Theor Appl Genet (2009) 118:1499–1508
DOI 10.1007/s00122-009-0998-1

ORIGINAL PAPER

A method for accelerated trait conversion in plant breeding

Ramsey S. Lewis · S. P. Kernodle

Received: 17 November 2008 / Accepted: 13 February 2009 / Published online: 6 March 2009

Abstract Backcrossing is often used in cultivar development to transfer one or a few genes to desired genetic backgrounds. The duration necessary to complete such 'trait conversions' is largely dependent upon generation times. Constitutive overexpression of the *Arabidopsis thaliana* gene *FT* (*FLOWERING LOCUS T*) induces early-flowering in many plants. Here, we used tobacco (*Nicotiana tabacum* L.) as a model system to propose and examine aspects of a modified backcross procedure where transgenic *FT* overexpression is used to reduce generation time and accelerate gene transfer. In this method, the breeder would select for an *FT* transgene insertion and the trait(s) of interest at each backcross generation except the last. In the final generation, selection would be conducted for the trait(s) of interest, but against *FT,* to generate the backcross-derived trait conversion. We demonstrate here that constitutive *FT* overexpression functions to dramatically reduce days-to-flower similarly in diverse tobacco genetic backgrounds. *FT*-containing plants flowered in an average of 39 days, in comparison with 87–138 days for non-*FT* plants. Two *FT* transgene insertions were found to segregate independently of several disease resistance genes often the focus of backcrossing in tobacco. In addition, no undesirable epigenetic effects on flowering time were observed once *FT* was segregated away. The proposed system would reduce the time required to complete a trait conversion in tobacco by nearly one-half. These features suggest the possible value of this modified backcrossing system for tobacco or other crop species where long generation times or photoperiod sensitivity may impede timely trait conversion.

Communicated by H. T. Nguyen.

R. S. Lewis (✉) · S. P. Kernodle
Department of Crop Science, North Carolina State University, Campus Box 7620, Raleigh, NC 27695, USA
e-mail: ramsey_lewis@ncsu.edu

Introduction

The backcross breeding method is often used in cultivar development programs when the goal is to introduce only one or a few genes from a donor genotype into a desired genetic background. The procedure provides a precise way of improving existing materials considered to be elite for a large number of attributes but deficient in a few characteristics (Allard 1960). Nuclear genes controlling disease resistance, morphological characteristics, or biochemical traits are frequently the focus of backcrossing. In addition, the method is typically used to establish male-sterile versions of elite lines by transferring nuclear genomes to cytoplasms contributing to this trait. The technique has likely gained greater application in recent years because of increased efforts in transferring transgene insertion events to elite commercial inbred lines. The process of transferring one or a few genes to a previously existing line using the backcross breeding procedure is sometimes referred to as 'trait conversion.'

In the backcross method, an individual plant of the gene donor is initially hybridized with an elite recipient line, also referred to as the recurrent parent. Progeny possessing the gene(s) or trait(s) of interest are then identified and 'backcrossed' to the recurrent parent. These steps are typically repeated four or five additional times followed by self-pollination to establish homozygosity for alleles conditioning the trait(s) of interest. The general objective of the backcross breeding method is to retain the gene(s) of

interest and to eliminate the remainder of the donor parent genome as rapidly and efficiently as possible. With each successive backcross (in the absence of background selection), the average percentage of the genome of the offspring that is derived from the donor line decreases and the percentage of the total recurrent parent genome increases according to the formula

$$\% \text{ recurrent parent genome} = 100\left[1 - (0.5)^{n+1}\right],$$

where $n =$ the number of backcross generations (Fehr 1987). The typical goal is to restore greater than 98% of the recurrent parent genome in this process, and 5–6 backcrosses are therefore generally made to establish a line nearly identical to that of the recurrent parent except for the introgressed gene(s) and closely linked genes (Stam and Zeven 1981).

A goal in plant breeding is to establish new and improved cultivars as quickly and efficiently as possible. The duration necessary to complete a trait conversion using the backcross procedure is largely dependent upon generation time (duration from seed to seed) for a given crop plant species. For cultivated tobacco (*Nicotiana tabacum* L.), for example, this interval is typically between 120 and 170 days in the field or greenhouse. More than 3 years would therefore often be required to generate BC_5F_3 seed for this species (Fig. 1). Methods for accelerating flowering would have utility for reducing generation time and the duration required to complete a trait conversion using the backcross breeding method.

Some plant species can be induced to flower through application of specific photoperiod and/or temperature combinations. Other species may be generally nonreactive

