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ABSTRACT

The *FLOWERING LOCUS T* (*FT*) gene is the essential integrator of flowering regulatory pathways in angiosperms. The paralogs of the *FT* gene may perform antagonistic functions, as exemplified by *BvFT1*, that suppresses flowering in *Beta vulgaris*, unlike the paralogous activator *BvFT2*. The roles of *FT* genes in other amaranths were less investigated. Here, we transformed *Arabidopsis thaliana* with the *FLOWERING LOCUS T like* (*FTL*) genes of *Chenopodium ficifolium* and found that both *CfFTL1* and *CfFTL2–1* accelerated flowering, despite having been the homologs of the *Beta vulgaris* floral promoter and suppressor, respectively. The floral promotive effect of *CfFTL2–1* was so strong that it caused lethality when overexpressed under the *35S* promoter. *CfFTL2–1* placed in an inducible cassette accelerated flowering after induction with methoxyphenozide. The spontaneous induction of *CfFTL2–1* led to precocious flowering in some primary transformants even without chemical induction. The *CqFT2–1* homolog from *Chenopodium quinoa* had the same impact on viability and flowering as *CfFTL2–1* when transferred to *A. thaliana*. After the *FTL* gene duplication in Amaranthaceae, the *FTL1* copy maintained the role of floral activator. The second copy *FTL2* underwent subsequent duplication and functional diversification, which enabled it to control the onset of flowering in amaranths to adapt to variable environments.

SUMMARY

The *FLOWERINGLOCUS T like 2–1* gene of *Chenopodium ficifolium* and*Chenopodium quinoa* acts as a strong activator of flowering in Arabidopsis, triggering flowering at cotyledon stage and causing lethality when overexpressed.

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Chenopodium; flowering; *FLOWERING LOCUS T like* genes; floral induction; lethality

Introduction

The decision when to flower is one of the most important commitments in a plant's life since it directly impacts evolutionary success of the species. The formation of flowers and seeds requires re-allocation of resources from the entire plant to maximize reproductive success, which is often followed by senescence in annuals. The proper timing of flowering helps the plant to balance reproductive cost and benefit. The onset of flowering is precisely controlled by environmental conditions (daylength, cold temperature in winter, ambient temperature, abiotic stress) as well as by endogenous factors (age, phytohormone concentrations, carbohydrate status).[1–3]

The central position at the crossover of the signaling pathways is occupied by the FLOWERING LOCUS T (FT) protein, which is the important part of the long-sought florigen.[4] in *Arabidopsis thaliana* (hereafter Arabidopsis).[5,6] and other species.[7,8] The FT protein is produced in the phloem companion cells of the leaves and transported to the apical meristem to trigger flowering.[9] The *FT* gene underwent duplications during the evolution of angiosperms and its paralogous copies occasionally acquired the opposite function as flowering suppressors.[10] The pair of floral integrators in *Beta vulgaris*, the sugar beet, which includes the BvFT2 protein as a floral promoter and BvFT1 as a floral repressor,[11] exemplifies this dual functionality of FT. The *BvFT1* and *BvFT2* genes repressed or promoted flowering, respectively, when ectopically expressed in sugar beet and Arabidopsis. The reversal of the function from the activation to the inhibition of flowering was caused by three amino acid substitutions in the functional domain of the fourth exon of the *BvFT* genes.[11]

The orthologs of *BvFT2* and *BvFT1* were found in all members of the family Amaranthaceae so far analyzed.[12] The *CrFTL1* gene in *Oxybasis rubra* (syn. *Chenopodium rubrum);*[13] promoted flowering in Arabidopsis in the same way as its sugar beet ortholog *BvFT2*.[14] After the early duplication, which gave rise to the *FT1* and the *FT2* paralogs, a subsequent gene duplication took place after the ancestor of *Beta* had diverged from the ancestors of *Oxybasis* and *Chenopodium*. This event generated two *FTL2* copies, *FTL2–1* and *FTL2–2*,[15] which are next to each other in the quinoa (*Chenopodium quinoa*) genome.[16] as evidence of this tandem duplication. The annotation of the *FTL* genes in *C. quinoa*.[16] follows the denomination derived from sugar beet genes.[11] The

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CfFTL1 gene has two counterparts in tetraploid *C. quinoa* - *CqFT2A* and *CqFT2B*.[17] *CfFTL2*-1 is homologous to the *CqFT1A* and *CqFT1B1–1* genes, and finally, the *CfFTL2–2* gene is homologous the *CqHD3AA* and *CqFT1B–2* genes in *C. quinoa*,[17] where A and B in the gene name refer to A or B subgenome. In our work, we use the gene nomenclature derived from *Oxybasis rubra*,[13] because the amaranth *FT* genes were discovered here first (Table 1). Another reason is the better correspondence of the annotation to the phylogeny. The *FTL2–1* and *FTL2–2* names indicate the duplication of the ancestral *FTL2* gene.[12]

Unlike the *BvFT1* gene, which was shown to act as floral repressor,[11] the function of its homologs in *Chenopodium* is less known. A detailed gene expression study of numerous flowering-related genes in numerous *C. quinoa* accessions with contrasting photoperiod response was conducted by Patiranage et al.[17] The expression of the *FTL* genes in the course of floral induction in seedling stage was investigated in *C. ficifolium* and *C. suecicum*,[18,19] the close diploid relatives of the donor of the subgenome B of tetraploid *C. quinoa*.[20,21] Whereas *CsFTL2–1* in *C. suecicum* was highly activated by short days, inducing flowering, negligible expression of this gene was observed in *C. ficifolium* under both short and long photoperiods. The low expression of *CfFTL2–1* was particularly noteworthy in the long-day accession *C. ficifolium* 283, which flowered earlier under long days without the apparent activation of any *CfFTL* gene.[18] The *CrFTL2–1* homolog in *O. rubra* was completely silenced,[18] which excludes any role in floral induction in this species. Thus, the expression of the *FTL2–1* paralog varied among the *Chenopodium-Oxybasis* species and accessions.

