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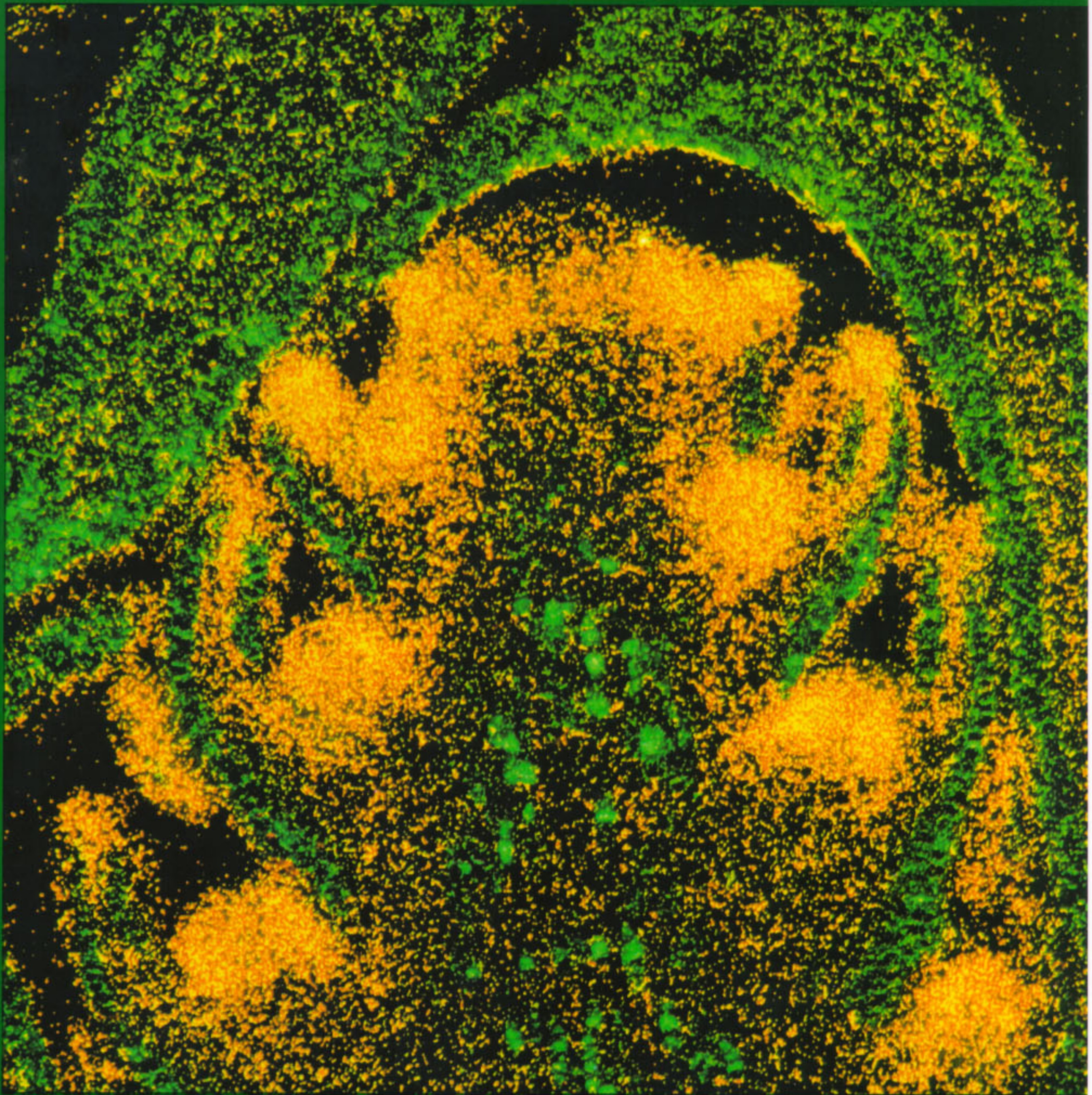

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Diverse effects of overexpression of *LEAFY* and *PTLF,* a poplar (Populus) homolog of *LEAFY/FLORICAULA,* in transgenic poplar and *Arabidopsis*

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Summary

***PTLF,* the *Populus trichocarpa* homolog of *LEAFY (LFY)* and *FLORICAULA,* was cloned to assess its function in a dioecious tree species. In situ hybridization studies showed that the gene was expressed most strongly in developing inflorescences. Expression was also seen in leaf primordia and very young leaves, most notably in apical vegetative buds near inflorescences, but also in seedlings. Although ectopic expression of the *PTLF* cDNA in *Arabidopsis accelerated* flowering, only one of the many tested transgenic lines of Populus flowered precociously. The majority of trees within a population of 3-year-old transgenic hybrid Populus lines with PTLF constitutively expressed showed few differences when compared to controls. However, phenotypic effects on growth rate and crown development, but not flowering, were seen in some trees with strong PTLF expression and became manifest only as the trees aged. Competence to respond to overexpression of LFY varied widely among Populus genotypes, giving consistent early flowering in only a single male *P. tremula* x *P, tremuloides* hybrid and causing gender change in another hybrid genotype. PTLF activity appears to be subject to regulation that does not affect heterologously expressed LFY, and is dependent upon tree maturation. Both genes provide tools for probing the mechanisms of delayed competence to flower in woody plants.**

Introduction

Herbaceous model species, especially Arabidopsis and Antirrhinum, have provided a *wealth of information about the* genes involved in *floral induction and the development of* inflorescences and flowers (reviewed by Levy *and Dean, 1998;* Pidkowich etal., *1999).* Mutations in several types of regulatory genes cause *abnormal* flowers to *be* formed or prevent flowering altogether, and a number of these genes have been cloned and extensively characterized. The remarkable conservation of the structures and functions of floral genes between highly divergent species suggests that many of their properties would remain unchanged across large phylogenetic distances.

The genus Populus is an important model system for the molecular biology of woody plants. Poplars include aspens (species of Populus, section Populus), cottonwoods (species of sections *Tacamahaca* Spach and *Aigeiros* Dubyh and various hybrids among and within sections. Cottonwoods and aspens are in well separated sections of the genus, and have been diverging for at least 20 million years (Eckenwalder, *1996).* These fast-growing trees have relatively small genomes, are generally easy to regenerate *in vitro,* and are susceptible to transformation with *Agrobacterium (Han etal.,* 2000). *We are* interested in determining the *basis* of the development of Populus flowers *and* inflorescences, both *to understand evolution* of floral form and eventually to enable control of flowering in these economically important species.

Floral development in poplars differs significantly from that of a typical hermaphroditic annual (e.g. Boes and Strauss, *1994).* The apices of the branches do not become

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inflorescences. The flowers are borne on axillary inflorescences or catkins, with male and female flowers found on separate trees, although occasionally mixed inflorescences or hermaphroditic flowers are seen. The inflorescences appear from dormant buds in the spring, usually occurring from about 5 years of age. Instead of four concentric whorls of organs (sepals outermost followed by petals, then stamens surrounding one or more carpets in the center), the Populus flower has only two whorls (a reduced perianth cup surrounding either stamens or carpels). Populus does not normally initiate male organs in female flowers, or vice versa (Boes and Strauss, 1994; Sheppard, 1997). After releasing pollen or seeds, the entire inflorescences are shed (Kaul, 1995). By late spring the inflorescence buds for the next year's flowers have already been initiated in the axils of the current year's leaves, and will develop for several more months before becoming dormant.