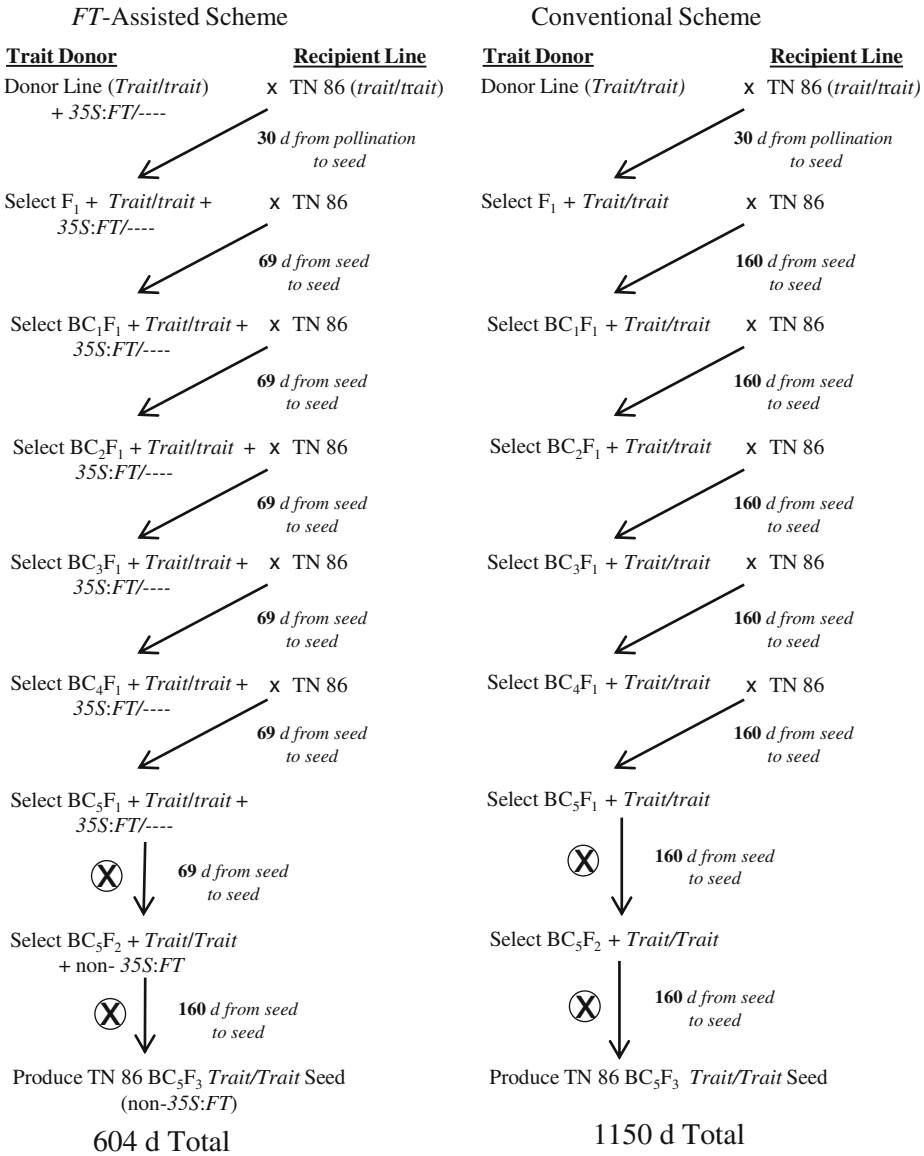

Fig. 1 Comparison of backcross breeding schemes for transferring a single dominant gene (*Trait*) from a donor line to representative burley tobacco cultivar 'TN 86.' Presentations assume ~160 days from seed to seed for commercial burley tobacco, ~69 days from seed to seed for *35S:FT* plants, continuous greenhouse space availability, and ability to detect the presence of the trait before flowering

to such treatments. Tobacco is considered to be a photoperiod insensitive plant species (although short-day, photoperiod sensitive mutants do exist), but flowering can be stimulated somewhat through exposure to specific environmental conditions (Kasperbauer 1966, 1969; Kasperbauer and Lowe 1966) or through grafting (Lang et al. 1977). These methods do not work efficiently for triggering flowering at a very early developmental stage, however, and improved methods could dramatically reduce the time required per backcross generation. Knowledge about the molecular biology, genetics, and physiology of flowering time in plants has accumulated rapidly in the last 10 years (Koornneef et al. 1998; Mouradov et al. 2002; Jaeger et al. 2006). Mapping experiments have led to the cloning of genes involved in flowering response in a number of species such as *Arabidopsis thaliana*, barley, wheat, tomato, and rice (Yano et al. 2000; El-Din El-Assal et al. 2001; Yan et al. 2004; Abe et al. 2005; Turner et al. 2005; Wigge et al. 2005; Lifschitz et al. 2006). One major gene that has been isolated is the *A. thaliana* gene *FT* (*FLOWERING LOCUS T*) (Kardailsky et al. 1999). This gene has been shown to encode for a graft-transmissible signal and may be common to most, if not all, flowering plants. Recent research has suggested that the FT protein, rather than *FT* transcript, is the likely mobile long-distance signal that is transported from the leaves, through the phloem, and to the shoot meristem to induce flowering in *A. thaliana* (Corbesier et al. 2007). When expressed constitutively or via viral vectors, *FT* and its orthologues can induce very early-flowering in tobacco, tomato, and other plants (Lifshitz and Eshed 2006; Lifschitz et al. 2006; Hsu et al. 2006; Lin et al. 2007).

A transgenic approach involving constitutive *FT* overexpression could be useful for accelerated backcross breeding for certain crops. Here, we use tobacco as a model system to propose and examine aspects of a breeding system where selection for an *FT* transgene insertion and the trait(s) of interest would be conducted in each backcross generation except the final generation. In the final backcross generation, selection would be conducted for the trait(s) of interest, but against the *FT* transgene to generate the backcross-derived trait conversion. Desired features of such a system would be applicability to diverse genetic backgrounds, independent segregation between *FT* and the gene(s) controlling the trait(s) of interest, and absence of *FT* transgene-induced epigenetic effects on flowering time after the transgene is segregated away. In experiments described here, tobacco was transformed with *FT* under control of the CaMV *35S* promoter. Experiments were conducted to (1) examine the effectiveness of *FT* overexpression for stimulating early-flowering in diverse genetic backgrounds, (2) demonstrate independent segregation between several *35S*:*FT* transgene insertions and four disease resistance genes which are often the focus of backcrossing in tobacco breeding, and (3) investigate the possibility of lingering epigenetic effects on flowering time once *FT* is segregated away.