The second paralog *FTL2–2* varied in expression profiles across the species, too. It was strongly upregulated in *C. suecicum* and in the long-day accession *C. ficifolium* 283 under the floral induction conditions.[18] In contrast, the *CrFTL2–2* gene of *O. rubra* exhibited invariant expression, not correlated with flowering. It also did not promote flowering in Arabidopsis, which indicated no participation in floral transition.[14] The *FTL2–2* gene underwent dynamic structural evolution. Unlike the *FTL2–1* paralog, which contains four conserved exons and three introns similarly to the other angiosperm *FT* genes, the *FTL2–2* gene acquired an additional exon and intron.[12] Whereas the complete *FTL2–2* gene exists in *O. rubra* and *C. suecicum*, the large deletion of 130 bp shortened the fourth exon of *CfFTL2–2* in *C. ficifolium* 283 and the entire *CfFTL2–2* gene was deleted in *C. ficifolium* 459. The changes in gene expression and structure, which affected *FTL2* paralogs after its duplication, might have influenced their function and led to sub- or neo-functionalization.

To better understand the function of the *Chenopodium FTL* genes, we transferred the *CfFTL1, CfFTL2–1*, and *CfFTL2–2* genes of *C. ficifolium* to both wild types and *ft*$^{-}$ mutants of Arabidopsis and analyzed the flowering phenotypes of the transformants. The *CfFTL1* overexpression accelerated flowering in all Arabidopsis genetic backgrounds (wild types and *ft* mutants), while the *CfFTL2–2* overexpression had no effect on flowering. Surprisingly, *CfFTL2–1* overexpression was lethal in Arabidopsis and the vector with the inducible expression of the *CfFTL2–1* gene had to be constructed to observe the impact of this gene on flowering in Arabidopsis after chemical induction.

As *C. ficifolium* is closely related to *C. quinoa*, we were curious, whether the surprisingly strong effect of *CfFTL2–1* on flowering in Arabidopsis would be also observed with its *C. quinoa* homolog *CqFTL2–1* (described as *CqFT1B–1)* by.[17] We transformed Arabidopsis with *CqFTL2–1* and found exactly the same flowering phenotypes as with *CfFTL2–1*. Our results indicate that *CfFTL1*, *CfFTL2–1* and *CqFTL2–1* promote flowering in Arabidopsis.

Materials and methods

Preparation of gene constructs for the transformation of Arabidopsis

All constructs used in this work were assembled using the GoldenBraid standard.[22] The sequences of the *Chenopodium FTL* genes can be found under the following GenBank accession numbers: (*CfFTL1* - MK212025; *CfFTL2-1* - MK212027; *CfFTL2-2* - MK212026, *CqFTL2–1* - XM_021919867). The open reading frames (ORF) were amplified from *C. ficifolium* 283.[18] et or *C. quinoa* QQ74.[16] cDNA using Phusion polymerase (Thermo Scientific) and primers designed using the GB-domesticator on the GBcloning website (https://gbcloning.upv.es) (Supplementary Table S1). Forty ng of the amplified and column-purified (Qiagen) DNA was cloned into the universal domestication plasmid pUPD2 by restriction ligation reaction with BsmBI and T4 ligase (both Thermo Scientific), and selected clones were verified by Sanger sequencing (Eurofins, Germany). The first set of plasmids was designated for constitutive expression of the respective *CfFTL* gene in Arabidopsis. In these vectors the *CfFTL* ORFs were under transcriptional control of the *CaMV 35S* promoter and terminator. The expression levels were increased by Tobacco mosaic virus Omega leader sequence. For inducible expression we modified the methoxyfenozide inducible system VGE.[23] to comply with GoldenBraid standard. The cassette containing the inducible CfFTL2–1 gene was flanked by two tobacco Matrix attachment region (MAR) elements TM2.[24] and RB7.[25] They were designed to reduce position effect and stabilize the variation of transgene

Table 1. The names of the *FTL* genes in the family Chenopodiaceae, arranged in the chronological order of the respective references. Two names for the *C. quinoa* genes are given in the last column, because Patiranage et al..[17] labeled the homeologs differently.

Species	*Oxybasis rubra*	*Beta vulgaris*	*Oxybasis rubra*	*Chenopodium ficifolium*	*Chenopodium quinoa*	*Chenopodium quinoa*
Reference	13	11	18	18	18	18
HomologousGenes	*CrFTL1*	*BvFT2*	*CrFTL1*	*CfFTL1*	*CqFTL1, CqFTL2–2*	*CqFT2A, CqFT2B*
Genes	*CrFTL2*	*BvFT1*	*CrFTL2–1*	*CfFTL2–1*	*CqFTL2–1*	*CqFT1A, CqFT1B1–1*
			CrFTL2–2	*CfFTL2–2*	*CqFTL2–2*	*CqHD3AA, CqFT1B–2*