The ability of the Arabidopsis floral meristem identity gene, LEAFY (LFY1, to accelerate flowering when overexpressed (Weigel and Nilsson, 1995) - even in heterologous plants such as aspens- has generated considerable interest in its role in the vegetative-to-reproductive phase transition, and in the potential use of LFY for controlling flowering in plants important to agriculture and forestry. Floral initiation involves a large assortment of genes which take part in responses to the autonomous maturation process, day length, and chilling (Levy and Dean, 1998; Pidkowich etal., 1999). Which of these genes might be involved in preventing young trees from flowering, or in maintaining the vegetative state of apical tips of branches, is unknown. Even in herbaceous species there is limited information on the extent of evolutionary diversity in regulation of competence to flower and floral meristem identity. It seems likely that there is important variation among species in how LFY and its homologs interact with other genes to regulate flowering.

To begin to study genetic controls on reproductive development in a dioecious tree, we have isolated the Populus trichocarpa (section Tacamahaca) homolog of the LFY and FLORICAULA (FLO) genes, which affect the inflorescence-to-floral meristem transition (Coen etal., 1990; Weigel etal., 1992). Within dicotyledonous angiosperms, LFY homologs appear to occur as a single functional locus per genome (Busch etal., 1999), facilitating interpretations of their structural and functional homologies among species.

Results

Structure of the gene and predicted polypeptide

A partial cDNA of the *P. trichocarpa* Torr. and Gray (also classified as *Populus balsamifera* ssp. *trichocarpa*; Brayshaw, 1965) homolog of LFY was isolated using PCR, as outlined in Experimental procedures. A cDNA library from developing inflorescences from a wild female tree was probed, yielding several near-full-length clones. One cDNA was used as a hybridization probe to identify several overlapping genomic clones from a library derived from an unrelated wild male tree. Figure 1(a) shows the structure of the cloned 12.5 kb genomic region. The gene sequence, including 2638bp upstream of the initiation codon and 457 by downstream of the poly(A) site, is available as GenBank accession number U93196.

An abridged gene sequence plus the predicted translation of the coding region are shown in Figure 1(b). The genomic sequence differed from the cDNA at only one base transition that resulted in a synonymous codon (base 1085 G to Al. Cloning-derived sequences (between the 5'EcoRl site and the initiation codon and between the poly(A) sequence and the 3'-Kpnl site) were contained in transformation constructs described below and are therefore included in Figure 1(b).

Hybridization analysis of genomic DNA was performed using a PCR fragment corresponding to the highly conserved third exon of PTLF as a probe. A single band was seen with each of several enzymes, demonstrating that there is only one copy of this gene present in P. trichocarpa (data not shown). No additional bands were seen at reduced stringency.

Figure2 shows the comparison of the deduced PTLF amino-acid sequence with several LFY homologs from a range of dicots. The Eucalyptus homolog (ELF, Southerton etal., 1995) and LFY appear to be especially divergent from the other sequences. In the most conserved (boxed) segments of Figure 2 there are 22 residues where ELF is the only sequence that doesn't match, and 12 sites where only LFY doesn't match, compared to an average of four such sites for the remaining polypeptides. PTLF has a smaller number of acidic residues in the charged central region than any of the other sequences.

In situ *hybridization*

The expression patterns of *PTLF* in a range of tissues are shown in Figure 3. Hybridization corresponds to the white or yellow regions; the bright blue areas seen in several images are darkly staining, thick-walled cells that refracted light strongly. Figure3(a) shows a negative control, a section of a male inflorescence hybridized with a sense *PTLF* probe. The *PTLF* antisense probe hybridized strongly to the floral meristems and developing flowers of both male and female plants (Figure3b-g). *PTLF* expression was not detected in apical inflorescence meristems, but was seen in flanking nascent floral meristems (Figure 3b,c). Developing flowers showed expression in immature carpets (Figure 3d) and anthers (Figure 3g). Both male

and female flowers exhibited some hybridization on the inner (adaxial) rim of the perianth cup during the midstages of development. This can be seen more clearly by comparing the *PTLF* hybridization patterns in Figure 3(d,g) with the PTAG 1/21P. trichocarpa *AGAMOUS)* hybridization pattern in Figure3(h). PTAG112 are a pair of recently duplicated orthologs to *AGAMOUS* and are detected only in the whorls that produce gametes (Brunner, 19981. No significant differences between sense and antisense probes were found with anthers of mature male flowers, or with stigmas, ovaries or ovules of mature female flowers (unpublished results).

PTLF clearly showed hybridization to bracts. In bract primordia and partially developed bracts, expression was seen throughout their structures, but as the bracts matured expression became limited to their tips and margins (Figure 3b). Hybridization to the margins of bracts can also be seen in Figure3(f,g), immediately above the flowers. No signal was seen in the most fully developed bracts.

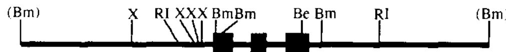
Hybridization was also observed with vegetative buds from mature branches (Figure 3j). The pattern of hybridization showed that there was RNA in the axils of newly formed leaves, but not in the center of the vegetative meristem. There was also significant expression in the tips of leaf primordia, and in some portions of surrounding developing leaves. Figure 36) shows the negative control. Even when the vegetative tissue was obtained from seedlings, weak hybridization was still seen in leaf primordia (Figure 3k). Figure 3(l) shows the bright-field image of the sample in Figure 3(k), to make the structures more evident.

Vegetative expression of *PTLF* was further characterized with competitive reverse transcription PCR (Wang and Mark, 19901. The level of *PTLF* RNA in developing vegetative buds of mature trees was 5% of the level found in developing inflorescences, decreasing sharply as the vegetative shoots expanded. Expression in shoots of juvenile and mature trees was less than 1% of that in developing inflorescence, and no expression was detectable in roots (data not shown).

Overexpression of PTLF in Populus infrequently causes early flowering

To test whether expression of *PTLF* could be altered to affect reproductive development of Populus, we trans

(a)



(b)

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...tagagatggaaaaggaactcaattaccctcaataataaaattggatggaaatcatct
agatggtggtccagtagtaagattttgggactaaaaggtttgttctctttgtggtctcag   -541
gttcgagccatgtggttgcttatatgatgaccactgaaaatttacatggtcgttaacttc
agggcccgtgggattagtcgaggtgcgtcaagttagtctggacacccatattaatctaaa   -421
aaaaaaaattaaatggcaaaaaatatttgaatgttgaagtaaaaaaagtgaaagggagg
tagtaaaacaatatacgacctaacaggagaggagtccaatcaagtagatcatgtgtcaag   -301
agatgagtggatagaagaacttcaagtgaagaatgtatgcagggaaccaaatgtgtgaat
gacacaaagatctgactagttcgatttcaactgtccagttccgaagaaacatcaaaaccc   -181
tttaattctgttagcttcccaatacatacaaaaaagaaaaaaagacaaaaaactcgtcct
gttaagggcagttttggtatataaataaaacaagaagctcacttgtctttatatatctac    -61
caaatccaagacatgcacctgtgaaagatcacagagagagagacaaggGGGCAGATAGAT
                  (cDNA sequence = gaattcggcacgagggcagata...)

