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NFL1, a *Nicotiana tabacum LEAFY*-Like Gene, Controls Meristem Initiation and Floral Structure

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The *Arabidopsis LEAFY* (*LFY*) gene product induces cells of the shoot apical meristem to differentiate into floral primordia by acting as a master regulator of downstream floral homeotic genes. Tobacco, an allotetraploid, possesses two homologous genes, *NFL1* and *NFL2*, which are 97% identical in amino acid sequence and share 73% amino acid sequence identity with *LFY*. In order to test whether the highly conserved tobacco orthologue, *NFL1*, shares functional identity with *LFY*, we created transgenic tobacco and Arabidopsis plants that constitutively express the *NFL1* cDNA. Our results indicate that *NFL1* plays a critical role in the allocation of meristematic cells that differentiate lateral structures such as leaves and branches, thereby determining the architecture of the wild-type tobacco shoot. *NFL1* also regulates floral meristem development and does so through the control of cell proliferation as well as cell identity. Surprisingly, unlike ectopic *LFY* expression, which can act as a floral trigger, ectopic *NFL1* expression does not promote severe precocious flowering in *Nicotiana tabacum* suggesting that variations in amino acid sequence among members of the *LFY*-like gene family have led to divergence in the functional roles of these genes.

Key words: *LEAFY*-like genes — Shoot meristem — Flowering.

Introduction

All visible structures of a mature plant shoot system, including leaves, branches, and floral organs, originate from the shoot apical meristem (Steeves and Sussex 1989). A central function of the shoot apical meristem is to selectively allocate cells that differentiate into organs and structures according to the appropriate plant developmental stage. These organs and structures emerge according to a species-specific reiterative pattern of precursor cell differentiation that changes precisely with developmental phase. For example, during the vegetative phase *Nicotiana tabacum* (tobacco) leaves emerge from the shoot apical meristem in a spiral phyllotaxy with a predictable angular degree of separation and a characteristic internode distance (Steeves and Sussex 1989, Poethig 1997). With the transition to the reproductive phase the apical meristem continues to produce structures in spiral mode until 5–7 inflorescence branches are initiated. Following this period floral meristems, borne on the inflorescence branches, allocate primordial cells in groups that develop mostly simultaneously in a whorled pattern. Coordinated emergence of the organs in a floral whorl is required for proper flower morphology and function.

Genetic and morphological analyses of mutants in which the shoot apical meristem fails to initiate the correct floral organs in the appropriate pattern have facilitated identification of genes that direct or participate in these crucial developmental programs. The *Floricaula* (*FLO*) gene of *Antirrhinum majus* (snapdragon) is required for proper phyllotaxy as well as floral meristem identity; plants carrying mutations in the *FLO* gene fail to shift from spiral to whorled phyllotaxy after the reproductive transition and do not produce normal flowers (Coen et al. 1990, Carpenter et al. 1995). The Arabidopsis *LFY* gene controls the timing of the vegetative-to-reproductive transition as well as floral organ identity; *LFY* mutations prolong the vegetative phase and cause leafy shoots to form in place of flowers (Schultz and Haughn 1991, Huala and Sussex 1992, Weigel et al. 1992). The *Aberrant Leaf and Flower* (*ALF*) gene of petunia regulates the ability of inflorescence meristems to become floral meristems; plants mutant for *ALF* produce leafy shoots in place of floral organs (Souer et al. 1998). Nucleic acid sequence comparisons of *LFY*, *FLO*, and *ALF* indicate that these genes are orthologues of the tobacco *NFL* genes (Kelly et al. 1995). Because tobacco is a tetraploid it possesses two nearly identical copies of the *NFL* gene, one from each ancestral diploid progenitor (Goodspeed 1954, Smith 1979, Okamuro and Goldberg 1985, Kelly et al. 1995).

Three criteria that can help evaluate whether orthologous genes of different species share a common function are: (1) conservation of amino acid sequence, (2) similarity of mRNA transcription patterns during development, and (3) phylogenetic relationships. The *NFL* genes share 93% amino acid identity with *ALF* (Souer et al. 1998), and 81% and 73% amino acid identity with *FLO* and *LFY* respectively (Kelly et al. 1995). Transcription of *FLO* mRNA occurs primarily in inflorescence and floral meristems (Coen et al. 1990). *LFY* mRNA expression in lateral primordia is continuous from the vegetative to the reproductive phase but increases in intensity with time (Blázquez et al. 1997). *NFL1* and *NFL2*, and the petunia *ALF* mRNAs are found in vegetative as well as floral meris-

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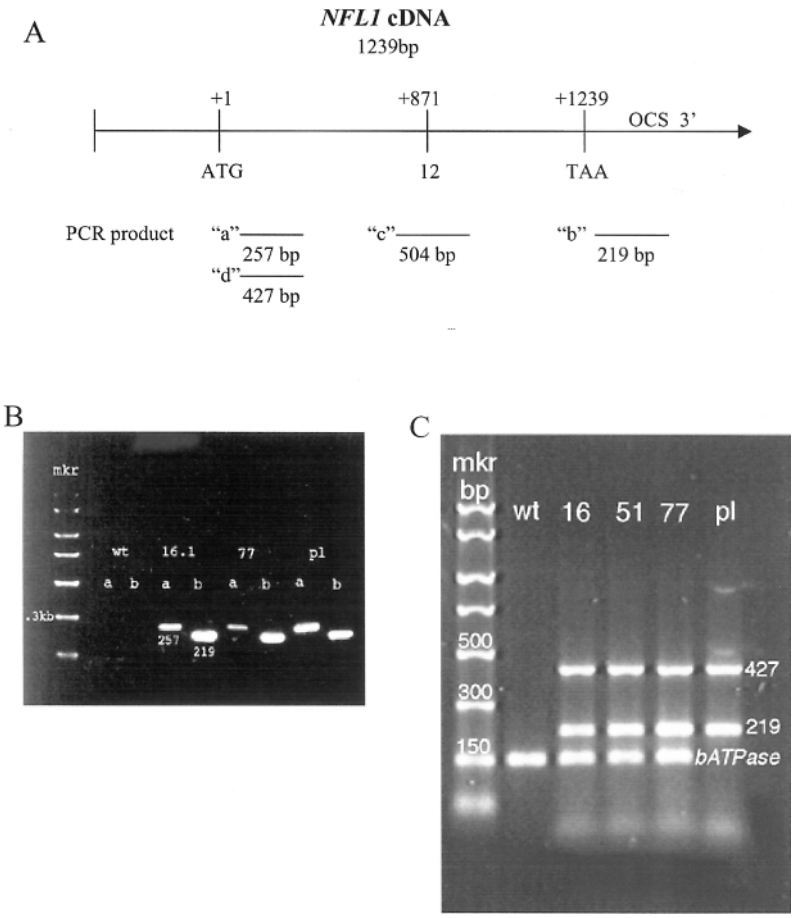

