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FLOWERING LOCUS T indel variants confer vernalization-independent and photoperiod-insensitive flowering of yellow lupin (Lupinus luteus L.)

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Abstract

Ongoing climate change has considerably reduced the seasonal window for crop vernalization, concurrently expanding cultivation area into northern latitudes with long-day photoperiod. To address these changes, cool season legume breeders need to understand molecular control of vernalization and photoperiod. A key floral transition gene integrating signals from these pathways is the Flowering locus T (FT). Here, a recently domesticated grain legume, yellow lupin (Lupinus luteus L.), was explored for potential involvement of FT homologues in ablation of vernalization and photoperiod requirements. Two FTa (LlutFTa1a and LlutFTa1b) and FTc (LlutFTc1 and LlutFTc2) homologues were identified and sequenced for two contrasting parents of a reference recombinant inbred line (RIL) population, an early-flowering cultivar Wodjil and a late-flowering wild-type F2B213. Large deletions were detected in the 5′ promoter regions of three FT homologues. Quantitative trait loci were identified for flowering time and vernalization response in the RIL population and in a diverse panel of wild and domesticated accessions. A 2227 bp deletion found in the LlutFTc1 promoter was linked with early phenology and vernalization independence, whereas LlutFTa1a and LlutFTc2 indels with photoperiod responsiveness. Comparative mapping highlighted convergence of FTc1 indel evolution in two Old World lupin species, addressing both artificial selection during domestication and natural adaptation to short season environmental conditions. We concluded that rapid flowering in yellow lupin is associated with the de-repression of the LlutFTc1 homologue from the juvenile phase, putatively due to the elimination of all binding sites in the promoter region for the AGAMOUS-like 15 transcription factor.

Introduction

Synchronization of flowering time with a particular season is essential for the reproductive success of plants growing in climates experiencing significant annual cycles. Knowledge of the regulatory network underlying flowering control would facilitate the breeding of new plant varieties that are better adapted to target agroecosystems. This issue is becoming more urgent in the era of climate change consecutively narrowing the time window for spring sowing of vernalization-responsive crops [1, 2]. It is due to the reduction of the lengths of winter and spring in the Northern Hemisphere mid-latitudes, where temperate climate crops are cultivated [3]. As legume species have generally low tolerance to freezing temperatures [4], in colder regions of temperate climate, that include also major European cultivation areas, vernalization-responsive species are sown in early spring rather than in autumn to fulfill vernalization requirements without an excessive risk of frost damage. Nevertheless, higher temperature in winter resulting from climate change may also lead to incomplete fulfillment of vernalization requirement in some winter plant species, resulting in delayed flowering or a failure of floral induction [5]. Indeed, a long-term study based on 47-year record (1954–2000) of phenological changes revealed a progression of delayed flowering in natural populations with high vernalization requirements [1], whereas analysis of inter-annual sensitivity of winter wheat yields to vernalization degree days during 1975–2009 revealed its potential vulnerability to warming-mediated vernalization variations in temperate climate [6]. Moreover, spring heat waves, that may occur more frequently in warmer climates, can erase epigenetic marks of vernalization, resulting in de-vernalization and delayed flowering [7, 8].

Genetic and molecular regulation of flowering induction is best known in the model plant, Arabidopsis thaliana (L.) Heynh. Flowering induction pathways address both environmental factors such as vernalization, high temperature, photoperiod and light quality...
as well as endogenous signalling, such as the gibberellin pathway, ageing and carbohydrates [9]. These pathways converge in the transcriptomic regulation of floral integrator genes. The key floral pathway integrator responding to environmental signals (low and high temperature, photoperiod and light quality) is the FLOWERING LOCUS T (FT) gene [10, 11]. Arabidopsis has only two FT-like genes (FT and a one close homolog, TWIN SISTER OF FT), whereas legume genomes usually encode higher number of FT-like genes, assigned into three subclades, FTa, FTb and FTC [12, 13]. While FT retains this basic role in all flowering plants studied to date, it is unclear how many homologues perform the role of floral integrator in legumes. Moreover, involvement of particular FT homologues in photoperiod or vernalization response considerably vary between legume species [12, 14–18]. In the present study, we selected yellow lupin (L. luteus L.), an annual legume with wild winter annual and domesticated spring annual forms, as a model to explore the sequence and functional divergence of FT homologues.

Yellow lupin (L. luteus L.) is a grain legume crop natively distributed primarily across the coastal region of the Iberian Peninsula [19]. Yellow lupin evolved numerous adaptations to deal with the dry-summer Mediterranean climate (such as drought avoidance by early phenology) and biotic pressures (i.e. anthracnose and aphid resistance) occurring in this environment [20–23]. Yellow lupin has a very short history of domestication as compared to other legumes, because all the key milestones in converting the wild types to domesticated forms were achieved in the 20th century [24]. Modern yellow lupin cultivars have low alkalioid and high protein content in seeds, and as such are currently considered as a highly nutritional alternative to soybean meal in animal diets [25]. Yellow lupin phenology studies revealed high variability of the vegetative period resulting mainly from differences in vernalization requirements and long photoperiod preferences [26, 27]. Depending on the application (green manure, biomass, or grain production) as well as climatic constraints (spring or winter sowing, the need of drought escape), breeding pressure on vernalization requirements and early phenology is different [28].

As the juvenile photoperiod-nonresponsive phase is relatively short in lupins [29], matching of periods with conditions ensuring fulfillment of vernalization and photoperiod requirements becomes challenging due to climate change. Determining the molecular components underlying existing variability in vernalization response and phenology would help to address this issue, enabling molecular-assisted breeding. Moreover, such a knowledge could facilitate studies in other vernalization-responsive legumes. Indeed, vernalization control in this large plant family differ from the Arabidopsis model because the majority of legumes do not have a key gene from the vernalization pathway, FLOWERING LOCUS C (FLC), whereas soybean, that has retained one such homologue, is vernalization independent because it evolved in a warm sub-tropical environment [30]. Early flowering based on thermoneutrality (vernalization independence) is the key agro-nomic trait of lupins, enabling their successful cultivation in temperate climates (spring-sown in Northern Europe or autumn sown in the mild Mediterranean-like climates of Australia) [29].

