

Ectopic expression of *NtEF1* and *NtEF2* promotes flowering and alters floral organ identity in *Nicotiana tabacum*

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Abstract A previous study revealed that *IbEF1* from sweet potato promoted early flowering in transgenic tobacco and acted as a novel regulator involved in flowering time control. Two putative ortholog genes were found in *N. tabacum*: *NtEF1* and *NtEF2*. Subcellular localization of NtEF1 and NtEF2 with an in vivo targeting approach revealed that NtEF1 and NtEF2 were correctly targeted to the cytosol in protoplasts when expressed as a green fluorescent fusion protein (GFP). *NtEF1* and *NtEF2* are not thought to act as transcription factors. In this study, the expression of *NtEF2* was compared with that of *NtEF1* in the apical buds of *N. tabacum* of various ages. *NtEF2* was

only detected in open flowers that were 100 days old, whereas *NtEF1* was expressed in apical buds of 60-, 70-, 80-, 90- and 100-day-old plants. *NtEF1* was expressed in all floral organs, although its level of expression varied in sepals, petals, stamens and carpels. In contrast, *NtEF2* mRNA expression was only detected in the sepals. Transgenic tobacco plants overexpressing *NtEF1* and *NtEF2* were generated to elucidate the physiological function of *NtEF1* and *NtEF2*. The *35S::NtEF1* and *35S::NtEF2* transgenic lines showed early-flowering phenotypes. Moreover, the constitutive expression of *NtEF1* and *NtEF2* occasionally engendered an abnormal morphological alteration of anthers. It is assumed that there are only a few differential flowering regulations occurring in tobacco compared to *Arabidopsis*, indicating that *NtEF1* and *NtEF2* interfere with the flowering time and floral development.

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time · *NtMADS5* · *Nicotiana tabacum*

List of Abbreviations

API	APETALA1
FUL	FRUITFULL
LFY	LEAFY
FT	FLOWERING LOCUS T
AGL20	AGAMOUS-LIKE20
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
SEP	SEPALLATA
CaMV35S	Cauliflower Mosaic Virus 35S
CTAB	Cetyl trimethylammonium
RACE	Rapid amplification of cDNA ends
PCR	Polymerase chain reaction
ORF	Open reading frame
NAA	1-Naphthaleneacetic acid

BAP	Benzylaminopurine
HPT	Hygromycin B phosphotransferase
GFP	Green fluorescent protein
sGFP	Synthetic green fluorescent protein
smGFP	Soluble-modified green fluorescent protein
SAM	Shoot apical meristem
IM	Inflorescence meristem
SPLs	SQUAMOSA PROMOTER BINDING LIKEs

Introduction

The transition to flowering is a very important developmental step in higher plants. Flowering in plants consists of two different phases: an initial vegetative phase and a subsequent reproductive phase. Responses to environmental and internal signals trigger flowering initiation through a process controlled by several pathways (Jack 2001; Mouradov et al. 2002; Simson and Dean 2002; Fornara et al. 2010). Extensive genetic and physiological studies in the plant model *Arabidopsis thaliana* have enabled the identification of many genes involved in the regulation of flowering, approximately 180 of which have been shown to be related to flowering time control (Fornara et al. 2010). Floral induction occurs when floral meristem identity genes, such as *APETALA1* (*API*), *FRUITFULL* (*FUL*), and *LEAFY* (*LFY*), are activated by floral pathway integrators, such as *FLOWERING LOCUS T* (*FT*), *AGAMOUS-LIKE20* (*AGL20*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in *Arabidopsis* (Jack 2001; Simson and Dean 2002). *LFY*, which stimulates diverse floral homeotic genes with other floral regulators, directly activates *API*, which is associated with the floral meristem specification and acts as a floral organ identity gene that belongs to class A of the ABC model (Weigel and Meyerowitz 1994; Mandel et al. 1992; Wagner et al. 1999). When the floral organ identity genes, which comprise the proposed ABC model to explain floral organ development, are up-regulated by the floral meristem identity genes, flowering is completed (Jack 2001; Glover 2007). However, the flowering mechanism is very complex and moreover, there are many unknown genes which are regarded as regulators that interact with other floral-related genes in a flowering pathway.

Constitutive expression of many genes involved in flowering has shown early-flowering phenotype and morphological alteration of the flower. The ectopic overexpression of *Triticum aestivum VRN1* (*TaVRN1*), a member of the *API* subfamily, represents the early-flowering phenotype and causes various levels of defects in the

morphology of *Arabidopsis* (Adam et al. 2007). *AGL24*, which is closely related to *SOC1*, one of the floral integrators, stimulates flowering in *Arabidopsis*. The constitutive expression of *AGL24* results in diverse forms of abnormal flowers containing bract-like sepals and ectopic inflorescence formation (Michaels et al. 2003; Yu et al. 2004). The overexpression of *AGL6* related to *API/FUL*-like genes and *SEPALLATA* (*SEP*) promoted flowering and homeotic conversion (Reinheimer and Kellogg 2009; Koo et al. 2010). The genes involved in flowering participate in various processes, such as transition to the reproductive stage and floral organ development, in the complicated interaction with many other flowering genes.

In a previous study, a novel gene, *IbEF1* (GenBank ID: KF286541), was isolated from a sweet potato at levels equivalent to the most abundant mRNA present in root tissue under salt stress. *IbEF1* was considered to be a novel regulator involved in the control of flowering because transgenic tobacco (*Nicotiana tabacum* L. 'Xanthi') containing *IbEF1* showed an early-flowering phenotype (Kim et al. 2011). *IbEF1* was initially derived from expressed sequence tags (ESTs) of dehydration-treated white fibrous roots of sweet potato and its sequence information was assigned to pathogen-related gene by alignment with the translated sequences of GenBank nucleotide sequence database (Kim et al. 2009). However, this annotation was merely classified as putative characteristic of genes by bioinformatics analysis, and other genes such as pathogen-related protein-like (NCBI accession number: XP_002285489), conserved hypothetical protein (GenBank ID: EEF33891), and predicted protein (GenBank ID: EEF11635) which show relatively high identity of deduced amino acid sequence processed by BLASTX through NCBI database were also not identified clearly. Kim et al. (2011) revealed characteristic of the gene and proved that overexpression of *IbEF1* gene from sweet potato confers early flowering in tobacco. So, it was devised that some homologue genes which have same function with *IbEF1* would exist in *Nicotiana tabacum*.

In this study, putative homologues of *IbEF1*, *NtEF1* and *NtEF2* were screened for in *Nicotiana tabacum*. A comparison of both *35S::NtEF1* and *35S::NtEF2* transgenic tobacco to wild-type tobacco, based on genetic and molecular analysis of floral-related genes such as *NtFUL*, *NFL1*, and *NtMADS5*, demonstrated that the constitutive expression of *NtEF1* and *NtEF2* promotes earlier floral-related gene accumulation. Although there is no difference between the expression patterns of *35S::NtEF1* and *35S::NtEF2*, *NtEF1* and *NtEF2* are certainly involved in the flowering time because all transgenic lines showed the accumulation of *NtMADS5*, an orthologue of *API*, and a molecular indicator of transition to the reproductive phase, earlier than the wild-type. In addition, the results

demonstrated that a constitutive expression of these genes promotes flowering and causes the formation of abnormal flowers under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter in tobacco.

Materials and methods

Plant materials

Tobacco plants (*Nicotiana tabacum* L. ‘Xanthi’, photoperiod independent) were used in this study. The plants were grown in a climate chamber at 25 ± 3 °C under a 16 h light/8 h dark photoperiod. Transgenic T₂ plants were grown under both 460 nm blue and 630 nm red LED light (Dynebio, Korea).

Cloning of full-length *NtEF1* and *NtEF2* cDNA

Total RNA was isolated from the roots of *N. tabacum* exposed to 150 mM NaCl using the modified cetyl trimethylammonium (CTAB) method (Kim and Hamada, 2005). cDNA synthesis was conducted using the RACE (Rapid Amplification of cDNA Ends) system (Clontech, Palo Alto, CA, USA) for further finding of 5' and 3'-UTR regions, according to the manufacturer's instructions. 3'-RACE cDNA was used as template to search *IbEF1* (Kim et al., 2011) orthologue from tobacco by polymerase chain reaction (PCR) using two primers (EF-gene-search-F; 5'-TTTGAAGAAGGCAGAACTAA-3' and EF-gene-search-R; 5'-ATCCATGTAACCCCAATGCCT-3') which were designed on the basis of similar sequence through BLAST search results of *IbEF1* gene (GenBank ID: KF286541). After obtaining *NtEF1* and *NtEF2* genes from tobacco, 5'-RACE-PCR and 3'-RACE-PCR was performed to find 5'- and 3'-UTR regions using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. The amplified fragments were cloned into T&A cloning vector (Real Biotech, Taiwan) and subsequently sequenced. Moreover, detailed comparison and alignment of sequences were performed using the CLC sequence viewer program (CLC Bio, Denmark) and the BLAST protein search program in the NCBI database.