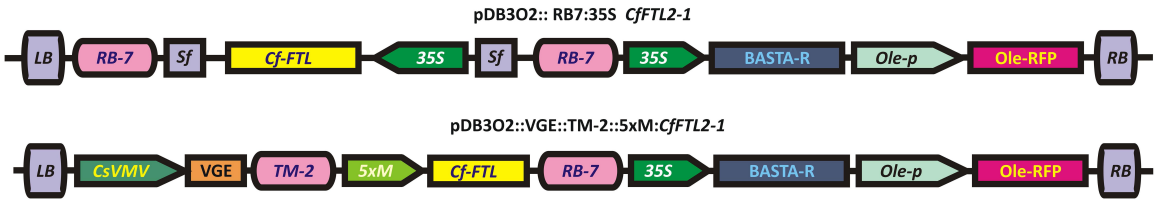

Figure 1. Schematic representation of T-DNA constructs used for the transformation of Arabidopsis. *LB*, *RB* – left and right T-DNA borders respectively; RB-7, TM-2 –matrix attachment regions from tobacco; *Sf*- short stuffer fragment 35 bp, *Cf-FTL* – *C. ficifolium FTL* ORF, *35S* – Cauliflower mosaic virus 35S promotor; BASTA-R phosphinothricin N-acetyltransferase gene conferring tolerance to Basta herbicide; *Ole-p* - oleosin promotor from Arabidopsis, *Ole-RFP* – gene for RFP reporter protein fused to Arabidopsis oleosin; *CsVMV* promotor from Cassava vein mosaic virus; *VGE* - chimeric transcription factor VGE reactive to methoxyfenozide, *5×M* – minimal *35S* promoter fused with 5 copies of Gal4 binding domain. Not drawn to scale.

expression among individual transgenic lines. They also reduced the likelihood that the transgene might trigger gene silencing, resulting in a gradual loss of transgene expression in T2 and further generations (Figure 1). All used components are summarized in Supplementary Table S2. The final binary constructs used for Arabidopsis transformation were assembled using the extended set of vectors alpha 11–14.[26]

Arabidopsis transformation

The plasmid vectors with a cassette were transferred into *Agrobacterium tumefaciens* strain EHA105.[27] using the freeze-thaw method of.[28] Arabidopsis wild types (Landsberg *erecta* L*er* or Columbia-0 Col-0) or *ft* mutants (CS56 *ft-1*, Cs185 *ft-3*) were transformed by the floral dipping method.[29] Primary transformants (T1 generation) were selected by spraying 120 mg l^{-1} BASTA© (Glufosinate-ammonium; Bayer, Germany, 150 g l^{-1}) three times at 3–7 day-intervals, starting with 7 day-old seedlings grown on soil. T1 plants were self-pollinated to produce T2 generation. T2 seeds carrying the insertion were identified based on red fluorescence using LEICA microscope (DM5000B) with LEICA CTR5000 light source. The segregation ratio was 3 : 1 (fluorescent : non-fluorescent seeds), which corresponds to Mendelian segregation ratio and is consistent with the presence of the single insertion. T3 progeny was obtained by self-pollination from the T2 homozygous lines, which produced homogenous progeny (all seeds were fluorescent). The presence of transgenes was verified by PCR amplification with BAR_F and BAR_R primers, and with the primers targeted to the *FTL* genes (Suplementary Table S1).

Plant growth conditions and phenotypic scoring

Arabidopsis seeds were stratified for 2 days at 4°C and sown on Jiffy-7 tablets (41 mm diameter, Jiffy Products International AS, Norway). At 10 days, seedlings were transplanted individually to new Jiffy-7 tablets. The donor plants used for floral dipping were grown in a cultivation room under long days (16 h : 8 h light : dark) at 20°C. Transgenic and control plants were grown in cultivation chamber E-36L2 (Percival Scientific, Perry, IA, USA) under 12 h : 12 h light : dark, 130 µmol m^{-2} s^{-1} light intensity, and 70% relative humidity 23°C at day and 22°C at night since germination until flowering. To measure flowering time, the number of rosette leaves at bolting was counted. The data were analyzed by one-way ANOVA, honestly significant differences (HSD) were determined by Tukey test, implemented in IBM SPSS Statistics.

CfFTL2–1 induction in transgenic Arabidopsis

Transgenic plants carrying the *CfFTL2–1* gene under the control of methoxyphenozide-inducible transcription factor (*VGE: TM-2:5×M:CfFTL2–1*), which were capable of reproduction to produce the T3 generation (30 individuals of each line), were subjected to induction treatment. Plants were grown in the Percival growth chamber under cultivation conditions as described above. A solution of 65 µM methoxyfenozide (Integro, Corteva).[23] was sprayed on plants three times with three-day intervals between applications, starting at the 6–9 leaf-stage (at the age of 4 weeks). The control plants were not chemically treated. The same experiment was conducted with untransformed Arabidopsis of the same genetic background as transgenic plants. Leaves for RNA extraction were sampled from six randomly selected plants immediately before the application of methoxyfenozide and from the same plants again at bolting, when leaf number was also determined.

RNA extraction and cDNA preparation

Total RNA was extracted using the Plant RNeasy Mini kit (Qiagen, Valencia, CA, USA). DNA contamination was eliminated by DNase I treatment according to the manufacturer's protocol (DNA-free, Ambion, Austin, TX, USA). If necessary, the DNase treatment was repeated to remove any traces of genomic DNA. RNA quality and concentration were checked on a 0.9% agarose gel and by NanoDrop (Thermo Fisher Scientific, Vantaa, Finland). RNA was heated together with oligo dT primers (500 ng) for 5 min at 65°C, chilled on ice and mixed with Transcriptor buffer (Roche, Diagnostics, Mannheim, Germany), 0.5 µl of Protector RNase Inhibitor (Roche), 2 µl of 10 mM dNTPs and 10 units of Transcriptor Reverse Transcriptase (Roche). Single-strand DNA (cDNA) was synthesized from 1 µg of RNA at 55°C for 30 min.

Quantitative PCR

qPCR was performed on the LightCycler 480 platform (Roche) with LC SYBR Green I Master (Roche) in a final volume of 10 µl with 500 nM of each of the primers (Supplementary Table S1). The program was: 10 min of initial denaturation at 95°C, then 40 cycles for 10 s at 95°C, 10 s at 60°C (at 58°C for

AtUBQ10), followed by 15 s at 72°C. Stable expression of the reference gene *AtUBQ10* was confirmed previously as described by Libus and Štorchová.[30] The PCR efficiencies were estimated based on serial dilutions of cDNAs and used to calculate relative expression using the formula E_R^{CpR}/E_T^{CpT}, where ET/ER represents the PCR efficiencies of the sample and reference, respectively, and CpT/CpR represents the cycle number at the threshold (crossing point).