Materials and methods

Transformation

The *A. thaliana FT* cDNA clone (Kardailsky et al. 1999) was kindly provided by Dr. Detleif Weigel of the Max Planck Institute for Developmental Biology, Tubingen, Germany. The binary Ti vector pBI121 (Jefferson et al. 1987) was used for transformation experiments. The *GUS* gene of this vector was replaced with the *FT* cDNA sequence to generate the new expression construct pSPK100 where expression of *FT* is driven by the CaMV *35S* promoter. The selectable marker gene in this construct is *npt*II. The new construct was electroporated into *Agrobacterium tumefaciens* strain LBA4404, and standard leaf disk transformation of burley tobacco cultivar 'TN 90LC' was performed according to the procedures of An et al. (1986). Regenerated *35S*:*FT* transformants (R_0 generation) exhibiting very obvious early-flowering phenotypes were retained for pollinations and further characterization.

Initial progeny testing

Regenerated TN 90LC *35S*:*FT* R_0 transformants exhibiting early-flowering phenotypes were hybridized as pollen parents with non-transformed TN 90LC. Eighteen to 54 F_1 progeny per cross were then evaluated for the early-flowering phenotype in a laboratory growth room maintained at 24–26°C and a photoperiod of 16 h light/8 h dark. Chi-Square tests (Steel et al. 1997) were performed to determine if observed ratios of early-flowering: normal-flowering plants conformed to that expected if there were one or two independently segregating *35S*:*FT* transgene insertions. Based on these results, progeny from two R_0 transformants predicted to likely possess single *35S*:*FT* insertions were selected for further characterization.

Early-flowering F_1 plants corresponding to the two selected R_0 transformants were backcrossed to non-transformed TN 90LC. Data were collected for days to flowering (reported throughout this investigation as days to first anther dehiscence) for 61 and 63 plants from the two BC_1F_1 progeny groups, respectively. Plants were initially grown in a laboratory growth room and data for early-flowering plants were collected in this environment. Non-early-flowering plants were transplanted to a field location (Central Crops Research Station, Clayton, NC) 50 days after seeding where flowering dates were also recorded.

PCR testing was also performed on each BC_1F_1 plant to detect the presence/absence of *35S*:*FT* using forward primer 5′-CTGGAACAACCTTTGGCAAT-3′ and reverse primer 5′-AGCCACTCTCCCTCTGACAA-3′ which were expected to amplify a 219 bp PCR product.

In order to test for the possibility of inactive or fragmented foreign DNA insertions in normal-flowering BC_1F_1 plants (an important aspect of the modified backcross procedure), PCR reactions were also performed to test for the presence/absence of a 195-bp region of the CaMV *35S* promoter amplified by forward primer 5′-GCTCCTACA AATGCCATCA-3′ and reverse primer 5′-GATAGTG GGATTGTGCGTCA-3′. PCR reactions were also performed to test for the presence/absence of a 206-bp region of the nopaline synthase (NOS) terminator sequence using forward primer 5′-TCGTTCAAACATTTGGCAAT-3′ and reverse primer 5′-TTGCGCGCTATATTTTGTTTT-3′.

All PCR reactions were performed using a 96-well PTC 100 thermal cycler (MJ Research, Watertown, MA) in 20 µl volumes containing 100 ng genomic DNA, 1X PCR buffer (New England Biolabs, Ipswich, MA), 2 mM $MgSO_4$, 250 µM of each dNTP, 1.25 units of Taq DNA polymerase (New England Biolabs), and 0.5 µM for each primer. Reaction parameters for *FT* detection were a single cycle of 95°C for 2 min, 52°C for 1 min, and 72° for 2 min; 30 cycles of 95°C for 15 s, 52°C for 1 min, and 72°C for 2 min; and a final step of 72°C for 5 min. Reaction parameters for the *35S* and NOS PCR tests were a single cycle of 94°C for 150 s, 58°C for 1 min, and 72°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min; and a final step of 72°C for 5 min.

Effect of constitutive *FT* overexpression in diverse genetic backgrounds

Early-flowering F_1 progeny (TN 90LC/TN 90LC *35S*:*FT* R_0) corresponding to the two selected R_0 transformants were also used to evaluate the effect of *FT* overexpression in diverse genetic backgrounds. Early-flowering *35S*:*FT* F_1 individuals were crossed with six genetically diverse tobacco cultivars or genetic stocks that exhibit a wide range of flowering times, leaf numbers, and plant types: 'Petite Havana,' 'Connecticut Shade 8212,' 'TN 86,' 'NC 2326,' 'Speight 168,' and 'Xanthi.' Petite Havana is a very early-flowering genetic stock of tobacco with very low leaf number. Connecticut Shade 8212 is a cigar wrapper tobacco cultivar with high plant height and relatively high leaf number. TN 86 is a burley tobacco cultivar that flowers relatively late and has a high leaf number. NC 2326 and Speight 168 are flue-cured tobacco cultivars that produce, on average 16 and 22 leaves, respectively. Xanthi is an oriental tobacco cultivar that produces a relatively high number of smaller leaves on an average-size stalk.

Twenty-two to 36 individuals from each of the 12 resulting progeny sets were evaluated for days to flowering. Plants were initially grown in a laboratory growth room and data for early-flowering plants were collected in this environment. Non-early-flowering plants were transplanted to a field location (Central Crops Research Station, Clayton, NC) 45 days after seeding where flowering dates were recorded.

