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Retroelement insertions at the Medicago *FTa1* locus in *spring* mutants eliminate vernalisation but not long-day requirements for early flowering

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SUMMARY

Molecular-genetic control of the flowering time of temperate-climate plants is best understood in Arabidopsis and the cereals wheat and barley. However, key regulators such as *FLC* and cereal *VRN2* are not found in legumes. Therefore, we used forward genetics to identify flowering time genes in the model legume *Medicago truncatula* (Medicago) which is induced to flower by vernalisation and long-day photoperiods. A screen of a *Tnt1* retroelement tagging population yielded two mutants, *spring2* and *spring3*, with a dominant early flowering phenotype. These mutants overexpress the floral activator *FTa1* and two candidate downstream flowering genes *SOC1a* and *FULb*, similar to the *spring1* somaclonal variant that we identified previously. We demonstrate here that an increase in the expression of *FTa1*, *SOC1a* and *FULb* and early flowering does not occur in all conditions in the *spring* mutants. It depends on long-day photoperiods but not on vernalisation. Isolation of flanking sequence tags and linkage analysis identified retroelement insertions at *FTa1* that co-segregated with the early flowering phenotype in all three *spring* mutants. These were *Tnt1* insertions in the *FTa1* third intron (*spring3*) or the 3′ intergenic region (*spring2*) and an endogenous *MERE1-4* retroelement in the 3′ intergenic region in *spring1*. Thus the *spring* mutants form an allelic series of gain-of-function mutations in *FTa1* which confer a spring growth habit. The *spring* retroelement insertions at *FTa1* separate long-day input from vernalisation input into *FTa1* regulation, but this is not due to large-scale changes in *FTa1* DNA methylation or transcript processing in the mutants.

Keywords: *Medicago truncatula*, *spring*, *FTa1*, *SOC1a*, *FULb*, retroelement, early flowering, *Tnt1*, photoperiod, vernalisation.

INTRODUCTION

Flowering time is a key adaptive trait in plants because coincidence of flowering with optimal conditions is important for robust seed and fruit development (Putterill *et al.*, 2004; Andres and Coupland, 2012). *FLOWERING LOCUS T* (*FT*) orthologs encode a crucial flowering trigger in many plants which has been shown to function as a mobile florigen (Turck *et al.*, 2008; Turnbull, 2011). Multiple environmental and internal cues regulate *FT* transcription (Srikanth and Schmid, 2011). In many temperate-climate plants, *FT* expression is increased in plants exposed to extended winter cold (vernalisation) followed by the warm, long days of spring (Trevaskis *et al.*, 2007; Pin *et al.*, 2010; Laurie *et al.*, 2011; Andres and Coupland, 2012). This ensures that they overwinter vegetatively and flower in milder spring conditions (Michaels *et al.*, 2005; Kim *et al.*, 2009).

Despite important commonalities such as *FT*, some of the upstream flowering-time regulatory cascades such as vernalisation pathways appear to be less conserved (Amasino, 2004; Andres and Coupland, 2012). Vernalisation stably represses expression of *Arabidopsis thaliana* (Arabidopsis) *FLOWERING LOCUS C* (*FLC*) (Kim *et al.*, 2009). This relieves FLC inhibition of *FT* and the floral integrator gene *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Michaels *et al.*, 2005). The long-day activator of flowering CONSTANS (CO) is then able to directly upregulate *FT* as

the days lengthen in spring (Andres and Coupland, 2012). The general theme of inhibition of a repressor directly or indirectly by vernalisation is maintained in plants such as the eudicot sugar beet and the cereals wheat and barley (Trevaskis *et al.*, 2007; Pin *et al.*, 2010; Andres and Coupland, 2012). However, the underlying molecular mechanisms differ. In wheat, vernalisation upregulates the *FRUITFULL-like* gene *VERNALISATION1* (*VRN1*) which then represses expression of the repressor *VRN2*, a grass-specific CCT-like gene. This relieves repression of the wheat *FT* orthologue *VRN3* which in turn further activates *VRN1* and floral development (Trevaskis *et al.*, 2007; Chen and Dubcovsky, 2012). Interestingly, in sugar beet a vernalisation-repressed *FT* gene (*BvFT1*) encodes a flowering time repressor that inhibits a *FT* floral activator (*BvFT2*) (Pin *et al.*, 2010).

The differences between vernalisation pathways raise the interesting and important question of how flowering time is controlled in other plant groups, but much less is known about this regulation (Kim *et al.*, 2009; Putterill *et al.*, 2013). We were drawn to investigating control of flowering time in the legume model *Medicago truncatula* (Medicago) as it has a number of attractive advantages. Flowering in Medicago is regulated by vernalisation and long day length, it has diploid in-breeding genetics, a moderate-sized sequenced genome of about 550 Mb and large mutant populations (Clarkson and Russell, 1975; Benlloch *et al.*, 2006; Rose, 2008; Young *et al.*, 2011). The latter include about 19 000 lines each carrying around 25 stably inserted copies of the tobacco *Tnt1* retroelement available for reverse and forward genetic mutant screens (Tadege *et al.*, 2008, 2009). In addition, numerous candidate flowering time genes have been mined out of the Medicago genome by their predicted amino acid sequence similarity with regulators from Arabidopsis (Hecht *et al.*, 2005; Yeoh *et al.*, 2011; Jung *et al.*, 2012).

Use of the Medicago genomic and mutant resources resulted in the first functional characterisation of an important Medicago flowering time gene, a *FT* orthologue called *FTa1* (Laurie *et al.*, 2011). *FTa1* is one of five *FT* genes identified in the Medicago genome sequence (Hecht *et al.*, 2005; Laurie *et al.*, 2011; Yeoh *et al.*, 2011). However, it is the only one whose expression is upregulated by the combination of the floral cues of vernalisation followed by long-day photoperiods (LD) (Laurie *et al.*, 2011). Two null *fta1 Tnt1* insertion mutants were identified by reverse genetics. These plants are late flowering after exposure to vernalisation and long day lengths. *FTa1* overexpression from the CaMV 35S promoter strongly promoted flowering in transgenic Medicago and Arabidopsis (Laurie *et al.*, 2011; Yeoh *et al.*, 2011). Taken together, these results confirm a significant function for *FTa1* in the control of flowering time in Medicago.

However, the molecular mechanisms by which the environmental cues of LD and vernalisation upregulate *FTa1* are not yet known. These pathways are likely to differ from those regulating Arabidopsis *FT* (Putterill *et al.*, 2013). For example, LD induction in Medicago results in expression of *FTa1* transcript throughout the day–night cycle. This is unlike *FT* which has a diurnal cycle with a peak in the late afternoon (Laurie *et al.*, 2011; Andres and Coupland, 2012). The role of *CO-like* genes in the LD response in Medicago is currently unclear (Putterill *et al.*, 2013). The FLC/MAF clade is not found in legumes and no repressors of flowering have been functionally identified to date (Putterill *et al.*, 2013). None of the Medicago *FT* genes are repressed by vernalisation, indicating that they do not appear to function as vernalisation-repressed inhibitors of flowering as observed in sugar beet (Pin *et al.*, 2010; Laurie *et al.*, 2011).