 M  D  P  E  A  F  T  A  S  L  F  K  W  D  T  R  A  M  V  P    (20)
ATGGATCCGGAGGCTTTCACGGCGAGTTTGTTCAAATGGGATACGAGAGCAATGGTGCCA     60

 H  P  N  R  L  L  E  M  V  P  P  P  Q  Q  P  P  A  A  A  F    (40)
CATCCTAACCGTCTGCTTGAAATGGTGCCCCCGCCTCAGCAGCCACCGGCTGCGGCGTTT    120

 A  V  R  P  R  E  L  C  G  L  E  E  L  F  Q  A  Y  G  I  R    (60)
GCTGTAAGGCCAAGGGAGCTATGTGGGCTAGAGGAGTTGTTTCAAGCTTATGGTATTAGG    180

 Y  Y  T  A  A  K  I  A  E  L  G  F  T  V  N  T  L  L  D  M    (80)
TACTACACGGCAGCAAAAATAGCTGAACTCGGGTTCACAGTGAACACCCTTTTGGACATG    240

 K  D  E  E  L  D  E  M  M  N  S  L  S  Q  I  F  R  W  D  L   (100)
AAAGACGAGGAGCTTGATGAAATGATGAATAGTTTGTCTCAGATCTTTAGGTGGGATCTT    300

 L  V  G  E  R  Y  G  I  K  A  A  V  R  A  E  R  R  R  L  D   (120)
CTTGTTGGTGAGAGGTATGGTATTAAAGCTGCTGTTAGAGCTGAAAGAAGAAGGCTTGAT    360

 E  E  D  P  R  R  R  Q  L  L  S  G  D  N  N  T  N  T  L  D   (140)
GAGGAGGATCCTAGGCGTAGGCAATTGCTCTCTGGTGATAATAATACAAATACTCTTGAT    420

 A  L  S  Q  E                                                 (145)
GCTCTCTCCCAAGAAGgtttggtt...(intron 1 = 521 bases)...tgtgttca   1016

 G  F  S  E  E  P  V  Q  Q  D  K  E  A  A  G  S  G  G  R  G   (165)
gGTTTCTCTGAGGAGCCAGTACAGCAAGACAAGGAGGCAGCAGGGAGCGGTGGAAGAGGG   1076

 T  W  E  A  V  A  A  G  E  R  K  K  Q  S  G  R  K  K  G  Q   (185)
ACATGGGAGGCAGTGGCAGCGGGGGAGAGGAAGAAACAGTCAGGGCGGAAGAAAGGCCAA   1136

 R  K  V  V  D  L  D  G  D  D  E  H  G  G  A  I  C  E  R  Q   (205)
AGAAAGGTGGTGGACCTTGATGGAGATGATGAACATGGTGGTGCTATCTGTGAGAGACAG   1196

 R  E  H  P  F  I  V  T  E  P  G  E  V  A  R  G  K  K  N  G   (225)
CGGGAGCACCCATTCATTGTAACAGAGCCTGGTGAAGTGGCACGTGGCAAAAAGAACGGT   1256

 L  D  Y  L  F  H  L  Y  E  Q  C  R  D  F  L  I  Q  V  Q  S   (245)
CTTGATTACCTCTTCCATTTATATGAACAGTGTCGTGATTTCTTGATCCAAGTCCAAAGC   1316

 I  A  K  E  R  G  E  K  C  P  T  K                           (257)
ATTGCGAAGGAGAGGGGAGAAAAATGCCCCACTAAGgtacgaagagtcagcttcgcgagg   1376

gattgattttattta...(intron 2 = 689 bases)...ttgtgtttggttatag    2040

 V  T  N  Q  V  F  R  Y  A  K  K  A  G  A  S  Y  I  N  K  P   (277)
GTGACAAATCAGGTGTTTAGGTATGCCAAGAAGGCAGGAGCAAGCTACATCAACAAGCCC   2100

 K  M  R  H  Y  V  H  C  Y  A  L  H  C  L  D  E  D  A  S  N   (297)
AAAATGAGACACTACGTGCATTGCTATGCTTTACATTGCCTCGATGAGGACGCATCCAAT   2160

 A  L  R  R  A  F  K  E  R  G  E  N  V  G  A  W  R  Q  A  C   (317)
GCACTTAGGAGAGCGTTCAAGGAGAGAGGAGAAAATGTTGGAGCATGGAGACAGGCTTGT   2220

 Y  K  P  L  V  A  I  A  S  R  Q  G  W  D  I  D  S  I  F  N   (337)
TACAAGCCCCTTGTAGCCATCGCATCACGCCAAGGCTGGGACATAGATTCCATTTTCAAT   2280

 A  H  P  R  L  A  I  W  Y  V  P  T  K  L  R  Q  L  C  Y  A   (357)
GCTCATCCTCGGCTTGCCATTTGGTATGTGCCGACCAAGCTCCGTCAACTTTGTTATGCA   2340