Fig. 1 *NFL1* overexpression construct and DNA analysis of transgenic plants. (A) *NFL1* overexpression construct. +1 represents the start of the translation of the *NFL1* cDNA; I2 represents the position of the second intron in the *NFL1* genomic sequence; +1239 represents the position of the stop codon of the *NFL1* cDNA. PCR products used to analyze the transgenic plants are indicated as spanning the CaMV *35S::NFL1* junction (257 bp, primer set "a"), or as spanning the borders of the second intron (504 bp, primer set "c"). (B) Gel electrophoresis of PCR products following amplification of genomic DNA from leaves of *35S::NFL1* T2 plants. "a" and "b" represents primer sets corresponding to the predicted PCR products described in (A). (C) Gel electrophoresis of RT-PCR products amplified from mRNA isolated from wild-type progenitor plants and *35S::NFL1* Class 1 plants. Amplified fragments were generated with primers located on either side of the CaMV *35S::NFL1* junction (427 bp fragment represented as "d" in (A)) and the 3′ junction of *NFL1* and OCS sequence (represented as the 219 bp fragment "b" in (A)). The ~150 bp band was generated by *N. plumbaginifolia* β-ATPase primers used as an internal mRNA standard (Boutry and Chua 1985, Kelly et al. 1990). Mkrs, markers; wt, wild-type. 16, 51 and 77 are three representative independent *35S::NFL1* Class 1 lines; pl, the *35S::NFL1* plasmid derived from *E. coli*.

tems (Kelly et al. 1995, Souer et al. 1998). Tobacco and petunia belong to the same phylogenetic family, the Solanaceae, and these two share the same subclass as snapdragon, the Asteridae. Arabidopsis is, the most distantly related of these species, belongs to a separate subclass, Dilleniidae (Cronquist 1988). Taken together, these observations suggest that the *NFL* genes from tobacco and *ALF* from petunia might share functional similarity, whereas *FLO* and *LFY* might have different roles in development. Tobacco plants bearing mutations in the *NFL* genes have not been isolated and therefore the role of these genes in development has not been determined.

NFL mRNA is transcribed in a ring-like pattern in a unique subset of cells in the organogenic peripheral zone of the tobacco vegetative meristem (Kelly et al. 1995), and therefore its presence in the meristem is not sufficient to promote flowering. This suggests that *NFL* may play a role in vegetative development in the meristem. In order to test this possibility, we created transgenic tobacco and Arabidopsis plants which constitutively transcribe *NFL1* cDNA from the *CaMV 35S* promoter. The results of these experiments indicate that *NFL1* plays a critical role in controlling the initiation of leaves and inflorescence branches in the wild-type tobacco shoot, and also regulates floral organ development. Surprisingly, unlike ectopic *LFY* expression which acts as a floral trigger, ectopic expression of *NFL1* did not promote severe precocious flowering in the *Xanthi* cultivar of tobacco or in Arabidopsis. Taken

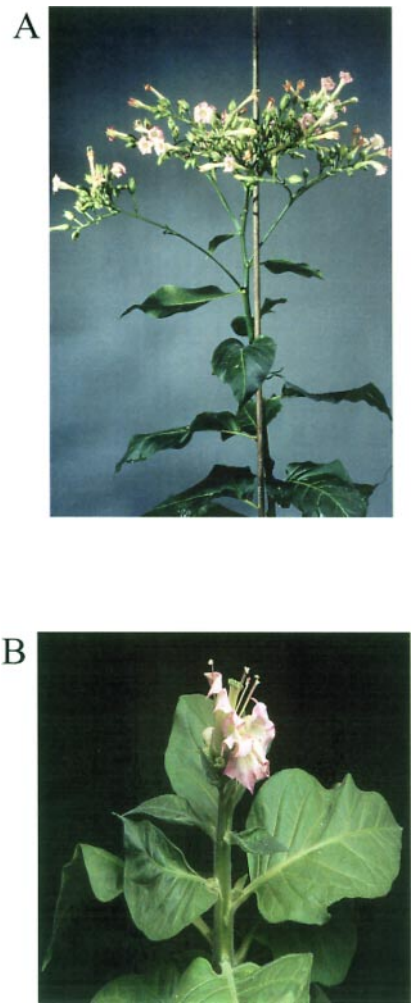

Fig. 2 Wild-type inflorescence and *35S::NFL1* Class I terminal flower structure. (A) Photograph of mature inflorescence of *N. tabacum* cv Xanthi showing inflorescence branch structure. (B) Photograph of terminal flower formed by *35S::NFL1* Class I Xanthi plants showing enlarged and malformed solitary flower that terminates the main shoot axis.