We have been taking a forward genetic approach to gain a better understanding of the control of flowering time in Medicago. Recently, we described fine mapping of the *spring1* early flowering Medicago mutant with elevated *FTa1* expression (Yeoh *et al.*, 2013). Its dominant phenotype suggested a gain-of-function mutation in an activator of flowering. The *FTa1* gene was the only gene in the *spring1* mapping interval that was mis-expressed in the *spring1* mutant. The greater abundance of *FTa1* transcript in both heterozygous and homozygous *spring1* plants was consistent with the dominant early flowering phenotype. However, DNA sequencing showed that *spring1* did not correspond to a mutation in the *FTa1* promoter or genomic sequence. Our microarray analysis also identified two candidate floral activators, *SOC1a* and *FULb,* whose expression was upregulated in *spring1* (Yeoh *et al.*, 2013). *SOC1a* and *FULb* transcripts were also elevated in transgenic Medicago plants overexpressing *FTa1*, suggesting that they were likely to be elevated in the *spring1* mutant as a consequence of the increased *FTa1* levels (Teper-Bamnolker and Samach, 2005; Yeoh *et al.*, 2013).

Here we report that we have identified two additional *spring* mutants, *spring*2 and *spring3*, with similar phenotypes to *spring1* as they have dominant early flowering and elevated expression of *FTa1, SOC1a* and *FULb*. We show that the flowering time of the *spring* mutants is sensitive to photoperiod regulation as they flower much more rapidly in LD than in short day photoperiods (SD), but early flowering does not require vernalisation. *FTa1, SOC1a* and *FULb* are expressed at much higher levels in *spring* mutants in LD than in SD which correlates with flowering time. Thus, the *spring* mutations separate vernalisation and long day inputs into *FTa1* regulation. We demonstrate that retroelement insertions in the third intron or in the proximal 3′ intergenic region of *FTa1* show 100% co-segregation with the dominant early flowering phenotype of the three *spring* mutants. Consistent with the conditional activation of *FTa1* expression, we did not detect increased DNA methylation at the *FTa1* locus in the *spring* mutants compared with wild-type R108 plants.

RESULTS

Identification of the *spring2* and *spring3* early flowering *Tnt1* mutants

We identified two early flowering *Tnt1* mutant lines in forward mutant screens in LD and these were named *spring2* and *spring3*. Next we tested the inheritance and dominance of *spring2* and *spring3* mutations using backcrosses to R108 wild-type plants (Figure 1a–c and Table S1 in Supporting Information). The results indicated that *spring*2 and *spring3* conferred dominant early flowering in LD. F_1 plants flowered at a similar time to the *spring* parent and much earlier than R108 when flowering was measured in days after germination to flowering or in node number at flowering (Figure 1a,b). In the F_2 generation, the plants segregated into two groups with early flowering plants similar to the *spring* parent and late-flowering plants that flowered like R108 (Table S1). The experimental hypothesis of a single dominant gene in *spring*2 was supported by an approximate segregation ratio of 3:1; 159 F_2 plants flowered early while 51 were late (χ^2 = 0.0.04; $0.5 < P < 0.9$). The experimental hypothesis of a single dominant gene in *spring3* was also supported by an approximate segregation ratio of 3:1 as 171 F_2 plants flowered early while 52 were late (χ^2= 0.22; $0.5 < P < 0.9$).

Finally, quantitative (q)RT-PCR showed that *spring*2 and *spring3* overexpress *FTa1* compared with R108 (Figure 1d). The increase in *FTa1* transcript levels in *spring3* is consistent with more severe early flowering phenotypes seen in the F_1 and F_2 plants from the backcross (Figure 1a,b, Table S1). Thus like *spring1*, *spring2* and *spring3* show early upregulation of *FTa1* expression in LD compared with R108 and a dominant early flowering phenotype.

The effect of photoperiod and vernalisation on flowering time

We measured the flowering time of the *spring* mutants and R108 plants in LD and SD conditions, with and without vernalisation (Figure 2 and Figure S1, Table S1). As expected, *spring* mutants showed accelerated flowering in LD compared with R108. *Spring3* plants were the most rapid flowering, followed closely by *spring1*, then *spring2* and finally R108. The flowering time of all of the *spring* mutants in SD was much slower. *Spring3* plants flowered like R108 in SD, while s*pring1* and *spring2* flowered a little more quickly, but were much delayed compared with LD. Therefore, the early flowering of all *spring* mutants is sensitive to the photoperiod, with *spring3* having the strictest dependence on LD for early flowering. The behaviour of the *spring* mutants contrasts with transgenic Medicago plants that express *FTa1* constitutively (*OX::FTa1*). We previously found that *OX::FTa1* plants flower very early in LD (Laurie *et al.*, 2011) and demonstrated here that they also flower very early in SD (Figure 2c,d).

Vernalisation modestly promoted flowering of the weaker *spring2* mutant in LD and all of the genotypes in SD (Figure 2a,b). The flowering of the strong *spring 1* and *spring3* mutants was not accelerated by vernalisation in LD (Figure 2a,b). This was also seen in a second experiment with *spring1* and R108 in LD, where a stronger vernalisation treatment strongly promoted the flowering of R108, but not of *spring1* (Figure S1a,b). These LD *spring1* plants flowered at a similar time and shared similar plant architecture to the strongly vernalised long-day grown (VLD) R108 plants with both types of plants having a longer primary axis and shorter side branches than LD R108 (Figure S1a–f).

Expression of flowering genes in response to photoperiod and vernalisation

Given the flowering time results, we hypothesized that *FTa1* expression in the *spring* mutants was sensitive to photoperiod. The qRT-PCR analyses indicated that indeed *FTa1* transcript was almost undetectable in the leaves of all genotypes in SD (Figure 3a). A very low level of *FTa1* transcript was detected in shoot apices in *spring1* and *spring2* (Figure 3a). In LD, *FTa1* transcript was increased in leaves and apices of the *spring* mutants compared with LD R108 (Figure 3a). The levels correlated very well with the acceleration of flowering time, with *spring1* and *spring3* having the highest expression of *FTa1* and flowering more rapidly than *spring2*.

We also examined the expression of *FULb* and *SOC1a* which are candidate downstream targets of *FTa1* as their expression was upregulated in LD *spring1* and *OX::FTa1* plants (Yeoh *et al.*, 2013). In LD, both transcripts were more abundant in leaves and shoot apices of *spring1* and *spring3* than in R108, correlating with the higher *FTa1* expression in these mutants (Figure 3b,c). There was little effect on expression of these genes in LD in leaves of the weaker *spring2* mutant, but moderate elevation in shoot apical samples compared with R108 (Figure 3b,c). In SD, *FULb* and *SOC1a* were expressed at lower levels in leaves and apices than in LD. Similar levels of both transcripts were seen in all genotypes in SD, apart from *spring1* which had a mild increase in *SOC1a* in shoot apices. Elevation of *FTa1*, *SOC1a* and *FULb* expression was also observed in R108 plants in VLD, correlating with their rapid flowering (Figure S1). In addition, our microarray analysis of LD *spring*1 and VLD R108 indicated that these genotypes generally shared very similar global patterns of expression as few differences in gene expression were detected (Table S3).

