Plant Biology. In the article “NEEDLY, a Pinus radiata ortholog of FLORICAULA/LEAFY genes, expressed in both reproductive and vegetative meristems” by Aidyn Mouradov, Tina Glassick, Britt Hamdorf, Lawrence Murphy, Beth Fowler, Soma Marla, and Robert D. Teasdale, which appeared in number 11, May 26, 1998, of Proc. Natl. Acad. Sci. USA (95, 6537–6542), the authors request that the following corrections be noted. In the Discussion on page 6541, the fourth, fifth, and sixth sentences should read as follows: “These domains are variable between FLO/LFY-like proteins. The proline-rich domain is not well pronounced in NLY and PrFLL. The acidic domain of gymnosperm FLO/LFY-like proteins is not as strong as corresponding domains of angiosperm homologs. Because the proline-rich and acidic domains are located within the variable regions, they may be subject to evolutionary changes.” Also, we would like to point out that in Fig. 1, the first 44 amino acids of the PEAFLF sequence were missing. A corrected figure and its legend appear below.

**Fig. 1.** Sequence comparison of FLO/LFY-like proteins (accession numbers in parentheses): PrFLL from *P. radiata* (U92008); NLY from *P. radiata* (U76757); BOFH from *Brassica oleracea* (718362); LFY from *Arabidopsis thaliana* (M91208); NFL1 and NFL2 from *Nicotiana tabacum* (U16172 and U16174, respectively); PEAFLF from *Pisum sativum* (AF010190); FLO from *Antirrhinum majus* (M55525); PtFL from *Populus balsamifera* (U93196); and RFL from *Oryza sativa* (AB005620). Black boxes indicate identical amino acids, shaded boxes indicate amino acids with similar properties, and dots indicate gaps introduced to optimize alignment. C1 and C2, conserved regions; V1 and V2, variable regions. Positions of the proline residues within the proline-rich region are indicated by asterisks. Acidic domain indicated by dashed line.
NEEDLY, a Pinus radiata ortholog of FLORICAULA/LEAFY genes, expressed in both reproductive and vegetative meristems

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ABSTRACT The LEAFY/FLORICAULA genes from Arabidopsis and Antirrhinum are necessary for normal flower development and play a key role in diverse angiosperm species. A homologue of these flower meristem-identity genes, NEEDLY (NLY), has been identified in Pinus radiata. Although the NLY protein shares extensive sequence similarity with its angiosperm counterparts, it is lacking the proline-rich and acidic motifs thought to function as transcriptional activation domains. NLY already is expressed during vegetative development at least 5 years before the transition to the reproductive phase. Expression of NLY in transgenic Arabidopsis promotes floral fate, demonstrating that, despite its sequence divergence, NLY encodes a functional ortholog of the FLORICAULA/LEAFY genes of angiosperms. Expression of the LFY::NLY transgene can largely complement the defects in flower development caused by a severe ify allele.

Molecular and genetic studies have shown that the mechanisms controlling flower development largely are conserved across distantly related angiosperm plants (1). The first step in flower development is the switch from the vegetative phase, during which shoots and leaves are produced, to the reproductive phase, during which flowers are initiated. Once this switch has been made, flower meristem-identity genes promote the initiation of individual flowers. In Arabidopsis, these genes include LEAFY (LFY) (2), APETALA1 (API) (3), CAULIFLOWER (CAL) (4), APETALA2 (AP2) (5), and UNUSUAL FLORAL ORGANS (UFO) (6). At least two of these genes, LFY and API, not only are required for flower initiation but are also sufficient to induce flowering in lateral shoots when overexpressed in transgenic plants (7, 8).