Independent segregation between *FT* and major disease resistance genes

A desired feature of an *FT*-assisted backcrossing system would be non-linkage between a specific *35S*:*FT* transgene insertion and the gene(s) controlling the trait(s) of interest. *35S*:*FT* transgene insertion events corresponding to the two selected R_0 transformants were tested for linkage with four disease resistance genes that are frequently the focus of backcrossing in tobacco breeding: the *Tobacco Mosaic Virus* (TMV) resistance gene *N* derived from *N. glutinosa* (Lewis et al. 2005), a major blue mold (caused by *Peronospora tabacina*) resistance quantitative trait locus (QTL) designated as *BMR* likely introgressed from *N. debneyi* (Milla et al. 2005), the Race 0 black shank (caused by *Phytophthora parasitica* var. *nicotianae*) resistance gene *Ph* transferred from *N. plumbaginifolia* (Johnson et al. 2002b), and the root knot nematode (*Meloidogyne incognita*) resistance gene *Rk* (Yi et al. 1998). All of these genes exhibit complete or partial dominance.

Crosses were made to generate segregating progenies suitable for determining the possibility of linkage between the *35S*:*FT* transgene insertions derived from the selected R_0 transformants and the four major disease resistance genes described above. Materials involved in these crosses were TN 90LC (*N*/*N*, *bmr*/*bmr*, *ph*/*ph*, *rk*/*rk*), flue-cured tobacco cultivar Speight 168 (*n*/*n*, *bmr*/*bmr*, *Ph*/*Ph*, *Rk*/*Rk*), and burley tobacco cultivar 'NC 2000' (*N*/*N*, *BMR*/*BMR*, *ph*/*ph*, *rk*/*rk*). Early-flowering TN 90LC/TN 90LC *35S*:*FT* R_0 F_1 individuals were initially crossed with both Speight 168 and NC 2000. Early-flowering progeny were subsequently crossed to either untransformed TN 90LC or Speight 168 to establish eight progeny groups (Table 3) to be used for linkage tests.

For each progeny group, 47–48 plants were phenotyped for early-flowering and genotyped for the presence/absence of the *35S*:*FT* transgene. In addition, plants were genotyped with a resistance gene-specific marker, or genotyped with a DNA marker closely linked to the resistance gene of interest. For TMV resistance conferred by *N*, plants were genotyped using gene-specific primer pairs E1 and E2 (Lewis et al. 2005). For blue mold resistance controlled

by *BMR*, plants were genotyped with SCAR marker $SOPR06_{268}$ previously identified as being closely linked to *BMR* by Milla et al. (2005). For Race 0 black shank resistance, plants were genotyped with RAPD marker $OPZ5_{770}$ previously identified as being closely linked to *Ph* on an introgressed *N. plumbaginifolia* chromosome segment (Johnson et al. 2002a). For root knot nematode resistance conferred by *Rk*, plants were genotyped with RAPD marker $UBC482_{700}$ previously identified as being closely linked to *Rk* by Yi et al. (1998). Chi-square tests for independence using 2 × 2 contingency tables were used to test the hypothesis of independent segregation between the *35S*:*FT* transgene insertions and each of the four DNA markers.

Testing for epigenetic effects

Epigenetic effects refer to modifications to genes other than changes in the DNA sequence, and may include DNA methylation and histone acetylation (Zhang 2008). The possibility that *35S*:*FT* may cause undesirable epigenetic effects on flowering time in progeny after it is segregated away was investigated. BC_1F_1 plants corresponding to each of the two selected R_0 transformants were used. The two BC_1F_1 groups were developed by crossing early-flowering TN 90LC/TN 90LC *35S*:*FT* R_0 F_1 individuals with untransformed TN 90LC. Non-early-flowering segregants (18 and 13 individuals for progeny derived from R_0 transformants P54a and P211a, respectively), were transplanted to a field environment along with 28 non-transformed TN90LC individuals. The three sets of plants were evaluated and compared for days-to-flower and plant height (cm to the height of the lowest floral branch at the time of flowering). PROC TTEST of SAS (SAS Institute, Cary, NC) was used to perform simple *t* tests to test for significant differences between TN 90LC and transgenic-derived individuals.

Results

Plant transformation and initial progeny testing

A total of 22 independent TN 90LC R_0 transformants exhibiting a very early-flowering phenotype were regenerated from tissue culture. These plants typically flowered at the two- or three-leaf stage (Fig. 2). The cultivar TN 90LC typically requires approximately 145 days to flower in normal greenhouse environments and produces more than 25 leaves. In order to estimate the number of active *35S*:*FT* transgene insertions that were present in these R_0 transformants, each was crossed with untransformed TN 90LC to produce 22 F_1 progeny sets that were examined for segregation of the early-flowering phenotype. Three primary transformants exhibited segregation ratios of early-flowering: normal-flowering plants for which Chi-square suggested the presence of a single *35S*:*FT* transgene insertion (Table 1). The remainder exhibited segregation ratios suggesting the presence of two or more segregating *35S*:*FT* transgene insertions. Throughout experiments

Fig. 2 Untransformed burley tobacco cultivar TN 90LC (*left*) and TN 90LC possessing *35S*:*FT* transgene (*right*). The former flowers in approximately 145 days, while the transgenic plant flowered in 40 days

Table 1 Segregation for early-flowering phenotype in progeny derived from crossing TN 90LC R_0 transformants with non-transformed TN 90LC