However, the relative tissue specificity of *FTa1* expression differed between LD *spring* mutants and VLD R108 plants. *FTa1* transcript levels were similar in leaves of LD *spring* plants and VLD R108 plants (Figure S1 g). *FTa1* expression was also high in shoot apices of the *spring*

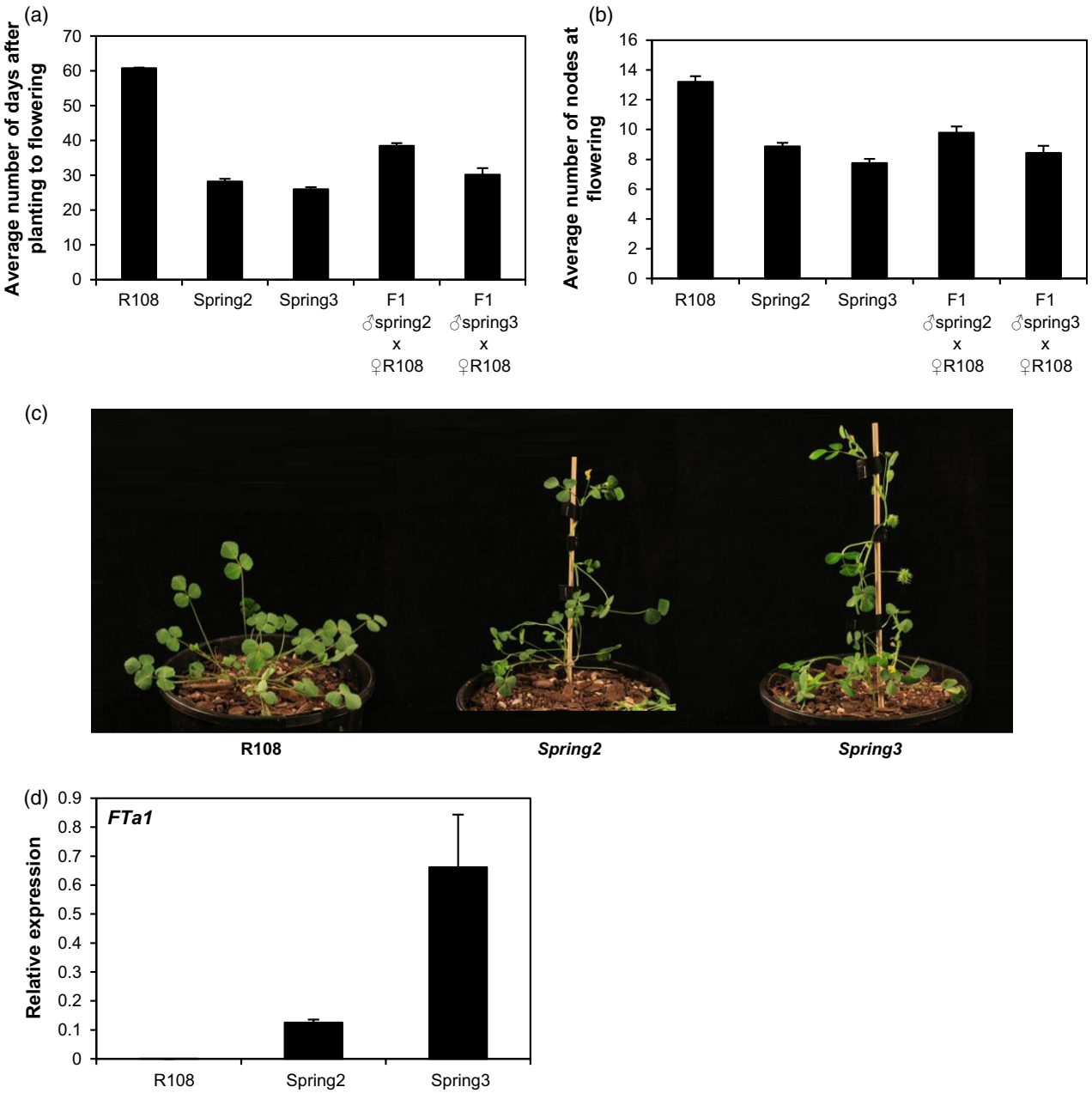

Figure 1. The *spring2* and *spring3* mutants have elevated *FTa1* expression and dominant early flowering in long day (LD) photoperiods.
The *spring2* and *spring3* mutants were backcrossed to R108 wild-type plants and grown in LD conditions.
(a,b) Graphs showing the flowering time of the *spring2* and *spring3* mutants and F_1 plants from the backcross (a) scored as the days to flowering after germination or (b) the number of nodes on the primary axis at the time of flowering. The mean ± SE is presented (n = 5–12).
(c) Photographs of 35-day-old R108, *spring2* and *spring 3* plants.
(d) The Relative expression of *FTa1* transcript in the first trifoliate leaf of 12–14-day-old seedlings was measured by quantitative RT-PCR with normalization with *PDF2*. The mean ± SE of three biological replicates is shown.

mutants (about two times lower than in leaves), but was very low in shoot apices of R108 plants (about 40 times lower than in leaves) (Figure 3a and Figure S1j). The relative tissue specificity of *FULb* and *SOC1a* did not appear to be much affected in the *spring* mutants (Figure 3b,c).

Retroelement insertions at the *FTa1* locus co-segregate with *spring* early flowering phenotypes

Using retroelement flanking sequence tags (FSTs) and linkage analysis we demonstrated that retroelement insertions in or downstream of *FTa1* in *spring1–3* showed 100% cosegregation with the early flowering phenotypes (Figure 4).