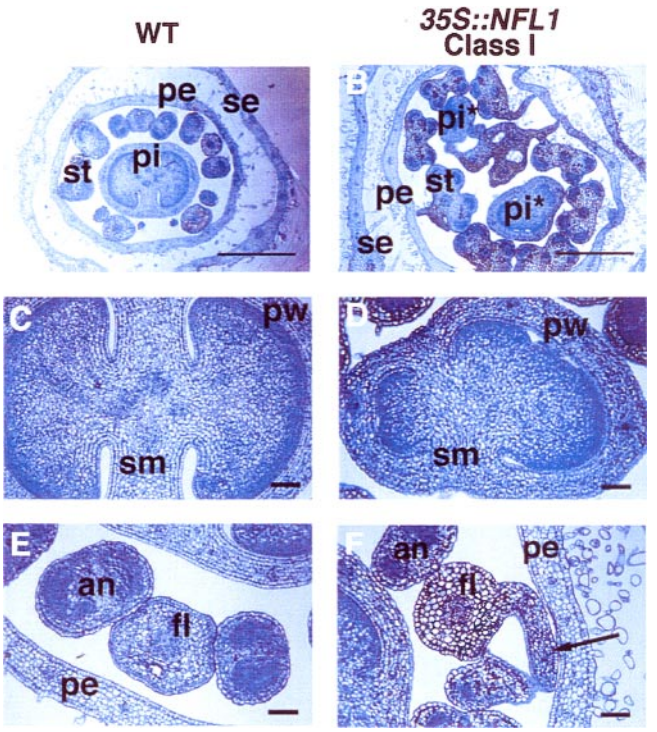

Fig. 3 Cellular phenotypes of developing wild-type and *35S::NFL1* Class I floral buds. (A, B) Transverse sections through buds of wild-type (A) and *35S::NFL1* Class I (B) floral buds showing supernumerary sepals, petals, stamens, and carpels in the *35S::NFL1* Class I floral bud. (C, D) Transverse sections through wild-type (C) and *35S::NFL1* (D) pistils showing asymmetry of the locules in the *35S::NFL1* Class I pistil. (E, F) Transverse section through wild-type (E) and *35S::NFL1* Class I (F) filaments and anther lobes showing the development of a petalloid stamen in the *35S::NFL*1 Class I flower (arrow). Floral buds were at stage number –2 as defined by Koltunow et al. (1990). Se, sepal; pe, petal; st, stamen; pi, pistil; pi*, abnormal pistil; sm, septum; pw, pistil wall; an, anther; fl, filament. Bar (A, B) 1 mm; bar (C–F) 100 μm.

together, our results indicate that variations in nucleic acid sequence among members of the *LFY*-like family of genes has led to divergence in the functional roles of these genes in plant development.

Results

Ectopic expression of NFL1 in tobacco prevents inflorescence branching and promotes terminal flower formation

Unlike the indeterminate Arabidopsis, day-neutral *N. tabacum* exhibits a determinate growth habit. The primary shoot meristem produces an average of 38 leaves, followed by 5–6 inflorescence branches, and then is consumed with the formation of a terminal flower approximately 75 d after germination in summer greenhouse conditions.

We created eight transgenic lines (T1) of *35S::NFL1* (constitutively transcribed *NFL1*) *N. tabacum* c.v. *Xanthi* and allowed these to self-pollinate. Analysis of hundreds of T2 progeny from each of the eight lines revealed that all the lines segregated individuals of the same two phenotypic classes (described below) as well as phenotypically wild-type plants. The presence of the *35S::NFL1* transgene (Fig. 1A) in each of the eight transgenic lines was confirmed with Southern analysis and PCR (Fig. 1B). RT-PCR (Fig. 1C) and RNA in situ analysis (data not shown) demonstrated that *35S::NFL1* RNA was expressed at high levels in leaves and apical meristems of Class I T3 plants. It is important to note that none of these eight *35S::NFL1* lines produced T2 or T3 progeny that displayed severe early precocious flowering as is observed with *35S::LFY N. tabacum* transgenic plants (see below).

35S::NFL1 phenotypic classes

Class I: 70% of the *35S::NFL1* plants from individual T2 lines produced an average of 24 leaves before the shoot terminated with the production of a large terminal flower. Untransformed *Xanthi* tobacco grown in identical greenhouse conditions produced an average of 38 leaves before producing 5–6 inflorescence branches bearing numerous (>30) flowers (Fig. 2A). In contrast to wild-type tobacco plants, the *35S::NFL1* Class I plants produced no inflorescence branches, and the terminal flower emerged directly from the shoot apex (Fig. 2B). These terminal flowers were 3–5 times larger than wild-type tobacco flowers, and exhibited supernumary floral organs in all four whorls, including supernumary stamens and multiple carpels rather than two fused carpels normally found in wild-type tobacco (Fig. 3). The normal separation of whorls was lost and the resulting chimeric structures include sepaloid-petals between whorls 1 and 2, and petaloid-stamens between whorls 2 and 3.

T3 plants produced by self-pollination of Class I T2 flowers developed into either Class I, Class II, or wild-type plants. T3 plants from the phenotypically wild-type T2 plants produced only wild-type progeny.

Class II: 5–6% of the *35S::NFL1* tobacco plants from individual T2 lines fell into a separate phenotypic class. These plants germinated with fused cotyledons (Fig. 4A) and usually died. Scanning electron microscopy (SEM) failed to detect the presence of a shoot apical meristem. However, close to 20% of these seedlings survived and eventually produced a meristem and leaf-like structures. These plants developed compact bush-like architecture and rarely produced flowers (Fig. 4B). SEM analysis of the apical meristems from the surviving *35S::NFL1* Class II plants (Fig. 5) showed simultaneous (as opposed to sequential) emergence of developmentally equivalent leaf primordia, and the meristems often bifurcated to produce multiple vegetative shoots. This chaotic pattern of lateral organ emergence ultimately resulted in a branched shoot architecture with multiple equivalent indeterminate shoot structures rather than a cymose architecture that produces the central primary shoot normally found in wild-type tobacco.