R_0 transformant	# plants early-flowering	# plants non-early-flowering	χ^2 ($P > \chi^2$)	
			Single segregating *FT* insertion	Two segregating *FT* insertions
TN 90LC *35S*:*FT* P21a	18	0	18.00 (<0.0001)[a]	6.00 (0.0143)
TN 90LC *35S*:*FT* P181a	14	4	5.56 (0.0184)	0.07 (0.7855)
TN 90LC *35S*:*FT* P22a	16	2	10.89 (0.0010)	1.85 (0.1736)
TN 90LC *35S*:*FT* P115a	15	3	8.00 (0.0047)	0.67 (0.4142)
TN 90LC *35S*:*FT* P31a	35	1	32.11 (<0.0001)	9.48 (0.0021)
TN 90LC *35S*:*FT* P171a	18	0	18.00 (0.0001)	6.00 (0.0143)
TN 90LC *35S*:*FT* P222a	39	15	10.67 (0.0011)	0.22 (0.6374)
TN 90LC *35S*:*FT* P146a	14	4	5.56 (0.0184)	0.07 (0.7855)
TN 90LC *35S*:*FT* P112a	0	18	18.00 (<0.0001)	54.00 (<0.0001)
TN 90LC *35S*:*FT* P182a	14	4	5.56 (0.0184)	0.07 (0.7855)
TN 90LC *35S*:*FT* P161a	14	4	5.56 (0.0184)	0.07 (0.7855)
TN 90LC *35S*:*FT* P192a	10	26	7.11 (0.0077)	42.82 (<0.0001)
TN 90LC *35S*:*FT* P193a	13	5	3.56 (0.0593)	0.07 (0.7855)
TN 90LC *35S*:*FT* P203a	16	2	10.89 (0.0010)	1.85 (0.1736)
TN 90LC *35S*:*FT* P53a	0	18	18.00 (<0.0001)	54.00 (<0.0001)
TN 90LC *35S*:*FT* P191a	15	3	8.00 (0.0047)	0.67 (0.4142)
TN 90LC *35S*:*FT* P91a	0	18	18.00 (<0.0001)	54.00 (<0.0001)
TN 90LC *35S*:*FT* P211a[b]	31	23	1.19 (0.2763)	8.91 (0.0028)
TN 90LC *35S*:*FT* P54a[b]	28	26	0.07 (0.7855)	15.43 (<0.0001)
TN 90LC *35S*:*FT* P223a	13	5	3.56 (0.0593)	0.07 (0.7855)
TN 90LC *35S*:*FT* P221a	12	6	2.00 (0.1573)	0.67 (0.4142)
TN 90LC *35S*:*FT* P113a	31	23	1.19 (0.2763)	8.91 (0.0028)

[a] Number in parenthesis indicates probability of observing the data under the given model

[b] Progeny derived from this R_0 transformant were selected for further study

described here, early-flowering plants were easily distinguishable from normal-flowering plants prior to actual flower development because of their characteristic shorter leaf trichomes leading to smoother leaf surfaces, and because the last emerging leaves exhibited more of an upright leaf habit.

Progeny corresponding to R_0 transformants P211a and P54a were selected for further characterization based on Chi-square based likelihoods of single segregating *35S:FT* loci ($P > 0.05$). Early-flowering F_1 plants corresponding to these two R_0 transformants were backcrossed to non-transformed TN 90LC to produce two groups of BC_1F_1 progeny. Sixty-one and 63 plants from the two groups, respectively, were evaluated for flowering time and also genotyped for the presence/absence of *FT*, the CaMV *35S* promoter, and the NOS terminator region. Complete co-segregation was observed between the early-flowering phenotype and the expected 219 bp *FT* PCR product in both progeny groups. No PCR product was observed for *FT*, the CaMV *35S* promoter, or the NOS terminator region in any of the normal-flowering types. The average days-to-flower (days to first anther dehiscence) for *35S*:*FT* plants from BC_1F_1 progeny groups corresponding to R_0 transformants P54a and P211a were 41.1 and 41.8 days, respectively. In contrast, the average days-to-flower for non-*35S*:*FT* plants were 131.4 and 135.2 days for the two groups, respectively.

Effect of constitutive *FT* overexpression in diverse genetic backgrounds

Early-flowering TN 90LC F_1 progeny corresponding to the two selected R_0 transformants were crossed with six diverse tobacco types to evaluate the effect of *35S*:*FT* in diverse genetic backgrounds. No obvious differences were observed for the effect of *35S*:*FT* among the different crosses. Average days-to-flower for early-flowering progeny within each group ranged from 36.1 to 40.3 days (Table 2). Averaged over all crosses, early-flowering progeny exhibited an average days-to-flower of 38.8 days. Over all early-flowering plants, the range in days-to-flower was 34 to 45 days. Average days-to-flower for normal-flowering progeny

Table 2 Average days to flower (first anther dehiscence) for progeny from crosses between transgenic TN 90LC *35S*:*FT* plants and six diverse lines