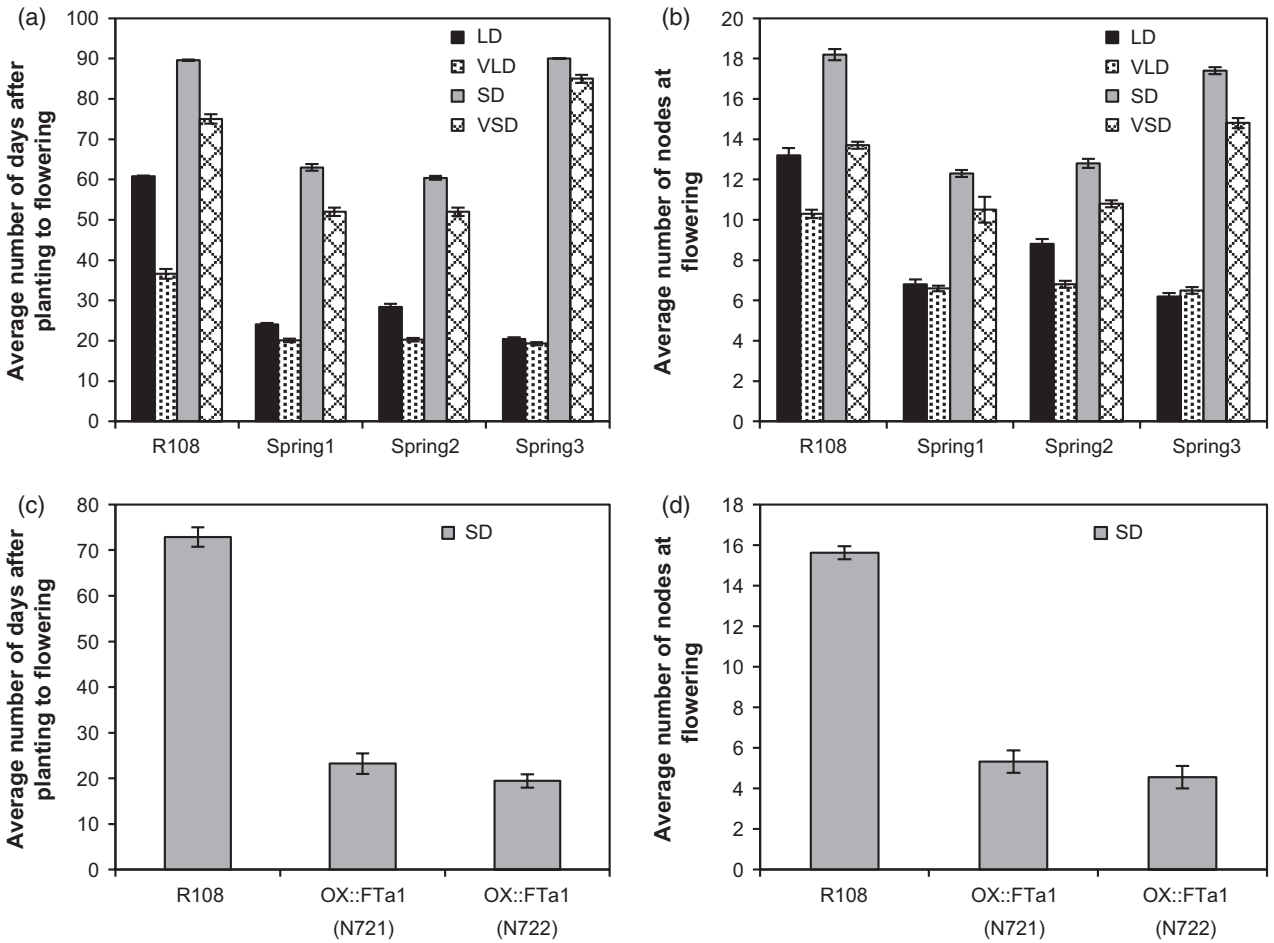

Figure 2. The *spring* mutants flower much earlier in long days (LD) than in short days (SD) and are less responsive to vernalisation than R108 in LD.
The *spring* mutants and controls were grown in LD or SD conditions either having been exposed to cold (vernalised, V) or not.
(a,b) Graphs showing the flowering time of the *spring* mutants (a) scored as the days to flowering after germination or (b) the number of nodes on the primary axis at the time of flowering. The mean ± SE is presented (n = 9–15).
(c,d) Transgenic plants that overexpressed *FTa1* (*OX::FTa1*) were grown in SD and flowering time was measured as described for (a) and (b). The mean ± SE is presented (n = 8–9).

spring1 *has a MERE1-4 retroelement insertion in the* FTa1 *3′ intergenic region*

Previously we found that the *FTa1* promoter and genomic coding sequence were identical in *spring1* mutants and R108 plants (Yeoh *et al.*, 2013). When PCR was used to amplify a 3′ intergenic fragment from R108 and *spring1* genomic DNA, the expected fragment of about 1.9 kb amplified from R108, but a much larger product of about 7.2 kb amplified from *spring1*. The DNA sequencing of both fragments identified a *MERE1-4* retroelement insertion positioned 694 bp downstream of the *FTa1* translation stop codon in *spring1*, but no other changes (Figure 4a). Linkage analysis confirmed that the *FTa1 MERE1-4* insertion showed 100% cosegregation with the *spring1* phenotype; 60 early flowering F_2 plants from a backcross with R108 had the *MERE1-4* insertion and nine late flowering plants we tested lacked it (Figure 4b) (Yeoh *et al.*, 2013). The insertion was also tightly linked to the *spring1* mutation in a second segregating population (present in 51 early flowering plants and missing from 40 late flowering plants tested).

spring2 *has a* Tnt1 *retroelement insertion in the* FTa1 *3′ intergenic region*

Using *Tnt1* transposon display, we identified 38 *Tnt1* FSTs from *spring*2 and developed PCR linkage markers for 20 of them. First, genotyping of a small group of plants segregating *spring*2 showed that two markers were linked; 3′_FST_15 and 5′_FST_05 were present in all eight early flowering progeny, but not in the four late flowering plants. Analysis of the DNA sequence of these two *spring*2-linked FSTs placed them both in the region of the *FTa1* gene. 3′_FST_15 was about 1.5 Mb from *FTa1*, while 5′_FST_05 was situated 2094 bp downstream from the *FTa1* translation stop codon. In the large F_2 population

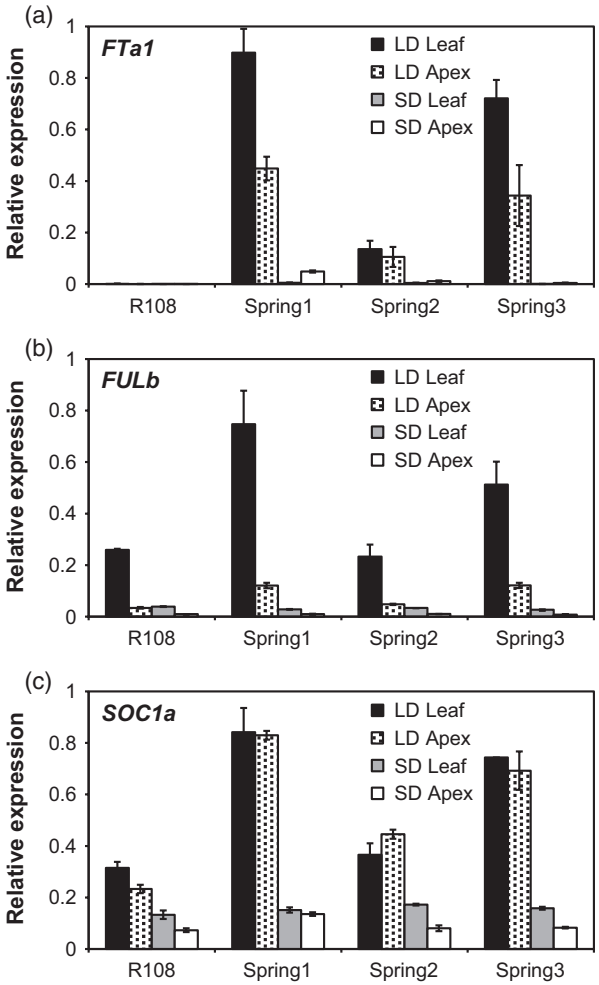

Figure 3. The *spring* mutants express *FTa1, FULb and SOC1a* at higher levels in long days (LD) than in short day (SD) conditions.
The *spring* mutants and R108 plants were grown in LD or SD conditions. Relative expression of transcripts in the first trifoliate leaf or shoot apices of 12–14-day-old seedlings of R108 and *spring* mutants was measured by quantitative RT-PCR with normalization with *PDF2*. Relative transcript abundance of *FTa1* (a), *FULb* (b) and *SOC1a* (c) is presented. The mean ± SE of three biological replicates is shown.