Quantitative trait loci (QTL) mapping revealed that flowering time in yellow lupin is controlled by several QTLs, with one major locus for vernalization response [31]. In the closely related species, narrow-leafed lupin, vernalization insensitivity is also based on a single locus, conferred by natural mutations that occurred during domestication period (Ku and Jul) [32, 33]. These mutations constitute two overlapping deletion variants in the promoter region of one of the FT homologues, LanFTc1 gene [18, 34]. Recent yellow lupin mapping studies revealed collinearity between the linkage group carrying the major QTL for vernalization response and the narrow-leafed lupin genome scaffold carrying LanFTc1 sequence [35, 36]. Flowering time in another related species, white lupin, was found to be under quantitative control, with two QTLs associated with FTa and FTC gene-based markers [37–40]. All these findings improved the knowledge on the key role of FT in flowering time control in legumes and driven our attention to the FT clade present in the yellow lupin genome.

In this study, the involvement of FT genes in flowering time control in yellow lupin was analyzed by several complementary approaches, including linkage and QTL mapping, gene sequencing and quantitative expression profiling under two contrasting photoperiod and vernalization conditions. Moreover, a yellow lupin germplasm diversity panel carrying wild and domesticated accessions was phenotyped for selected phenology traits and vernalization responsiveness in controlled conditions as well as genotyped for the presence of indel polymorphisms using PCR-based markers spanning the whole FT gene sequences and promoter regions. The study provided several independent lines of evidence to support the involvement of three FT homologues in flowering time control in yellow lupin, with the LlutFTc1 homologue playing the key role in domestication driven by reduction of juvenile phase and elimination of vernalization requirements to induce flowering.

## Results

### Yellow lupin phenology is strongly determined by genotype

Phenotypic data of phenology traits analyzed with and without pre-sowing vernalization under ambient long day photoperiod were obtained for 109 yellow lupin accessions in the 2016 trial and for 111 accessions in the 2017 and 2019 trials (Tables S1 and S2). High variability between particular accessions in phenology in the absence of vernalization was observed, ranging from 42.5 ± 2.2 (PRH444/14) to 82.4 ± 5.5 (Biscainhos-4) days to the first floral bud emergence, from 50.7 ± 1.4 (PRH444/14) to 91.8 ± 5.3 (Biscainhos-4) days to the onset of flowering and from 68.3 ± 2.7 (Idol) to 101.3 ± 4.7 (Biscainhos-4) days to the end of flowering on the main stem. Accessions in the diversity panel differed also in vernalization requirements, ranging from full thermoneutrality to very high responsiveness, manifested by acceleration of transition from vegetative to generative growth phases by 3 weeks. Estimated marginal mean pairwise comparisons revealed that 22 lines were fully thermoneutral for bud emergence, 29 for start of flowering, 18 for end of flowering, and 9 lines for all of these traits (Table S3). The broad sense heritability coefficients of phenology traits in the yellow lupin diversity panel were high in the absence of vernalization, ranging from 74.9% to 81.4% and moderately high with vernalization treatment, ranging from 55.6% to 58.9% (Table 1).

### Large indels series are present in the promoter regions of all yellow lupin FT homologues

BLAST analysis of yellow lupin draft genome assembly using L. angustifolius FT genes identified four FT homologues. Aligning these homologues with other legume FT homologues allowed the assignment of these homologues (by Bayesian inference) to the FTa clade (LlutFTa1a and LlutFTa1b) and the FTC (LlutFTc1 and LlutFTc2) clade (Fig. 1). This analysis confirmed also reported lineage-specific duplications of FTa and FTC homologues in lupins [41].
Table 1. Genetic parameters calculated by linear mixed-effect model for selected phenology traits observed in a diversity panel of 111 yellow lupin accessions in long day conditions

| Parameters                              | BE 1   | BE +v  | SF    | SF +v  | EF    | EF +v  |
|-----------------------------------------|--------|--------|-------|--------|-------|--------|
| Phenotypic variance                     | 97.1   | 75.5   | 93.3  | 69.1   | 69.0  | 51.5   |
| Broad-sense heritability (%)            | 78.8   | 55.6   | 81.4  | 58.5   | 75.0  | 58.9   |
| Heritability on the mean basis (%)      | 94.7   | 83.8   | 95.7  | 85.9   | 92.2  | 84.3   |
| Selective accuracy (%)                  | 0.97   | 0.92   | 0.98  | 0.93   | 0.96  | 0.92   |
| Genotype-environment correlation        | 0.58   | 0.70   | 0.54  | 0.66   | 0.73  | 0.78   |
| Genotypic coefficient of variation      | 14.25  | 11.93  | 12.71 | 10.24  | 8.85  | 7.24   |
| Residual coefficient of variation       | 4.80   | 5.85   | 4.10  | 4.99   | 2.66  | 2.83   |

Phenotypic trait abbreviations are as follows: the number days to floral bud emergence without vernalization (BE) and with vernalization (BE +v), the number of days to start of flowering without vernalization (SF) and with vernalization (SF +v), and the number of days to end of flowering without vernalization (EF) and with vernalization (EF +v).

Figure 1. Majority rule consensus of 12502 trees found in a Bayesian analysis of selected legume FLOWERING LOCUS T (FT) genes. Numbers are posterior probabilities. The length of coding sequence alignment was 537 nucleotides. Abbreviated species names are provided as follows: At, Arabidopsis thaliana; Gm, Glycine max; Lan, Lupinus angustifolius; Mt, Medicago truncatula; Llut, Lupinus luteus; Lalb, Lupinus albus. L. luteus genes were identified in this study, L. albus sequences were extracted from the reference genome [42], whereas the remaining sequences were derived from the recent phylogenetic studies [12,14,41]. NCBI accession numbers or L. albus gene names [42] were provided in parentheses.

Four yellow lupin accessions differing in domestication status and phenology were selected for further studies: PRH444/14 (Polish breeding line, very early flowering and thermoneutral), Wodjil (Australian cultivar, early flowering and near-thermoneutral), Parys (Polish cultivar, intermediate flowering and responsive to vernalization), and P28213 (Azorean wild population, late flowering and highly responsive to vernalization). Phenotyping in climatic chambers under two contrasting photoperiods confirmed variability in earliness and vernalization responsiveness observed between these accessions in greenhouse (Table 2).