Progeny set	Days-to-flower for early-flowering segregants		Days-to-flower for normal-flowering segregants	
	Average[a]	Range	Average[a]	Range
Speight 168/TN 90LC *35S*:*FT* P54a	38.9 (29)	37–41	112.0 (6)	103–114
Speight 168/TN 90LC *35S*:*FT* P211a	36.5 (24)	35–39	111.6 (10)	102–123
NC 2326/TN 90LC *35S*:*FT* P54a	37.2 (25)	34–40	113.0 (9)	104–129
NC 2326/TN 90LC *35S*:*FT* P211a	39.7 (33)	37–44	109.7 (3)	96–118
TN 86/TN 90LC *35S*:*FT* P54a	39.7 (17)	38–41	138.0 (5)	130–146
TN 86/TN 90LC *35S*:*FT* P211a	40.3 (28)	39–43	111.1 (8)	102–125
Connecticutt Shade 8212/TN 90LC *35S*:*FT* P54a	40.0 (27)	39–45	105.4 (9)	101–111
Connecticutt Shade 8212/TN 90LC *35S*:*FT* P211a	39.6 (27)	39–41	112.0 (9)	101–137
Xanthi/TN 90LC *35S*:*FT* P54a	39.5 (30)	37–42	96.0 (7)	93–98
Xanthi/TN 90LC *35S*:*FT* P211a	39.7 (31)	37–43	109.2 (5)	98–132
Petite Havana/TN 90LC *35S*:*FT* P54a	36.1 (25)	34–38	86.7 (11)	85–90
Petite Havana/TN 90LC *35S*:*FT* P211a	37.4 (28)	35–43	91.8 (8)	88–95

[a] Number in parenthesis indicates number of plants for which data were averaged

groups ranged from 86.7 days for progeny involving crosses with Petite Havana, to 138.0 days for progeny derived from crosses involving burley tobacco cultivar TN 86 (Table 2). Data for non-*35S*:*FT* plants were consistent with their pedigrees, where plants derived from crosses involving early-flowering lines such as Petite Havana flowered earliest, and pedigrees involving the later-flowering tobacco cultivar TN 86 generally flowered the latest. Advantage in terms of *35S*:*FT*-based reduction in days to flower was greatest for crosses involving burley tobacco cultivar TN86 (81.4 days and 67.0% reduction averaged over both transgenic events), and least for crosses involving the early-flowering genetic stock Petite Havana (52.1 days and 58.6% reduction averaged over both events).

Independent segregation between *FT* and major disease resistance genes

Transgene insertions in materials derived from primary transformants P211a and P54a (Table 3) were evaluated for linkage to each of four separate disease resistance genes. Presence of disease resistance genes was determined via genic (the case for TMV resistance gene *N*) or predicted via closely linked DNA markers (the case for blue mold resistance QTL *BMR*, the black shank resistance gene *Ph*, and the root knot nematode resistance gene *Rk*). Chi-square tests for independence suggested that neither of the *35S*:*FT* transgene insertions were linked to DNA markers for *Ph* or *Rk* (Table 3). The test suggested that the *35S*:*FT* transgene insertion event derived from R_0 transformant P54a was inserted in the same chromosome as *N* ($P = 0.024$), and that the transgene insertion event derived from R_0 transformant P211a was inserted in the chromosome carrying *BMR* ($P = 0.043$). Linkages were not to the extent that recombination between the two genes could not easily be identified, however (Table 3). Complete cosegregation was also observed between the early-flowering phenotype and the presence of the expected *FT* PCR product in each group. No *FT* PCR products were observed in normal-flowering individuals.

Testing for epigenetic effects

The possibility that *35S*:*FT* may cause unintended epigenetic effects on flowering time or plant height in progeny after the transgene is segregated away was investigated. Field-grown normal-flowering TN 90LC/TN 90LC *35S*:*FT*// TN 90LC BC_1F_1 individuals (see above) were compared to untransformed field-grown TN 90LC individuals. If the presence of *35S*:*FT* has a tendency to cause alterations in expression of genes affecting flowering time once it is segregated away, we would have expected to see differences in flowering time between the untransformed TN 90LC group and the transgenic-derived group. No significant differences were observed, however, between TN 90LC and normal-flowering segregants derived from either R_0 transformant for either flowering time or plant height ($P > 0.05$).

Discussion

The influence of constitutive overexpression of *FT* in tobacco has been published previously, with emphasis on understanding the role of this gene on the physiology of

Table 3 Tests for independent segregation between *35S:FT* transgene insertions and four disease resistance genes

35S:FT Insertion			Observed *FT* + Dis. resistant	Observed *FT* + Dis. susceptible	Observed non-*FT* + Dis. resistant	Observed non-*FT* + Dis. susceptible	χ^2	*P* value
Event	Disease resistance gene	Progeny set pedigree						
P54a	*Tobacco mosaic virus* resistance gene *N*	TN 90LC *35S:FT* P54a (*NN*)/Speight 168 (*nn*)//Speight 168 (*nn*)	9	13	19	7	5.073	0.024
P211a	*Tobacco mosaic virus* resistance gene *N*	TN 90LC *35S:FT* P211a (*NN*)/Speight 168 (*nn*)//Speight 168 (*nn*)	11	12	14	11	0.321	0.571
P54a	Blue mold resistance QTL *BMR*	TN 90LC *35S:FT* P54a (*bmrbmr*)/NC2000 (*BMRBMR*)//TN 90LC (*bmrbmr*)	11	13	12	12	0.084	0.773
P211a	Blue mold resistance QTL *BMR*	TN 90LC *35S:FT* P211a (*bmrbmr*)/NC2000 (*BMRBMR*)//TN 90LC (*bmrbmr*)	9	15	16	8	4.090	0.043
P54a	Race 0 black shank resistance gene *Ph*	TN 90LC *35S:FT* P54a (*phph*)/Speight 168 (*PhPh*)//TN 90LC (*phph*)	9	13	11	15	0.010	0.922
P211a	Race 0 black shank resistance gene *Ph*	TN 90LC *35S:FT* P211a (*phph*)/Speight 168 (*PhPh*)//TN 90LC (*phph*)	11	12	9	15	0.512	0.474
P54a	Root knot nematode resistance gene *Rk*	TN 90LC *35S:FT* P54a (*rkrk*)/Speight 168 (*RkRk*)//TN 90LC (*rkrk*)	13	9	13	12	0.238	0.626
P211a	Root knot nematode resistance gene *Rk*	TN 90LC *35S:FT* P211a (*rkrk*)/Speight 168 (*RkRk*)//TN 90LC (*rkrk*)	15	8	13	11	0.596	0.440