from the backcross of *spring2* with R108, 3′_FST_15 was separable from *spring2* by four recombination events, while 5′_FST_05 immediately downstream of *FTa1* showed 100% cosegregation with *spring2*; present in all of the 159 early flowering plants and absent in the 51 late flowering plants (Figure 4c).

spring3 *has a* Tnt1 *retroelement insertion in the third intron of* FTa1

We isolated 77 *Tnt1* FSTs using transposon display from *spring3* plants and carried out linkage analysis with 39 of them. In the first round of genotyping of 12 plants, we found one FST, 5′_FST_46, cosegregating with the *spring3* early flowering phenotype. Interestingly, DNA sequence analysis of the FST indicated that it was about 2.8 Mb from *FTa1*. However, linkage analysis in the large F_2 population from the backcross of *spring3* to R108 found that 5′_FST_46 was separated from *spring3* by four recombination events. We tested if *spring3* had an additional *Tnt1* insertion much nearer to *FTa1* using genomic PCR. This work uncovered a *Tnt1* insertion in the third and largest intron of the *FTa1* gene, 1227 bp downstream of the translation start codon (Figure 4a,c). It showed 100% cosegregation with the *spring3* phenotype as it was present in all of the 128 early flowering F_2 plants and absent from the 38 late flowering plants tested.

Genomic annotations of the approximately 2.5 kb *FTa1* region containing the three *spring* retroelement insertions did not predict any genes apart from *FTa1* itself (Yeoh *et al.*, 2013) and we found that that the region showed no significant homology to other nucleotide sequences in GenBank.

Analysis of retroelement expression, *FTa1* genomic DNA sequence and *FTa1* transcript processing in the *spring* mutants

Retroelement insertions can have a wide range of effects on gene expression, ranging between the extremes of repression to activation of transcription, conferring environmental control on the expression of adjacent genes as well as affecting transcript processing, amongst other features (Studer *et al.*, 2011; Butelli *et al.*, 2012; Lisch, 2013). As *FTa1* transcript levels were conditionally elevated in LD, we asked if this correlated with expression of *Tnt1* and *MERE1* retroelements. However, qRT-PCR indicated that the *Tnt1* and *MERE1* transcripts accumulated to similar levels in LD and SD (Figure S2a,b). Expression of *Tnt1* was greatly elevated in *spring3* compared with *spring2*, but this might reflect the higher number of *Tnt1* insertions that we detected in *spring3*. Expression of *MERE1* transcripts was slightly elevated in *spring1* compared with R108, possibly due to an increase in MERE copy number in the *spring1* mutant caused by transposition during plant regeneration (Rakocevic *et al.*, 2009). Solo long terminal repeats (LTRs) of the *Angela* family of the *Ty1/copia* superfamily retrotransposons, as positioned upstream of *FTa1* (Yeoh *et al.*, 2013), were expressed at a very low level in all genotypes in LD conditions (Figure S2c). Expression of all the retroelements was detectable in leaves and shoot apex samples, with *Tnt1* expression being higher in leaves than shoot apices in *spring3*. In summary, overall we did not observe strong correlations between the expression of the retroelement transcripts and *FTa1* accumulation in the *spring* mutants.

We also confirmed by PCR amplification and sequencing that the genomic sequences from the *spring2* and *spring3* mutants were identical to R108/*spring1* in the *FTa1* region apart from the retroelement insertions (Figure S3a) (Yeoh *et al.*, 2013). In addition, using genomic PCR we could not detect other retroelement insertions in the vicinity of the