Full FT sequences, including ~8 kbp promoter regions, were retrieved by sequencing and assembly of overlapping PCR products (Table S4). This revealed the presence of indels in the promoter and other regions. Then, PCR-based screening of the diversity panel was performed. From the 76 primer pairs tested, 21 revealed indel polymorphism detectable under the resolution of 2% agarose gel electrophoresis with the minor allele frequency ranging from 0.009 to 0.234 (Table S5). Eight different long (≥6 bp) indel variants were identified for the LlutFTa1a gene, seven for LlutFTa1b, four for LlutFTc1, and eleven for LlutFTc2 (Table 3, Fig. 2).

Sequencing performed for PRH444/14, Wodjil, Parys and P28213 accessions revealed, besides long indels, numerous SNPs and/or short (< 5 bp) indels, namely 48 in LlutFTa1a, 258 in LlutFTa1b (including one in the fourth exon), 32 in LlutFTc1 and 81 in LlutFTc2 (including one in the second exon and two in the fourth exon).
In localized in the promoters, accounting for 41, 213, and 24 SNPs, the third intron. FGENESH predictions for all nucleotide variants. The list of polymorphic

Table 2. Number of days from sowing to the first bud, flower and pod in four L. luteus lines selected for sequencing of FT genes

| Line          | Vernalization | Days to first bud | Days to first flower | Days to first pod |
|---------------|---------------|-------------------|----------------------|------------------|
| 8-hour photoperiod |               |                   |                      |                  |
| P28213        | -             | DNF²             | 60.3 ± 4.9          | 70.5 ± 5.2       | 78.4 ± 3.1       |
| Parys         | -             | 67.0 ± 1.2       | 74.1 ± 1.6          | 84.3 ± 1.5       |
| PRH444        | -             | 48.6 ± 3.3       | 54.6 ± 3.6          | 64.2 ± 2.8       |
| Wodjil        | -             | 40.6 ± 2.9       | 47.4 ± 3.1          | 56.1 ± 5.5       |
| Wodjil        | +             | 36.6 ± 8.1       | 42.1 ± 7.9          | 52.1 ± 9.1       |
| 16-hour photoperiod |   |                   |                      |                  |
| P28213        | -             | 73.0 ± 4.6       | 78.5 ± 4.2          | 86.5 ± 4.2       |
| Parys         | -             | 53.8 ± 1.3       | 60.8 ± 1.9          | 67.5 ± 1.5       |
| PRH444        | -             | 46.2 ± 6.3       | 54.3 ± 6.0          | 63.1 ± 6.0       |
| Wodjil        | -             | 40.7 ± 0.5       | 47.4 ± 0.5          | 52.3 ± 0.7       |
| Wodjil        | +             | 40.5 ± 2.1       | 48.0 ± 2.1          | 54.3 ± 2.2       |

¹plants did not flower. ²standard deviation.

Table 3. Major indels (length ≥ 6 bp) identified in FT genes in a diversity panel of 111 yellow lupin accessions

| Gene       | Indel no. | Region   | Locus (bp)¹ | Length (bp) |
|------------|-----------|----------|-------------|-------------|
| LlutFTa1a  | 1         | promoter | 2401        | 12          |
| LlutFTa1b  | 1         | promoter | 2219        | 24          |
| LlutFTc1   | 1         | promoter | 3590        | 2227        |
| LlutFTc2   | 1         | promoter | 7403        | 128         |

¹Position in the sequence alignment (see Supplementary File 1)

In LlutFTa1a, LlutFTa1b and LlutFTc1, the majority of SNPs were localized in the promoters, accounting for 41, 213, and 24 SNPs, respectively. In LlutFTc2, the majority of SNPs (56) were found in the third intron. FGENESH+ provided identical protein sequence predictions for all nucleotide variants. The list of polymorphic

loci is presented in Table S6 whereas FASTA alignments in Supplementary File 1.

LlutFTa1a, LlutFTc1 and LlutFTc2 indels are strongly associated with flowering time and vernalization responsiveness

Three indel markers from the LlutFTa1a, one from the LlutFTc1 and six from the LlutFTc2 revealed statistically significant correlation with all phenology traits observed in a 3-year series of greenhouse experiments, as well as with vernalization responsiveness (understood as a shift in the BLUP for number of days in vernalized variant versus non-vernalized) (Table S7, Fig. 2). Thus, a large indel from the LlutFTc1 promoter (2227 bp, indel 1) showed the highest association with phenology traits (Spearman’s rank correlation coefficient, rho[ρ]-value, from 0.70 to 0.72) and with vernalization responsiveness (ρ-value from −0.66 to 0.53) among all analyzed markers. Interestingly, this LlutFTc1 indel allele was found only in domesticated germplasm except one landrace originating from Palestine (Palestyna-5). It should be noted that two other LlutFTc1 promoter indel markers (indels 3 and 4) did not reveal significant correlation with any of the phenology traits.

Five LlutFTc2 indels, namely indels 1 and 2 from the promoter, indels 3 and 4 from the second intron and indel 9 from the third intron (carrying a large Copia-like retrotransposon insertion) also revealed significant correlation with phenology traits, but considerably lower than the large LlutFTc1 promoter indel 1 (ρ-value from 0.43 to 0.48) as well as moderate correlation with vernalization response (ρ-value from −0.33 to −0.28). The most 3’ LlutFTc2 indel 11, localized close to the fourth exon, showed significant but moderate correlation with phenology (ρ-value from 0.35 to 0.39) and vernalization responsiveness (ρ-value from −0.19 to −0.23). Three LlutFTa1 indels (indels 1 and 3 from the promoter and indel 7 from the third intron) had similar correlation with vernalization response as four LlutFTc2 indels (ρ-value from −0.33 to −0.25) and lower correlation with phenology (ρ-value from 0.35 to 0.39). Another two LlutFTa1a promoter indels (2 and 4) revealed significant correlation with one or three traits (ρ-value from 0.20 to 0.22), and vernalization response for the end of flowering (ρ-value −0.22 and −0.23). Two rare LlutFTa1 alleles (FTa1a_F6_R6 presence/absence and indel 6) and all studied
Sequence polymorphism revealed in the *Lupinus luteus* FLOWERING LOCUS T (*LlutFTa1a, LlutFTa1b, LlutFTc1* and *LlutFTc2*) genes. Black tags visualize SNP and short (≤ 5 bp) indel loci whereas red rectangles and blue triangles show exons and large (≥ 6 bp) indels, respectively. $P$-value of Spearman’s rank correlation coefficient, calculated for the phenology traits (BE, time to floral bud emergence; SF, time to start of flowering; and EF, time to end of flowering) and for the vernalization responsiveness of these traits (vBE, vSF and vEF, respectively), was shown in the following scheme: ***, $p < 0.0001$; **, $0.0001 \leq p < 0.001$; *, $0.001 \leq p \leq 0.05$; −, $p > 0.05$ (not significant); x, not analyzed.