Table 4 Estimated number of days to complete backcross trait conversions (generation of BC_xF_3 seed) for *x* backcrosses and six different hypothetical genetic backgrounds with different generation times

Genetic background generation time	# backcrosses							
	2	3	4	5	6	7	8	9
70	310[a]	380	450	520	590	660	730	800
	307[b]	376	445	514	583	652	721	790
	(1.0)[c]	(1.1)	(1.1)	(1.2)	(1.2)	(1.2)	(1.2)	(1.3)
100	430	530	630	730	830	930	1,030	1,130
	337	406	475	544	613	682	751	820
	(21.6)	(23.4)	(24.6)	(25.5)	(26.1)	(26.7)	(27.1)	(27.4)
130	550	680	810	940	1,070	1,200	1,330	1,460
	367	436	505	574	643	712	781	850
	(33.3)	(35.9)	(37.7)	(38.9)	(39.9)	(40.7)	(41.3)	(41.8)
160	670	830	990	1,150	1,310	1,470	1,630	1,790
	397	466	535	604	673	742	811	880
	(40.7)	(43.9)	(46.0)	(47.5)	(48.6)	(49.5)	(50.2)	(50.8)
190	790	980	1,170	1,360	1,550	1,740	1,930	2,120
	427	496	565	634	703	772	841	910
	(45.9)	(49.4)	(51.7)	(53.4)	(54.6)	(55.6)	(56.4)	(57.1)
220	910	1,130	1,350	1,570	1,790	2,010	2,230	2,450
	457	526	595	664	733	802	871	940
	(49.8)	(53.5)	(55.9)	(57.7)	(59.1)	(60.1)	(60.9)	(61.6)

Data are presented for (1) days required to complete trait conversion using conventional system, (2) days required to complete conversion using *FT*-based system, and (3) percent reduction in time required using *FT*-based system. Calculations are based on the assumption that the trait is controlled by a single dominant gene and that phenotyping or genotyping can be performed on vegetative tissues prior to flowering

[a] The first number indicates the number of days required to complete trait conversion using the conventional backcrossing system

[b] The second number indicates the number of days required to complete trait conversion utilizing the *FT*-based backcrossing system

[c] Number in parenthesis indicates percent reduction in days required using the *FT*-based system

flowering in plants (Lifschitz et al. 2006). In the current study, we have demonstrated that *35S*:*FT* transgene expression has a dramatic effect to stimulate early-flowering approximately 39 days after seeding in a diverse array of tobacco genetic backgrounds. Normal tobacco cultivars generally require 120–170 days to flower in field or greenhouse situations. Tobacco seed capsules typically require 21–30 days to mature following pollination (Wernsman and Matzinger 1980), although seeds can be germinated as soon as 14 days after pollination (Gwynn 1973). The *35S*:*FT* system can thus be used to reduce generation time to 55–69 days.

The substantial reduction in generation time conferred by *35S*:*FT* transgene expression points to the gene's possible utility in a modified backcross system that could dramatically lessen the time required to complete backcross trait conversions for some crop species. In the current investigation, we have demonstrated independent segregation between two *35S*:*FT* transgene insertions and several major disease resistance genes frequently deployed in tobacco cultivar development. In addition, no remnant foreign *FT*, CaMV *35S* promoter, or NOS terminator sequences were detected in normal-flowering segregants derived from the selected R_0 transformants using the PCR methods utilized here. Furthermore, we observed no evidence of undesirable epigenetic effects on flowering time after the transgene is segregated away. These are three desired features of a modified backcrossing system designed to take advantage of *35S*:*FT*-induced early-flowering. We therefore propose a breeding scheme in which the breeder would initially hybridize a gene donor carrying *35S*:*FT* and the trait(s) of interest with a desired recipient genotype. The breeder would then select for *35S*:*FT* and the trait(s) of interest at each backcross generation except for the final backcross generation. At the final backcross generation, the breeder would select for the trait(s) of interest, but against the presence of *35S*:*FT*, to generate the backcross-derived trait conversion. For a dominant gene of interest in tobacco, we estimate that use of this system would reduce the time required to complete five backcrosses and generate BC_5F_3 seed from 1,150 to 604 days (47.5% reduction), assuming a genetic background that typically exhibits a generation time of 160 days (Fig. 1). This represents a year and one-half reduction in time required for commercial release of a new cultivar. The method could also be of great value for reducing the time required to transfer the nuclear genome of an elite line to a cytoplasm contributing to male-sterility. The number of backcross generations used in cultivar development programs may differ and depend upon a number of factors including breeder preference, the genetic distance between the recurrent and nonrecurrent parents, and preliminary phenotypic or genotypic selections that may occur in early backcross generations. The advantage of the *FT*-assisted backcross method would increase as the average generation time increases (genetic background effect) and as the number of intended backcrosses increases (Table 4). An additional advantage may include a reduction in space required for backcross breeding efforts. For tobacco, the space requirements for *35S*:*FT* plants are small and pollinations can be made on numerous plants maintained in laboratory growth rooms.