PCR analyses of genomic DNA isolated from these *35S::NFL1* Class II bushy plants indicated that the *35S::NFL1* transgene was intact. However, unlike Class I *35S::NFL1* plants, no *NFL* mRNA was detected in leaves (Fig. 6) or shoot meristems (data not shown) of the Class II plants by RT-PCR or by in situ hybridization analysis of shoot meristems (data not shown). The introduction of altered forms of native genes into the plant genome can lead to a condition known as "co-suppression" wherein both the endogenous and transgenic copies of a gene are silenced, resulting in a loss-of-function phenotype (Napoli et al. 1990, Matzke and Matzke 1995, Meyer 1995, Voinnet et al. 1998). The inability to detect either *NFL1* or *NFL2* mRNA indicates that the Class II *35S::NFL1* plants are *NFL* co-suppressed plants and that the highly-branched non-flowering condition represents at least a partial loss-of-function phenotype for the *NFL* genes.

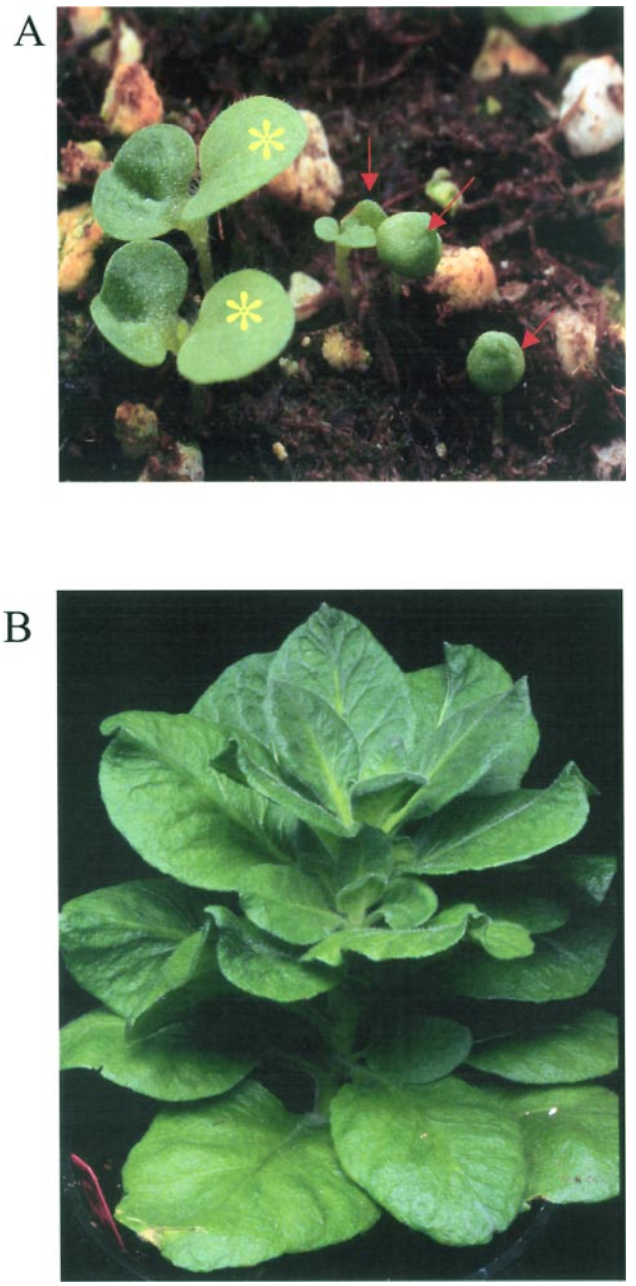

Fig. 4 Photographs of *35S::NFL1* Class II non-flowering phenotypes. (A) Segregation of wild-type and *35S::NFL1* Class II seedling phenotype. Transgenic Class II seedlings exhibit fused ball-shaped cotyledons as well abnormally formed cotyledons. (B) A *35S::NFL1* Class II non-flowering T2 co-suppressed *NFL1* plant photographed 70 d after germination. Note the dwarf stature, reduced internode length, and thickened leather-like leaves exhibiting abnormal vascular development. * indicates wild-type seedling; arrows indicate *35S::NFL1* Class II seedlings.