*LlutFTa1b* indels (2–7) did not reveal significant correlation with any trait. Marker grouping based on the distribution of FT indel polymorphism in analyzed yellow lupin lines revealed the presence of two major clusters, one carrying markers which revealed statistically significant correlation with all observed phenotypic traits and the other composed of the remaining markers (Fig. 3).

**LlutFTc1** and **LlutFTc2** genes co-localize with two major QTLs for yellow lupin flowering time

Based on the identified polymorphisms, 3 indel and 13 CAPS markers were developed for *LlutFTa1a*, two CAPS markers for *LlutFTa1b*, and single indel markers for the *LlutFTc1* and *LlutFTc2* (Table S8). Expected indel or and restriction enzyme cleavage products in parental lines were obtained for all markers. However, screening of the RIL mapping population revealed that two *LlutFTa1a* markers (*FTa1a_F5_R5* and *FTa1a_F13_R13*) and one *LlutFTa1b* marker (*FTa1b_M1_CAPS*) were monomorphic. Moreover, segregation was significantly distorted ($\chi^2$ p-value 1E-11) from the expected 1:1 ratio for the remaining *LlutFTa1* markers. The *LlutFTc1* indel marker was localized in the linkage group YL-21 (2.9 cM, LOD values to surrounding markers 26.5 and 23.1), the *LlutFTc2* marker in the linkage group YL-01 (40.8 cM, LOD values 25.9 and 23.1), whereas the *LlutFTa1b* marker (*FTa1b_F19_R20*) at the end of the linkage group YL-60 (5.1 cM, LOD value 16.9). Markers with distorted segregation remained unmapped. QTL mapping was performed using linkage map updated with these markers and published data on flowering time in yellow lupin RIL population [31, 35]. Six statistically significant QTLs (1000 permutation test p-value < 0.05) were identified (Table S9). These QTLs are localized on the linkage groups YL-01 (within the LODmax-1 range of 39.5–42.3 cM), YL-03 (20.9–25.7 cM), YL-06 (39.0–46.6 cM), YL-17 (38.0–
Figure 3. Heatmap showing marker clustering and Spearman’s rank correlation coefficients calculated for the diversity panel of 111 yellow lupin accessions using allelic diversity of \textit{LlutFTa1a}, \textit{LlutFTa1b}, \textit{LlutFTc1} and \textit{LlutFTc2} gene-based markers. Phenotypic trait abbreviations are as follows: BE, the number days to floral bud emergence (without vernalization); vBE, influence of vernalization on BE; SF, the number of days to start of flowering; vSF, influence of vernalization on SF; EF, the number of days to end of flowering; vEF, influence of vernalization on EF. Asterisk (*) indicates significant correlations in the following scheme: ***, $p < 0.0001$; **, $0.0001 \leq p < 0.001$; *, $0.001 \leq p \leq 0.05$.

Figure 4. Two linkage groups (YL-01 and YL-21) from the yellow lupin linkage map carrying newly developed \textit{LlutFTc1} and \textit{LlutFTc2} markers and major quantitative trait loci for flowering time observed in a recombinant inbred line mapping population. Boxes indicate LOD$_{\text{max}}$-1 intervals, whereas whiskers extends to LOD$_{\text{max}}$-2 intervals. 41.0 cM), YL-21 (0.0–5.0 cM) and YL-34 (3.6–8.9 cM). \textit{LlutFTc1} and \textit{LlutFTc2} markers were found to be localized directly in the major QTL peaks (Fig. 4), explaining approximately 11% (\textit{LlutFTc1}) and 25% (\textit{LlutFTc2}) of observed phenotypic variance (flowering time of non-vernalized plants). Moreover, \textit{LlutFTc1} marker matched the key locus for vernalization responsiveness in yellow lupin RIL population.

As a finished genome assembly is not available for yellow lupin, synteny with the better-characterized close relative, narrow-leaved lupin, was explored. All yellow lupin flowering time QTLs revealed patterns of shared collinearity (Table S10). The majority of these blocks carried known regulators from vernalization and photoperiod pathways. Thus, the QTL on linkage group YL-01 was in a collinear region of NLL-17 carrying the \textit{LanFTc2} gene and the QTL on YL-21 was in a collinear region of NLL-10 encoding the \textit{LanFTc1} gene. Moreover, the QTL on YL-03 matched the NLL-02 region containing the \textit{LanCOL-9} gene and the QTL on YL-06 was syntenic to two NLL-20 regions separated by a break of collinearity; one of these regions carries the \textit{LanFTa1} gene (Table S10).

\textbf{FT genes and alleles differ in responsiveness to vernalization and photoperiod}

Four yellow lupin lines differing in time to flowering and vernalization responsiveness (PRH444/14, Wodjil, Parys and P28213) were subjected to \textit{LlutFTa1a}, \textit{LlutFTa1b}, \textit{LlutFTc1} and \textit{LlutFTc2} gene expression profiling under two contrasting photoperiods (Tables S11 and S12, Fig. 5). Comparing mean values...
Figure 5. Expression profiles of *LlutFTa1a*, *LlutFTa1b*, *LlutFTc1* and *LlutFTc2* genes in yellow lupin accessions P28213 (Po), Parys (Pa), Wodjil (Wo) and PRH444/14 (PR). Numbers following accession abbreviations stands for sampling terms (Table S12). Three biological and three technical replicates were analyzed. Error bars show standard deviation. Two reference genes, a DEAD box RNA helicase 1 (*LlutDRH1*) and a beta tubulin 7 (*LlutTUB7*) were used for ΔΔCq normalization. *LlutFTa1a* and *LlutFTc1* graphs are shown in log scale whereas *LlutFTa1b* and *LlutFTc2* in linear scale. Significance of vernalization influence on gene expression is shown above the data points. Significance of photoperiod influence is presented below the x axis (on the left panels for non-vernalized plants, n, and on the right panels for vernalized plants, v).