Structural analysis of the *NtEF1* and *NtEF2* genes

Specific primers for the open reading frame (ORF) were used in the PCR analysis. PCR amplification was conducted using PCR reaction mixtures with a total volume of 50 μ L, and with 30 ng of DNA as the template, 5 μ L of 10 \times buffer, 4 μ L of 2.5 mM dNTPs, 1 μ L of 10 μ M primers, and 2.5 U of ExTaq DNA polymerase (Takara

Bio, Japan). The PCR reaction mixtures were heated at 94 °C for 5 min, after which they were subjected to 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C, followed by a 5 min final extension at 72 °C. The amplified PCR products were assayed using 1 % agarose gel electrophoresis; then the purified PCR fragments were cloned into a T&A cloning vector (Real Biotech, Taiwan). Sequence alignment and comparison were executed using the CLC Sequence Viewer program (CLC Bio, Denmark).

Plant transformation and molecular analysis

Tobacco leaves from in vitro grown plants were used for *Agrobacterium tumefaciens*-mediated tobacco transformation. Each coding region of *NtEF1* and *NtEF2* was fused to the cauliflower mosaic virus (CaMV) 35S promoter at the *KpnI* and *BamHI* sites of the pCAMLA vector (pCAMBIA 1300 + P35S-Tnos cassette) (Lee et al., 2005) (Fig. 5a). Further, the pCAMLA vector was transformed into *A. tumefaciens* strain EHA 105 via the freeze–thaw method (An 1987). Tobacco leaf discs inoculated with bacterial suspension at OD₆₀₀ = 0.6–1.0 for 5 min were washed with sterile water and dried on a sterile filter paper. The inoculated leaf discs were incubated in dark for 2 days and then transferred to an MS medium supplemented with 0.1 mg/L 1-naphthaleneacetic acid (NAA), 1.0 mg/L benzylaminopurine (BAP), 50 mg/L hygromycin and 500 mg/L cefotaxime for a shoot induction. Regenerated shoots were then rooted on a half-strength MS medium containing 50 mg/L hygromycin and 500 mg/L cefotaxime. Transgenic lines that were selected on the basis of hygromycin resistance were further identified by hygromycin-resistance gene-specific PCR and Southern hybridization analysis. For the Southern hybridization analysis, genomic DNA was isolated from the transgenic tobacco leaves using a modified CTAB method (Kim and Hamada, 2005). Briefly, more than 27 μ g of each DNA sample were digested overnight with restriction enzymes. The genomic DNA isolated from the transgenic tobacco was then digested with *HindIII*, after which the products were purified and separated by 0.8 % agarose gel electrophoresis. The gel was subsequently agitated in a tray containing 0.25 N HCl for 15 min and then rinsed briefly with dH₂O, after which it was agitated again in a tray, including 0.4 N NaOH for 30 min. The DNA was then transferred onto a positively charged nylon membrane (Roche Molecular Biochemicals, Germany) utilizing the capillary transfer method (Sambrook and Russell 2001). The transferred membrane was rinsed in 2 \times SSC for 5 min, after which it was allowed to dry in a dry oven set to 80 °C. Next, a hygromycin (HPT)-resistance gene fragment was labeled as a probe by two primers (forward; 5'-CCACTATCGGCGAGTACTTCY

AC-3' and reverse; 5'-CTCTCGATGAGCTGATGCTT TG-3') using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Germany). Hybridization, washing and detection were conducted according to the guidelines established for the DIG system (Roche Molecular Biochemicals, Germany). Finally, verified transgenic T₂ generation seedlings were transplanted into pots and grown in the growth room for further experiment.

Reverse transcription (RT)-PCR analysis

Total RNA was isolated from tobacco apical shoots of *NtEF1* and *NtEF2* T₂ lines containing apical leaves ≤ 2 cm in length according to the modified CTAB method (Kim and Hamada 2005). In addition, total RNA was isolated from the apical portion of wild-type tobacco harvested by day. Further, total RNA was also isolated from the leaves, stems and roots of 90-day-old plants that had floral buds or the sepals, petals, stamens and carpels of open flowers. Each total RNA sample was treated with DNase to remove the contaminated genomic DNA using DNase I (Takara Bio, Japan). One microgram of total RNA was then synthesized to the first-strand cDNA using a PrimeScript first-strand cDNA Synthesis Kit (Takara Bio, Japan) and oligo dT primer. The RT reaction mixture was then diluted with 100 μ L distilled water, after which the 1 μ L RT reaction mixture was used as a template in a 50 μ L PCR amplification reaction mixture composed of 5 μ L of 10 \times buffer, 4 μ L of 2.5 mM dNTPs, 1 μ L of 10 μ M primers and 2.5 U of ExTaq DNA polymerase (Takara Bio, Japan).

PCR consisted of 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C, with a 5 min final extension at 72 °C. For calibration of the transcript level, a primer set (forward; 5'-TGGACTCTGGTGATGGTGTC-3' and reverse; 5'-CCT CCAATCCAAACACTGTA-3') based on the *Nicotiana tabacum* actin gene sequence was used in the experiment with tobacco (Kim et al. 2011). To confirm the expression level of *NtEF1* and *NtEF2*, the following five specific primer sets were used (Fig. 8): *NtEF1*, 5'-TTCGCGTGGGAAGT GATAAACG-3' and 5'-TGGTATGGGCAGTGTGAGT GG-3'; *NtEF2*, 5'-CATGTTTATTCAGGACCACCAGA-3' and 5'-GTCTACACTTTTGGAGCCTGTT-3'; *NtFUL*, 5'-GGGAAGCATATCAGAGTAC-3' and 5'-CAAGGCTGA TAAAGATCAG-3'; *NFL1*, 5'-AAGGAGCGAGGAGAGA ATGTTG-3' and 5'-AGGAGGTAGATATAGGGGCAA GC-3'; *NtMADS5*, 5'-GCATCTTTCCTCCTACAACCA C-3' and 5'-GGGCATAACATTGTATTGAAC-3'. The hygromycin (hpt)-resistance gene was also amplified in wild-type tobacco and transgenic T₂ lines. The primer set of HPT (forward; 5'-CCCATTCCGACCGCAAGGAA-3' and reverse; 5'-CCGCTGTTATGCGGCCATTG-3') was designed based on the *hygromycin B phosphotransferase* (*HPT*) gene sequence used in the selection marker of the

plant transformation vector pCAMLA. The amplified PCR products were visualized by electrophoresis in 1 % agarose gel.

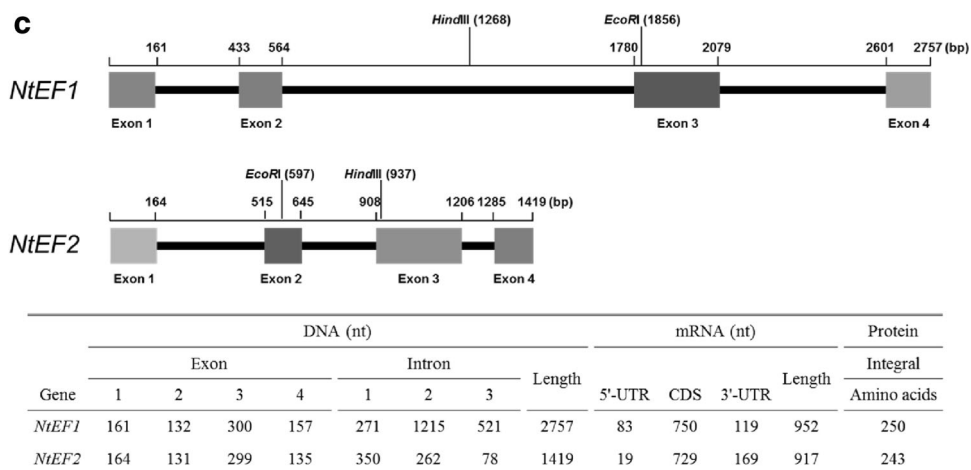
Subcellular localization of green fluorescent protein (GFP) fusion constructs