Class II *35S::NFL1* T2 plants rarely flower. Seed obtained

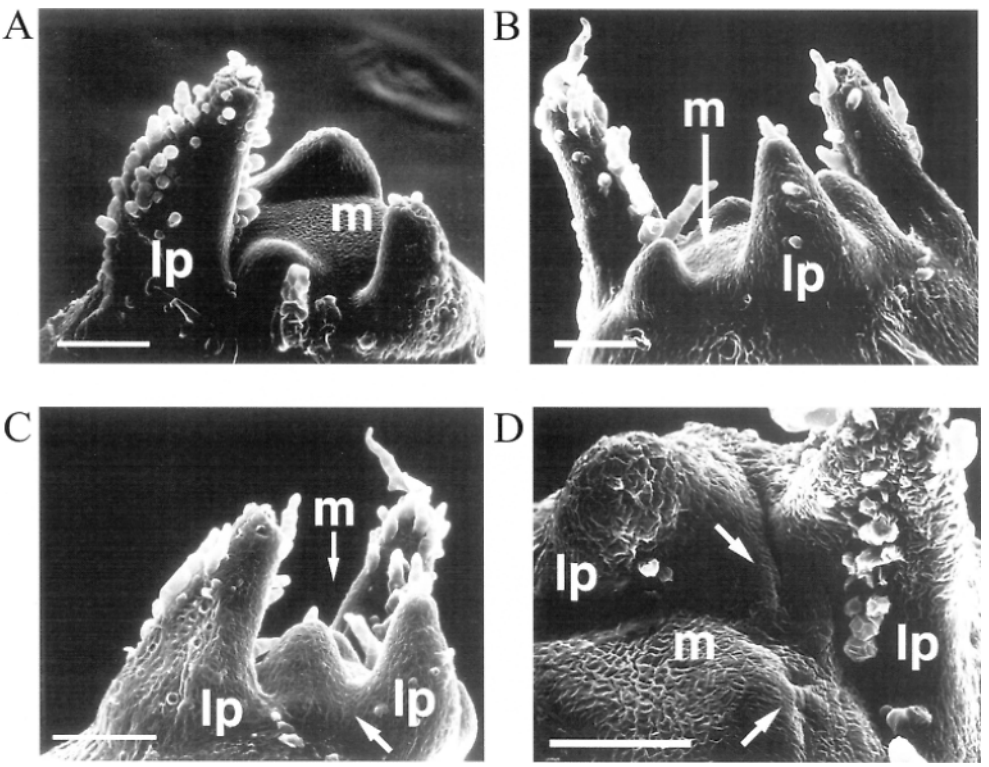

Fig. 5 Scanning electron micrographs of wild-type and *35S::NFL1* Class II non-flowering vegetative shoot apices. (A) Wild-type shoot apex showing normal leaf phyllotaxy. (B–D) *35S::NFL1* non-flowering shoot apex with abnormal leaf phyllotaxy and numerous leaf primordia. In (C) the arrow (lower right) indicates fused leaf primordia (lp). In (D) the arrows indicate a bifurcation of the shoot meristem. 1p, leaf primordium; m, meristem. Bar = 110 μm.

by self-fertilization of rare flowers from several older specimens yielded seedlings with fused cotyledons that developed into Class II plants.

The remaining 24–25% of the T2 progeny from the *35S::NFL1* T1 plants were wild-type and produced only wild-type progeny.

Ectopic expression of LEAFY in tobacco causes severe early flowering

Mutations in the Arabidopsis *LFY* gene prolong inflorescence development and prevent the production of flowers (Schultz and Haughn 1991, Huala and Sussex 1992, Weigel et al. 1992). In Arabidopsis, constitutive expression of *LFY* from the *CaMV35S* promoter attenuates both the vegetative and inflorescence phases of development by causing the precocious production of a terminal flower on the primary shoot (Weigel and Nilsson 1995). Lateral inflorescences also terminate prematurely with solitary flowers. Therefore, ectopic expression of *LFY* is sufficient to induce a developmental shift from vegetative to reproductive growth, and the level of *LFY* expression controls the number of leaf-like primordia that are permitted to initiate during the late vegetative and infloresence phases of development.

In order to test whether constitutive *LFY* expression could also act as a developmental switch in tobacco six independent *35S::LFY* transgenic *N. tabacum* c.v. *Xanthi* lines were created. Characterization of 375 T2 generation *35S::LFY* plants, including at least 42 plants from each line, demonstrated that 75% of the T2 plants could be placed into two distinct classes based on phenotypic traits. Table 1 and Fig. 7A compare the developmental characteristics of *35S::NFL1* Class I plants to *35S::LFY* Class I and Class II plants. Only *35S::LFY* Class II plants exhibit severe precocious flowering (Fig. 7B). The remaining 25% of the *35S::LFY* T2 plants appeared wild type and yielded only wild-type progeny.

Class I: 50% of the *35S::LFY* plants from individual T2 lines produced an average of 22 leaves before the shoot terminated with the production of an abnormally large terminal flower exhibiting supernumary floral organs in all four whorls. Like *35S::NFL1* Class I plants, *35S::LFY* Class I plants failed to produce inflorescence branches, but instead produced a large terminal flower directly from the primary shoot apex around 60 d after germination. Wild-type tobacco plants grown under the same conditions typically flower about 15 d later at 75 d post-germination. Thus in every phenotypic aspect, the *35S::LFY* Class I plants are identical to *35S::NFL1* Class I

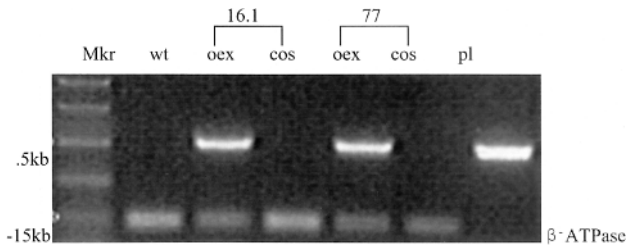

Fig. 6 Gel electrophoresis of RT-PCR products amplified from leaf mRNA of *35S::NFL1* T3 plants. RT-PCR amplification from sibling T3 generation plants of two (2) independent *35S::NFL1* transgenic lines 16.1 and 77. "oex" indicates a T3 plant with an overexpression phenotype that corresponded with to *35S::NFL1* Class I phenotype. "cos" indicates a sibling T3 plant with a phenotype corresponding to the *35S::NFL1* Class II non-flowering plant phenotype. The 504 bp fragment was generated by primers located on either side of intron 2 as shown in Fig. 1A (indicated as fragment "c"). These PCR primers will amplify both *NFL1* and *NFL2* sequence if it was present as mRNA. The ~150 bp fragment was generated by *N. plumbinaginifolia* β-ATPase primers for use as an internal mRNA standard (Boutry and Chua 1985, Kelly et al. 1990). Mkrs, markers; wt, wild-type non-transformed plant; pl, the 35S::NFL1 plasmid derived from *E. coli.*

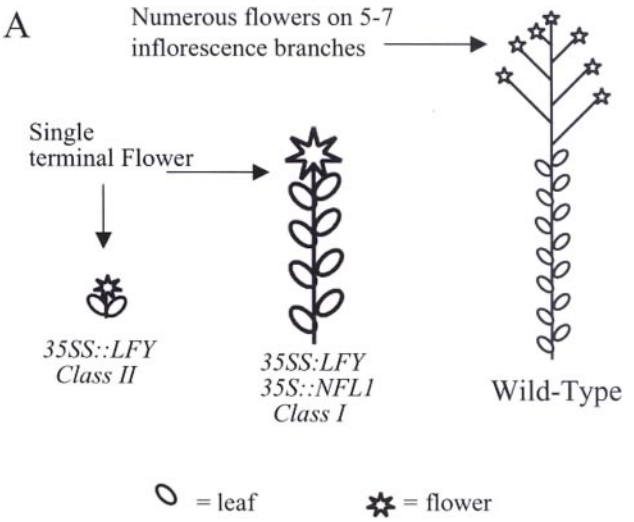

Fig. 7 Flowering phenotypes of *35S::LFY* and *35S::NFL1* tobacco. (A) Wild-type *N. tabacum* cv. Xanthi plants produce an average of 38 leaves before producing 5–7 infloresence branches bearing >30 flowers (see Fig. 2A). *35S::NFL1* and *35S::LFY* Class I plants make 22–24 leaves before producing terminal flower. However, *35S::LFY* Class II plants make an average of two leaves before producing an abnormal terminal flower. (B) Photograph of a *35S::LFY* Class II plant showing production of an aberrant terminal flower and severe early-flowering phenotype.

plants. T3 progeny from self-pollinated *35S::LFY* Class I T2 plants always segregated into both classes of *35S::LFY* plants (see below), as well as phenotypically wild-type segregants.