The expression of LFY orthologs has been studied in detail in four angiosperm species. Of these, only expression of the snapdragon gene FLORICAULA (FLO) is specific to the reproductive phase (9), whereas the others are expressed, to varying degrees, during the vegetative phase as well. LFY expression during the vegetative phase is initially low but increases with the age of the plant. Expression levels are highest upon entering the reproductive phase, suggesting that LFY levels are critical for the transition to flowering. This point has been confirmed by demonstrating that increasing the copy number of endogenous LFY reduces the number of leaves produced before the first flower is formed (10). Both the Nicotiana NFL and the pea PEAFL0 genes are expressed constitutively in emerging leaf primordia during the vegetative phase, as well as in floral organ primordia (11, 12). Although there is no evidence of a function of LFY and its ortholog in vegetative development of either Arabidopsis or Nicotiana, the situation in pea is different. Inactivation of PEAFL0 in the pea mutant unifoliata (uni) not only causes a floral phenotype that is similar to that seen in flo or ify mutants but also changes the morphology of the compound pea leaves (12).

Similar to many angiosperms, the “flowering” of P. radiata starts with the transformation of an indeterminate axillary apex into a determinate reproductive apex, which forms the strobili (cones) (13). A new long shoot terminal bud (LSTB) is formed at the tip of the rapidly elongating shoot during spring. The organogenic sequence of the apical meristem determines the fate of the shoot axis. The axillary apices that emerge on the sides of the apical meristem differentiate either as vegetative dwarf shoot buds (DSBs), reproductive pollen-cone buds (PCBs), or seed-cone buds (SCBs). SCBs become anatomically differentiated with the initiation of bracts (stage 1). Ovuliferous scale primordia are initiated from hypodermal cells on the adaxial base of bracts (stage 2). At stage 3, a fused bract-ovuliferous scale complex becomes displaced from the cone-bud axis. In differentiated PCBs, microsporangial initials appear in the peripheral zone of the axis (stage 1). During stage 2, microsporophyll initiation is complete and developed pollen mother cells are visible.

In contrast to angiosperms, our understanding of the molecular processes governing reproductive development in gymnosperms is very limited. A small family of MADS-box genes that is expressed in unisexual reproductive organs (and that shares similarity with floral organ-identity genes from angiosperms) has been isolated from two gymnosperms, Norway spruce (Picea abies) and Pinus radiata, and from their evolutionary ancestor, ferns (14–17). Here, we report the isolation and characterization of the first P. radiata gene belonging to the meristem-identity family of FLO/LFY-like genes. We show that the expression pattern of this gene, NEEDLY (NLY), is similar to that of these angiosperm genes, and we demonstrate with transgenic plants that it represents a true functional ortholog of the Arabidopsis LFY gene.

MATERIALS AND METHODS

P. radiata Samples. Female, male, and vegetative LSTBs were collected from an adult tree (~20 years of age and 30 m in height) in Victoria, Australia, from early March through June of 1996. Immature SCBs, PCBs, and DSBs were collected, placed on ice, dissected, and frozen in liquid nitrogen or fixed for in situ hybridization. Elongated needles also were collected.

Abbreviations: SCB, seed-cone bud; PCB, pollen-cone bud; DSB, dwarf shoot bud; LSTB, long shoot terminal bud; LD, long day; SD, short day.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U76757).

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Isolation of RNA. The total RNA from various tissues was isolated according to Chang et al. (18). Total RNA (5μg) from different organs were reverse transcribed by using the Ready-To-Go T-Primed First Strand Kit (Pharmacia). RT-PCRs were performed by using the set of NLY-specific primers, amplifying the 308-bp fragment: 5'-AGCATCTTTTACATTGTCACG-3' and 5'-CACAACATTACTCTCTCTTC-3'. As a control, a 520-bp PCR fragment of the rRNA was amplified by using a set of primers: 5'-AGTATAATCACTTTAATCACTT-3' and 5'-GATCCTCAGAAGACTAGGAGA-3'. PCR fragments were run on a 1% agarose gel, blotted onto nylon membranes, and hybridized to 32P-labeled fragments were run on a 1% agarose gel, blotted onto nylon membranes, and hybridized to 32P-labeled

Cloning of NLY. The 1.629-bp NLY cDNA clone (GenBank accession no. U76757) has a 1.212-bp ORF predicted to encode a 404-amino acid, 46-kDa protein. The predicted NLY protein aligns well with the sequences of other FLO/LFY-like proteins (Fig. 1). Sequence comparison revealed two large conserved regions (c1 and c2) and two shorter regions of lower similarity (variable regions v1 and v2). A proline-rich region near the amino terminus and an acidic central region are found in the variable regions of all angiosperm FLO/LFY-like proteins. Both of these domains are typical for transcriptional activators and may be important for the function of FLO/LFY-like proteins (2, 22). Unlike angiosperm proteins, NLY, as well as PrFLL, a product of another FLO/LFY-like gene from P. radiata (accession number U92008), does not contain either of these domains.