To construct the *NtEF1*:sGFP and *NtEF2*:sGFP, each coding region of *NtEF1* and *NtEF2*, without the natural termination codon, was amplified by PCR using two specific primers (*NtEF1*, 5'-CTCGAGATGGAAGGTGAAAA GGAAAGGAT-3' and 5'-CTCGAGTGTCTGGTATGG GCAGTGTGTA-3'; *NtEF2*, 5'-CTCGAGATGGCAAGTT TAGTAGGAGGGG-3' and 5'-CTCGAGATTTTCATGAA AGGACATTTTGAAGC-3'). *NtEF1* and *NtEF2* were each ligated in frame to the N terminus of the synthetic (s) GFP (Fig. 4a). To construct smGFP:*NtEF1* and smGFP:*NtEF2*, a full length of each *NtEF1* and *NtEF2* was prepared by PCR amplification using two specific primers (*NtEF1*; 5'-CTCGAGATGGAAGGTGAAAAGGAAAGGAT-3' and 5'-CTCGAGTTAGTTCTGGTATGGGCA-3', *NtEF2*; 5'-CTCGAGATGGCAAGTTTAGTAGGAGGGG-3' and 5'-CTCGAGTTATTTTCATGAAAGGACATTTTGAAG-3') and then fused to the C terminus of the soluble-modified (sm) GFP without the terminator codon (Fig. 4a). All chimeric GFP fusion constructs were placed under control of the CaMV 35S promoter in a pUC vector for expression in protoplasts. Plasmids were purified using Qiagen (Valencia, CA) columns according to the manufacturer's protocol. The fusion constructs were introduced into *Arabidopsis* protoplasts prepared from whole seedlings via polyethylene glycol-mediated transformation (Kang et al. 1998). The expression of the fusion constructs was monitored at various times after transformation, and images were captured with a cooled charge-coupled device camera using a Zeiss Axioplan fluorescence microscope (Jena, Germany). The filter sets used included XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and XF137 (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega Optical, Brattleboro, VT) for green and red fluorescent proteins, respectively. The images were then processed using Adobe Photoshop (Mountain View, CA, USA).

Scanning electron microscopy

The surface of the anthers of tobacco T₂ plants was observed using the scanning electron microscopy, as previously described (Kim 2008). Briefly, selected anthers were immersed in modified Karnovsky's fixative (Karnovsky 1965) consisting of 2 % (v/v) glutaraldehyde and 2 % (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) overnight at 4 °C, after which they were

Fig. 1 continued



washed with the same buffer three times for 10 min each. The specimens were then fixed in a second fixative, including 1 % (w/v) osmium tetroxide in the same buffer at 4 °C for 2 h. Next, the samples were washed briefly with distilled water two times and then dehydrated by submersion in 30, 50, 70, 80, 95, and 2× 100 % ethanol at room temperature for 10 min each. The specimens were subsequently treated with isoamyl acetate two times for 10 min each, then dried in a critical point drier (CPD 030; BAL-TEC Inc., Balzers, Liechtenstein). Finally, the specimens were mounted on metal stubs, sputter-coated with gold and observed using the scanning electron microscopy.

Results and discussion

Sequence analysis of *NtEF1* and *NtEF2*

The sequence analysis indicated the full lengths of *NtEF1* and *NtEF2*. Using reconstructed sequences, the cDNA derived from leaf RNAs of tobacco (*Nicotiana tabacum* L. ‘Xanthi’) was amplified, and the *NtEF1* and *NtEF2* cDNA was cloned in suitable vectors and sequenced (Fig. 1a, b). The two pairs of specific primers were designed in accordance with the coding regions of these two mRNA sequences and then used to amplify the genomic DNA fragments as well as the entire *NtEF1* and *NtEF2* genes. The fragments were sequenced, thereby enabling a complete reconstruction of the *NtEF1* and *NtEF2* genes with the annotation of introns and exons (Fig. 1c). The sequences were deposited in GenBank with accession numbers KF286542 (*NtEF1*) and KF286543 (*NtEF2*). The comparison of the two genes (Fig. 1c) demonstrated a strong conservation of the gene structure. The sequenced regions of the two genes were 2,757 and 1,419 bp long, respectively, for *NtEF1* and *NtEF2*. The two genes contained four exons and three introns. The coding sequences

were 750 bp for *NtEF1* and 729 bp for *NtEF2*. Although exons 1, 2 and 3 have a similar length, the lengths of all introns differed between the genes. The lengths of the first, second and third introns were 271, 1215 and 521 bp in *NtEF1*, and 350 bp, 262 bp and 78 bp in *NtEF2*, respectively. Moreover, the length of the four exons differed between the genes because they coded for regions in which the two proteins differed. The predicted protein sequences were 250 and 243 amino acids for *NtEF1* and *NtEF2*, respectively, and the alignment of the two protein sequences showed a 47 % residue identity (Fig. 2a). In addition, each deduced amino acid sequence of *NtEF1* and *NtEF2* displayed a high identity with *IbEF1* of *Ipomoea batatas* (KF286541; 71 and 75 %), the pathogen-related protein of *Vitis vinifera* (NCBI accession number: XP_002285489; 77 and 55 %), predicted protein of *Populus trichocarpa* (GenBank ID: EEF11635; 71 and 50 %), conserved hypothetical protein of *Ricinus communis* (GenBank ID: EEF33891; 75 and 54 %) and pathogenesis-related protein of *Arabidopsis thaliana* (GenBank ID: AAM66077; 71 and 50 %) (Fig. 2a, b). However, the *NtEF1* and *NtEF2* proteins had no predicted conserved domain based on the NCBI conserved protein domain database.

Expression patterns of *NtEF1* and *NtEF2*

To monitor the temporal expression patterns of *NtEF1* and *NtEF2*, the level of the corresponding mRNA in the apical shoots containing apical leaves from 60-, 70- and 80-day-old tobacco, in floral buds from 90-day-old tobacco, and in open flowers from 100-day-old tobacco was monitored by RT-PCR analysis. To confirm the spatial expression patterns of the *NtEF1* and *NtEF2* gene from tobacco, the level of the mRNA expression in different organs was examined by the RT-PCR analysis. The total RNA samples were isolated from the leaves, stems and roots of 90-day-old