Results

CfFTL1, *but not* CfFTL2–2, *accelerated flowering in Arabidopsis*

Primary transformants (T1 generation) of Arabidopsis in Col-0, L*er* and *ft-3* genetic backgrounds carrying *CfFTL1* under the control of the strong constitutive promoter *35S* flowered early, after forming about four rosette leaves (Table 2). However, only some of them were able to produce viable seeds and give rise to further generations, the rest of T1 plants died after early flowering without progeny (Table 2).

We estimated leaf numbers and measured expression of the *CfFTL1* transgene in independent lines in all three genetic backgrounds in T2 and T3 generations. We found significantly lower leaf number at flowering in the transformants compared with wild type or *ft-3* mutant, which implied accelerated flowering in the lines expressing the transgene (Figure 2). One of five Col-0 transgenic lines did not express *CfFTL1* in any generation and its flowering time did not differ from the wild type. Another line flowered earlier than the wild type only in the T2, not in the T3 generation, which agreed with transgene expression in the respective generation. The decline of transgene expression suggested its gradual silencing, which might have also caused the high variation observed in transgene expression levels among the lines. The absence of accelerated flowering in the lines not expressing the transgene indicates that the transgene activity is responsible for this effect in Col-0 (Figure 2a).

It was difficult to gain permanent *CfFTL1* transgenic lines in the *Ler* background. Repeated transformation yielded 12 primary transformants, but only three of them were able to produce progeny. They silenced the transgene, except one lineage, which exhibited *CfFTL1* expression in the T1 generation and, unlike the others, accelerated flowering (Figure 2b).

The promotion of flowering was particularly prominent in the CS185 *ft-3* mutant with the *CfFTL1* transgene (Figure 2c). Whereas *ft-3* mutants flowered very late after producing about 40 rosette leaves, the transgenic lines flowered early with 4–5 rosette leaves, similarly to transgenic plants derived from wild type genetic backgrounds and overexpressing the transgene. Alike the Col-0 and *Ler* primary transformants, the CS185 plants carrying the *CfFTL1* gene often died without progeny. We also transformed CS56 *ft-1* mutant with this gene, but we recovered no primary transformants.

The Col-0 transgenic lines with *CfFTL2–2* under the control of the *35S* promoter flowered at the same time as the Col-0 wild type, while the L*er* transgenic lines flowered later than the L*er* wild type, particularly in the T2 generation (Figure 3).

Some Arabidopsis seedlings with the inducible CfFTL2–1 *transgene flowered immediately after germination*

We were unable to recover Arabidopsis transformants with the *35S::CfFTL2–1* construct, despite repeated floral dipping experiments. Then, we noticed several seedlings dying somewhat later after the Basta application. The amplification of their DNA with specific primers (Supplementary Table S1) confirmed the presence of the *CfFTL2–1* transgene. Thus, transformation of Arabidopsis with *CfFTL2–1* gene under the *35S* promoter was lethal.

To understand the impact of *CfFTL2–1* on Arabidopsis, we placed this gene to the VGE inducible system,[23] which enables induction of the transgene by methoxyfenozide. Two types of primary transformants were obtained after the transformation with *VGE:TM-2:5×M:CqFTL2–1* (Table 2). About a half of primary transformants flowered immediately after expanding cotyledons. They produced tiny flowers, sometimes with well-developed stigmas, or small flower buds with prominent trichomes (Figure 4). All these plants died early without producing seed. As no methoxyphenozide was used, premature

Table 2. The numbers of all primary transgenic lines and the lines capable reproduction, obtained by the transfer of the *FTL* genes of *C. ficifolium* and *C. quinoa* to Arabidopsis wild types and *ft* mutants.

Transgene casette	Arabidopsis genetic background	Primary transgenic lines	Lines producing seed	Lines producing progeny
35S:CfFTL1	Col-0	16	12	5
	Ler	12	6	3
	CS185 ft-3	10	6	2
	CS56 ft-1	0	0	0
35S:CfFTL2–2	Col-0	10	10	10
	Ler	20	20	20
35S:CfFTL2–1	Col-0	0	0	0
	Ler	0	0	0
	CS185 ft-3	0	0	0
	CS56 ft-1	0	0	0
VGE:TM-2:5×M:CfFTL2–1	Col-0	>100	20	6
	Ler	24	4	2
	CS185 ft-3	3	0	0
	CS56 ft-1	28	3	2
VGE:TM-2:5×M:CqFTL2–1	Col-0	>100	23	15
	Ler	>50	22	18
	CS185 ft-3	3	0	0
	CS56 ft-1	>50	10	6