 E  R  N  S  A  T  S  S  S  S  V  S  G  T  G  G  H  L  P  F   (377)
GAGCGCAATAGTGCCACTTCTTCAAGCTCTGTCTCTGGTACTGGAGGTCACCTGCCGTTT   2400
 *
TGAGTTCTTAATTATGCCAAGATAAATACTCCTATCTCTATAAAATTGTCAAAATGTATG   2460
TTGTAGCGAGGTCAGGACAAAGTATTGGTTGATGGAGGATGGTTCATTAAATTTCACATC   2520
CTTGACTATTTATATATCATGATATGCTTAAAGGCTCTAAtcattgtttacgtcgatgga   2580
                              (...aaggctctaaaaaaaaaaaaaaaaaaacc
ggcacgagggggggcccggtacc = cDNA sequence)
actattatatttctaatttagttttcagggaagtctaggctgctggtgcctacagtgtcc
ataaatttgagcaaaatggccaaaaggggccaattgggacccactaaattaatttggtgg   2700
tgcagtccccccttacaatacgactgcatgtaatacttgtccaaaatttgagtgcagt...
```

Figure 7. Structure of the *PTLF* gene.

(a) Restriction map of a genomic clone of *PTLF,* covering regions that were subcloned and sequenced. The direction of transcription is left to right. The three exons are shown as black boxes. Bm, ***BamHI;*** RI, EcoRI; Be, ***BstEII; X, XbaI.*** Sites in parentheses are from the vector.

(b) Sequence of the *PTLF* gene and predicted translation. Base 1 of this figure corresponds to base 2639 of GenBank accession number U93196. Upper case letters indicate exon sequences. The 5' end of the cDNA clone, including the flanking EcoRI site, is given beneath bases -25 to -4, and the 3' end, including the flanking *KpnI site,* is given beneath bases 2521 to 2580. A TATA box and a CCAAT box that may function in the promoter are shown double underlined.

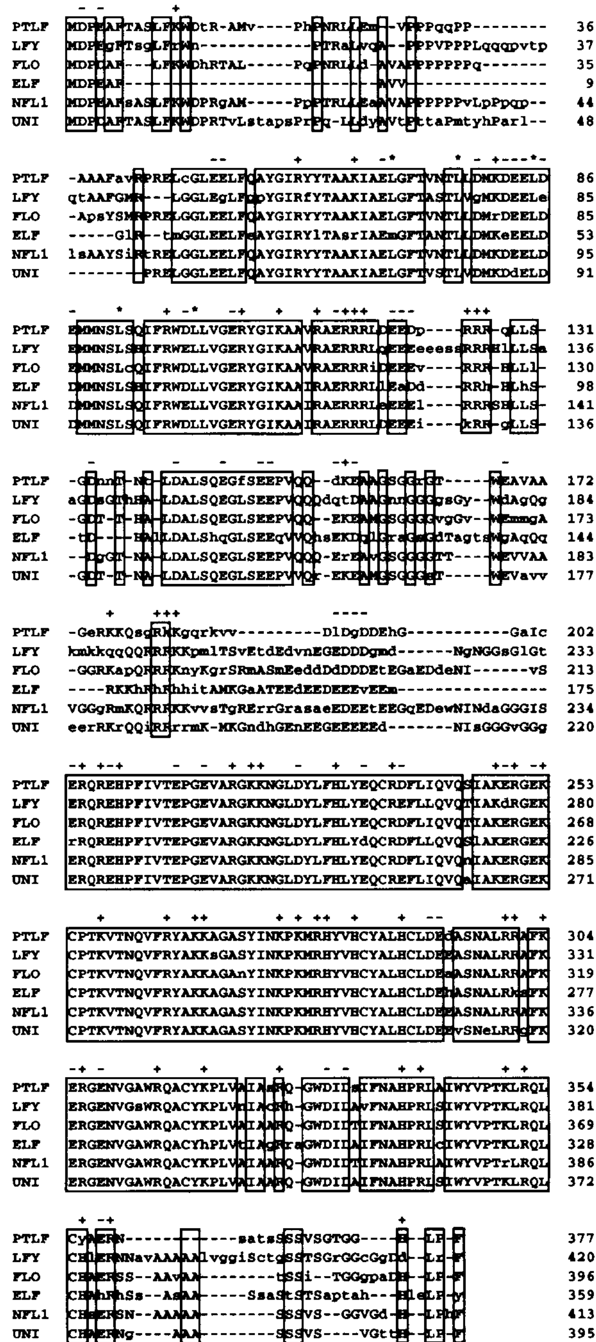


Figure2. Comparison of predicted amino-acid sequence of PTLF with homologs from five dicots.

ELF (Eucalyptus globulus homolog, Southerton et al., 19981; *FLO* (Coen etal., 1990); *LFY* (Weigel etal., 1992); NFL1 (Nicotiana tabacum homolog, Kelly etal., 1995); UNI (UNIFOLIATA of *Pisum sativa*, Hofer etal., 1997). Gaps introduced to maximize the alignment are shown as dashes. Amino-acid numbers are shown at the right. Residues with at least one match in the alignment are in upper case; those with no match are in lower case. Boxes indicate highly conserved regions where at least five out of six sequences match. + and - denote positions where basic and acidic residues, respectively, have been conserved in at least five out of six species. Asterisks denote positions of five conserved leucines with periodic spacing.

formed plants with the PTLF cDNA driven by the CaMV 35S promoter. Study of many 35S::GUS transgenic poplars showed that there was strong meristematic expression from this promoter (unpublished data). The *PTLF c*DNA (Figure 1b) was inserted behind the CaMV 35S promoter of pB1121, to produce p103S and p115AS, the sense and antisense orientations, respectively. Cottonwoods are relatively difficult to transform, and regulatory agencies may not permit transgenic cottonwoods to flower in field tests in Corvallis, Oregon, due to the abundance of wild relatives. Therefore, we used the P, *tremula x P. alba* hybrid aspen female clone INRA 717-1B4 and the P, *tremula x P. tremuloides* hybrid aspen male clone INRA 353-38 for our experiments. These genotypes are readily transformed with *Agrobacterium,* allowing a number of independent transgenic plants to be obtained in each hybrid, totaling 20 sense-PTLF(p103S) and 42 antisense-PTLFIp115AS) lines. Because several more years may pass before the antisense PTLFtrees reach reproductive maturity and possibly exhibit phenotypic differences compared to control plants, subsequent work focused on the sense-PTLF plants.

Although overexpression of *LFY in* aspens was reported to result in short, bushy plants that flower within a year (Weigel and Nilsson, 1995), no such obvious phenotypes were seen with p103S. During more than 1 year of growth in soil in a greenhouse, and an additional year at a field site in Corvallis, Oregon, no flowering or other differences were noted for any of the *PTLF transgenics* relative to control trees (data not shown). The presence of the transgene was verified through hybridization analysis of genomic DNA (data not shown). RNA blots were used to determine transcription levels, and showed a message size consistent with termination at the poly(A) site of the cDNA (data not shown). Starting in their third year, several p103S lines with high levels of *PTLF* RNA exhibited abnormal vegetative morphology. These lines will be discussed further below. The transformation was later repeated using p104S, a version of the sense construct that had extraneous sequences removed in order to place a nopaline synthase transcription terminator immediately after the cDNA (p103S relied solely on termination sequences present in the *PTLF* cDNAI. Sixteen PCR-confirmed transgenic lines were produced from clone 353-38. Three of these 16 lines showed evidence of a bushy phenotype 9 months after rooting and subsequent growth in the greenhouse, but only one produced flowers (Figure 4g,h).

Overexpression of PTLF in Arabidopsis *demonstrates that the gene is functional*

To exclude the possibility that a faulty construct or cDNA was responsible for the rarity of phenotype in Populus, p103S was tested in *Arabidopsis* (ecotype Columbia). T, seeds were selected for kanamycin resistance, resistant

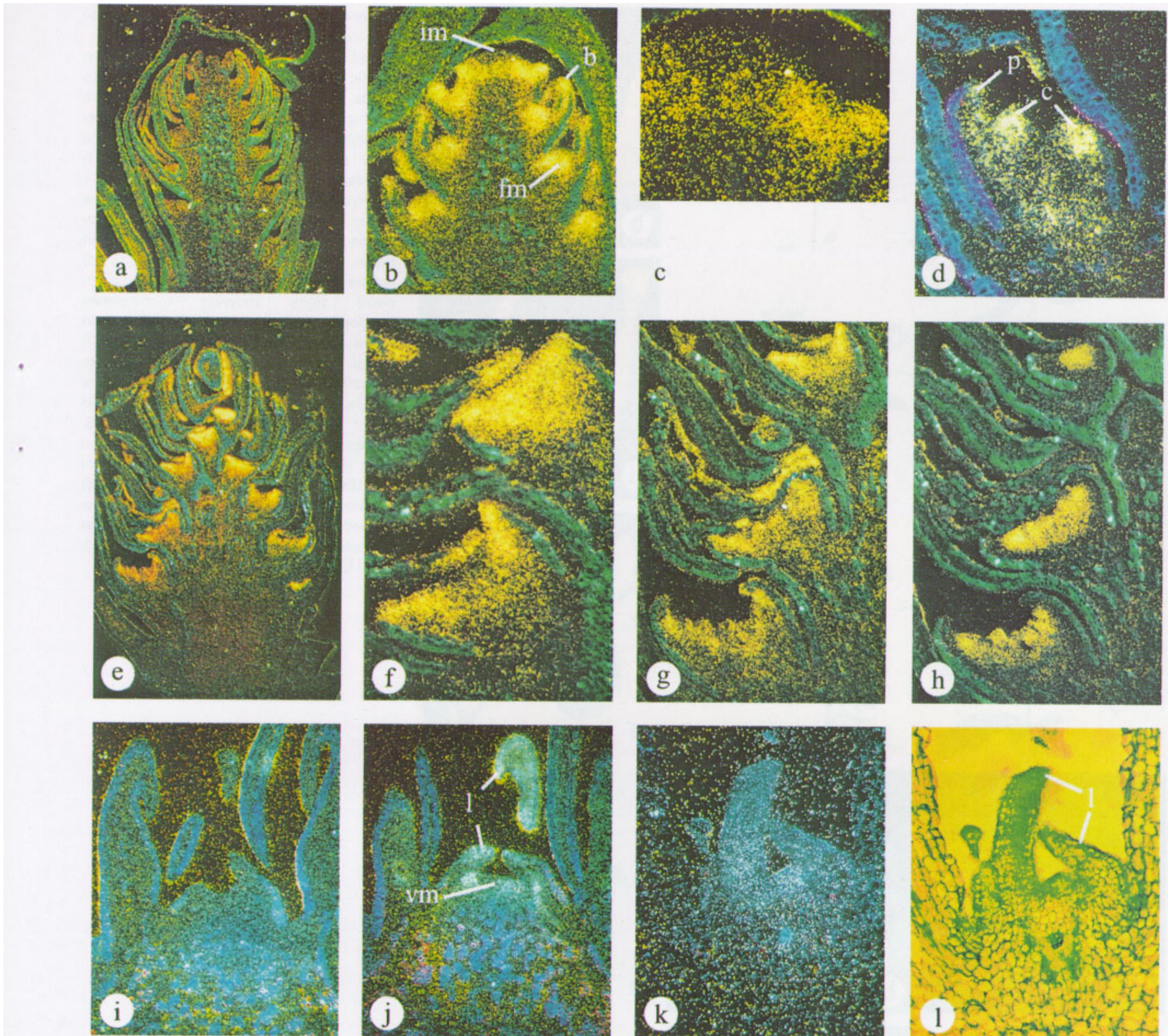


Figure3. In situ hybridization analysis of Mt

All images are longitudinal sections. Structures are indicated as follows: b, bract; c, carpet; fm, floral meristem; im, inflorescence meristem; l, developing leaf; p, perianth cup; vm, vegetative meristem.