To more closely determine the evolutionary relationship between FLO/LFY-like proteins, a phylogenetic tree was constructed (Fig. 2). This tree showed that the topology of these genes seems to be concordant with the topology of the species phylogeny and suggests that NLY is a gymnosperm ortholog of FLO/LFY-like genes.

Expression of NLY. The expression patterns of the NLY gene first were analyzed by RT-PCR. Cone sizes selected for this experiment approximately represent three stages of SCB and two stages of PCB development. The 308-bp NLY fragment was amplified from the mRNA isolated from differentiated SCBs and PCBs (Fig. 3). NLY expression also was detected in vegetative organs, such as DSBs and needles, from the adult tree as well as from 1-month-old seedlings. No expression was detected in roots.

In situ hybridization was used to localize NLY transcripts during the early stages of LSTB development and within differentiated SCBs and PCBs and reproductive PCBs and DSBs (Fig. 4). NLY transcript first was detected during early stages of LSTB development, in the first apices emerging on the side of the apical meristem. These axillary apices could later differentiate as DSBs, PCBs, or SCBs. In the large apex of LSTB, NLY expression was transient in the early, undifferentiated cone buds located at the top part of the LSTB. In the progressively more developed, still not differentiated, cone buds located at the base of the LSTB, NLY no longer accumulated uniformly but preferentially in the peripheral zones of the cone buds. No expression was observed in the apical meristem region nor in the fertile cataphylls surrounding the axillary apices.

Differentiation of the SCBs usually began in the most basal axillary apices and proceeded acropetally. In the large apex of differentiated SCBs, NLY transcripts initially were concentrated in numerous, regularly spaced groups of dividing cells in the peripheral zone (Fig. 4b). The hybridization signal extended 8–10 cells deep into the peripheral zone. Continued periclinal division of the cells in these basal pockets led to the formation of bract primordia. The number of cells expressing NLY increased in more developed bract primordia and was concentrated in their middle parts and adaxial sides (stage 1, Fig. 4c). This NLY expression pattern was transient.
stage 2 of development, the number of NLY positive cells within adaxial side bract primordia decreased, with their subsequent concentration in a group of hypodermal cells (6–8 cells deep) at the adaxial side of the bract primordia (Fig. 4d). Regulated division of the cells expressing NLY led to ovuliferous scale initiation. During stage 3, NLY expression was almost uniform within the ovuliferous scale primordia of the fused bract-ovuliferous scale complex, whereas NLY levels in bracts dramatically decreased (Fig. 4e).

Accumulation of NLY in differentiated PCBs is shown in Fig. 4f and g. During stage 1, NLY was expressed at low levels within microsporophyll primordia in a group of cells that gave rise to sporogenous tissue. A much higher level of expression was detected in developed pollen mother cells during stage 2, when microsporophyll initiation was complete (Fig. 4g). Expression of NLY in undifferentiated axillary buds that emerged within vegetative LSTB is shown in Fig. 4h. In differentiated DSBs, NLY transcripts were found in groups of cells within the peripheral zone on the side of the small DSB apex (Fig. 4i). Continued periclinal division of these cells indicated the initiation of needle primordia (arrowheads). Uniform and strong accumulation of NLY transcripts in two of the three to five needle primordia surrounding the apex is shown in Fig. 4j. Hybridization with control, sense probe, in all analyzed sections did not show any detectable signals (not shown).