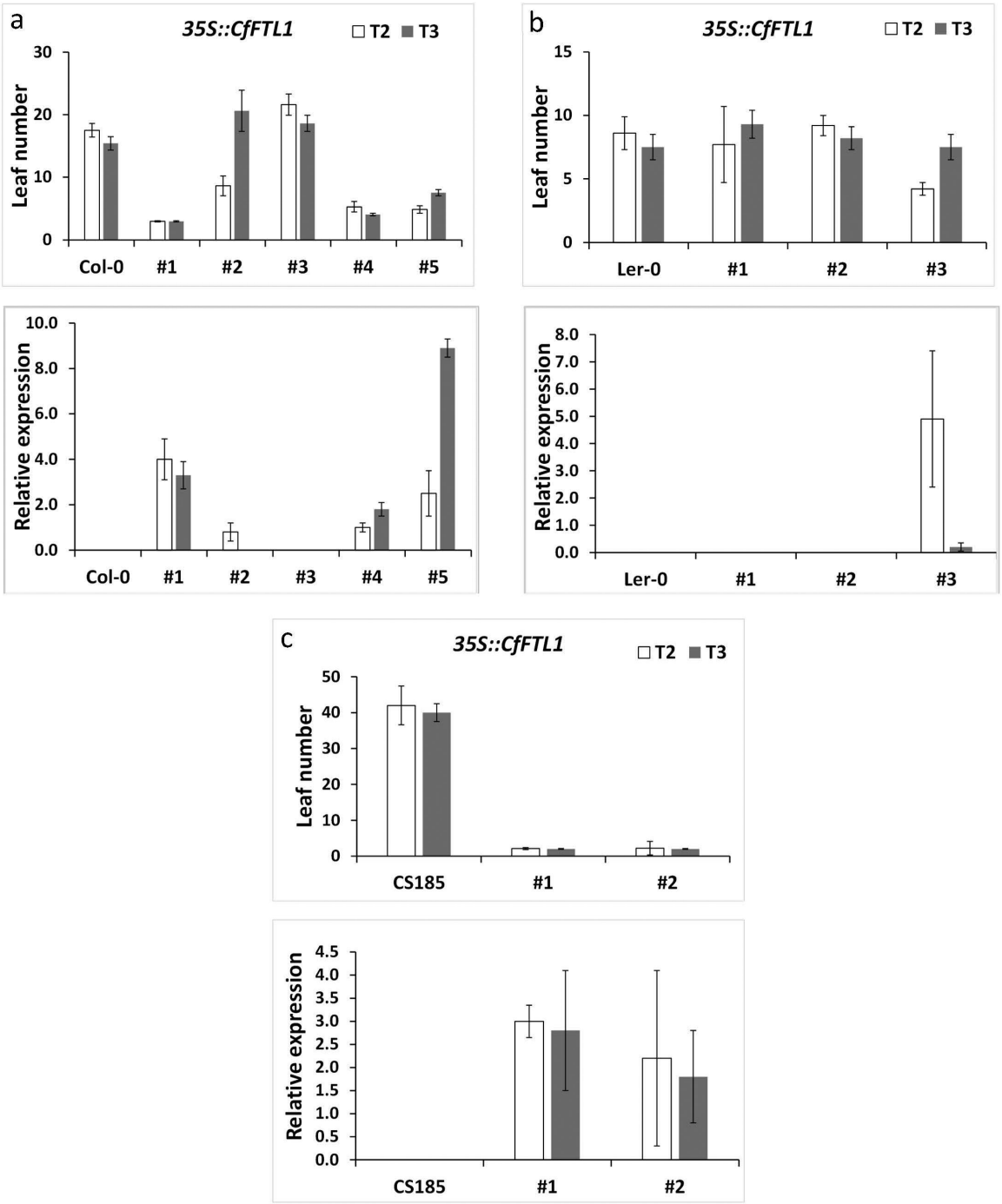

Figure 2. The number of rosette leaves and relative transgene expression at flowering time in Arabidopsis transformed with the *CfFTL1* gene under the *35S* promoter in the T2 and T3 generations. a. The *CfFTL1* transformants in the Col-0, b. Ler, and c. CS185 *(ft-3)* backgrounds. The averages and standard deviations were calculated from 20 to 35 plants of the respective independent lineages, which are labeled by the numbers on the x axis. Asterisks represent honestly significant difference (HSD) estimated by Tukey test.

flowering had to be caused by the spontaneous leakage in the *CfFTL2–1* expression. We estimated transgene expression in six early flowering transgenic seedlings (Figure 5). Other *CfFTL2–1* primary transformants did not differ from recipient plants (*ft* mutants or wild types) in their flowering phenotypes. These plants gave rise to transgenic lines, in which the transgene was not expressed without methoxyphenozid induction.

The transformation of Arabidopsis with the *VGE:TM-2:5×M:CqFTL2–1* construct, bearing the *CqFTL2–1* gene of *C. quinoa* QQ74, provided the same results as transformation with its *C. ficifolium* ortholog. Many primary transformants flowered just after germination and died (Table 2).

Arabidopsis carrying the inducible CfFTL2–1 *transgene accelerated flowering after methoxyfenozide application*

We selected transgenic lines with inducible *CfFTL2–1* in the Col-0 and mutant CS56 *ft-1* genetic backgrounds to investigate

