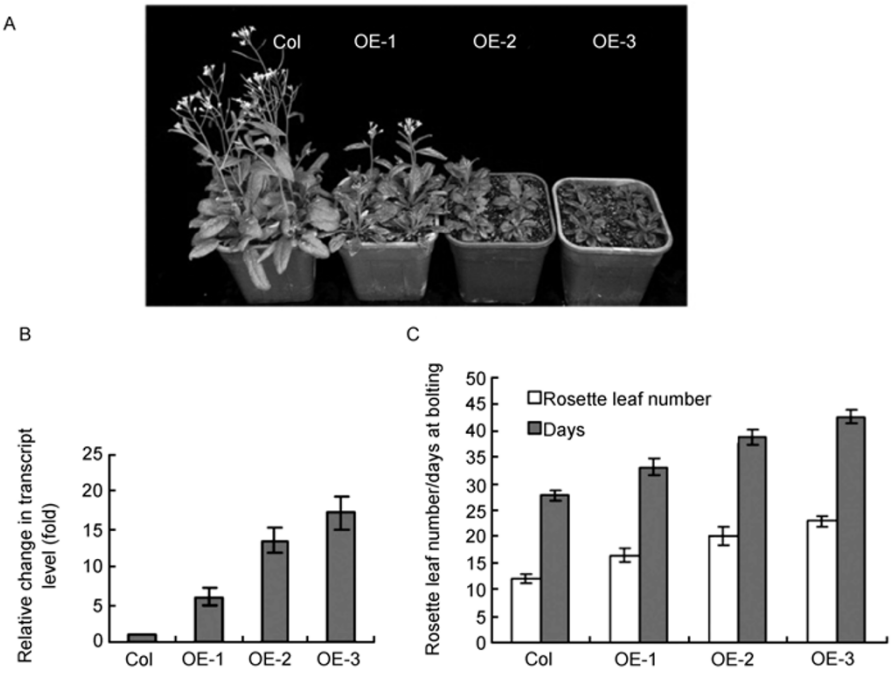

Figure 5 Transgenic plants overexpressing *ANAC089ΔC*. A, Flowering phenotypes of transgenic plants overexpressing *ANAC089ΔC* under long-day photoperiod conditions. B, Transcript levels of *ANAC089ΔC* in the transgenic plants (OE-1, OE-2 and OE-3). C, The number of rosette leaves and the days to the inflorescence stem height at 1 cm. Measurements of 30 plants were averaged. Bars denote standard error of the mean.

2.6 Expression patterns of flowering time genes in the *35S::ANAC089ΔC* transgenic plants

As *35S::ANAC089ΔC* plants exhibited a delayed flowering phenotype, we examined the expression patterns of several major flowering integrator genes [24] in the transgenic plants (OE-2 and OE-3) using RT-qPCR analysis. The transcript levels of *CO*, *SOC1*, *FT*, and *LFY* were greatly repressed in the transgenic plants (Figure 6). The expression levels of a major regulator in the photoperiod pathway, *CO*, were downregulated in OE-2 (38% of the control) and in OE-3 (27% of the control). The transcript levels of *SOC1*, *FT*, and *LFY* respectively decreased in OE-2 by 37%, 13%, and 35% of the control and in OE-3 by 23%, 9%, and 28% of the control. However, compared to wild-type plants, the *FLC* expression levels were respectively more than 3.23- and 4.06-fold higher in OE-2 and OE-3. The transcript level of *GAI* or *RGA* was not significantly impacted in either of the tested plants. These observations indicated that *ANAC089* negatively regulated flowering initiation by regulating *CO*, *FLC*, *FT*, *SOC1*, and *LFY* expressions.

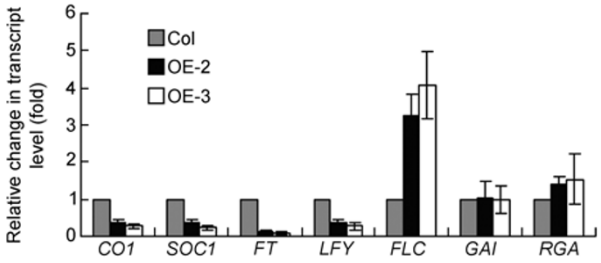

Figure 6 Transcript levels of flowering time genes. Transcript levels were measured by RT-qPCR and normalized by using *actin* as an internal reference. Bars denote the standard error of the mean. All experiments were independently repeated three times.

3 Discussion

Accumulating proofs suggest that MTTFs are associated with the intracellular membranes and released by controlled proteolytic cleavage. Such an adaptive strategy ensures plants respond to abrupt environmental stimuli and developmental changes [17,41,42]. Sequence analysis predicted that a TMD was located in the far C-terminal sequence of ANAC089. Thus we presumed that ANAC089 might be an MTTF. The following transient assay showed that the full-size ANAC089 (ANAC089F) localized to the ER and the truncated ANAC089 (ANAC089ΔC) were exclusively in the nucleus. The C-terminal region of ANAC089 and the C-terminal domain of ANAC089ΔC had transcription activation activity. The above results indicated that ANAC089 was a membrane-tethered transcription factor and the truncated form (ANAC089ΔC) was responsible for its physiological function as with other MTTFs [16].

Transgenic plants overexpressing *ANAC089ΔC* were late flowering in the long day condition. Consistent with the

delayed flowering phenotype, the transcriptional levels of *CO*, *FT*, *SOC1* and *LFY* decreased but *FLC* expression increased. We also obtained *ANAC089F*-overexpressing transgenic plants (*35S::ANAC089F*), but they only exhibited a slight degree of retarded growth (data not shown). This might be due to ANAC089F as a dormant form and indicated that the membrane release was essential for the *ANAC089* function. In addition, a T-DNA insertion mutant *FLAG066A02* obtained from Institute Jean-Pierre Bourgin did not show any discernible phenotypic changes compared with wide-type plants. This is possible due to a functional redundancy among the subfamily members. In Kim's studies the transgenic plants overexpressing *NTL8ΔC* also exhibited late flowering [5]. Both *NTL8* and *ANAC089* belong to the OsNAC8 subfamily in *Arabidopsis*. Owing to no informative knockout mutant of *ANAC089* being available, double mutant or RNA-induced gene silencing was used.

Because ANAC089 negatively regulates *CO* expression, a significant question is whether ANAC089 directly binds to a *cis*-acting element within the *CO* promoter. Tran *et al.* [13] identified the NAC recognition sequence containing CATGT and CACG as the core DNA binding site. There are seven CATGT motifs and four CACG motifs in the *CO* promoter region (1900 bp upstream of ATG, http://www.dna.affrc.go.jp/PLACE/index.html). Under the long-day condition the *CO* transcript accumulates in the vasculature of cotyledons and leaves to stimulate *FT* transcription. Considering that the strong GUS signal was located in the vascular tissues of leaves (Figure 4), we hypothesize that *CO* may be a target of ANAC089. A future study will be performed to reveal whether or not ANAC089 directly targets and activates *CO* as well as its DNA binding specificity.

Most of the identified MTTFs are involved in stress response [16,17]. It remains undetermined whether or not *ANAC089* is also involved in the environmental stress response. Considering ANAC089 as a membrane-tethered transcription factor, another question concerns what leads to the proteolytic cleavage of ANAC089.

In summary, our results suggest that ANAC089 is a membrane-tethered transcription factor and its truncated form is responsible for its physiological function in flowering time control. Yet the precise molecular mechanism by which ANAC089 regulates floral initiation awaits future research.

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