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**Fig. 1.** Sequence comparison of FLO/LFY-like proteins (accession numbers in parentheses): PrFLL from *P. radiata* (U92008); NLY from *P. radiata* (U76757); BOFH from *Brassica oleracea* (718362); LFY from *Arabidopsis thaliana* (M91208); NFL1 and NFL2 from *Nicotiana tabacum* (U16172 and U16174, respectively); PEAFL0 from *Pisum sativum* (AF010190); FLO from *Antirrhinum majus* (M55525); PtFL from *Populus balsamifera* (U93196); and RFL from *Oryza sativa* (AB005620). Black boxes indicate identical amino acids, shaded boxes indicate amino acids with similar properties, and dots indicate gaps introduced to optimize alignment. c1 and c2, conserved regions; v1 and v2, variable regions. Positions of the proline residues within the proline-rich region are indicated by asterisks. Acidic domain indicated by dashed line.

**Fig. 2.** Phylogenetic tree of the FLO/LFY gene family. A single most parsimonious tree was obtained (consistency index = 0.917; retention index = 0.760). Bootstrap values for 100 replicates are shown above each branch.

**Fig. 3.** RT-PCR analyses of NLY transcripts levels in PCB (stages 1–2), SCB (stages 1–3), DSB from an adult tree and 1-month-old seedlings and roots. PCR products were blotted onto nylon membranes and hybridized to 32P-labeled NLY cDNA clone. The RNA fragment was amplified from the same RNA samples as a positive control.
Constitutive NLY Expression Converts Arabidopsis Shoot Meristems into Floral Meristems. To determine whether NLY was a functional ortholog of the FLO/LFY-class of genes, we generated transgenic Arabidopsis (Columbia ecotype) in which the NLY gene was expressed under the control of the constitutive 35S promoter from cauliflower mosaic virus. In contrast to the wild-type, most of the transgenic 35S::NLY lines showed early flowering. In 35S::NLY-1, -3, -7, and -10 plants grown under LD conditions, floral buds were visible after an average of 13–15 days, with 9–10 rosette leaves (40–60 plants were analyzed for each line). The earliest line, 35S::NLY-7, flowered with 7.9 ± 0.4 rosette leaves (compared with 12.1 ± 0.3 leaves in wild-type Col plants). The reduction of flowering time was most dramatic when 35S::NLY plants were grown under SD conditions. Flowering time was reduced to 4–5 weeks after germination, with a much lower number of rosette leaves (22.8 ± 1.8) than control, wild-type plants (40.8 ± 1.8 rosette leaves).

In addition to early flowering, 35S::NLY plants showed the conversion of shoots into flowers. Although the severity and details of the phenotype varied among different lines, the most common 35S::NLY phenotype was a conversion of all lateral shoots into solitary flowers (and termination of the primary inflorescence shoot with an apical flower) in plants grown under either LD or SD conditions (Fig. 5 a-d). This phenotype was found in 12 35S::NLY lines among 36 kanamycin-resistant lines. In contrast, in wild-type Arabidopsis, lateral shoots developed in the axils of cauline leaves (bracts) and produced clusters of flowers (Fig. 5 a and e). In three lines (35S::NLY-1, -3 and -10), development of the primary shoot under LD conditions ceased prematurely with a terminal flower, resulting in a very short inflorescence (middle plant in Fig. 5 a). One extreme line (35S::NLY-7) formed a terminal flower immediately above the rosette (Fig. 5 e).

Control of Flowering Time by the Level of NLY Expression. The gain-of-function experiments presented above show that constitutive NLY expression can cause early flowering in transgenic Arabidopsis plants. To confirm that this effect indicated a specific function of NLY in controlling meristem identity, another set of transgenic plants was created in which NLY was expressed in a more restricted fashion. A transgene was constructed in which the NLY cDNA was expressed under the control of 2.3 kb of LFY 5′ upstream sequences. This promoter fragment confers levels of LFY expression that are sufficient to support normal flower development, and its activity mimics that of the endogenous LFY gene during the vegetative phase (10).
Among 28 independent transgenic lines, statistically significant differences with wild-type plants were observed in seven *LFY*::*NLY* lines. These lines flowered earlier than wild-type plants under both LD and SD growth conditions. The most severe phenotype was observed in lines *LFY*::*NLY*-4 and *NLY*-7. The average numbers of rosette leaves on *LFY*::*NLY*-4 and *NLY*-7 lines grown under LD conditions were 10.2 ± 0.3 and 10.7 ± 0.4, respectively, compared with 12.6 ± 0.2 in wild-type plants (50 plants were analyzed for each line). Acceleration of flowering was more dramatic under SD conditions, where the average numbers of rosette leaves on *LFY*::*NLY*-4 and *NLY*-7 lines grown under SD conditions were 28 ± 0.3 and 25 ± 0.7, respectively.