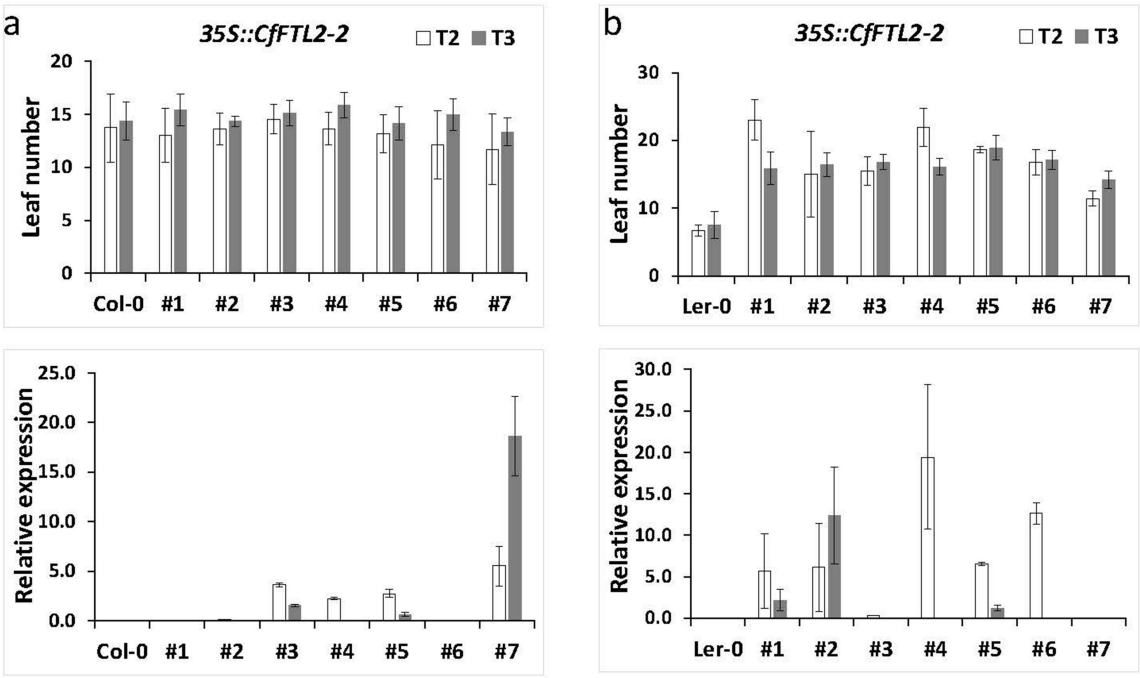

Figure 3. The number of rosette leaves and relative transgene expression at flowering time in Arabidopsis transformed with the *CfFTL2–2* gene under the *35S* promoter in the T2 and T3 generations. a. the *CfFTL2–2* transformants in the Col-0, and b. Ler backgrounds. The averages and standard deviations were calculated from 20 to 35 plants of the respective independent lineages, which are labeled by the numbers on the x axis. Asterisks represent honestly significant difference (HSD) estimated by Tukey test.

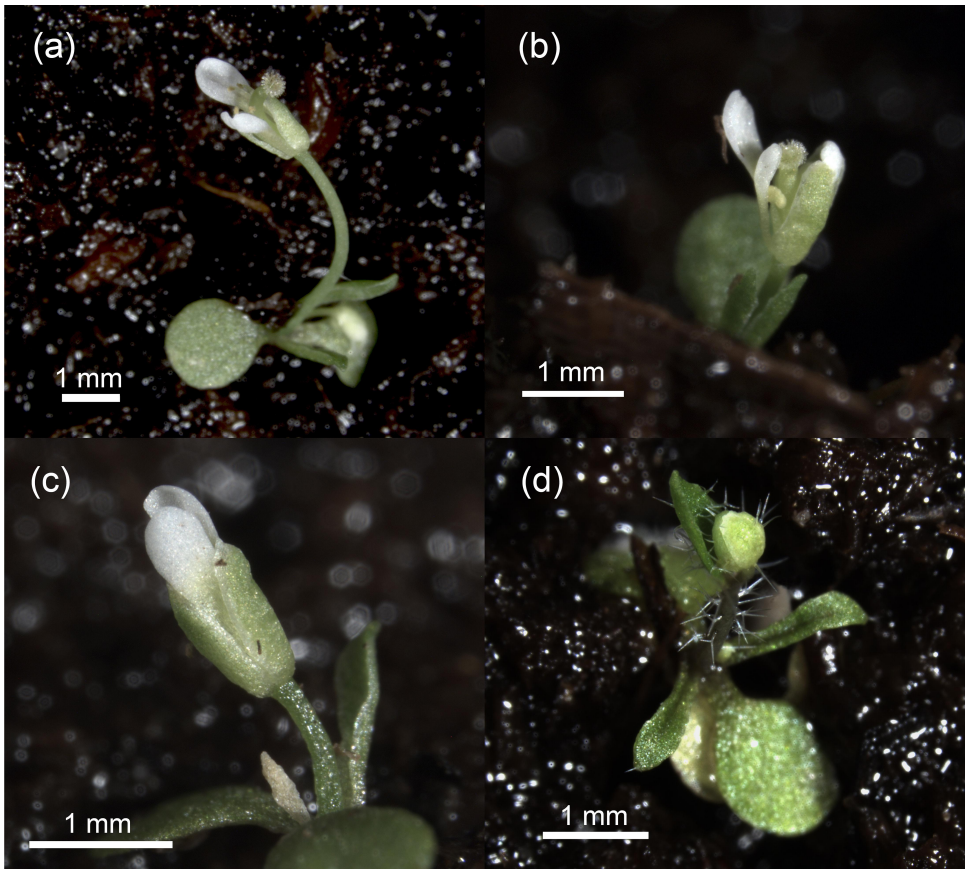

Figure 4. Phenotypes of primary transformants of Arabidopsis Col-0 carrying *CfFTL2–1* under the complex metoxyfenozide-inducible promoter (*Vge:TM-2:5×m:cfftl2–1*), which flowered without chemical induction. Plants started to bolt immediately after germination. Some of them formed minuscule flowers (a, b, c), others produced tiny flower buds with long trichomes (d). All the plantlets died without generating viable seed. Photo: Lukáš Synek.