∗, significant (p ≤ 0.05); no symbol, not significant; ×, not calculated due to very different variance between groups, †, not calculated due to the lack of corresponding data point for pairwise comparison.

from all data points, *LlutFTa1a*, *LlutFTc1* and *LlutFTc2* genes revealed approximately 40–70 times higher expression levels than the *LlutFTa1b* gene (6.2 ± 15.4, 9.0 ± 16.8 and 4.9 ± 5.8 vs 0.13 ± 0.17, respectively). *LlutFTa1a*, *LlutFTc1* and *LlutFTc2* were considered as good candidate genes due to revealed association of sequence polymorphism with phenology traits and vernalization responsiveness, therefore their expression profiles are described herein in this context. Comparative analysis of expression levels between genotypes, photoperiod, vernalization variants, and growth phases is provided in Table S13. Moreover, *LlutFTa1a*, *LlutFTc1* and *LlutFTc2* genes revealed strong association between gene expression and timing of transition from vegetative to generative phases (Fig. 5).

Differences in expression profiles between genotypes were significant. In the absence of vernalization (Fig. 5), *LlutFTa1a* gene expression in Wodjil was higher than in P28213 up to 79-fold.
under SD (short day photoperiod) and up to 168-fold under LD (long day photoperiod). Similar observation was made for the LlutFTa1a gene, reaching differences up to 225-fold and up to 42-fold under LD. Moreover, LlutFTa1a was also higher than in P28213 (up to 12-fold), and Parys (up to 5-fold).

Taking into consideration photoperiod responses, FT indels were found for AGL71 (two in the LlutFTa1a gene) and 92–218 for the LlutFTc2 gene. Analysis provided evidence for split into two clades before a polyploidy event.

### FT indels carry hypothetical binding sites of transcription factors from vernalization and photoperiod pathways

Promoter regions of LlutFTa1a, LlutFTc1 and LlutFTc2 genes were annotated for the presence of hypothetical binding sites of transcription factors (Table S14). The number of motif hits differing in the whole promoter sequences between P28213 and Wodjil reached 110–2260 for the LlutFTc1 gene, 116–242 for the LlutFTc2 gene and 92–218 for the LlutFTa1a gene. Analysis of polymorphisms found in the diversity panel revealed the presence of 11–228 motif hits for the LlutFTa1a gene and 6–704 motif hits for the LlutFTc1 gene. Variability of transcription factor binding sites was highly associated with the presence of large indels, i.e., 2231 hits for LlutFTc1 indel1, 693 hits for LlutFTc1 indel4, 349 hits for LlutFTc1 indel3, 215 hits for LlutFTa1a indel4. As the vast majority binding sites for particular transcription factors were present both in the polymorphic and monomorphic regions, the number of motifs found only in the polymorphic loci was much lower, reaching from 0 to 137 hits (Table 4).

As one polymorphic locus typically provided redundant hits, therefore the real number of candidate unique transcription factors even lower. Thus, just a few unique transcription factors could participate in regulation of flowering time in response to photoperiod and vernalization were identified. Taking into consideration the reports from other studies providing evidence for FT promoter binding and/or control of flowering time (see Discussion), a narrow list of candidate transcription factors was selected, including TARGET OF EAT2 (TOE2) for the LlutFTa1a gene, AGAMOUS-like 15 (AGL15) for the LlutFTc1 gene and MYB62 for the LlutFTc2 gene. (Table 4).

As lengths and positions of major LanFTc1 and LanFTc1 promoter indels are similar [34] it would be interesting to know if the sets of indel-specific transcription factor binding sites are also similar. Therefore, we analyzed LanFTc1 indels in the same way as the LlutFTc1 indels (Table S15). This analysis highlighted AGL15 as a candidate diversifying transcription factor for LanFTc1 gene (Fig. 6), revealing 3 binding sites in Pal indel, 4 sites in Ku indel, 5 sites in Jul indel, and one site in the monomorphic region with much lower similarity score (0.81–0.82) than the sites in the indels (0.96–1.00). Moreover, in the LanFTc1 indels, binding sites were found for AGL71 (two in the Ku, Pal and Jul indels, another two in monomorphic regions) and SUF4 (1 in Ku, Pal and Jul, 0 in monomorphic regions). No candidate binding site for VRN1 was found in the whole LanFTc1 promoter.

### Discussion

Yellow lupin duplicates of FTa and FTc homologues as remnants of a lineage-specific ploidy event.

The number of FTa and FTc homologues revealed in yellow lupin genome in the present study is the same as in the narrow-leaved and white lupin genomes [18, 39, 40]. Bayesian inference provided evidence for split into FTa, FTb and FTc clades before...
the divergence of major Papilionoideae lineages [41]. This observation supports the concept that a simultaneous divergence of all major legume subfamilies was associated with mass extinction at the Cretaceous–Paleogene boundary (66 million years ago) and a whole-genome duplication event [54–56]. Additional polyploidy events occurred later in downstream lineages, including lupin (hypothesized triplication) and soybean (duplication) [42, 57, 58]. Remnants of these processes can still be found in plant genomes in the form of additional gene copies arranged in collinear blocks. The mechanisms conferring the retention of duplicated genes are not well understood, nevertheless, it was revealed that high retention rates, include, among others, genes from flowering and cold-responsive pathways [59, 60].

Sub-functionalization of FTc1 into vernalization and FTa1 into photoperiod in lupins

The present study revealed sub-functionalization of LlutFTc1 into vernalization pathway (Fig. 2–4), whereas LlutFTa1 into photoperiod response (Fig. 5). A similar observation was made for LanFTc1 (wild allele) and LanFTa1 (Palestinian allele) genes in narrow-leaved lupin [18, 61]. Unfortunately, providing direct evidence for these functions by targeted reverse genetics is currently impractical due to the constraints of the lupin transformation system. In other legume species, FT duplicates also revealed functional divergence between photoperiod and vernalization pathways, such as genes MtFTb1 and MtFTk2 vs MtFTa2 in Medicago truncatula or genes PsFTb2 vs PsFTa1 in Pisum sativum, respectively [12, 14, 62]. Incorporation of different FT homologs into vernalization pathway in legumes is not surprising when the timeline of ancient climate changes is placed in the evolutionary context. Vernalization as a trait likely evolved in a response to major global cooling that peaked during the Eocene–Oligocene boundary 34 million years ago, as evidenced for temperate Pooidae grasses [63, 64]. Therefore, a general mechanism of vernalization response based on the FTc clade may have been established several million years before the ploidy event in the Lupinus lineage [58, 65].