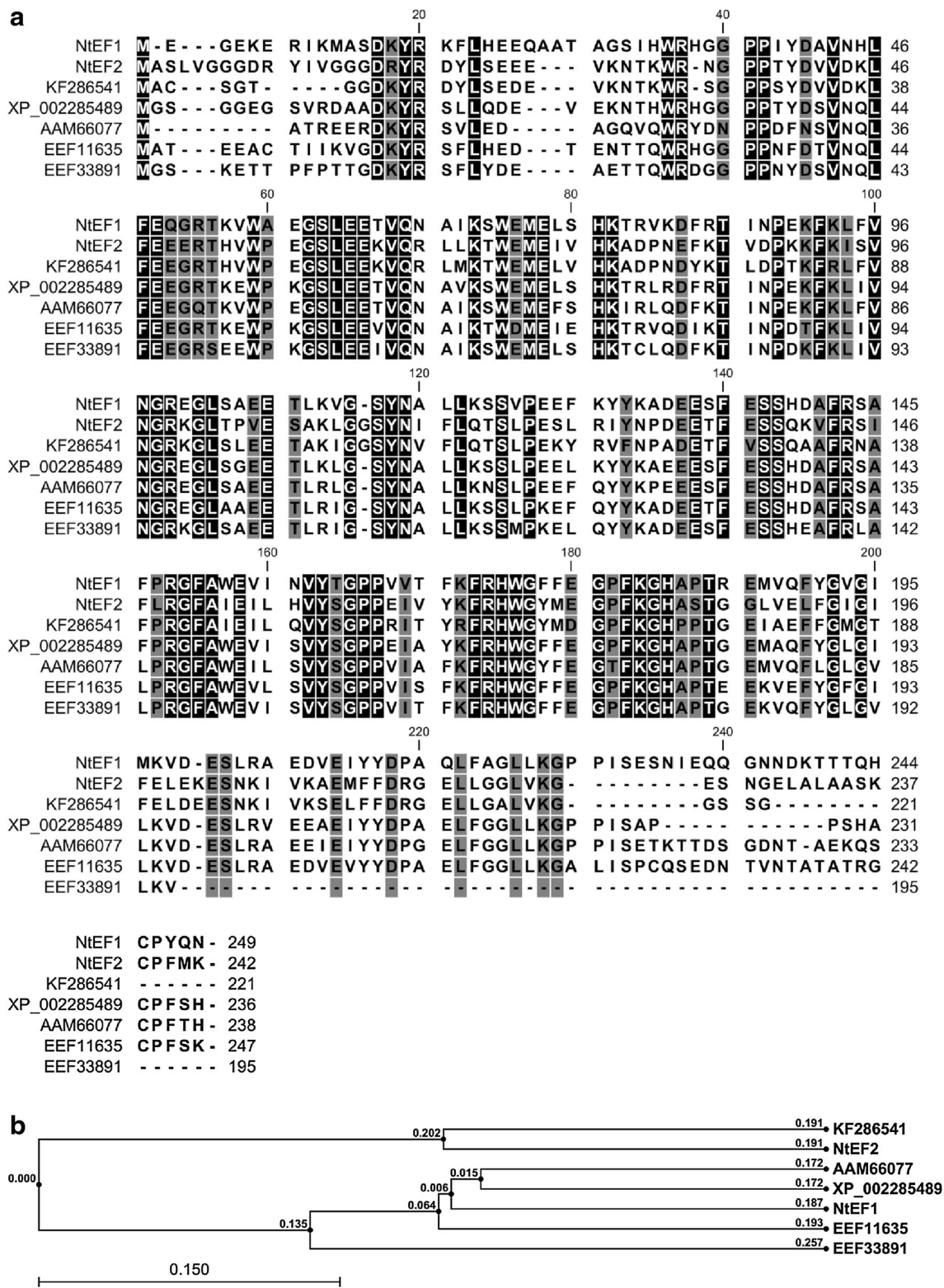


Fig. 2 Comparison of the amino acid sequences of NtEF1, NtEF2 and other homologous proteins. **a** Alignment of deduced amino acid sequences of NtEF1 and NtEF2 with highly homologous sequences, including IbEF1 of *Ipomoea batatas* (KF286541), pathogen-related protein of *Vitis vinifera* (XP_002285489), predicted protein of *Populus trichocarpa* (EEF11635), conserved hypothetical protein of

Ricinus communis (EEF33891), and pathogenesis-related protein of *Arabidopsis thaliana* (AAM66077). Identical amino acid residues in all seven sequences are indicated by white letters with black backgrounds. **b** Phylogenetic analysis of NtEF1, NtEF2 and homologous sequences via UPGMA

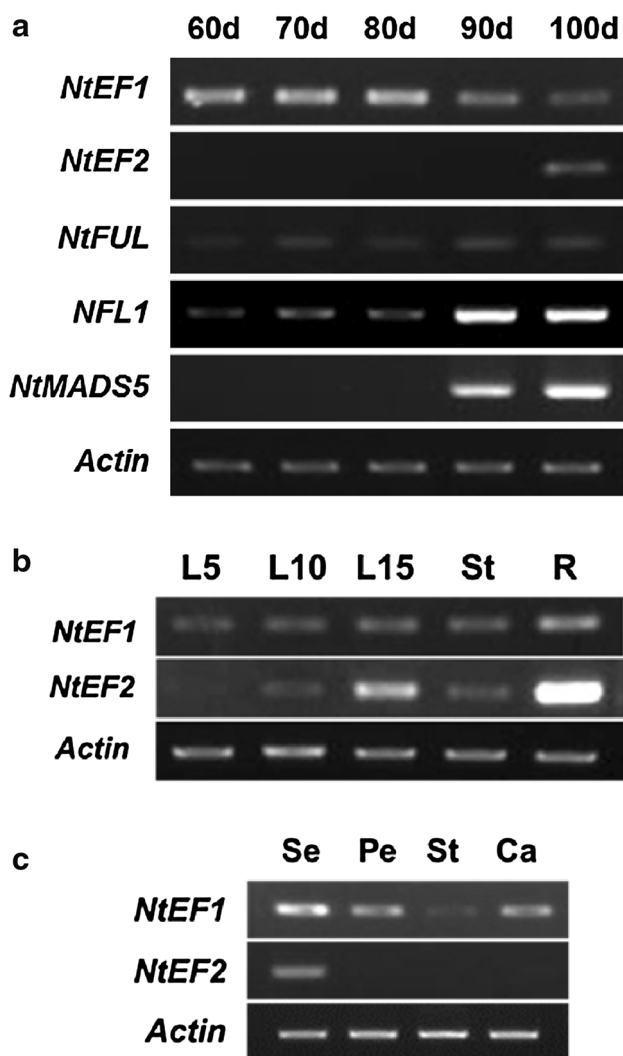


Fig. 3 RT-PCR analysis of wild-type tobacco. **a** Expression patterns of *NtEF1*, *NtEF2*, and flowering-related genes in apical shoots with young apical leaves of 60-, 70-, and 80-day-old tobacco, in floral buds of 90-day-old plants, and in open flowers of 100-day-old plants. The specific primer sets of nine genes, *NtEF1* (KF286542), *NtEF2* (KF286543), *NtFUL* (DQ534202), *NFL1* (U15798), and *NtMADS5* (AF068724), were used in the analysis. *Actin* was amplified as an internal control. **b** Expression of *NtEF1* and *NtEF2* in leaves (L), stems (St) and roots (R) of wild-type 90-day-old plants. L5: fifth leaf from flower bud, L10: tenth leaf from flower bud, L15: fifteenth leaf from flower bud. *Actin* was amplified as an internal control. **c** Expression of *NtEF1* and *NtEF2* in sepals (Se), petals (Pe), stamens (St), and carpels (Ca) of open flowers from 100-day-old plants. *Actin* was amplified as an internal control

tobacco with floral buds as well as from the sepals, petals, stamens and carpels of open flowers.

In the temporal expression patterns, *NtEF1* was expressed in the apical part, floral buds and open flower at all stages from 60 to 100 days, while *NtEF2* was only detected in open flowers of the 100-day-old plant (Fig. 3a). Before tobacco had a floral bud, the level of *NtEF1* expression in the apical parts was rather high at 60, 70 and 80 days (Fig. 3a).

In the spatial expression patterns of *NtEF1* and *NtEF2* from the 90-day-old plant with floral buds, the *NtEF1* mRNA expression level in the fifth, tenth and fifteenth leaf apart from the top was similar to that in the stem; however, the level in the roots was slightly higher (Fig. 3b). However, the *NtEF2* mRNA expression became more detectable as the location of the leaf was lower and the *NtEF2* mRNA level in the roots was comparatively higher (Fig. 3b). Further, the *NtEF1* mRNA expression levels differed among the sepals, petals, stamens and carpels; yet, it was expressed in all floral organs (Fig. 3c). In contrast, the *NtEF2* mRNA expression was only detected in the sepals (Fig. 3c).

Subcellular localization analysis

Cellular localization of NtEF1 and NtEF2 was examined by constructing a fusion protein with a GFP (sGFP or smGFP). The GFP was fused to the N or C terminus of NtEF1 or NtEF2 (Fig. 4a) to determine whether some amino acid regions of NtEF1 or NtEF2 had a function or not, even though NtEF1 and NtEF2 have no putative conserved domain based on the NCBI conserved protein domain database. Each fusion construct was introduced into protoplasts prepared from *Arabidopsis* tissues using the polyethylene glycol transformation method (Kang et al. 1998). After the transient expression of the fusion constructs, an individual protoplast was examined under a fluorescence microscope. As shown in Fig. 4b, the expression patterns of NtEF1:sGFP and smGFP:NtEF1 introduced into the protoplasts were the same, as were those of NtEF2:sGFP and smGFP:NtEF2. These findings indicate that the amino acid residues in the N or C terminus of NtEF1 and NtEF2 have no effect on the expression. The fluorescence of NtEF1 and NtEF2 protein fused to GFP was associated with cytosol, indicating that they were primarily targeted to the cytosol (Fig. 4b). These findings suggest that NtEF1 and NtEF2 do not function as transcription factors.

Phenotype of *NtEF1* and *NtEF2* transgenic plants

The *NtEF1* and *NtEF2* genes under the control of the CaMV 35S promoter were introduced into *N. tabacum*. Several overexpression lines were obtained. Specifically, 35S::*NtEF1* transgenic tobacco plants included ten independent lines, whereas 35S::*NtEF2* transgenic plants contained six independent lines. The insertion of T-DNA containing *NtEF1* and *NtEF2* genes (Fig. 5a) in the transgenic lines for constitutive expression was confirmed by the Southern blot analysis (Fig. 5b). The insertion was detected by probes based on a hygromycin resistance gene sequence that includes T-DNA as a selection marker