Class II: 20% of the *35S::LFY* T2 plants from individual T2 lines produced only 2–4 abnormal leaves followed by a single abnormal terminal flower that was visible approximately 16 d after germination (Fig. 7B). Supernumerary floral organs were always present in these flowers, and the sepal, petal and stamen whorls were not distinctly separate as in wild-type tobacco flowers but rather were often fused to form chimeric organs. These flowers usually failed to produce seed, but new "revertant" shoots often emerged to produce Class I type terminal flowers bearing viable seed. T3 seed from these flowers produced only early flowering *35S::LFY* Class II plants; the T3 generation did not produce phenotypically wild-type plants, suggesting that *35S::LFY* Class II plants possess more than one copy of the dominant *35S::LFY* transgene.

Constitutive NFL1 expression in Arabidopsis does not promote precocious flowering

Constitutive expression of *LFY* in both Arabidopsis and tobacco accelerates the transition from vegetative to floral development and promotes early flowering. Constitutive expression of *NFL1* in tobacco prevents inflorescence development, but does not cause severe early flowering. In order to test whether constitutively expressed *NFL1* could promote precocious flowering in Arabidopsis 16 independent lines of the Columbia ecotype containing the *35S::NFL1* transgene were created. The presence of ectopic *35S::NFL1* mRNA in young leaves was confirmed using RT-PCR on eight of these lines (data not shown). We observed 200 T2 plants, including at least 24 from each of the eight lines in LD (18 h light/6 h dark) and SD (9 h light/15 h dark) for phenotypes with regard to number of rosette leaves formed prior to the first visible floral bud, number of days to first visible floral bud, and vegetative and floral defects. As shown in Table 2, none of the *35S::NFL1* Arabidopsis plants differed significantly from wild-type plants in any of these characters under these growth conditions except for several of the *35S::NFL1* lines that exhibited abnormal leaf shape and rosette development in SD conditions (data not shown). All *35S::NFL1* Arabidopsis plants produced normal flowers and viable seed.

Table 1 Flowering characteristics of *35S::NFL1* and *35S::LFY* tobacco

	Number of days to flowering [a]	Number of leaves to flowering [b]	Stem height, cm [c]
Wild type	74.5 (0.96)	38 (1.6)	34.3 (1.5)
35S::NFL1 Class I	62.6 (1.5)	24.4 (2.4)	13.4 (0.76)
35S::LFY Class I	60.3 (1.5)	21.5 (2.2)	12.2 (0.71)
35S::LFY	16.3 (1.6)	2.3 (0.49)	0.8 (0.21)

Standard deviations are given in parentheses.
[a] Floral buds first visible to the unaided eye.
[b] Leaf nodes counted.
[c] Stem height measured from the base of shoot to the first inflorescent branch (for wild type) or terminal flower (for *35S::NFL1* and *35S::LFY*).

Ectopic expression of the 35S::NFL1 transgene fails to rescue Arabidopsis leafy mutants

If *NFL1* and *LFY* are functional orthologues then *NFL1* should be capable of replacing the missing *LFY* gene product in a homozygous *lfy* mutant background. In order to test this possibility we used pollen from a *35S::NFL1* Arabidopsis line to fertilize a recipient plant heterozygous for the *lfy*-16 allele (Shannon and Meeks-Wagner 1993). Twenty-five percent of the F2 plants segregated the *lfy* phenotype, and half of these leafy-phenotype plants possessed the *35S::NFL1* transgene as determined by PCR. RT-PCR analyses indicated that the *35S::NFL1* transgene was expressed at high levels in these plants (data not shown). These results indicate that ectopic expression of *35S::NFL1* was unable to restore wild-type flowering or floral development in a *lfy* mutant, and suggest that the *NFL1* and *LFY* gene products are not functionally equivalent with regard to flower initiation in Arabidopsis.

Discussion

The introduction of dominant gain-of-function mutations by ectopic mRNA expression has proven to be a powerful tool for understanding the normal function of a gene in development. Such a method facilitated the discovery that *LFY* is capable of causing severe precocious flowering in transgenic Arabidopsis, while loss-of-function mutations failed to indicate a role for *LFY* in the regulation of floral timing (Weigel and Nilsson 1995). This gain-of-function phenotype (precocious flowering) helped to confirm the role of *LFY* as a master regulator of downstream floral homeotic genes. Therefore, analysis of phenotypes caused by ectopic expression of other regulatory genes might likewise provide clues regarding potential downstream targets in a particular genetic pathway. In addition, the analysis of individuals from stably co-suppressed lineages which occasionally emerge from the over-expression lines can be extremely useful for the prediction of loss-of-function phenotypes (Napoli et al. 1990, Matzke and Matzke 1995, Meyer 1995, Voinnet et al. 1998).

NFL1 operates in the meristem to regulate organ initiation

In wild-type tobacco plants *NFL1* mRNA is expressed in a very specific pattern of cells in the organogenic peripheral zone surrounding the undifferentiated central zone of the shoot vegetative meristem (Kelly et al. 1995). Within this region, *NFL1* mRNA is not detected in the meristematic cells that give rise to incipient lateral primordia, suggesting that down-regulation of *NFL1* in specific meristematic cells precedes the emergence of lateral structures (Kelly et al. 1995). Recent studies with the rice *LFY/FLO* orthologue (*RFL*) indicate that *RFL* expression is also down-regulated at incipient branch sites and therefore may be necessary for panicle (inflorescence) branching (Kyozuka et al. 1998).