AGL15 as a candidate FTc1 transcription factor controlling vernalization requirement and vegetative phase duration

The present study evidenced the association between indel polymorphism in regulatory region of LlutFTc1 and vernalization-independent flowering (Fig. 2). A similar observation was reported for narrow-leaved lupin and the series of LanFTc1 indels [34]. In Arabidopsis, the FT promoter region is relatively long (~5 kbp) and carries numerous binding sites for regulatory agents from photoperiod, light quality, vernalization and aging pathways [43, 68]. Our study (Table 4) revealed that four transcription factors have specific candidate binding sites in the polymorphic regions of the LlutFTc1 promoter: AGAMOUS-like 15 (AGL15), AGL71, SUPPRESSOR OF FRI 4 (SUF4) and VERNALIZATION 1 (VRN1). Comparative analysis of candidate binding sites in LlutFTc1 and LanFTc1 promoters designated AGL15 as a candidate
transcription factor diversifying between particular structural variants (Fig. 6).

AGL15 is a MADS-box transcription factor that acts as a floral repressor during vegetative phase by binding FT promoter sequence at sites that partially overlap those bound by FLC and SHORT VEGETATIVE PHASE (SVP) proteins [43]. The LlutFTc1 indel carries all candidate AGL15 binding sites found in the whole promoter (Table 4). Therefore, hypothetical repression of the LlutFTc1 gene by AGL15 may occur in the late flowering Parys and L28213 lines whilst it should not occur in the early flowering lines lacking appropriate binding sites. High expression of LlutFTc1 observed in PHR444/14 and Wodijil lines beginning with the juvenile phase highlights LlutFTc1 as a major candidate for the LlutFTc1 indel-related early flowering. A similar conclusion can be made for the LanFTc1 (narrow-leaved lupin) indels.

The second candidate supported by in silico indel analysis in both lupin species, SUF4, controls vernalization dependence by binding FLC chromatin as a component of FRIGIDA transcription activator complex [44]. Nevertheless, to our knowledge, there is no evidence for SUF4 to bind the FT promoter. Moreover, in Arabidopsis, SUF4 activates transcription of the target, whereas in our study candidate binding site was found in the wild allele, indicating expected repressive activity. The third candidate, supported by indel analysis only in yellow lupin, VRN1, is a B3 domain carrying transcription factor associated with Arabidopsis flowering in response to vernalization [45]. VRN1 constitutes a hypothetically eudicot-specific component of PRC1-like complex, which is one of the two Polycomb complexes involved in epigenetic silencing of an FLC gene [46, 47]. PRC1-like activity is also linked with epigenetic control of FT, enabling temperature-responsive flowering time regulation [70]. Due to presence of VRN1 binding sites only in the wild allele, such vernalization-driven silencing of LlutFTc1 should lead to opposite expression and flowering time profiles than observed, therefore this mechanism is unlikely. The last candidate, AGL71, is a MADS-box transcription factor acting downstream of SOC1 and promoting flowering in the shoot apical and axillary meristems under the gibberellin-dependent pathway [48]. However, we disregarded this transcription factor due to presence of additional candidate binding sites also in the monomorphic region of the LanFTc1 promoter.

**Disruption of enhancer chromatin loop formation by FTc1 promoter indels is unlikely**

The other possible mechanism that could explain the observed difference in phenotypes associated with LlutFTc1 promoter variants is related with protein-mediated interaction between structural components of the FT promoter [71]. Two such motifs, CCAAT and RE-alpha, were also found in narrow-leaved lupin FT promoters at conserved positions [41]. In Arabidopsis, CCAAT sequences serve as binding sites for the NUCLEAR FACTOR Y (NF-Y) – CONSTANTS (CO) complex facilitating formation of long-distance chromatin loop bringing distal enhancer elements into close association with the proximal CO-responsive elements (CORE1 and CORE2) [72–74]. The functional consequence of this interaction is reduction of PcG protein levels at the FT promoter, relieving this region from Polycomb silencing under inductive photoperiod [75]. A large LlutFTc1 indel reported in this study carries six CCAAT elements (Table S14), whereas LanFTc1 indel variants carry from one to several such motifs [41]. Nevertheless, in both species there were additional CCAAT elements identified, flanking these indels, which may eventually participate in chromatin loop formation. Moreover, eventual disruption of chromatin looping at LlutFTc1 or LanFTc1 promoters by indels should result in the opposite phenotypic effects than observed.

**TOE2 as a candidate LlutFTa1a transcription factor controlling photoperiod response**

The present study identified three candidate transcription factors (Table 4) for the LlutFTa1a gene: BASIC LEUCINE ZIPPER 52 (bZIP52), GROWTH REGULATING FACTOR 6 (GRF6) and TARGET OF EAT 2 (TOE2). In Arabidopsis, bZIP52 protein is involved in heat stress response [49]. GRF6, known as a 14-3-3 protein, induces rice flowering by interaction in shoot apical meristem with FT and FLOWERING LOCUS D (FD) proteins to activate the floral promoter SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) and downstream floral meristem identity genes [50]. However, no evidence for binding FT promoter by GRF6 was found in literature data. The latter transcription factor, TOE2, is a component of photoperiodic pathway and represses FT transcription by binding to its chromatin, preventing flowering under short days [51, 52]. The presence of a TOE2 candidate binding site only in the wild (P28213) LlutFTa1a promoter allele supports the hypothesis on the involvement of TOE2 in photoperiod-related flowering control in yellow lupin.