(a) Early male inflorescence, sense probe (15x); (b) very early female inflorescence, antisense probe (30x); (c) magnification of the inflorescence meristem region of panel B (120x); (d) partially developed female flower, antisense probe (15x); (e) early male inflorescence, antisense probe (15x); (f) magnification of panel E (90x); (g) partially developed male flowers, antisense probe (60x); (h) partially developed male flowers, antisense PTAG1 (Brunner, 1998) probe (60x); (i) vegetative meristem from developing terminal bud, sense probe (30x); (j) vegetative meristem from developing terminal bud, antisense probe (30x); (k) seedling vegetative meristem, antisense probe (30x); (l) bright-field image of seedling vegetative meristem from panel (k) (30x).

plants grown to maturity under long days (see Experimental procedures), and the number of days to formation of the first flower bud was recorded. *PTLF* caused *Arabidopsis* to flower an average of 5 days earlier than control plants, regardless of whether grown in soil or *in vitro* (Figure 5). No marked differences in appearance between p103S-transformed plants and controls were observed; p103S-transformed plants averaged one fewer rosette leaf than controls (data not shown). As in the case of Populus, many of the *Arabidopsis* did not appear to respond to *PTLF,* in the trial where plants were transferred to soil only 17 out of 49 p103S-transformed plants flowered 2 or more days before the earliest control plants (Figure 5a).

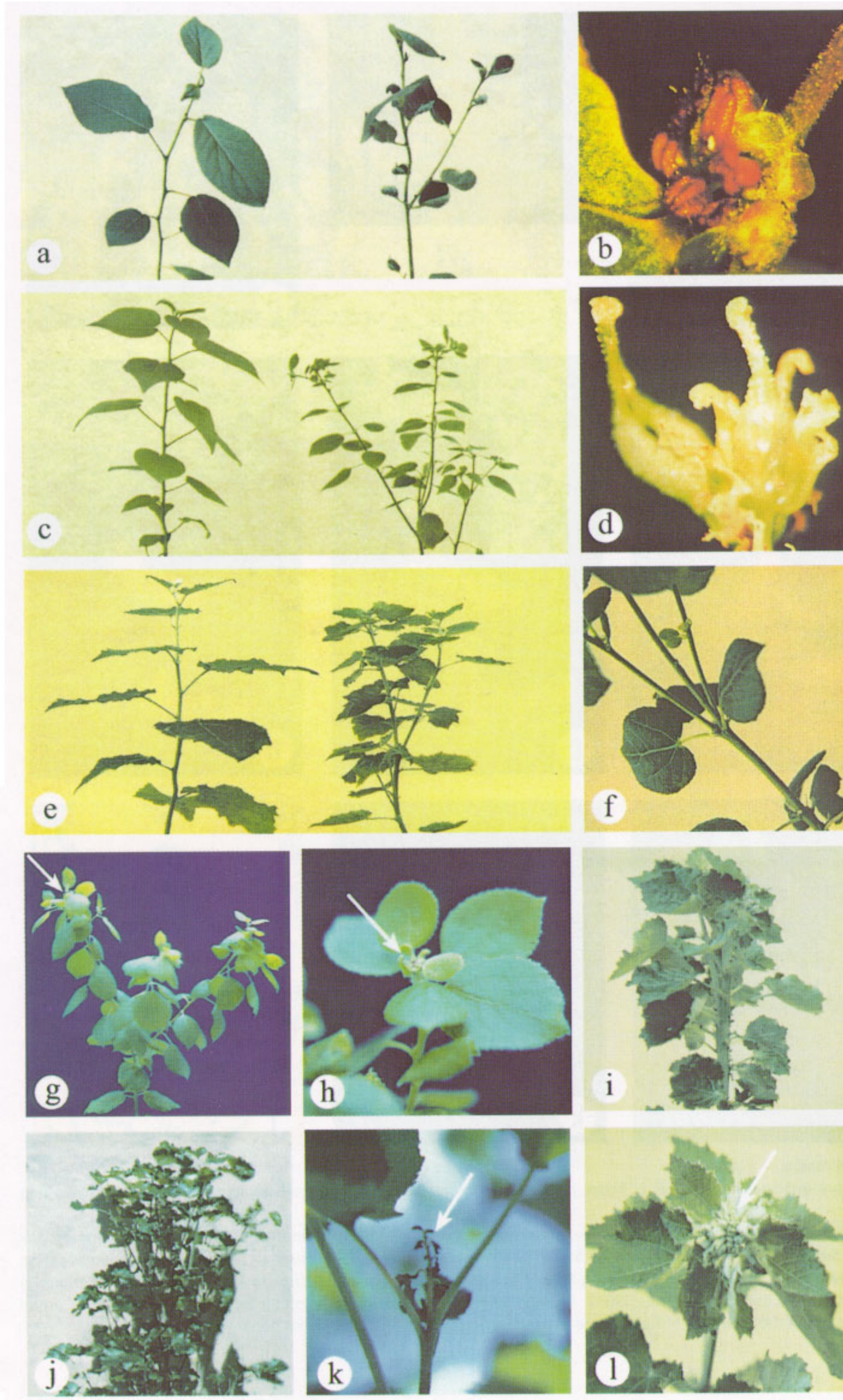
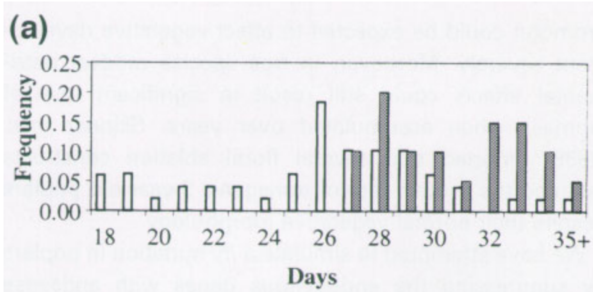
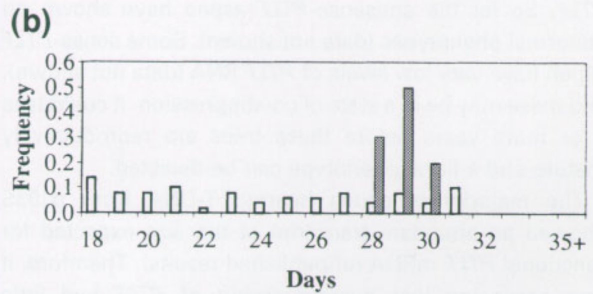


Figure4. Phenotypic effects in Populus of constitutive *LFY* and *PTLF* expression.
a,c,e,i,l) 4-month-old greenhouse-grown plants; panels; (j,k) 2-year-old field-grown plants. In (a,c,e) non-transformed plants are on the left, and *pDW151135S:LFY) trans*formed plants are on the right. Panels in the right column (b,d,f,i,l) depict pDW151transformed plants.
(a) Hybrid cottonwood clone 184-402 (line 31 on right); (b) male flower produced by hybrid aspen clone 353-38, line 5; (c) hybrid aspen clone 353-38 (line 9 on right); (d) female flower produced by a hybrid cottonwood clone showing incompletely developed carpellate flowers; (e) hybrid aspen clone 717-1B4 (line 36 on right); (f) lateral branches produced after flower formation in hybrid aspen clone 353-38, line E; (g,h) male flowers at apex of clone 353-38 under *PTLF* overexpression with p104S; arrows show location of flower; (i) fasciated main stem in a ramet of hybrid aspen clone 717-1B4, line 112; (j,k) *sense-PTLF* (*p103*S) transformed 717-1B4; (1) pDW151-transformed 717-1B4 showing extreme shortening of internodes at apex (arrow), line 112.

Overexpression *of* LFY does not produce early flowering in all genotypes *of Populus*

Because the aspen hybrids used in the above experiments were not the same as described in the original report of LFY overexpression (Weigel and Nilsson, 1995), we tested whether the failure of 717-1B4 and 353-38 to respond to PTLF overexpression was matched by a lack of response to LFY. Clones 717-1B4 and 353-38 were both transformed with plasmid pDW151, in which the LFY coding region is driven by the 35S promoter (Weigel and Nilsson, 1995). Only two of 19 transgenic lines of clone 717-1B4 exhibited





Figures. Acceleration of flowering by constitutive expression of PTLF in *Arabidopsis.*
(a,b) Frequency of transgenic *Arabidopsis initiating* flowering plotted versus days after germination when flowering began. In (a•, grown in soil, n=49 for p103S-transformed plants (white bars) and n=20 for untransformed control plants (gray bars). In (b•, grown in *vitro,* n=50 for p103S-transformed plants (white bars) and n=20 for untransformed plants (gray bars. All plants were grown under 16 h days.

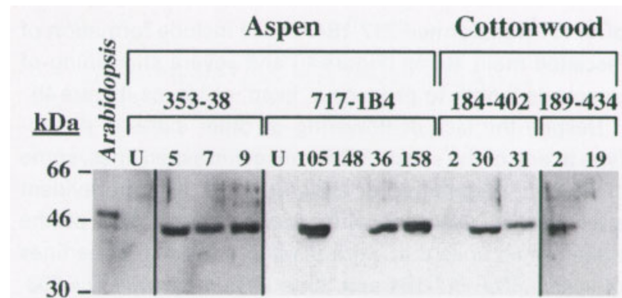


Figure6. Immunoblot analysis of *LFY* expression levels in various Populus clonal backgrounds.
Total protein extracts (25pg *35S::LFY Arabidopsis* or 100pg *35S::LFY* Populus) were fractionated by SIDS-PAGE and immunodetected using a polyclonal antibody to *LFY.* The migrations of molecular weight markers are indicated at the left. *Arabidopsis, 35S::LFY Arabidopsis. U,* untransformed aspens. The identities of the transformed lines are indicated under the clone numbers.

precocious flowering after growing as rooted plants for more than 2 years. However, in both lines anthers rather than carpets were produced in this normally female clone. In contrast, each of 20 transgenic lines of male clone 35338 produced flowers within 6 months of transfer to soil, all of which bore stamens. Figure 4(b) shows an example of the individual male flowers formed in place of inflorescences of transformed 353-38. Using the very limited amounts of pollen that could be extracted from their nondehiscing anthers, we pollinated female flowers of wild *P. tremuloides* in a greenhouse. Viability was assessed by staining for pollen tubes (Martin, 1959). Two of the 20 pDW151-transformed lines tested showed pollen tube germination, but at a very low frequency (unpublished observations.

Five *P. trichocarpa* x *P. deltoides hybrid* cottonwood genotypes, clones 17-50, 19-53, 24-305, 184-402 and 189434, were also transformed with pDW151. In total, 39 transgenic lines were produced, of which five showed precocious flowering. After 8 months' growth in a greenhouse, flowering of cottonwood was observed only in lines from female clones 17-50 and 184-402. The flowers had abnormal structures, with unfused carpets and only rudimentary ovules (Figure4d).