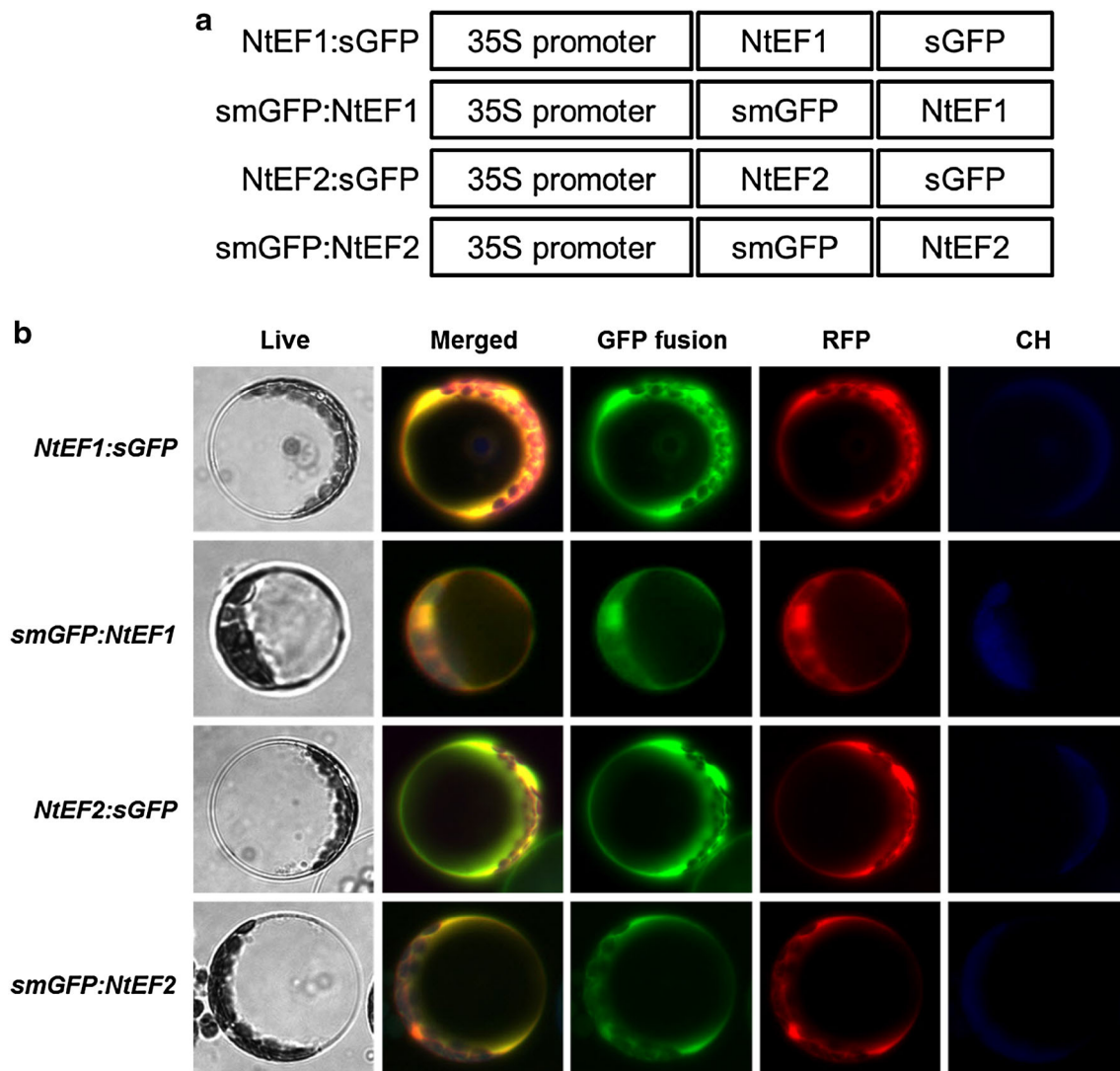


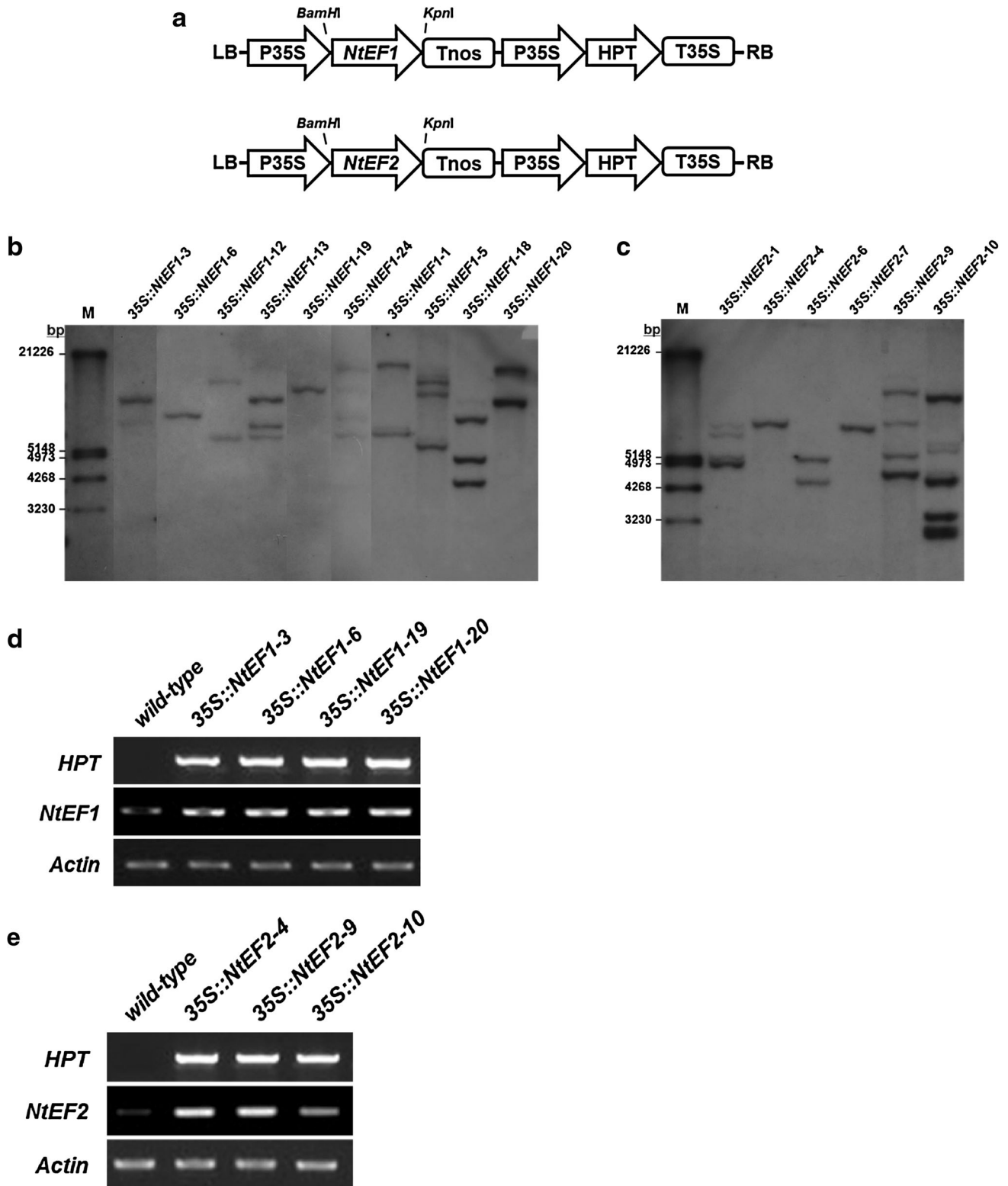
Fig. 4 a Scheme showing the various fusion constructs used in subcellular localization analysis. NtEF1:sGFP, NtEF1 coding region without the natural termination codon was ligated in frame to N-terminus sGFP under control of the CaMV 35S promoter. smGFP:NtEF1, NtEF1 coding region was ligated with C-terminus smGFP removing terminator codon under control of the CaMV 35S promoter. NtEF2:sGFP, NtEF2 coding region removing the natural termination codon was ligated to N-terminus sGFP under control of the CaMV 35S promoter. smGFP:NtEF2, NtEF2 coding region was ligated with C-terminus smGFP without the terminator codon under control of the CaMV 35S promoter. **b** Subcellular localization of the

GFP fusion proteins in protoplasts. The fusion constructs were introduced into *Arabidopsis* protoplasts. The pictures in the first row are bright-field live images (live). The pictures in the second row represent the overlap of green, red, and blue fluorescent signals of GFP, RFP, and chlorophyll, respectively (merged). The photos in the third row show each GFP fused to NtEF1 or NtEF2 in the transformed protoplasts (GFP fusion). The pictures in the fourth row show cytosolic marker RFP in transformed protoplasts (RFP). The photos in the fifth row indicate the auto-fluorescent signal of chlorophyll colored blue (CH)

because *NtEF1* and *NtEF2* are endogenous genes in tobacco. Lines 3, 6 and 19 of *35S::NtEF1* contain one copy of the T-DNA, while the other lines have multiple copies (Fig. 5b). Lines 4 and 7 of *35S::NtEF2* contain one copy, but the other lines contain multiple copies (Fig. 5b).