Table 2 Flowering characteristics of wild-type and *35S::NFL1* Arabidopsis

	Number of days to flowering [a]	Number of leaves to flowering [b]	Number of plants
COL – 0 **LD**	23.4 (3.2)	9.8 (1.1)	(*n*=27)
35S::NFL1 **LD**	24.6 (2.5)	10.1 (0.4)	(*n*=126)
COL – 0 **SD**	109.3 (10.5)	61.5 (7.2)	(*n*=15)
35S::NFL1 **SD**	102.1 (8.6)	65.3 (4.4)	(*n*=36)

Standard deviations are given in parentheses.
[a] Days from sowing to 1-cm inflorescence.
[b] Number of rosette leaves on plants with a 1-cm inflorescence.
LD, long day photoperiod conditions; SD, short day photoperiod conditions.

Results presented here support the idea that *NFL1* functions in wild-type tobacco to control allocation and placement of the lateral meristematic cells that produce organs such as the branches of the inflorescence shoot, floral organs, and the cotyledons of the embryonic seedling. In the *NFL1* overexpression plants (*35S::NFL1* Class I), high levels of *NFL1* mRNA in the apical meristem are associated with suppression of lateral meristem development. These plants fail to initiate branches and therefore the primary shoot terminates with a flower. In contrast, the *NFL1* co-suppressed plants (*35S::NFL1* Class II) exhibit the opposite phenotype: unregulated initiation of lateral meristems that determine the placement of leaves and branches. These plants develop an indeterminate bush-like architecture and continue to produce branches and leaves in an irregular phyllotactic pattern indefinitely. *NFL1* cosuppressed *N. tabacum* plants display phenotypes similar to *lfy/flo/alf* mutants of Arabidopsis, *Antirrhinum* and petunia, respectively, and to *N. benthamiana* plants in which a viral vector was used to silence NFL expression (Ratcliff et al. 2001), but exhibit additional phyllotaxis defects. *NFL1* cosuppressed plants rarely flower, and the few seeds obtained from these plants produce seedlings with fused cotyledons. Unpublished results from previous experiments indicate that antisense *NFL1* lines also produced seedlings with fused cotyledons which developed into plants that produced flowers with severely fused floral organs. Taken together, these results suggest that the ability to regulate the initiation of floral primordia is conserved among the Arabidopsis, snapdragon, petunia, and tobacco leafy-like genes. However, *NFL1* has an additional function in regulating the allocation and placement of precursor cells that differentiate into lateral structures at the vegetative shoot apical meristem.

Mis-expression of NFL1 or LFY in tobacco uncouples phyllotaxy from cellular identity

The allocation of cells to organ primordia at the shoot apical meristem appears to operate within the confines of a tightly controlled spatial and temporal regulatory framework, so that precursor cells are subjected to the appropriate developmental cues and thus differentiate correctly (for review see Meyerowitz 1997). In wild-type tobacco plants, primordia which are fated to differentiate as leaves emerge from the flanks of the vegetative meristem with reiterative spiral phyllotaxy, each individual leaf separated from the next by a species-specific pause in leaf primordia initiation. In contrast, primordia which are fated to become floral organs emerge simultaneously within whorls from the floral meristem and are commonly positioned closely to facilitate the joining of organs to form a single structure such as the tube-like tobacco corolla. It follows that the developmental processes which imprint cellular fate must be tightly coordinated with the mechanisms that regulate phyllotaxy of organ placement.

Our results demonstrate that constitutive expression of either *35S::NFL1* or *35S::LFY* in tobacco uncouples these two developmental processes. Both *35S::NFL1* and *35S::LFY* tobacco plants commonly produce leaf primordia in partial-whorled phyllotaxy resulting in cup-shaped leaf structures, as well as floral organ primordia in partial-spiral phyllotaxy resulting in the formation of continuous spiral flowers reminiscent of those observed in *flo* mutants (Carpenter et al. 1995). This suggests that the *FLO/LFY/NFL* gene products act to specify the boundaries of primordia proliferation zones. The absence of *NFL* and *RFL* mRNA (see above) from incipient branch primordia of wild-type tobacco and rice, respectively, may indicate that transcription of these genes is down-regulated within allotted differentiation zones to permit the initiation of organogenesis at the meristem.

The LFY-like genes exhibit functional divergence

Species that share fundamental developmental processes often share highly conserved orthologous genes that regulate these processes (Sussex 1989, Coen and Nugent 1994, Ma 1994, Manak and Scott 1994). Such orthologues invariably exhibit genetic variation in their amino acid sequences and these changes – mutations acquired as species diverge – present opportunities for modifying the original functional role of a particular gene. Our results indicate that *LFY* and its tobacco orthologue, *NFL1*, are functionally divergent with regard to the ability to initiate the transition from vegetative to reproductive development in angiosperms. While ectopic *35S::LFY* expression can promote severe precocious flowering in transgenic day-neutral *N. tabacum* and in Arabidopsis, ectopic *35S::NFL1* expression is less effective at doing so in tobacco and completely unable to do so in Arabidopsis. These results indicate that *LFY* is able to regulate downstream target genes of *NFL1*, but not vice versa. Unpublished data (Weigel and Coen 1997) indicates that *FLO* under the control of the *LFY* promoter only partially rescues the Arabidopsis *lfy* mutant phenotype, even though *lfy* and *flo* null phenotypes clearly show that they are functional orthologs. Recently, Kyozuka et al. (1998) have shown that a monocot orthologue of *LFY*, *RFL* from rice, does not promote precocious flowering in Arabidopsis, demonstrating that *RFL* function is also distinct from that of *LFY*.