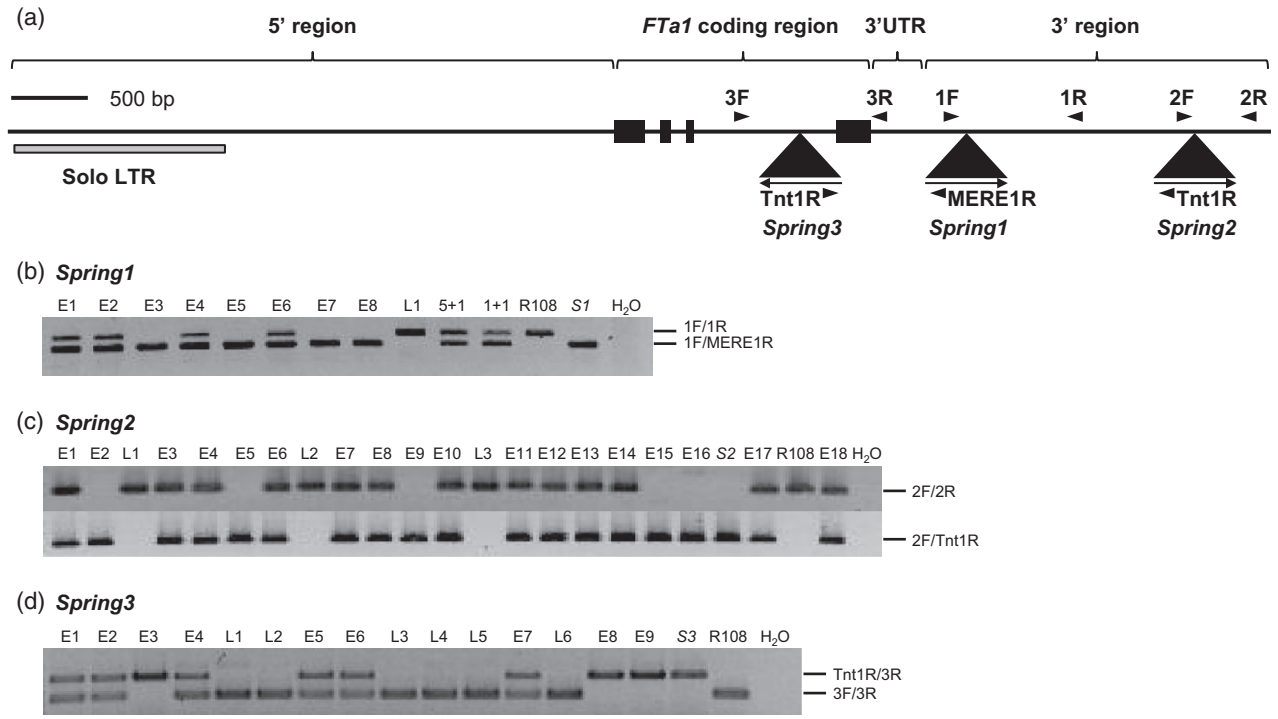

Figure 4. Retroelement insertions at the *FTa1* gene show 100% cosegregation with the early flowering phenotype of *spring* mutants.
(a) Diagram of the *FTa1* genomic region showing the position of retroelement insertions (black triangles) in the *spring* mutants. The direction of the retroelement open reading frame is shown with an arrowed line. Black boxes are *FTa1* exons. The positions of primers used for PCR genotyping are shown as black arrowheads, with gene-specific primers numbered 1 for *spring1*, 2 for *spring 2* and 3 for *spring 3*. LTR, long terminal repeat; UTR, untranslated region.
(b–d) Polymerase chain reaction (PCR) was used to amplify genomic DNA from segregating populations of *spring1* (b), *spring2* (c) or *spring3* (d) F_2 plants and controls including *spring* mutants (*S1* to *-3*) or R108 as the wild type. The plants were scored as early (E) or late flowering (L).
Representative examples of PCR fragments separated by agarose gel electrophoresis are shown. In (b) the L1 sample is a pool of genomic DNA of six independent late flowering plants. The control lanes confirm that PCR is able to amplify a detectable fragment from the *spring1* TNT1 insertion when it is present at one part in six (5 + 1) or in equal parts (1 + 1) with R108 genomic DNA.

FTa1 gene. We also carried out *FTa1* 3′ rapid amplification of cDNA ends (RACE) to compare transcript processing in the *spring* mutants and R108. All genotypes produced full-length, spliced and polyadenylated *FTa1* transcripts that encoded the predicted FTa1 protein of 176 amino acids (Figure S3b) (Laurie *et al.*, 2011). This confirmed that the *Tnt1* retroelement in the third intron was spliced out of the *FTa1* transcript in *spring3* plants.

We also detected a rare alternative splicing variant of *FTa1* amongst the R108 and *spring1* clones which is predicted to encode a protein with a small internal deletion of 14 amino acids (Figure S3b). The length of the most common 3′ untranslated region (UTR) was 349 nucleotides with some variation in all genotypes (Figure S3c). Thus overall, genomic and cDNA sequence analysis indicated that *FTa1* sequence and transcript processing were similar in the *spring* mutants and R108.

DNA methylation at the *FTa1* locus is not altered in the *spring* mutants

Retroelement insertions are often associated with silencing of a locus by a heterochromatisation process involving small interfering (si) RNAs and blocks of increased DNA methylation (Lippman *et al.*, 2004). Methylation-dependent McrBC restriction digestion and mock digestions of genomic DNA were used to analyse cytosine methylation in the *spring* mutants and R108 (Figure 5). The mock-digested DNA samples amplified to similar levels in all genotypes, indicating that there was no difference in copy number of these DNA sequences between the genotypes. Using semi-quantitative PCR higher amounts of methylation were indicated in some regions (I to III) as no fragments were detectable after PCR amplification of the digested samples (Figure 5a,b). We also used qPCR to amplify small fragments (numbered 1–8) (Figure 5a,c). In this case, the solo LTR qPCR amplifications were not specific to the solo LTR upstream of *FTa1*, and thus other copies were amplified from the genome (Yeoh *et al.*, 2013). Again, all genotypes were similar to each other. Thus the McrBC digestions revealed no differences between *FTa1* DNA methylation patterns in the *spring* mutants and R108.

Second, bisulphite sequencing of a DNA fragment specific to the solo LTR upstream of *FTa1* in *spring1* and R108 indicated that the CG and CHG residues were generally

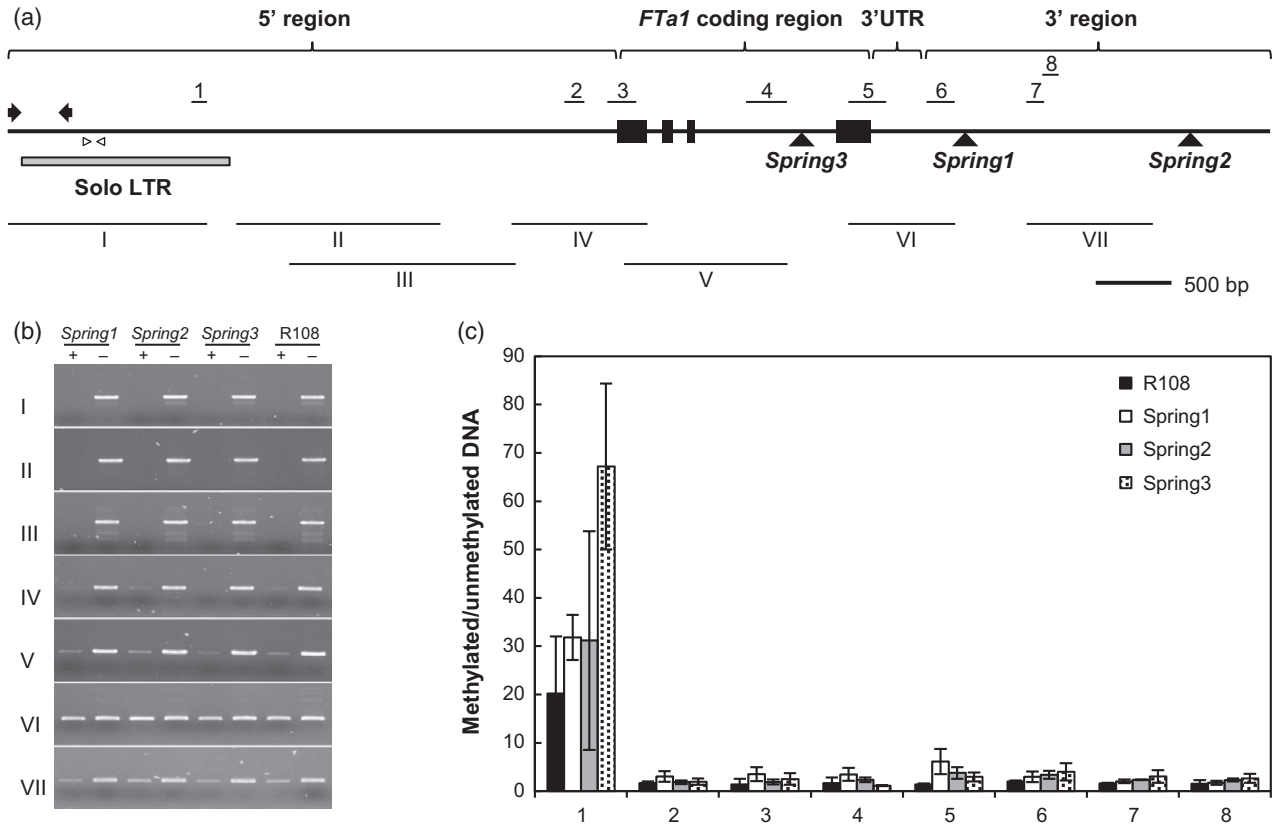

Figure 5. Analysis of methylation of DNA in the *FTa1* genomic region in the *spring* mutants in long days (LD).
(a) Diagram of the *FTa1* genomic region showing the position of retroelement insertions (black triangles) in the *spring* mutants (*spring1*, *spring2*, *spring3*). Black boxes are *FTa1* exons. Amplicons analysed by semi-quantitative or quantitative (q) PCR after McrBC digestion are indicated by fragments numbered I–VII and 1–8 respectively. LTR, long terminal repeat; UTR, untranslated region.
(b,c) Genomic DNA from leaves of 19-day-old plants in LD was digested with the methylation-dependent restriction enzyme McrBC and amplified by PCR. (b) Semi-quantitative PCR was carried out on McrBC digested (+) or mock treated (−) genomic DNA and amplicons were visualised using agarose gel electrophoresis. The PCR amplifications after 28 cycles (I, II and IV–VII) and 35 cycles (III) are shown. (c) Real-time qPCR was carried out on McrBC-digested or mock-treated genomic DNA. The bars represent the average ratio of methylated versus unmethylated DNA of two or three biological replicates ± SE.