The presence of LFY was verified in representative Populus transformants by Western analysis. Figure6 shows the levels of LFY polypeptide detected in 12 individuals. For all of the Populus hybrids, LFY migrated slightly more rapidly than when expressed in *Arabidopsis.* Untransformed hybrid aspen plants were used as negative controls. Line 148 of hybrid aspen 717-1B4 and line 19 of hybrid cottonwood 189-434 failed to express detectable LFY, although they carried the selectable marker gene, and line 2 of hybrid cottonwood 184-402 showed only weak expression. Three lines of hybrid cottonwood that demonstrated expression of LFY polypeptide failed to flower precociously. The antibody did not cross-react efficiently with PTLF (data not shown), so it was not possible to extend this experiment to test the sense-PTLF transgenics.

Effects *of overexpression of* LFY *and* PTLF *on* vegetative growth *of Populus*

Figure 4 demonstrates the effects of constitutive expression of LFY and *PTLF on* the phenotypes of Populus plants. Figure 4a,c,e show 4-month-old plants (untransformed on the left, pDW151-transformed on the right). The hybrid cottonwood 184-402 line 31 produced smaller, deformed leaves that were often cup-like as a result of *LFY over*expression (Figure 4a). Hybrid aspen 353-38 line 5, which did flower precociously, also developed a bushier growth habit with more frequent branching, shorter internodes and significantly smaller, more rounded leaves (Figure 4c). Figure 4(f) shows a plant where a flower formed at the apex, then growth resumed from lateral meristems. These effects were found in the majority of transformants of this hybrid. In some cases, LFY-overexpressing hybrid aspen 717-1B4 did produce extra branches and also had somewhat shorter internodes and smaller leaves (Figure 4e), but flowers were only rarely detected. Several other developmental abnormalities were occasionally noted in lines of

pDW151-transformed 717-1B4. These include formation of fasciated main stems (Figure 4i) and severe shortening of internode length to produce a 'head' of leaves (Figure 4l).

Despite the lack of flowering or other extreme phenotype in any of the sense-PTLFtransgenic aspen lines, some changes in vegetative development did become evident after several years' growth. Approximately half of the transformed lines that were planted outdoors, three lines of sense-PTLF717-1B4 and three lines of sense-PTLF35338 began to show increased ramification of branches from the current year's growth after being in the field for 2 years. Figure 4(j) shows the crown from one line of a 2-year-old tree in clone 717-1B4, and Figure4(k) shows the dysgenic apical shoot development that led to the proliferation of lateral shoots. Winter mortality of these abnormal shoots was also seen in all six transformed lines that demonstrated excess branching. Similar abnormalities had never been seen before in any of the several hundred transgenic lines of poplars produced in our laboratory, indicating that this was not a somaclonal effect from transformation.

It was noted that these lines were growing more slowly than average. Height and diameter were therefore measured for three ramets of each of the 11 transformed sense-PTLF lines and for 10 untransformed ramets of each line (planted in a randomized block design). A significant (P<0.05) negative correlation (t2=0.34) was seen between expression levels of *PTLF RNA* and tree volume index (height x diameter z, adjusted for differences between clones). Sense-PTLFtrees averaged 20% less growth than untransformed control trees (data not shown).

Discussion

The expression pattern of *PTLF in* Populus (Figure3) showed general congruence with those seen in *Antirrhinum* and *Arabidopsis* (Blazquez etal., 1997; Coen etal., 1990; Weigel etal., 1992). Strongest expression was in the lateral floral meristems of developing inflorescences. *PTLF* was clearly expressed in non-floral tissues, including bracts, flanks of vegetative meristems, and very young leaves (Figure 3b,e,j). The expression near the vegetative meristem was probably due to incipient leaf primordia. *PTLF* RNA was not seen in the centers of apical meristems, whether inflorescence or vegetative (Figure 3c,j). One of the original goals of this research was to determine if *PTLF* was regulated specifically enough that its promoter could be used in synthesizing a sterility transgene (Strauss etal., 19951. Although Nilsson etal. (1998) have shown that the *LFY promoter can* be used in a genetic ablation construct to generate *Arabidopsis* plants that are apparently vegetatively normal and completely lack flowers, the expression pattern seen for *PTLF* indicates that a similar construct driven by the *PTLF* promoter could be expected to affect vegetative development severely. Moreover, in tree species modest detrimental effects could still result in significant loss of biomass when accumulated over years. Skinner etal. (1999) reported that several floral ablation constructs retarded the growth rate of transgenic 3-year-old poplars despite their normal vegetative morphology.

We have attempted to simulate a Ify mutation in poplars by suppressing the endogenous genes with antisense *PTLF. So* far the antisense-PTLF aspen have shown no abnormal phenotypes (data not shown). Some sense-PTLF aspen have very low levels of *PTLF RNA* (data not shown), and these may be in a state of co-suppression. It could take 5 or more years before these trees are reproductively mature and a floral phenotype can be detected.