There was no phenotypic difference between the wild type and transgenic lines in the juvenile plant stage, except for early flowering in transgenic plants. After inflorescence,

most of the *35S::NtEF1* and *35S::NtEF2* transgenic lines had normal morphology relative to that of wild-type tobacco. A few plants of several overexpressing T₁ lines conveyed an abnormal morphological alteration of the stamen (Fig. 6a) and therefore could not undergo the self-pollination problem, although the phenotype frequency was very low. Specifically, only one or two of the eight plants in a single transgenic line showed an alteration of the stamen shape; some lines showed no plants with



morphological alteration (data not shown). The morphological alteration of the stamen containing petals appeared somewhat more frequently in *35S::NtEF1* than in *35S::NtEF2* (Fig. 6a).

The T_2 progeny of the *35S::NtEF1* and *35S::NtEF2* lines was obtained via self-pollination. Four lines of *35S::NtEF1* and four lines of *35S::NtEF2* were selected for the flowering time analysis; however, these lines had fewer

Fig. 5 Southern blot and RT-PCR analysis of the *NtEF1* and *NtEF2*. **a**, Schematic diagrams of the expression vectors. *Top* T-DNA region of pCAMLA containing the *NtEF1* coding region. *Bottom* T-DNA region of pCAMLA containing the *NtEF2* coding region. **b**, **c** Southern blot analysis of genomic DNA from transgenic T₂ tobacco plants. Genomic DNA was digested with *Hind*III. Hybridizations were carried out with a hygromycin-specific probe. **b** Southern blot of genomic DNA from transgenic tobacco overexpressing *NtEF1*. M: DIG-labeled DNA molecular weight marker. *Each lane* represents an individual line of the transgenic tobacco including *NtEF1* and *NtEF2*. M: DIG-labeled DNA molecular weight marker. *Each lane* represents an individual line of the transgenic tobacco including *NtEF2*. **c**, **d** RT-PCR analysis in 70-day-old wild-type tobacco and transgenic T₂ tobacco lines for analysis of flowering time. *Actin* was amplified as an internal control. **c** Expression patterns of the hygromycin *B phosphotransferase* (*HPT*) gene and *NtEF1* gene in 70-day-old wild-type and *35S::NtEF1* lines. **d** Expression patterns of the *HPT* gene and *NtEF2* gene in 70-day-old wild-type and *35S::NtEF2* lines

copies of T-DNA or abnormal flower morphology in the T₁ generation. In addition, the transgenic T₂ plants with wild-type tobacco were grown in a climate chamber at 25 ± 3 °C under a 16 h light/8 h dark photoperiod. The transgenic lines were obtained by hygromycin selection and Southern blot analysis. Moreover, the overexpression of *NtEF1* and *NtEF2* was confirmed by checking the mRNA expression levels of *HPT*, *NtEF1* and *NtEF2* (Fig. 5c, d). As shown in Fig. 5c, d, the *HPT* mRNA expression only appeared in the *35S::NtEF1* and *35S::NtEF2* transgenic lines. Each gene under the control of the CaMV 35S promoter contained the *HPT* gene positioned in the T-DNA for a constitutive expression of *NtEF1* and *NtEF2* in transgenic tobacco (Fig. 5a). *NtEF1* and *NtEF2* were confirmed to be overexpressed in each transgenic line because their mRNA expression levels were higher in transgenic plants than in wild-type tobacco (Fig. 5c, d).

In transgenic T₂ plants, the abnormal alteration of the stamens with petals was only observed in *35S::NtEF1-20* plants (Fig. 6b). The abnormal stamen in the *35S::NtEF1-20* showed a normal-shaped filament and an unusual shaped anther covered with petals (Fig. 6b). Some transgenic plants had slim-shaped anthers, a reduced amount of pollen or shorter filaments compared to the round-shaped anthers and length of filament of wild-type tobacco stamens (Fig. 6b). The unusual shaped anthers of the *35S::NtEF1* and *35S::NtEF2* plants were identified by the naked eye, after which the surfaces were observed by a scanning electron microscopy (SEM). There was not a very distinct difference between *35S::NtEF1-3* and wild-type tobacco anthers, except for the volume and length (Fig. 6c). The shape of *35S::NtEF2-7* anthers was twisted and showed an abnormal structure relative to the control (Fig. 6c). The *35S::NtEF1-20* aborted anther covered with petals appeared to have a problem with the differentiation of pollen sacs (Fig. 6c). Self-pollination problems in a few

transgenic plants were considered to result from the morphological alteration of anthers. This phenotype corresponded to *gai*-induced male-sterile phenotype in transgenic tobacco (Huang et al. 2003; Al-Ahmad and Gressel 2005). Although *NtEF1* and *NtEF2* are not directly involved in gibberellin acid, the constitutive expression of *NtEF1* and *NtEF2* may affect floral organ development. However, these phenotypic frequencies were also very low in transgenic T₂ plants. It is assumed that these unexpected phenotypes are involved with the environmental stimuli, T-DNA insertion copy number or a different expression level of *NtEF1* and *NtEF2* because the floral development pathway is very complex.

Kim et al. (2011) found that the *IbEF1* sweet potato gene promotes rapid flowering through the ectopic constitutive expression in tobacco. To confirm if the *NtEF1* and *NtEF2*, searched as homologue genes of *IbEF1* in tobacco, is related to early flowering, the flowering time of *35S::NtEF1* and *35S::NtEF2* transgenic tobacco T₂ grown in a climate chamber at 25 ± 3 °C under a 16 h light/8 h dark photoperiod was analyzed. When compared to wild-type tobacco, the *35S::NtEF1* and *35S::NtEF2* lines showed earlier bolting followed by the formation of the floral bud. No wild-type tobacco plants showed floral buds, whereas transgenic *35S::NtEF1* and *35S::NtEF2* plants formed floral buds early; further, the inflorescence of some transgenic plants was completely finished within 100 days of planting (Fig. 7; Table 1). The flowering time was assessed based on node numbers and days at floral bud formation. On average, wild-type tobacco plants first formed floral buds approximately 30 days later than the overexpression lines of *35S::NtEF1* and *35S::NtEF2* (Table 1). Due to the early transition to flowering occurring in the transgenic lines, there was no distinct difference in the number of nodes between wild type and transgenic lines; however, the stem height of transgenic lines was slightly shorter than that of the wild type after inflorescence. These early-flowering phenotypes of *35S::NtEF1* and *35S::NtEF2* were confirmed at the molecular level by RT-PCR, which were conducted to determine if the mRNA expression of floral-related genes occurred earlier in transgenic lines than in wild type.

The flowering pathway is very complex, and plants initiate flowering after the transition from the vegetative to the reproductive stage (Mandel et al. 1992; Araki 2001; Mouradov et al. 2002). Floral induction related to the coordination of many endogenous factors and environmental stimuli is needed during this process; moreover, the floral induction process has been shown to be a genetic and molecular mechanism by diverse studies of *Arabidopsis* (Mandel et al. 1992; Araki 2001; Mouradov et al. 2002; Fornara et al. 2010). During a floral transition, the meristem identity is changed, and the shoot apical meristem

(SAM) is converted into an inflorescence meristem (IM) upon floral induction. The floral transition is controlled by key regulators, including the transcription factors *LFY*, *FUL*, and *API*, which specify the floral meristem identity (Benlloch et al., 2007; Sablowsky 2007; Litt and Irish 2003). *LFY* is a master regulator of flowering because *lfy* mutation induces a delayed transition to the reproductive phase and development of leafy shoots instead of flowers, while the constitutive expression of *LFY* confers early flowering and the conversion of shoots into flowers aberrantly in *Arabidopsis* (Schultz and Haughn 1991; Huala and Sussex 1992; Weigel et al. 1992; Weigel and Nilsson 1995). *LFY* is believed to occur downstream in all identified

flowering pathways, to be controlled by diverse input signals and to regulate many other genes involved in flowering time and floral meristem identity (William et al. 2004; Fornara et al. 2010; Srikanth and Schmid 2011). *FUL* also functions as a transcription factor associated with the transition to the reproductive phase and the MI transition together with other genes. Specifically, the loss-of-function phenotype in *Arabidopsis* engenders a change of transition to flowering, whereas a constitutive expression of *FUL* promotes flowering (Ferrandiz et al. 2000a, b; Teper-Bamnolker and Samach 2005). *FUL* is believed to occur downstream in the photoperiod pathway, where *FT* and *FD* participate as regulators; however, *SQUAMOS PROMOTER BINDING*