Generation time in some plant species can be reliably reduced through modification of environmental conditions such as light and/or temperature. For other species, however, environmentally induced premature flowering may be erratic or not applicable. The *FT*-based system proposed here may have utility for species besides *N. tabacum* where shorter generation times are desired for plant breeding procedures such as backcrossing. Advantages would be greatest for species with long generation times or species where photoperiod sensitivity or vernalization requirements may be complicating factors. *FT* orthologues have been demonstrated to function to cause early-flowering in other plant species (Lifschitz et al. 2006; Bohlenius et al. 2006; Hsu et al. 2006; Lin et al. 2007; Tamaki et al. 2007), and these gene products may play a role in regulation of flowering time in many angiosperm species. The system could be especially valuable for tree species where many years are sometimes required for initial floral structure formation (Bohlenius et al. 2006; Hsu et al. 2006).

References

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator *FT* at the shoot apex. Science 309:1052–1056

Allard RW (1960) Principles of plant breeding. John Wiley and Sons, Inc., New York

An G, Watson BD, Cheng CC (1986) Transformation of tobacco, tomato, potato, and *Arabidopsis thaliana* using a binary Ti vector system. Plant Physiol 81:301–305

Bohlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O (2006) *CO*/*FT* regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 312:1040–1043

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle L, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316:1030–1033

El-Din El-Assal S, Alonso-Blanco C, Peeters AJ, Raz V, Koornneef M (2001) A QTL for flowering time in *Arabidopsis* reveals a novel allele of *CRY2*. Nat Genet 29:435–440

Fehr WR (1987) Principles of cultivar development. Vol. 1—theory and technique. Macmillian Publishing Company, New York, NY

Gwynn GR (1973) Effect of maturity on germination of seed of six tobacco cultivars. Tob Sci 17:108–109

Hsu C-Y, Liu Y, Luthe DS, Yuceer C (2006) Poplar *FT2* shortens the juvenile phase and promotes seasonal flowering. Plant Cell 18:1846–1861

Jaeger KE, Graf A, Wigge PA (2006) The control of flowering in time and space. J Exp Bot 57:3415–3418

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Johnson ES, Wolff MF, Wernsman EA (2002a) Marker-assisted selection for resistance to black shank disease in tobacco. Plant Dis 86:1303–1309

Johnson ES, Wolff MF, Wernsman EA, Atchley WR, Shew HD (2002b) Origin of the black shank resistance gene, *Ph*, in tobacco cultivar Coker 371-Gold. Plant Dis 86:1080–1084

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer *FT*. Science 268:1962–1965

Kasperbauer MJ (1966) Interaction of photoperiod and temperature on flowering of burley tobacco. Tob Sci 10:119–120

Kasperbauer MJ (1969) Photo- and thermo-control of pretransplant floral induction in burley tobacco. Agron J 61:898–902

Kasperbauer MJ, Lowe RH (1966) Flowering of three types of *Nicotiana tabacum* under controlled-environments. Tob Sci 10:107–108

Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W (1998) Genetic control of flowering time in *Arabidopsis*. Ann Rev Plant Physiol Plant Mol Biol 49:345–370

Lang A, Chailakhyan MKh, Frolova IA (1977) Promotion and inhibition of flower formation in a dayneutral plant in grafts with short-day and a long-day plant. Proc Natl Acad Sci USA 74:2412–2416

Lewis RS, Milla SR, Levin JS (2005) Molecular and genetic characterization of *Nicotiana glutinosa* L. chromosome segments in tobacco mosaic virus-resistant tobacco accessions. Crop Sci 45:2355–2362

Lifschitz E, Eviatar T, Rozman A, Shalit A, Goldshmidt A, Amsellem Z, Alvarez JP, Eshed Y (2006) The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc Natl Acad Sci USA 103:6398–6403

Lifshitz E, Eshed Y (2006) Universal florigenic signals triggered by *FT* homologues regulate growth and flowering cycles in perennial day-neutral tomato. J Exp Bot 57:3405–3414

Lin M-K, Belanger H, Lee Y-J, Varkonyi-Gasic E, Taoka K-I, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ (2007) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell 19:1488–1506

Milla SR, Levin JS, Lewis RS, Rufty RC (2005) RAPD and SCAR markers linked to an introgressed gene conditioning resistance to *Peronospora tabacina* D.B. Adam in tobacco. Crop Sci 45:2346–2354

Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: Interacting pathways as a basis for diversity. Plant Cell 14(Suppl):S111–S130

Stam P, Zeven C (1981) The theoretical proportion of the donor genome in near-isogenic lines of self-fertilizers bred by back-crossing. Euphytica 30:227–238

Steel RG, Torrie JH, Dickey DA (1997) Principles and procedures of statistics. A biometrical approach. McGraw-Hill, NewYork

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator Ppd-H1 provided adaptation to photoperiod in barley. Science 310:1031–1034

Wernsman EA, Matzinger DF (1980) Tobacco. In: Fehr W, Hadley H (eds) Hybridization of crop plants. American Society of Agronomy and Crop Science Society of America, Madison, pp 657–668

Wigge PA, Kim M, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in *Arabidopsis*. Science 310:1056–1059

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dobcovsky J (2004) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1643

Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene CONSTANS. Plant Cell 12:2473–2482

Yi Y-H, Rufty RC, Wernsman EA (1998) Mapping the root-knot nematode resistance gene (*Rk*) in tobacco with RAPD markers. Plant Dis 82:1319–1322

Zhang X (2008) The epigenetic landscape of plants. Science 320:489–492