The majority of aspen bearing T-DNA from p103S showed an abundant transcript at the size expected for functional *PTLF* mRNA (unpublished results). Therefore, it was surprising that overexpression of *PTLF* had little effect. Based on the acceleration of flowering seen in p103S-transformed *Arabidopsis (Figure* 5), and the vegetative phenotypes of the 6 p103S-transformed aspen lines that began to show increased branching and some branch mortality as they aged, we infer that a functional PTLF polypeptide is being synthesized in these trees. The observation of early flowering in a single line of p104Stransformed aspen supports the conclusion that PTLF is functional. However, PTLF seems to be less active than LFY in all species tested. *Arabidopsis* transformed with *35S::PTLF* did not appear to exhibit the terminal flowers and conversion of axillary meristems to flowers that was seen with *35S::LFY* (unpublished results; Weigel and Nilsson, 1995).

Multiple lines of Populus overexpressing either *LFY* or *PTLF* showed alterations in development (Figure 4), but generally not the early flowering phenotype that was expected. There was a clear distinction between the hybrid aspens; 353-38, a male clone similar to the one used by Weigel and Nilsson (19951, showed precocious flowering at very high frequency, while clone 717-1B4 flowered rarely. Hybrid cottonwood appears able to respond to LFY to an intermediate extent. It is not known how much the response seen in a given species or hybrid was due to the sex or genotype of the clone studied; however, *LFY* overexpression did cause both male and female poplars to flower precociously to some extent.

Frohlich and Parker (2000) have proposed that *LFY* evolved from a gene that promoted male flowering in ancestral gymnosperms, which is intriguing in light of the fact that a male aspen clone was most responsive to overexpression of *LFY* and *PTLF*. This could also provide an explanation for the observed change in gender caused by *LFY* overexpression, resulting in male flowers on normally female aspen clone 717-1B4. However, *LFY is*

clearly able to induce female reproductive development in Arabidopsis and at least one cottonwood clone (Figure4).

Because phenotypic changes correlated with PTLF overexpression appear only after several years of growth, negative regulatory factors that constrain PTLF function may be involved in juvenility. Such a gene may function similarly to HASTY, which decreases the ability of 35S::LFY to accelerate flowering in Arabidopsis (Telfer and Poethig, 1998). Precocious flowering of 35S::LFY Arabidopsis was not due to shortening of the juvenile phase (Weigel and Nilsson, 1995), but there are no data to indicate whether this was true in Populus. One explanation for the observations that hybrid aspen 353-38 readily flowers precociously with LFY but rarely flowers with PTLF, while Arabidopsis responds to both genes, is that aspen expresses a gene that inhibits the action of PTLF but not LFY. Possibly the divergent sequence of LFY (Figure 2) makes it unable to fully interact with the endogenous Populus inhibitor. The minimal flowering response of the 717-1B4 hybrid to LFY could be explained if the P. alba parent of 717-1B4 contributed a form of the negative regulatory factor (not found in *P. tremula* or *P. tremuloides*) that is better able to interact with LFY, at least to prevent the final stages of floral induction. Genes that normally prevent Populus apical vegetative meristems from becoming inflorescences could also affect the initiation of the ectopic floral meristems. Finally, there may be significance in the altered migration seen for Populus-expressed LFY during gel electrophoresis (Figure 6). Post-translational modification, such as phosphorylation, could account for the difference in mobility and could obviously affect LFY function. Components of long-day, short-day, and autonomous pathways have been shown to genetically interact with LFY (Levy and Dean, 19981; involvement of kinases or other protein-modifying factors in the various signal transduction cascades that lead to flowering and converge on LFY would not be surprising. While the putative modification to LFY in Populus compared to Arabidopsis could affect regulation of some LFY functions, altered migration was not correlated with flower induction. All poplar genotypes studied had a similar change in LFY mobility yet their frequency of floral induction varied widely.

The varying responses of different Populus hybrids to LFY indicate that a complex set of interactions, involving both positive and negative regulators, is probably taking place. As pointed out by Haughn etal. (1995), the numerous genes that regulate response to day length are strong candidates for interactions with LFY. Because Arabidopsi's is a facultative long-day plant, while the timing of Populus floral development adheres to a strict schedule that begins in early spring (Boes and Strauss, 1994), some of the differences noted between constitutive expression of LFY and PTLF may be a result of the day-length response pathway. We suspect that the branch tip mortality observed in six lines of PTLF-overexpressing aspen (Figure 4) is a consequence of reduced cold tolerance, which could be due to a disrupted response to the short days of autumn.

Despite many similarities among the structures and expression patterns of homologs of LFY, our results provide further evidence for important diversity in their expression and function. Analysis of the genes whose products modulate PTLF function, and the gene regions required for these interactions, should provide fresh insights into the causes of juvenility in trees and other plants with long non-flowering phases.