Fig. 6 Phenotypic alterations of the transgenic tobacco plants overexpressing *NtEF1* and *NtEF2*. **a** Unexpected phenotypic alteration of transgenic tobacco T₁ plants overexpressing *NtEF1* and *NtEF2*. A few plants of each transgenic line showed abnormal morphological alteration of the stamen. **b**, **c** Unusual morphological alteration of transgenic tobacco T₂ plants overexpressing *NtEF1* and *NtEF2*. **b** Longitudinal sections or open flowers that showed different shape anthers of transgenic lines. **c** Scanning electron microscopy analysis of surface cell morphology in wild-type tobacco anther and transgenic tobacco anther showing unusual shape

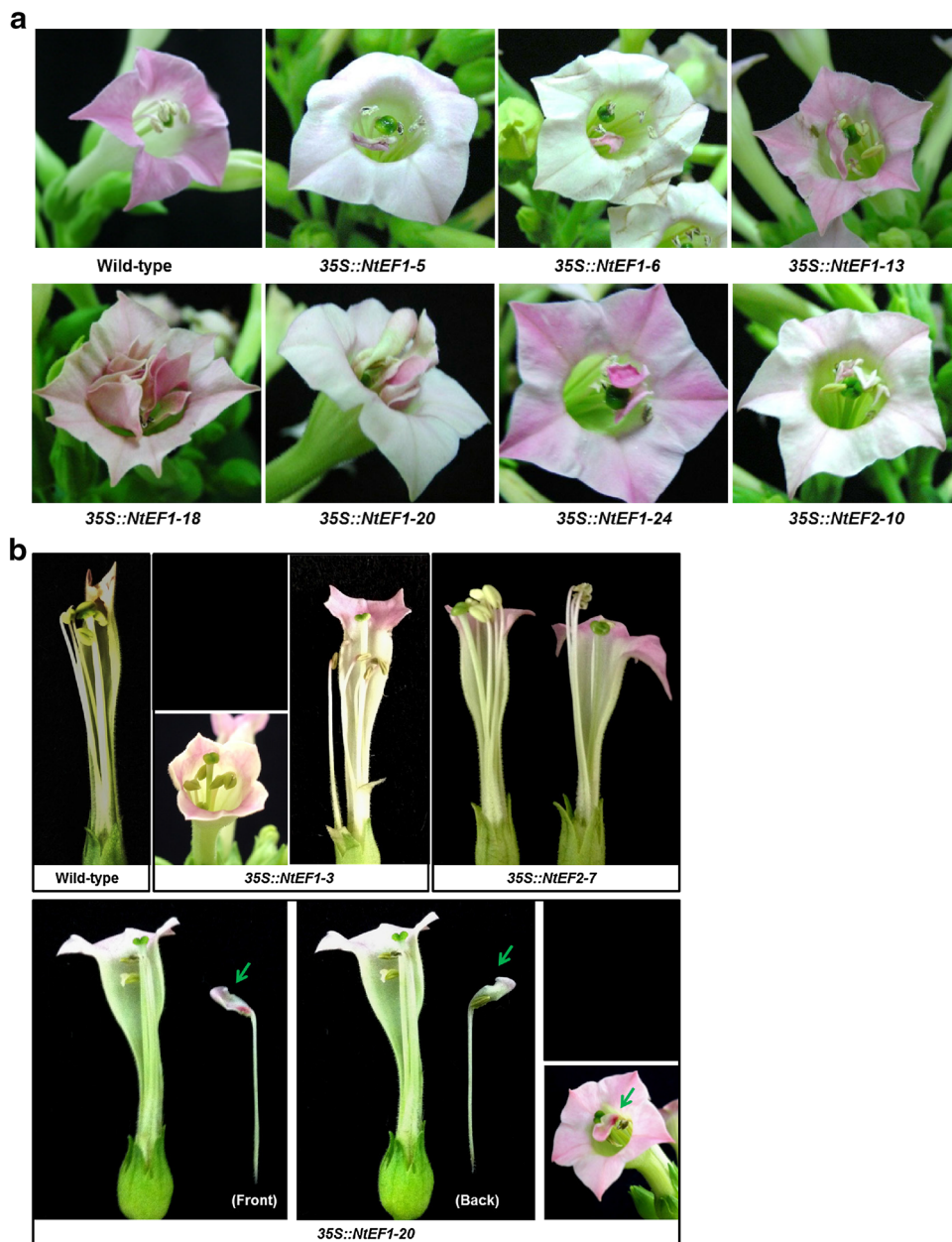
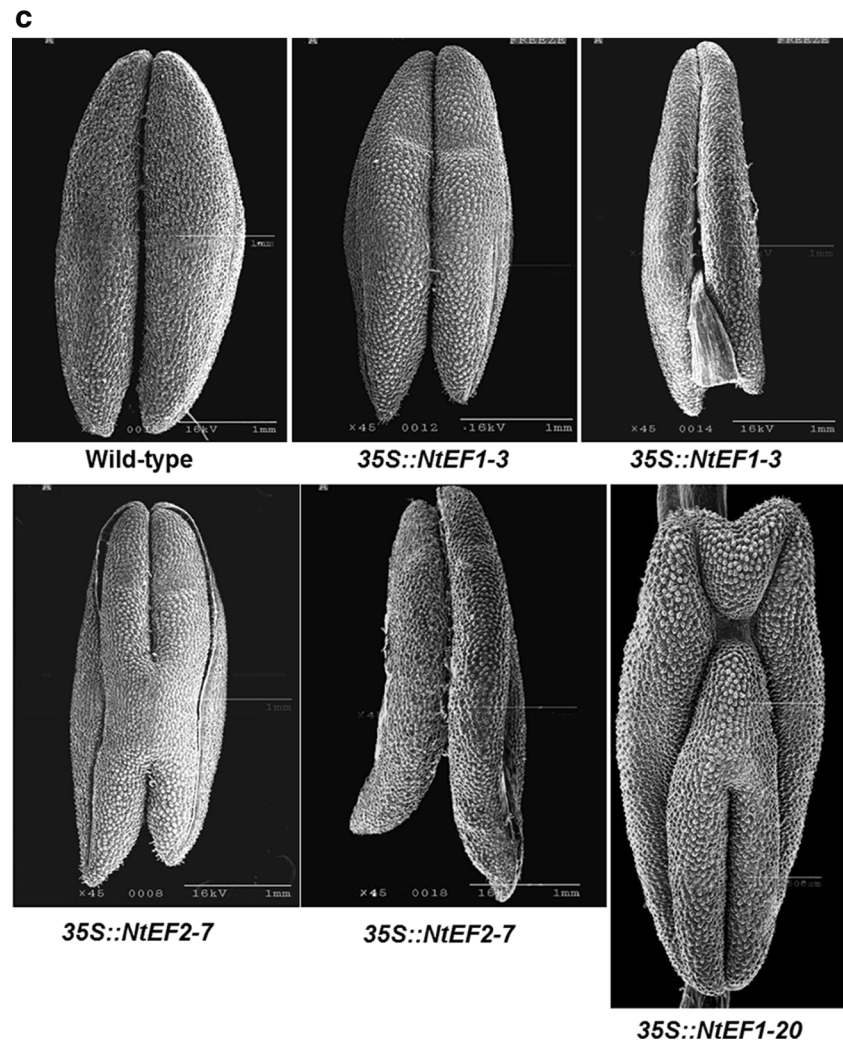


Fig. 6 continued



LIKEs (*SPLs*) transcription factors related to the aging pathway directly activate *LFY* and *API* in addition to *FUL* (Abe et al. 2005; Teper-Bamnolker and Samach 2005; Yamaguchi et al. 2009; Srikanth and Schmid 2011). Moreover, *API* plays an important role in the specification of floral meristem identity and the floral homeotic gene (Bowman et al. 1993; Schultz and Haughn 1993). Accordingly, a suitable regulation of *LFY*, *FUL* and *API* accumulation plays key roles in the initiation of flower formation, in that increased levels of *LFY*, *FUL* or *API* promote MI transition and floral development (Ferrandiz et al. 2000a; Mandel and Yanofsky 1995; Weigel and Nilsson 1995; Yamaguchi et al. 2009). As shown in Fig. 8, the floral-related genes analyzed by RT-PCR are *NtFUL*, *NFL1* and *NtMADS5*, which are the orthologues of *FUL*, *LFY* and *API*, respectively (Smykal et al. 2007; Ahearn et al. 2001; Jang et al. 2002). mRNA expression of *NtMADS5*, the *API* orthologue, generally does not occur in apical buds during all vegetative stages; however, *NtMADS5* mRNA can be

detected when tobacco is transformed from the vegetative to the floral stage (Smykal et al. 2007). *NtMADS5* accumulation can be a molecular indicator of the transition to the reproductive phase during the life cycle of tobacco. The transgenic lines of *35S::NtEF1* and *35S::NtEF2* undergo the transition to the reproductive stage earlier than wild type at 80, 90 and 100 days because *NtMADS5* mRNA expression appears more rapidly in transgenic lines (Fig. 8a, b). These results correlate with the early-flowering phenotypes in transgenic lines, in which *NtEF1* and *NtEF2* are overexpressed. When *NtMADS5* mRNA expression is not detectable, the tobacco plant is in the vegetative stage. Transcripts of *NtFUL* and *NFL1*, or upstream genes of *NtMADS5*, were visible during the vegetative phase because *NtMADS5* mRNA expression was not detected during this phase (Fig. 8a, b). These expression patterns correspond to the results reported in previous studies. *FUL* and *LFY* are both expressed in *Arabidopsis* during the vegetative stage, and their orthologue genes, *NtFUL* and *NFL1*, also accumulate