very highly methylated (>80%) in both genotypes (Figure S4c). Methylation at the CHH residues was lower, but similar between *spring1* and R108 (Figure S4b). The abundant DNA methylation detected at the solo LTR upstream of *FTa1* was consistent with the results of the McrBC digestions. In summary, we did not detect any changes to DNA methylation at the *FTa1* locus in *spring* mutants compared with R108 plants in LD.

DISCUSSION

In this study, we showed that *spring1*, *spring2* and *spring3* appear to form an allelic series of gain-of-function mutations caused by retroelement insertions in or 3′ of the floral activator *FTa1*. The insertions lead to an increased abundance of *FTa1* transcript and dominant early flowering in LD that is independent of vernalisation. The observed upregulation of *FTa1* in the *spring* mutants is expected to be sufficient to accelerate flowering, as we showed previously that *OX::FTa1* transgenic plants flower rapidly in LD in the absence of vernalisation (Yeoh *et al.*, 2013). However, the photoperiod responsiveness of the *spring* mutants is unlike *OX::FTa1* plants which flower rapidly in LD and SD.

Spring2 and *spring3* contain *Tnt1* insertions at *FTa1*, while *spring1* has an endogenous *MERE1-4* retroelement inserted at *FTa1*. We previously predicted that *spring1* was a somaclonal variant that had arisen during regeneration in plant tissue culture (Yeoh *et al.*, 2013). The endogenous retroelement *MERE1-4* was not previously shown to transpose during tissue culture in Medicago (Rakocevic *et al.*, 2009), but this is likely to have occurred in the *spring1* mutant. The three retroelement insertions are located within about 2.5 kb of each other at the *FTa1* locus in what may be an insertional hot spot, but which is not predicted to contain any genes apart from *FTa1.* They show 100% cosegregation with the *spring* mutant phenotypes in linkage analysis. These linkage results are consistent with our independent fine mapping and microarray experiments

which implicated *FTa1* in the *spring1* phenotype (Yeoh *et al.*, 2013).

The effect of the *spring* retroelement insertions at the *FTa1* locus is to eliminate vernalisation, but not LD requirements for upregulation of *FTa1* expression and rapid flowering. Thus it is possible that the vernalisation pathway and the LD pathways regulate *FTa1* expression independently. *Spring3* plants with a *Tnt1* insertion in the third *FTa1* intron retain full responsiveness to photoperiod, indicating that this genomic region is not required for LD control of flowering time. Insertions in the 3′ intergenic region in *spring2* and *spring3* have a mild impact on photoperiod sensitivity. The s*pring2* mutant, which has the insertion most distal to *FTa1*, has the leakiest photoperiodic phenotype, i.e. it is the least early flowering in LD and has slightly accelerated early flowering in SD relative to R108. Turning to the effect of vernalisation, *spring1* and *spring3* have the strongest early flowering phenotype in LD and do not flower much earlier if vernalised. Since *spring* mutants share many phenotypic features with vernalised R108 plants in LD, it is possible that the *spring* retroelements interfere with the same regulatory processes that are affected by vernalisation. On the other hand, *spring* mutants are not perfect mimics of vernalised R108 plants as the relative abundance of *FTa1* transcript in leaves and shoot apices strongly differs between *spring* and wild-type R108 plants. This may be indicative of a more general release of negative regulatory constraints on *FTa1* in the *spring* mutants. *Spring* mutants are not defective in their ability to sense vernalisation, as all genotypes responded to vernalisation with a mild acceleration of flowering in SD.

Several repressors of *FT* are known in Arabidopsis that bind to the promoter, intron or 3′ intergenic regions (Pin and Nilsson, 2012). For example, the AP2-domain SCHLAFMUTZE (SMZ) flowering-time repressor binds about 1.5 kb downstream of the *FT* coding region (Mathieu *et al.*, 2009) and the repressor FLC represses FT by binding the first large intron of FT (Hepworth *et al.*, 2002). Although SMZ and FLC orthologs have not been identified in Medicago, transcriptional regulators of *FTa1* may bind similarly to regulatory sequences within intron or downstream sequences to repress expression of *FTa1*. Alternatively, since the *spring* retroelement insertions are near each other, but not in identical positions, they might interrupt longer-range negative regulatory chromatin interactions at *FTa1*, rather than specific repressor-binding sites (Schubert *et al.*, 2006; Jiang *et al.*, 2008; Adrian *et al.*, 2010; Farrona *et al.*, 2011). Our results so far indicate that misexpression of *FTa1* in the *spring* mutants does not involve large-scale changes in *FTa1* genomic DNA methylation in any of the genotypes. Nor does it appear to result from the effects of copy number variation of the *FTa1* gene in the *spring* mutants. *FTa1* transcript also appears to be processed as observed in R108 wild-type plants. We did discover a rare alternatively spliced *FTa1* transcript in R108 and *spring1* predicted to encode a FTa1 protein with an internal deletion. The deletion does not appear to overlap with regions shown to be critical for FT function as a floral activator, but the functional significance of this modified FTa1 protein would be worth investigating (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006; Laurie *et al.*, 2011).

The Medicago *spring* mutant phenotypes are reminiscent of spring or summer annual varieties of other temperate plants. Such plants have a spring growth habit as they germinate, grow and rapidly reproduce in spring or summer without requiring vernalisation (Kim *et al.*, 2009). In Arabidopsis, natural accessions with summer annual phenotypes usually result from mutations in flowering repressors such as *FLC* and *FRIGIDA* (*FRI*) (Kim *et al.*, 2009). For example, in contrast to the effect of the *spring FTa1* retroelements, reduction in *FLC* expression in the rapid cycling summer annual Landsberg is caused by an intronic retroelement insertion. This attracts siRNAs and repressive chromatin modifications leading to *FLC* gene silencing (Liu *et al.*, 2004). In the temperate cereals, where winter and spring varieties are important crops, mutations at several loci including *FT* have been found to underlie the spring growth habit. These include increases in gene copy number from one to four copies of the barley *FT* orthologue *HvFT1* (*VRN-H3*) that are sufficient to confer a spring lifestyle (Yan *et al.*, 2006; Nitcher *et al.*, 2013). These plants have earlier *HvFT1* expression and dominant early flowering that is vernalisation independent, but remains LD dependent. Other spring varieties, including some domesticated varieties of barley and the legumes pea and lentils, have a spring growth habit due to a different mechanism. Changes in their circadian rhythms have reduced daylength sensitivity so that they can flower in SD, an adaptation that has extended their geographic range enabling them to be successfully cropped in the short growing seasons typical of high latitudes (Faure *et al.*, 2012; Weller *et al.*, 2012).