**Copia-like retrotransposon insertion at the LlutFTc2 gene may delay flowering under non-inductive photoperiod**

A large (5269 bp) insertion of a Copia-like retrotransposon in the third intron of the LlutFTc2 was associated with delayed flowering (Fig. 2 and 4). Moreover, LlutFTc2 gene expression in P28213 line carrying this insertion was significantly reduced under short days (Fig. 5). A similar phenomenon was observed in soybean, where insertion of a Copia-like element (6224 bp) in the first intron of the soybean FT homologue (GmFT2a) was associated with decreased expression of this gene and delayed flowering [17]. A Tgm-like transposon insertion in the third intron of the GmFT2c gene that occurred at the early stage of soybean domestication also caused later flowering than the wild allele [76] Similarly, insertion of the Tnt1 retrotransposon within the first intron of the M. truncatula FT homologue (MtFTa1) resulted in a late-flowering phenotype [12]. In Arabidopsis, insertion of the Mutator-like transposable element in the first intron of the FLC gene conferred early flowering phenotype based on epigenetic silencing of FLC, mediated by short interfering RNAs [77]. Thus, the first intron of the FLC gene is relatively long and it is frequently targeted by transposons and retrotransposons in Brassicaceae species, providing significant transcriptional and phenotypic variation [78]. In A. thaliana most long introns are enriched with heterochromatic transposable element sequences [79]. Interestingly, the third introns of FT genes in three lupin species with sequenced genomes (L. angustifolius, L. albus and L. luteus) are also relatively long (from about 1.5 kbp to 6.5 kbp), therefore a similar mechanism like in Arabidopsis FLC may be expected. Apart from commonly observed deleterious effects, insertion of transposable elements can provide adaptive variation and facilitate evolutionary response to rapid environmental changes [80].

**FT indel polymorphism provides high flexibility in modification of yellow lupin phenology by traditional breeding**

In this study, LlutFTc1 indel allele conferring vernalization independence was found only in domesticated yellow lupin germplasm except one landrace from Palestine. A similar scenario was revealed for LanFTc1 indels in narrow-leaved lupin
To our knowledge it is the first example of such a convergence of FT indel evolution addressing artificial selection during domestication process and adaptation to environmental conditions that favors short season (Palestinian allele) between two related species. Both species (yellow lupin and narrow-leafed lupin) had lost vernalization requirement through large deletions in the promoter regions of the same FTc1 homologue, a central integrator of flowering time. This provides a unique opportunity to explore the molecular regulation of flowering time in these related species. It also provides motivation to prospect for additional examples of FTc1 deletions in other lupin species. This study provides for the first time a molecular marker that is perfectly predictive of vernalization responsiveness in yellow lupin. This can be used to facilitate introgression of new genetic diversity into domesticated germplasm (without vernalization requirement) from wild types (primarily with vernalization requirement). Moreover, the yellow lupin diversity panel offer very high flexibility for selection for vernalization and photoperiod responsiveness/independence due to the presence of lines carrying different allelic combinations of LlutFTa1a, LlutFTc1 and LlutFTc2 indels. High variability in allelic composition resulted in large phenotypic variance of flowering time and vernalization responsiveness, including numerous intermediate phenotypes with upgraded domestication status awaiting further exploitation by classic breeding.

Materials and methods

Yellow lupin germplasm material

A panel of yellow lupin accessions was assembled representing the full spectrum of diversity for this species with seed provided by the Poznań Plant Breeders Ltd. (Wiatrowo, Poland) and Plant Breeding Smolice Ltd. (Przędzalno, Poland). The panel (Table S1) comprised 111 accessions (3 wild types, 5 landraces, 4 mutants, 33 cross-derivatives / breeding lines and 66 cultivars). A mapping population of 97 recombinant inbred lines (RILs) along with parental controls (P28213 and Wodjil) was provided by the Department for Primary Industries and Regional Development (South Perth, Australia).

Phenotyping of yellow lupin phenology and vernalization responsiveness

Vernalization was performed by placing seeds for 21 days at 5°C on moist filter paper in Petri dishes in darkness. Non-vernalized control plants were sown four days before the end of vernalization treatment and grown at 24°C to maintain similar thermal time. Plants were cultivated in a greenhouse located at the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland (52°26′N 16°54′E) during the growing seasons of 2016 (sowing date 23.03), 2017 (sowing date 27.03) and 2019 (sowing date 25.03) under ambient long day photoperiods (~12–17 h). Phenology observations included bud emergence (counted as days from sowing to the first bud appearance), start of flowering (recorded when the first fully colored petal was observed) and end of flowering (recorded when most of petals on the main stem faded). The number of observed replicates varied between 3 and 10 (mean value of 6.2) depending on germination rate and plant survival during the experiments.

Calculation of heritability and interactions

The linear mixed-effect model was used to estimate variance components and predict the genetic values via single-trait BLUP (best linear unbiased prediction). A lmer function was used to fit the model [81] from lmer4 (version 1.1–29) R 4.1.0 package [82]. Using variance components, the phenotypic variance, the broad-sense heritability, the heritability on the mean basis, the selective accuracy (the correlation between the predicted and true genotypic values), a genotype-environment correlation, a genotypic coefficient of variation and a residual coefficient of variation were calculated [83]. The vernalization effect on flowering time in investigated genotypes was tested using the estimated marginal means method [84]. Using the emmeans function from emmeans (version 1.5.4) R package, a combination of effect of genotypes and vernalization from a linear mixed-effect model was used in pairwise comparison. As a multiplicity adjustment method “tukey” was applied.

Sequencing of the yellow lupin FT homologues

Coding sequences of L. angustifolius FT homologues, LanFTc1, LanFTc2, LanFTa1a and LanFTa1b [41] were aligned to the yellow lupin genome scaffolds (N=2458, N50=1.5 Mbp, unpublished) using progressive Mauve algorithm with gapped aligner MUSCLE 3.6 [85, 86] implemented in Geneious v8.1 [87]. Gene features in selected scaffolds were annotated in FGENESH+ [88] using the Glycine max model and L. angustifolius FT protein sequences as references. The nucleotide sequences of FT homologues were analyzed in four yellow lupin accessions differing in flowering time and vernalization responsiveness (PRH444/14, Wodjil, Parys and P28213). Young leaves were collected from 5-week-old plants cultivated in a greenhouse. DNA was isolated using DNaseasy Plant Mini Kit (Qiagen). Based on FT sequence annotations, a series of overlapping PCR primer pairs covering the entire gene sequences from ~8 kbp promoter to 3′ untranslated regions were designed (Table S4). The lengths of targeted genomic regions were 12 197 bp for the LlutFTa1a gene, 11 103 bp for the LlutFTb1b, 15 260 bp for the LlutFTc1 and 17 236 bp for the LlutFTc2. Standard sized (up to 2 kbp) PCR products were amplified using GoTaq G2 Flexi DNA Polymerase (Promega, Mannheim, Germany) whereas longer products used GoTaq® Long PCR Master Mix (Promega). Amplicons were directly Sanger-sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 96-capillary 3730xl DNA Analyzer (Applied Biosystems) by Genomed (Warsaw, Poland). Final FT sequences were assembled using de novo assembler in Geneious and aligned to each other using a progressive Mauve algorithm. Bayesian inference of FT coding sequences [12, 14, 41, 42] was performed as previously described [41].