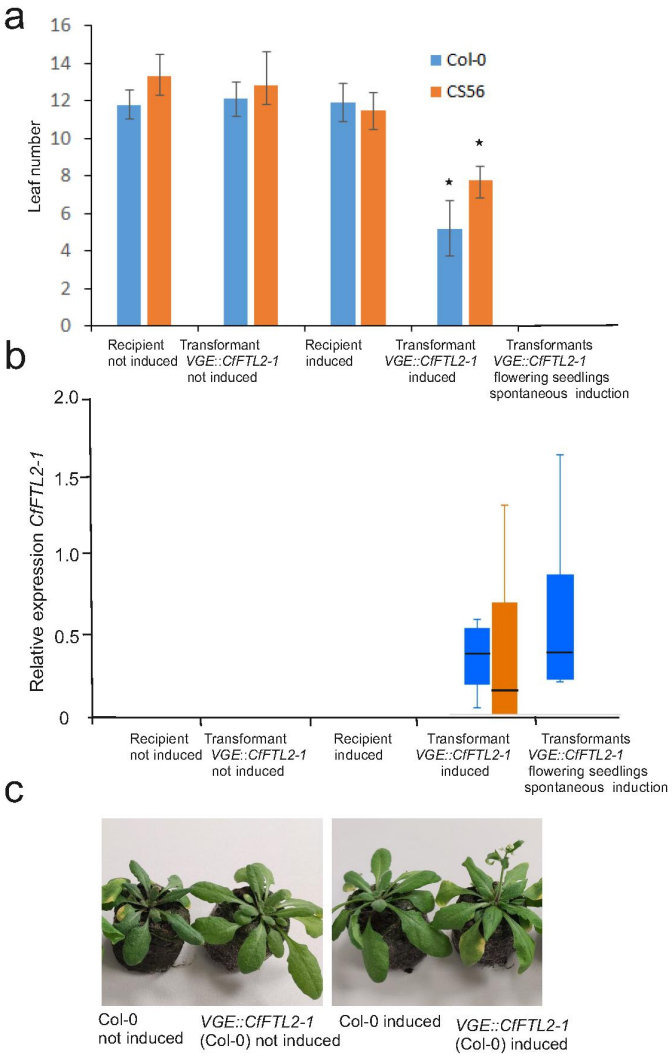

Figure 5. The acceleration of flowering and the *CfFTL2–1* gene expression in Arabidopsis carrying the *CfFTL2–1* transgene. a. The number of rosette leaves formed since the time of metoxyfenozide treatment till flowering in Col-0 and CS56 (Ler *ft-1*) backgrounds, calculated as the average with standard deviation from 20–30 plants of the same homozygous transgenic line. Asterisks represent honestly significant difference (HSD) estimated by Tukey test. The seedlings (6 primary transformants in Col-0 background) with the spontaneously induced transgene flowered without forming rosette leaves. b. The *CfFTL2–1* gene expression relative to the reference *AtUBQ10* in induced and not induced plants and in spontaneously induced transformants (6 individuals) at flowering time. Median, the first and third quartile, maximum and minimum values are shown. c. The pictures of Col-0 and transgenic Arabidopsis plants taken 9 days after the metoxyfenozide treatment.

the function of this gene. We wished to demonstrate the induction by methoxyfenozide in both Arabidopsis ecotypes – Col-0 and Ler. We chose the mutant CS56 *ft-1* derived from Ler, which flowered later than Ler wild type (and approximately at the same time as Col-0 wild type), making the proper timing of the induction easier and comparable between the two genetic backgrounds. The transgenic plants flowered significantly earlier after the application of methozyphenozide than the untransformed Col-0 and CS56 *ft-1* plants after the same application, or the transgenic plants not induced by methozyphenozide (Figure 5a). The acceleration of flowering was accompanied by activation of the transgene (Figure 5b). Whereas *CfFTL2–1* expression was negligible before methoxyfenozide application, it increased dramatically after this treatment. The transgene transcript levels varied considerably among individual plants of the same line, as documented by Figure 5b. However, the plants with both high and low *CfFTL2–1* expression flowered approximately at the same time after forming similar numbers of leaves. The rather uniform effect of variable transgene expression may be explained by the existence of a threshold value necessary for floral induction. After crossing the threshold, additional *CfFTL2–1* expression did not further accelerate flowering.

Discussion

The CfFTL1 *and* CfFTL2–1 *genes activate flowering in Arabidopsis, but their impacts differ*

The *CfFTL1* and *CfFTL2–1* expression in Arabidopsis promoted flowering in both wild types and *ft* mutants, which is consistent with their roles as floral activators. This finding is not unexpected, because the CfFTL1 and CfFTL2–1 proteins share the same sequence with most angiosperm FT floral activators, including the sugar beet floral promoter BvFTL2, in the functionally important region in external loop of the protein.[18] They do not possess amino acids Asn(N)134, Gln(Q) 141, and Gln(Q)142 responsible for the suppression of flowering in the sugar beet floral inhibitor BvFT1.[11] The three amino acid substitutions that converted BvFT1 function in sugar beet from the activation of flowering to its opposite most likely occurred after the *Beta* ancestor had diverged from the *Chenopodium* ancestor.

The impact of the *CfFTL1* and *CfFTL2–1* expression on Arabidopsis development differed substantially between the two genes. The overexpression of *CfFTL1*, driven by the constitutive *35S* promoter, accelerated flowering in transgenic lines. In contrast, the overexpression of *CfFTL2–1* was lethal for Arabidopsis seedlings. To estimate its function, we had to place this gene into the inducible cassette and to induce it with methoxyfenozide. Because the selection of primary transformants occurred in the absence of the chemical inducer, we expected the same flowering behavior in both transformants and recipient plants. Surprisingly, a large proportion of transformants started to flower immediately after expanding cotyledons and died without forming seeds. The VGE casette with the *CfFTL2–1* gene is protected against transcription from adjacent DNA by tobacco MAR elements.[24,25] However, even such isolation from the genomic background is not absolute and may lead to leaky transgene expression in some primary transformants, depending on the specific site of insertion. Premature flowering exhausts resources, which prevents transgenic plants from the production of viable seeds. The sudden reprogramming from vegetative growth to the reproduction in the very early developmental stage can be also responsible for the lethality of the *CfFTl2–1* overexpression driven by the *35S* promoter. In this case, seedlings died after germination, having formed only cotyledons.