Thus our future work will be directed at how the Medicago *spring* retroelement insertions contribute to *FTa1* regulatory diversity to give new and potentially useful flowering time variants with different responses to the environment.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and vernalisation treatments

All plants used are in the R108_C3 (R108) wild-type background; R108 is a genotype of *M. truncatula* Gaertn (barrel medic), subspecies *tricycla.* The *spring1* mutant and *OX::FTa1* were previously reported (Laurie *et al.*, 2011; Yeoh *et al.*, 2013). The *spring2* and *spring3* mutants were identified in a 2010 glasshouse screen of *Tnt1* insertion mutants at the Samuel Roberts Noble Foundation (Ardmore, OK, USA). Plants for flowering time experiments and gene expression measurements were grown under LD (16-h light/8-h dark) or SD (8-h light/16-h dark) in a growth room or plant

growth cabinets with about 200 μmol m^{-2} sec^{-1} cool white fluorescent light at about 22°C as described previously (Yeoh *et al.*, 2013). For vernalisation (V), germinated seeds were exposed to 2 weeks of cold in the dark at either about 7°C (stronger vernalisation) or 4°C (weaker vernalisation) before planting out.

Plant crosses and scoring flowering time

Backcrosses were carried out between the *spring* mutants and R108 to investigate the inheritance of the *spring2* and *spring3* mutations, to generate *spring* mutant lines for phenotyping and to generate large segregating F_2 populations for linkage analysis with DNA markers. Multiple crosses between *spring2* and *spring3* and R108 plants were done in both directions and plants grown up and scored for flowering time (Table S1) as described previously for *spring1* (Yeoh *et al.*, 2013). Homozygous *spring2* or *spring3* mutant F_3 plants after one backcross, or after two backcrosses in the case of *spring1*, were used for the major flowering time and corresponding gene expression experiments. Figure S1 used a true breeding line of *spring1* prior to backcrosses and similar results were obtained.

Analysis of gene expression by qRT-PCR and microarray analysis

Trifoliate leaves or shoot apices were harvested with scissors and dissected by eye as needed using a scalpel from 13–14-day-old plants grown as described in the text. The RNA extraction, cDNA synthesis and qRT-PCR were carried out as previously described (Yeoh *et al.*, 2013) using primers listed in Table S2. Each data point was derived from three biological replicates and each replicate consisted of a pool of tissues from three independent plants. *PROTODERMAL FACTOR 2* (*PDF2*) was used as a normaliser. For the microarray analysis, total RNA was extracted from VLD R108 and LD *spring1* from the first trifoliate leaf when seedlings were 12–14 days old and at the three true-leaf stage (one monofoliate leaf and two trifoliate leaves). Three biological replicates were harvested in parallel, each consisting of a pool of leaves from three independent plants. The cDNA synthesis from each of the biological replicates, labelling, hybridisation to the Affymetrix *Medicago* GeneChip arrays and statistical analysis of the results were performed according to the manufacturer's instructions (Affymetrix, http://www.affymetrix.com/) and as described previously (Yeoh *et al.*, 2013).

FTa1 3′ RACE analysis and DNA sequencing of the *FTa1* genomic region in *spring* mutants and R108

First-strand cDNA of LD *spring* mutants and VLD R108 was synthesized from RNA from three biological replicates with an oligo dT primer (Frohmann *et al.*, 1988) (Table S2). The cDNA samples from the three biological replicates were pooled and cDNAs were PCR amplified (Table S2). The PCR products were purified, cloned, insert DNA amplified by colony PCR, directly sequenced and aligned with the R108 *FTa1* genomic and cDNA sequence. The *FTa1* genomic sequence from *spring2* and *spring3* plants was amplified by PCR (Table S2). Five PCR reactions of each type were pooled and directly sequenced.

Identification of *Tnt1* flanking sequence tags by transposon display and linkage analysis

Tnt1 FSTs were isolated from genomic DNA of the *spring2* and *spring3* mutants using the transposon display method as previously described (Ratet *et al.*, 2010). The resulting sequences were aligned with the *M. truncatula* Mt3.5 Genome assembly (http://medicagohapmap.org/), the NCBI Nucleotide Collection High Throughput Genomic Sequences (HTGS) database using MegaBLAST (optimized for highly similar sequences) and the Non-redundant Protein Sequences database using BLASTX. Flanking sequence tags were used to develop DNA markers and then used in linkage analyses on segregating F_2 *spring* populations from the backcrosses to R108. The FSTs 100% linked (Table S2) to the *spring* mutations would be expected to be present in all early flowering plants and absent in the late flowering segregants. The corresponding R108 amplicons would be present in all of the late flowering plants and in early flowering heterozygotes.

DNA methylation analysis of the solo LTR by bisulphite sequencing

Bisulphite sequencing was used to determine DNA methylation status by converting unmethylated cytosine residues to uracil, leaving methylated cytosines unaffected (Henderson *et al.*, 2010). Genomic DNA was extracted from the first trifoliate leaves of *spring1* and R108 13–14-day-old plants in LD using the DNeasy® Plant Mini kit (Qiagen, http://www.qiagen.com/). Each sample consisted of a pool of three leaves, each one taken from an individual plant. Two hundred nanograms of genomic DNA from *spring1* and R108 was subjected to bisulphite conversion using the EpiTect® Bisulfite Kit (Qiagen) and amplified by PCR (Table S2). The PCR products were purified (High Pure PCR Product Purification Kit, Roche, http://www.roche.com/) and cloned. Twenty-three independent clones were sequenced for both *spring1* and R108.

McrBC-based methylation analysis

McrBC cuts DNA containing methylcytosine residues on one or both strands. The two half-sites (G/A)^{m}C recognised by the enzyme can be up to 3 kb apart, but the enzyme cuts close to one of them. Genomic DNA was extracted with a urea-based extraction buffer from leaf tissue of a pool of 19-day-old LD plants and digested with McrBC (New England Biolabs, https://www.neb.com/) (Martin *et al.*, 2009) and amplified by PCR (Table S2). Semi-quantitative PCR was performed on one sample for each genotype. Quantitative RT-PCR was also performed. The level of methylation was calculated using the $2^{\Delta C}{}_{t}$ method, where ΔC_t is the difference between C_t values of digested and mock samples. Results were presented as averages of three biological replicates except for *spring2* where two biological replicates were used (± SE).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Comparison of *spring1* phenotypes with vernalised and non-vernalised R108 plants in long days.

Figure S2. Analysis of expression of retroelements.

Figure S3. DNA sequencing of *FTa1* genomic region and cDNA clones.

Figure S4. Analysis of DNA methylation of the solo long terminal repeat by bisulphite sequencing.

Table S1. Flowering time measurements.

Table S2. List of primers.

Table S3. Microarray analysis of genes differentially expressed in long day *spring1* compared to VLD R108.

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