**DISCUSSION**

The analysis of the protein structures showed that *NLY*, as well as PrFLL, are distinct from their angiosperm homologues. The products of *FLO*/*LFY*-like genes have been proposed to be transcription factors, based on their structure and their nuclear localization (22, 23). Among their characteristics are a proline-rich domain at the amino terminus and an acidic region in the middle part of the proteins. Surprisingly, these domains are missing in *NLY* and PrFLL. Because the proline-rich and acidic domains are located within the variable regions, they may be subject to evolutionary changes after the separation of the angiosperm and gymnosperm lineages. The common ancestor of *FLO*/*LFY*-like proteins might have had (i) gymnosperm-type structure, and separation and evolution of the angiosperm lineage were associated with elaboration of their gene structure; (ii) angiosperm-type structure, and separation was associated with loss of certain protein domains; or (iii) an intermediate structure that was elaborated during angiosperm evolution and simplified in the evolution of gymnosperms.

The radiata pine genome contains two *FLO*/*LFY*-like genes. Surprisingly, the *NLY* and PrFLL proteins are less closely related to each other (50–55%) than the *FLO* and *LFY* gene products from the distantly related angiosperm species *Arabidopsis* and *Antirrhinum* (70%). However, divergence between the two pine genes apparently has not occurred at an equal rate throughout the coding sequences. Within the conserved c2 domain, *NLY* and PrFLL share several nonconservative changes, such as H->D, D/E->K, and Y->H, suggesting that they diverged more rapidly from each other within c1, as well as v1 and v2, than angiosperm proteins did after the gymnosperm/angiosperm split.

**LFY Can Function as a Flower Meristem-Identity Gene in an Angiosperm.** The expression pattern of *NLY* in young emerging primordia, on both vegetative and reproductive apices, is very similar to that of its angiosperm homologues. We have demonstrated that *NLY* is likely to act in a conserved
network of regulatory genes; it functions in transgenic Arabidopsis in a very similar manner to the endogenous LFY gene.

Strikingly similar to overexpression of LFY, overexpression of NLY caused the conversion of lateral shoots into solitary flowers, as well as the truncation of the main shoot with a terminal flower. Moreover, similar to LFY, the effect of ectopic NLY activity differs for primary and secondary shoot meristems. Secondary meristems of transgenic plants produced only a solitary flower, whereas primary meristems produced leaves before they switched to the formation of bractless lateral flowers and finally a terminal flower. The final aspect that NLY shares with LFY is its ability to induce early flowering, most dramatically under SD conditions. Recently, Blázquez and colleagues (10) demonstrated that LFY not only has properties of a flower meristem-identity gene but also of a flowering-time gene. Thus, changing the copy number of wild-type LFY affects the number of leaves produced before the first bractless flower was initiated. This fact confirms that transcriptional regulation of LFY is an important determinant of flowering time. Similarly, we found that expression of NLY under control of the endogenous LFY promoter reduces flowering time, without ectopic transformation of shoot into flower meristems. The effect of NLY on early flowering, when expressed from a physiologically relevant promoter, confirms that the in vivo activity of NLY is indeed very similar to that of LFY. The observation that the LFY::NLY transgene can largely complement the defects in flower development caused by a severe lfy allele strongly supports the conclusion that NLY is a pine ortholog of LFY.

Apart from their role in flower development, FLO/LFY-like genes in angiosperms can have other functions, as demonstrated by the leaf phenotype of uni mutants in pea. The latter observation suggests that the more generalized function of angiosperm FLO/LFY genes is in maintaining a transient phase of indeterminacy before lateral derivatives of an apical meristem are specified. Although this seems less likely in perennials such as pine trees, which have a prolonged vegetative phase of many years, functional studies of NLY in pine are needed to assess its function in the development of vegetative and reproductive primordia.

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