Transformation of Arabidopsis with the *CfFTL1* gene under the control of the *35S* promoter generated some permanent transgenic lines, but many primary transformants did not produce viable seed. The successfully reproducing lines often silenced the *CfFTL1* transgene, which effect persisted to the next generation. It is therefore likely that the *CfFTL1*

overexpression also interfered with growth and reproduction in Arabidopsis, similarly to the *CfFTL2–1* transgene, although this effect was much weaker than in the case of *CfFTL2–1*. Gene silencing could have been transmitted to the next generation by epigenetic mechanisms.[31]

The FTL2 *gene duplication in* Chenopodium *enabled the diversification of their functions*

The *CfFTL2–1* gene is one of two products of the *FTL2* gene duplication, which occurred after the divergence of *Chenopodium* from *Beta*. The second duplicate *CfFTL2–2* harbors a large deletion, which removed 130 bp of the fourth exon including the motifs necessary for the function of this gene.[18] The observation that the *CfFTL2–2* overexpression did not affect flowering in Arabidopsis Col-0 is in line with this notion. On the other hand, the delay in flowering was found in the L*er* background, no matter whether the transgene was expressed or not. Thus, this delay cannot be caused by the activation of *CfFTL2–2*, but rather by the effect of some other component of the construct. The difference between the Ler and Col-0 could have been caused by distinct pace of floral induction in the two ecotypes. Ler wild type flowered earlier than Col-0 wild type and thus the mode of interaction with the transgene expression might have occurred differently.

Unlike *CfFTL2–1*, the *CfFTL2–2* paralog in *C. ficifolium* did not accelerate flowering in Arabidopsis, most likely due to the large deletion removing functionally important amino acids. However, it is possible that this particular gene participates in the regulation of flowering in other *Chenopodium* species, *e.g.* in *C. suecicum*, where it is present in a complete form and is rhythmically expressed during floral induction.[18]

The *FT* gene sequences are highly conserved among angiosperms and thus are expected to maintain their function when transferred to phylogenetically unrelated species. For example, overexpression of *PnFT1* of *Pharbitis nil*,[7] *CrFTL1* of *O. rubra*,[13] *BvFT2* of sugar beet,[11] or *GmFT2a* of soybean.[32] promoted flowering in Arabidopsis. However, we have not found any report of lethality caused by the ectopic expression of an angiosperm *FT* gene in Arabidopsis. The underlying cause of lethality due to *CfFTL2–1* overexpression may be immediate floral induction during germination of Arabidopsis seedlings, which is stronger and faster than the activation of flowering controlled by other angiosperm *FT*s, including *CfFTL1*. Because *Chenopodium* is recalcitrant to stable transformation with *Agrobacterium*, we were unable to effect transformation in a homologous system. We are currently running the experiments with virus-induced gene silencing in *Chenopodium* to confirm our conclusions.

As *CfFTL2–1* acts as a powerful promoter of flowering, then we may better understand the results of the study of photoperiodic floral induction in *C. ficifolium*.[18] The accession 459 highly upregulated *CfFTL1* under short days when its flowering was accelerated, which was consistent with the promotional role of this gene. In contrast, the long-day accession 283 flowered earlier under long days without apparent activation of any *FTL* gene. However, when *CfFTL2–1* encodes a very strong promoter of flowering, even a very low increase in *CfFTL2–1* transcription, not detected by RT qPCR, could accelerate flowering under long days. We are now testing this hypothesis by the comprehensive analysis of the global transcriptomes during photoperiodic floral induction in *C. ficifolium* 283.

Chenopodium ficifolium was proposed as a potential diploid model species for the genetic analyses of the tetraploid crop *C. quinoa*.[33] We have therefore transformed Arabidopsis with the inducible *CqFTL2–1* gene of *C. quinoa* to see whether this would result in the same outcome as the transfer of its *C. ficifolium* ortholog. The CfFTL2–1 and CqFTL2–1 proteins differ only in two amino acid substitutions, located outside the functionally important regions. Hence, it is not unexpected that the transformation of Arabidopsis with inducible *CqFTL2–1* would produce the same results as with the inducible *CfFTL2–1* gene; namely, the appearance of many tiny, precociously flowering primary transformants. However, the *CqFT1B–1* (identical to *CqFTL2–1*) was considered unlikely to function as flowering time regulators in *C. quinoa* based on its transcriptional profile.[17] If *CqFTL2–1* functions as a strong floral activator in *C. quinoa*, even small hard to detect changes in gene expression could promote flowering. The identification of floral activators in *C. quinoa* may also have practical importance for quinoa breeding, particularly as the crop spreads to areas of the globe where short- and long-day flowering responses would be advantageous for increasing yields through heat-stress avoidance during the normal flowering period. Our work supports the usefulness of *C. ficifolium* as a diploid model to be compared with tetraploid *C. quinoa*.

Our results are also interesting from the perspective of the evolution of gene function. The BvFT1 protein became the repressor of flowering after the genus *Beta* had diverged from *Chenopodium* within the Chenopodiaceae-Amaranthaceae, whereas the CfFTL2–1 protein retained its original function as a floral promoter. The prevailing hypothesis is that activation of flowering was the most likely ancestral role of FTL2 because CfFTL2–1 shares three functionally important amino acids with FT activators, not with its ortholog BvFT1. The *FTL2–1* genes of *C. ficifolium* and *C. quinoa* triggered precocious flowering in Arabidopsis seedlings despite being homologs of the *BvFT1* floral repressor. This finding illustrates the distinct evolutionary trends of two *FT* paralogs which diverged early during the evolution of in the family Amaranthaceae.[12] The *BvFT2*, *CfFTL1* genes and their orthologs retained a conserved gene structure and floral activator function. In contrast, *BvFT1, CfFTL2–1, CfFTL2–2* and their orthologs underwent prominent structural changes including exon acquisition, large deletions or

complete loss, and functional diversifications. Thus, the *FT1*/*FTL2* lineage became a versatile toolkit of the evolution enabling the adaptation of annual fast-cycling amaranths to variable environments.

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HS: conceptualization; OAJA, CB, TM, D G-L, KE, ZV and HS: methodology; OAJA, MK and DG-L: resources; HS: writing – original draft; OAJA, DG-L, TM, MK and HS: writing – review & editing; HS: supervision; HS: funding acquisition.

Abbreviations

CaMV, Cauliflower Mosaic Virus; *FT*, *FLOWERING LOCUS T*; *FTL*, *FLOWERING LOCUS T like*; MAR, Matrix attachment region; *UBQ*, *UBIQUITINE*;

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