Experimental procedures

Collection of tissues

Tissues were collected from individual wild trees in the vicinity of Corvallis between *1991* and *1994.* Immature inflorescence tissue was collected from trees in mid- to late May. Reproductive buds were dissected to remove the young bud scales, and the entire inflorescences were collected. Terminal vegetative buds from flowering branches were collected at the same time, also with bud scales removed. Mature inflorescences were collected in mid- to late February as they emerged from buds, *1-2* days before anthesis. Expanding vegetative shoots and mature, flowering branches were collected in late February or early March. All tissues used for RNA extraction were frozen in liquid nitrogen as soon as possible after collection and stored at -80°C for up to 2 years. Tissues for hybridization analysis were immediately placed in fixative, then prepared for embedding in Paraplast (Kelly etal., 1995).

Construction of cDNA and genomic libraries

RNA for cDNA synthesis was extracted from developing inflorescence buds of a single female P. trichocarpa using the method of Baker etal. (19901, and purified by centrifugation through a 5.7m CsCI pad. Poly(A)+ RNA was selected using oligo(dT) cellulose columns (mRNA Separation Kit, Clontech). The cDNA library was prepared using the Lambda-ZAP cDNA cloning kit (Stratagene). The genomic library was constructed from DNA that had been isolated from dormant vegetative buds of a single male tree using a modified CTAB technique (Wagner etal., 19871. The vector used was Stratagene's LambdaGem-12 with partially filled-in Xhol sites. Packaging of the DNA into phage particles was performed with GigaPack Gold II (Stratagene).

PCR of cDNA for PTLF

A hybridization probe for the Populus *LFY* homolog was obtained by touchdown PCR (Don etal., 1991) of the cDNA library with a degenerate primer specific to a highly conserved region of *LFY* (5'-CGGAATTCATG(C/A)GICA(T/CITA(T/C)GTICAIT/C•TG(T/C)TA (T/C)GC-3'), and 5'-C6CTCGAGT,$-3'. The 480bp fragment was gel-purified and subcloned into pBluescript SK(-) for further characterization.

Sequence analysis

Sequencing of the cDNA was performed using the Sequenase 2.0 kit (United States Biochemical Corp.), according to the methods

described by the manufacturer. Sequencing of the gene was done at the Oregon State University Central Services Laboratory, using an AB1370 sequencer. Sequence analysis was performed using PCGENE (Intelligenetics) and GENERUNNER (Hastings Software Inc.).

Hybridization analysis

Standard methods were used for plaque hybridizations, genomic DNA blots and RNA blots (Sambrook etal., 1989). For copy number analysis of transgenic Populus DNA, signal intensities were analyzed using a Molecular Dynamics Phosphorimager and IMAGEQUANT software.

In situ hybridization analysis

Tissue samples from various sources were fixed, embedded, sectioned, and hybridized as described by Kelly etal. (1995), with the following modifications. Sections were 10pm in thickness. Probes were not alkaline hydrolyzed. Slides were photographed using a dark-light illuminator and/or a dark-field stop in the substage condenser.

Overexpression and antisense constructs of PTLF *cDNA*

Plasmids p103S and p115AS were generated by excising the *PTLF* cDNA using EcoRI and *Kpnl,* polishing the ends with T4 DNA polymerase, and ligating the fragment into Smal-digested pB1121 (Jefferson etal., 1987). Clones of each orientation were identified by PCR. Plasmid p104S, an additional *PTLF* sense construct, was generated by digesting p103S with Sad, polishing the site with T4 DNA polymerase, then digestion with Smal (regenerated during p103S construction) to excise the GUS gene, and ligation. The structures of the sense constructs were verified by PCR and sequencing.

Transformation of plants

P. tremula x P. alba hybrid 717-1B4 and *P. tremula x P. tremuloides* hybrid 353-38 were transformed with pDW151 (Weigel and Nilsson, 19951, p103S, and p115AS using disarmed Agrobacterium tumefaciens strain C58pMP90 (Leple etal., 19921. Hybrid cottonwood was transformed in a similar manner, with modifications described by Han etal. (20001. Agrobacterium transformation of *Arabidopsis (ecotype* Columbia) was performed by the in planta method of Bechtold etal. (19931. Transformed seedlings (Ti) were selected on solid medium containing 0.7% Phytagar (Gibco-BRL) 1% sucrose, 0.5 x Murashige and Skoog salts (Sigma), and 50 mg ml-1 kanamycin. For flowering-time experiments, untransformed control seeds were plated at the same time on the above medium minus kanamycin. T1 Kan R seedlings and untransformed controls were grown under a 16h photoperiod (fluorescent Sylvania Cool Whites) at 23-25°C and either left to flower on agar medium (in vitro-grown) or transferred (soil-grown) after 15days to Arabidopsis Potting Medium (Lehle Seeds).

Western analysis of LFY *expression*

Whole-cell extracts were prepared from leaf tissue of greenhousegrown *Arabidopsis,* aspen and cottonwood and analyzed by the method of He etal. (19941. Samples (100pg) of total protein were fractionated by SDS-PAGE on 10% acrylamide gels (Laemmli,

19701. Immunological detection was performed using a poly
clonal antibody against purified *LFY,* horseradish peroxidaseconjugated goat anti-rabbit secondary antibody (Kirkegaard and Perry Laboratories), and the Enhanced Chemiluminescence Detection Kit (Amersham).

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