Fig. 7 Early flowering of overexpression plants at 100 days from planting. **a** Left wild-type, right *35S::NtEF1*. **b** Left wild-type, right *35S::NtEF2*



Table 1 Flowering characteristics of wild-type, *35S::NtEF1* and *35S::NtEF2* tobacco

Line	Number of days to flowering ^a (range)	Number of nodes (range)	Stem height, cm ^b (range)	Number of plants
Wild type	135.9 ± 12.9 (112–151)	40.3 ± 3.2 (36–46)	81.8 ± 6.3 (69.2–89.5)	(n = 8)
<i>35S::NtEF1-3</i>	98.6 ± 10.2 (87–116)	34.8 ± 2.7 (32–39)	64.5 ± 6.5 (53.4–69.8)	(n = 7)
<i>35S::NtEF1-6</i>	111.0 ± 8.5 (100–121)	37.3 ± 3.8 (33–40)	77.9 ± 11.2 (65.2–86)	(n = 5)
<i>35S::NtEF1-19</i>	111.6 ± 8.9 (96–118)	39.4 ± 2.6 (36–43)	72.9 ± 4.8 (65.2–76.5)	(n = 10)
<i>35S::NtEF1-20</i>	93.8 ± 14.3 (79–122)	32.0 ± 4.0 (28–41)	72.9 ± 6.9 (64.8–84.3)	(n = 12)
<i>35S::NtEF2-4</i>	95.5 ± 9.4 (81–116)	33.3 ± 2.9 (29–38)	69.8 ± 7.9 (60.5–78.8)	(n = 8)
<i>35S::NtEF2-7</i>	91.6 ± 11.5 (77–114)	30.1 ± 3.1 (26–34)	64.7 ± 8.7 (51.7–80.8)	(n = 7)
<i>35S::NtEF2-9</i>	91.7 ± 12.0 (79–109)	34.5 ± 4.3 (28–41)	75.0 ± 11.2 (63.5–92.6)	(n = 6)
<i>35S::NtEF2-10</i>	92.7 ± 13.6 (76–115)	34.4 ± 5.3 (30–45)	69.9 ± 8.6 (61.0–86.7)	(n = 8)

Values are mean ± SD

^a Floral buds first visible to the unaided eye

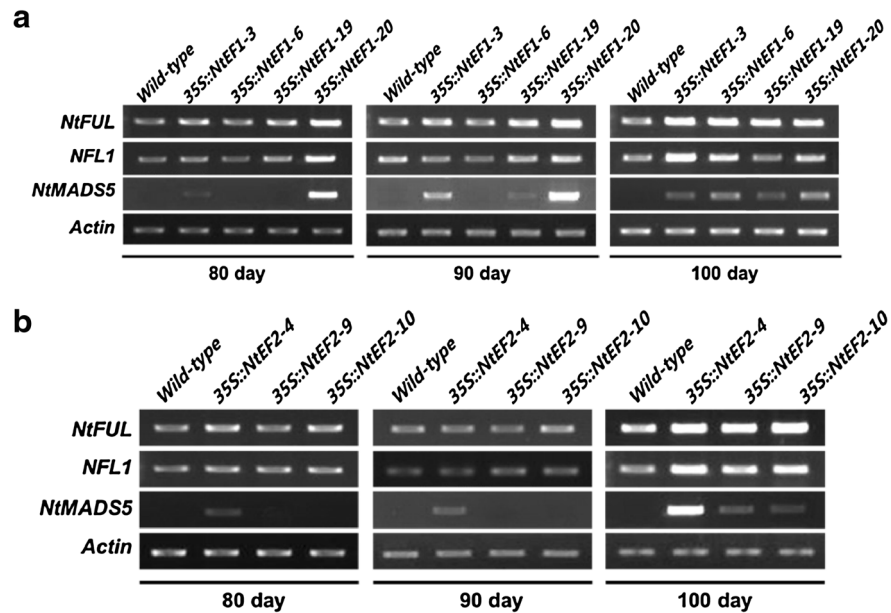
^b Stem height measured from the base of shoots to the first leaf node below the flower

in tobacco during the vegetative phase; however, they are all drastically up-regulated in the apical buds after transition to the floral stage (Kelly et al. 1995; Mandel et al. 1995; Blazquez et al. 1997; Borner et al. 2000; Smykal et al. 2007). *NFL1* is crucial to the control of the initiation of leaves and inflorescence branches in tobacco shoots, while also acting as a regulator of floral organ development (Ahearn et al. 2001). Although *NFL1* is an orthologue of *LFY*, the function of *NFL1* and *LFY* differs because the ectopic *NFL1* expression did not show severe early-flowering phenotype when compared to the tobacco and *Arabidopsis* with ectopic *LFY* expression (Kelly et al. 1995; Ahearn et al. 2001). Molecular and genetic studies of flowering time regulation in tobacco are still limited; however, the results of the present study suggest that a slightly different flowering regulation system exists more in tobacco than *Arabidopsis*.

Although the temporal and spatial mRNA expression patterns of the two genes are different and the identity of their two proteins is not high, *NtEF1* and *NtEF2* may regulate flowering because a constitutive expression of *NtEF1* and *NtEF2* showed early flowering in tobacco. Further, the phenotype was confirmed by the mRNA expression of floral regulator genes, such as *NtMADS5*. However, it is not certain as to whether *NtEF1* and *NtEF2* are transcription factors that directly upregulate or suppress floral-related genes in tobacco. Further studies are needed to demonstrate how *NtEF1* and *NtEF2* participate in the flowering pathway of tobacco and interact with other floral-related genes, as well as if there is a different action between the *NtEF1* and *NtEF2* genes.

Above all, this study revealed that constitutive expression of *NtEF1* and *NtEF2* genes, respectively, promotes

Fig. 8 Expression of the floral-related genes in apical shoots including young apical leaves of *35S::NtEF1* and *35S::NtEF2* transgenic plants at different stages was determined by RT-PCR using the primers shown in the table below. **a** Total RNA isolated from 80-, 90-, and 100-day-old wild-type tobacco and *35S::NtEF1* transgenic plants was used as a template. *Actin* was amplified as an internal control. **b** Total RNA isolated from 80-, 90-, and 100-day-old wild-type tobacco and *35S::NtEF2* transgenic plants was used as a template. *Actin* was amplified as an internal control



cDNA	Foward primer	Reverse primer	Accession No.
<i>NtEF1</i>	TTCGCGTGGGAAGTGATAAACG	TGGTATGGCAGTGTGAGTGG	KF286542
<i>NtEF2</i>	CATGTTTATTCAGGACCACCAGA	GTCTACACTTTGGAGCCTGTT	KF286543
<i>NtFUL</i>	GGGAAGCATATCAGAGTAC	CAAGGCTGATAAAGATCAG	DQ534202
<i>NFL1</i>	AAGGAGCGAGGAGAGAATGTTG	AGGAGGTAGATATAGGGCAAGC	U15798
<i>NtMADS5</i>	GCATCTTCTCTCAACCCAC	GGGCATAACATTGTATTGAAC	AF068724

early flowering. These days worldwide many countries produce biodiesel which is common name representing an alternative fuel made from crop oils or animal fats (Avinash et al. 2014). Biodiesel has been magnified that it is main substitute and attractive energy source compared to fossil fuel because the biodiesel is not limitative source that could be continuously produced and it has lower emission of combustion gas causing global warming (Ahmad et al. 2011). Most biodiesel is produced from bio-fuel crops such as soybean, sunflower, rubber seed, canola, castor, and rapeseed in various countries (Avinash et al. 2014). Since these oil seed crops have high potential for biodiesel production, promoting early flowering to produce seeds is very important thing besides selection crop cultivar, land use, and modification of oil-extraction methods to increase production efficiency of biodiesel. Thus, it is suggested that *NtEF1* and *NtEF2* to cause early flowering could be used in gene modification of the oil seed crops, and *NtEF1* and *NtEF2* genes have industrial utility value on the perspective of biotechnological application.

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