Linkage mapping of FT genes and flowering time QTL loci

Molecular markers anchored in FT polymorphisms were designed to localize FT homologues on the yellow lupin linkage map [31] (Table S8). Standard agarose gel electrophoresis was used for visualization of indel markers whereas Cleaved Amplified Polymorphic Sequence (CAPS) approach [89] for single nucleotide polymorphisms (SNPs). Restriction sites and corresponding enzymes were identified using dCAPS Finder 2.0 [90]. Restriction enzymes were supplied by Thermo Fisher Scientific (Warsaw, Poland) and New England Biolabs (Ipswich, USA). Chi-square (χ2) values for Mendelian segregation in F2 RILs were estimated using the expected 1:1 segregation ratio (disregarding heterozygotes). Published marker segregation data [31] and those developed in this study were imported to Map Manager QTXb20 [91] and distributed under p-value of 0.001 to the positions at which their insertions caused the greatest increase in the sum of LOD linkage scores for adjacent loci. Kosambi function was used
to calculate map distances. Data on flowering time [31] and the updated linkage map from this study were exploited for composite interval mapping using Windows QTL Cartographer V2.5 (window size 10 cM and walk speed 0.5 cM). To test the stability of identified QTLs, calculations were performed within the range from 1 to 10 background control markers. Using the same parameters, permutation tests (x1000) were performed to establish LOD thresholds. Linkage groups were drawn using MapChart [92].

**Correlation between genotype (FT indel polymorphism) and phenotype (phenological traits)**

To survey distribution of FT indel polymorphism and find eventual novel variants in lines with contrasting phenology, yellow lupin diversity panel was screened by PCR and agarose gel electrophoresis with the same primers as those used for FT sequencing (Table S4). Putatively novel indel alleles were confirmed by Sanger sequencing. Reference alleles (Wodjil) were coded as 1, alternative alleles as 2, additional alternative alleles (the least frequent) as 3, whereas heterozygotes as 1.5 (presence of alleles 1 and 2) or 2.5 (presence of alleles 2 and 3). Association between genotype and phenotype (flowering time and vernalization response) was calculated as Spearman’s rank correlation between alleles and BLUP values. To check if the revealed associations could be considered as statistically significant by normal standards, p-value was calculated using cor.test R base function. Correlation values were visualized using heatmap function from Complexheatmap (version 1.10.2) R package [93]. Promoter regions of FT genes were annotated for hypothetical transcription factor binding sites using the Plant Promoter Analysis Navigator 3.0 [69].

**Expression profiling of yellow lupin FT genes in response to photoperiod and vernalization**

Vernalization and sowing procedures were as described above. Plants were cultivated in climatic chambers with controlled humidity (40–50% day, 70–80% night) and temperature (22°C day, 18°C night). Two levels of photoperiod were applied, short day (SD, 8 h) and long day (LD, 16 h). Young leaves were sampled every week one hour before the end of the light phase, covering the period from about 2–3 weeks before floral bud emergence until flowering (Table S12). Plant material was immediately frozen in liquid nitrogen and stored at −80°C. SV Total RNA Isolation System (Promega) was used for RNA isolation. Concentration and quality were measured using a NanoDrop 2000 (ThermoFisher Scientific). Additional quality control was performed for 60 isolates using Experion™ Automated Electrophoresis System and Experion RNA StdSens Analysis Kit (Bio-Rad, Hercules, CA, USA). The first-strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad) and 1 μg of total RNA per sample. The set of analyzed genes (Table S16) included *LlutFTa1a*, *LlutFTa1b*, *LlutFTc1* and *LlutFTc2* genes and two references – a homolog of DEAD box RNA helicase 1 (*LlutDRH1*) and beta tubulin gene (*LlutTUB7*). Gene expression profiling was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). Standard curves were developed following previously reported protocol [61]. R [2] and PCR efficiency values (Table S16) were calculated using Bio-Rad CFX Manager 3.1. Three biological replicates (each with three technical replicates) including inter-run calibration samples (*LlutTUB7*) and no-template controls were analyzed. High resolution DNA melting was performed after PCR to control the specificity of amplification. Calculations of ΔΔCq included both reference genes. Effects of growth phase (expression at analyzed date divided by expression at the first date), vernalization (x-fold change of expression after vernalization), photoperiod (x-fold change of expression of SD versus LD or vice versa) and genotype were analyzed. Statistical significance was tested using t test for mean ratio [94,95]. Calculations were made in R with custom script using “t.test.ratio” function from the matrions (version 1.4.2) package. First, the equal of variance was tested; if this condition was satisfied the classical t-test was used; otherwise, the Welch’s t-test formula was used [96]. If variances were significantly different (p-value <0.001) it was assumed that the results come from different populations and calculation was not performed [97]. To evaluate stability of reference genes during vernalization, mean efficiency-corrected Cq values obtained for reference genes were compared between vernalized and non-vernralized accesses revealing non-significant differences for all studied lines (Table S17).

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**Contributions**

MK designed the experiments. PP, SR, MMI and IJM-B performed data analysis and interpretation. MK and MNN wrote the manuscript.

**Data availability statement**

Annotated *LlutFTa1a*, *LlutFTa1b*, *LlutFTc1* and *LlutFTc2* gene sequences with promoter regions assembled for Wodjil, PRH444/14, Parys and P28213 lines as well as PCR product sequences representing other FT indel variants found in diversity panel and regions of reference genes used in quantitative PCR were deposited in the DNA Data Bank of Japan under accession numbers LC663825-LC663836, LC664023-LC664026, LC664167-LC664174, LC666892-LC666899. Custom R scripts were deposited in GitHub repository (https://github.com/igrilupin/Yellow-lupin-paper).

**Conflicts of interest**

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**Supplementary data**

Supplementary data is available at Horticulture Research online.
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