In addition, our results show that some functional conservation does exist between *LFY* and *NFL1*. For example, *LFY* is capable of functioning like *NFL1* by suppressing the formation of inflorescence branches when overexpressed in tobacco. Both *LFY* and *NFL1* also promote the formation of supernumerary floral organs when expressed ectopically in tobacco, but *35S::NFL1* is not as effective as *35S::LFY* in activating the floral homeotic genes. Perhaps *NFL1* is necessarily less potent than *LFY* because of the additional functions of *NFL1* in primordia allocation and phyllotaxy throughout plant development. Recent evidence suggests that the ability of *LFY* to promote floral organogenesis is governed by its interactions with other gene products and that the concentration of *LFY* and the proteins it interacts with must reach a critical threshold before the floral transition can occur. Blázquez et al. (1997) have shown that *LFY* expression increases in the Arabidopsis meris-

tem as the plant progresses through the vegetative and floral phases and that the relative levels of *LFY* affect the timing of the transition to flowering. In contrast, Kelly et al. (1995) found that the levels of *NFL* expression in the tobacco shoot apical meristem remain constant throughout development. This suggests a model for floral initiation in tobacco in which other factors that interact with *NFL1* to prevent flowering gradually decrease during vegetative growth until a critical concentration threshold is reached and the floral cascade initiates. Several Arabidopsis early-flowering mutants, such as the *EMF* (embryonic flower), *ELF3* (early flowering), and *TFL1* (terminal flower) mutants, have been shown to play roles in the repression of flowering (Sung et al. 1992, Zagotta et al. 1992, Shannon and Meeks-Wagner 1991). The *N. tabacum* orthologues of these genes may also be involved in regulating floral initiation in tobacco, as has been shown for the tobacco orthologue of *TFL1* (Amaya et al. 1999).

Our results indicate that the *LFY*-like genes not only regulate floral organ development, but may also play an important role in controlling the initiation of lateral organs and structures from the shoot apical meristem in other plant species. The identification of downstream target genes regulated by *LFY* and *NFL1*, and of the divergent functional domains of the *LFY* and *NFL1* proteins, will be important in elucidating the precise mechanisms by which the *LFY*-like genes control fundamental aspects of shoot development.

Materials and Methods

Construction of transgenes

NFL1 genomic DNA was used to construct the overexpression vector. A full-length *NFL1* cDNA was constructed by joining two sequential *NFL1* fragments isolated from an expression library made from *N. tabacum* c.v. *Samsun* shoot apices (Kelly et al. 1995). The resulting fragment, which consisted of a 1239 base pair open reading frame, was ligated into pSLJ4D4, a *CaMV 35S* expression vector (Jones et al. 1992). The integrity of the resulting *CaMV 35S-NFL1*-OCS construct was confirmed by sequencing the entire promoter/cDNA region to rule out point mutations and it was then cloned into the plant transformation vector pMON505 which allows for kanamycin selection in plants (Horsch and Klee 1986). The resulting NFL/pMON505 plant transformation vector was electroporated (Dower et al. 1988) into *Agrobacterium* GV3101 (Fraley et al. 1985). The *35S::LFY* cDNA construct (Weigel and Nilsson 1995) contains the same *CaMV 35S*/promoter region as the *35S::NFL* constructs (Benfey and Chua 1990).

Plant material and growth conditions

For tobacco transformation, leaf discs from *Xanthi* tobacco cultivars were transformed according to Horsch et al. (1985). The initial (T1) transformants were grown in long day (LD) (18 h light/6 h dark) at 27°C for 6 weeks and then transferred to long-day summer greenhouse conditions. Natural light was supplemented with 400 watt sodium vapor lamps to ensure a minimum of 18 h light d^{-1}. The plants were allowed to self-pollinate and the T2 seeds were collected and sown on kanamycin plates and in soil.

For Arabidopsis transformation the primary and secondary inflorescences of ecotype Columbia plants were excised, and after numerous unopened buds were visible the plants (T0) were dipped into a solution of *Agrobacterium* (OD_{600} = 0.8) suspended in 10 mM $MgCl_2$, 2.5% sucrose, and 0.01% Silwet L-77 (Lehle Seeds); plants were grown in LD and permitted to self-pollinate. T1 seeds were collected and sown on soil soaked with kanamycin (60 mg ml^{-1}), and green seedlings were allowed to self-pollinate. Transgene presence and expression was confirmed by Southern analysis (Sambrook et al. 1989) and/or PCR and reverse-transcription PCR (RT-PCR) (Kelly et al. 1995).

Tissue fixation and microscopy

Tissue samples were fixed as previously described (Shannon and Meeks-Wagner 1993). For histological analysis, samples were embedded in JB-4 (Polysciences Inc., Warrington, PA, U.S.A.) according to the manufacturer's instructions. Sections were cut with an LKB Ultratome III microtome and adhered to glass slides coated with Vectabond adhesive (Vector Labs, Burlingame, CA, U.S.A.). Sections were stained with 0.1% methylene blue, 0.1% azure A in 0.02 M phosphate buffer pH 4.2. Coverslips were sealed with Permount (Fisher Scientific). A Zeiss Universal microscope was used to examine tissue sections and observations were recorded using Kodak, Ektachrome 64T color slide film. Photographic slides were scanned with a Polaroid Sprint Scan 35 and cropping of scanned images was performed in Photoshop 4.0 (Adobe). Scanning electron microscopy was performed as previously described (Pickett et al. 1996).

In situ hybridization

In situ hybridization experiments with digoxigenin nonradioactive probes were performed essentially as described by Drews et al. (1991) and Jackson (1991) with modifications from Kelly et al. (1995). Longitudinal sections, 8 μm thick, were made of *N. tabacum* c.v. *Xanthi* shoot apical meristems. Both antisense and sense cRNA probes were synthesized for the cloned *NFL1* gene. Slides were incubated at 45°C with hybridization solution without probe for 3 h prior to the hybridization experiment. Light microscopic images were obtained as described above.

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