Received: 8 September 2021 | Accepted: 27 September 2021

DOI: 10.1111/mpp.13151

Molecular Plant Pathology WILEY

ORIGINAL ARTICLE

A role of flowering genes in the tolerance of *Arabidopsis thaliana* to cucumber mosaic virus

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Funding information
Agencia Estatal de Investigación, Spain, Grant/Award Number: PID2019-104249GB-I00, RTI2018-094302-B-I00, RTI2018-097749-B-I00 and RYC-2013-14689; Erasmus Mundus EU programme, Grant/Award Number: 2013-2536/001-001

Abstract

The genetic basis of plant tolerance to parasites is poorly understood. We have previously shown that tolerance of *Arabidopsis thaliana* to its pathogen cucumber mosaic virus is achieved through changes in host life-history traits on infection that result in delaying flowering and reallocating resources from vegetative growth to reproduction. In this system we analyse here genetic determinants of tolerance using a recombinant inbred line family derived from a cross of two accessions with extreme phenotypes. Three major quantitative trait loci for tolerance were identified, which co-located with three flowering repressor genes, *FLC*, *FRI*, and *HUA2*. The role of these genes in tolerance was further examined in genotypes carrying functional or nonfunctional alleles. Functional alleles of *FLC* together with *FRI* and/or *HUA2* were required for both tolerance and resource reallocation from growth to reproduction. Analyses of *FLC* alleles from wild accessions that differentially modulate flowering time showed that they ranked differently for their effects on tolerance and flowering. These results pinpoint a role of *FLC* in *A. thaliana* tolerance to cucmber mosaic virus, which is a novel major finding, as *FLC* has not been recognized previously to be involved in plant defence. Although tolerance is associated with a delay in flowering that allows resource reallocation, our results indicate that *FLC* regulates tolerance and flowering initiation by different mechanisms. Thus, we open a new avenue of research on the interplay between defence and development in plants.

KEYWORDS
cucumber mosaic virus, *FLC*, life-history traits, plant defence, plant–virus interactions, resource reallocation

1 | INTRODUCTION

Tolerance, defined as the ability of hosts to limit the damage caused by a given parasite burden (Little et al., 2010; Pagán & García-Arenal, 2020), is a major defence response of plants to parasites. In most known cases, tolerance to plant parasites has a polygenic inheritance (Pagán & García-Arenal, 2020), which, among other causes (Jeger et al., 2006), has resulted in tolerance rarely having been bred into crops, with exceptions (Desbiez et al., 2003). The limited use of tolerance in plant disease control probably explains that, compared to resistance, the genetics and mechanisms of tolerance remain poorly studied and understood (Pagán & García-Arenal, 2020).

Tolerance may be related to the host's ability to alter its life-history programme on infection (Gandon et al., 2002; Hochberg et al., 1992).

Life-history theory predicts that parasitized hosts may modify optimal resource allocation by increasing the reproductive effort and/or altering temporal life-history schedules to maximize fitness (Forbes, 1993; Perrin et al., 1996). Experimental support for this hypothesis derives mostly from studies of invertebrate animals (e.g., Barribeau et al., 2010; Blair & Webster, 2007; Chadwick & Little, 2005; Fredensborg & Poulin, 2006; Michalakis & Hochberg, 1994; Polak & Starmer, 1998; Vale & Little, 2012). Evidence from plants is less abundant but it has been shown in different systems that tolerance to herbivory or parasitism could be explained by reallocation of host resources to reproduction (Agrawal, 2000; Fellous & Salvaudon, 2009; Strauss & Agrawal, 1999). Examples include tolerance of wheat to *Septoria tritici* (Collin et al., 2018); *Senecio vulgaris* to *Puccinia lagenophora* (Paul & Ayres, 1986); *Urtica dioica* to *Cuscuta europaea* (Koskela et al., 2002); and *Arabidopsis thaliana* to *Hyaloperonospora arabidopsidis* (Salvaudon & Shykoff, 2013), cucumber mosaic virus (CMV), and turnip mosaic virus (TuMV) (Montes et al., 2020; Pagán et al., 2008). Changes in life-history traits in tolerant genotypes of plants may also involve temporal rescheduling of development, either by accelerating reproduction, as in *A. thaliana* tolerance to *Pseudomonas viridiflava*, *H. parasitica*, or highly virulent genotypes of TuMV (Goss & Bergelson, 2006; Montes et al., 2020; Salvaudon & Shykof, 2013), or by delaying reproduction, as in *A. thaliana* tolerance to *Verticillium dahliae*, CMV, or mild strains of TuMV (Hily et al., 2016; Montes et al., 2020; Pagán et al., 2008; Shukla et al., 2018; Veronese et al., 2003).

Our group has studied the tolerance of *A. thaliana* (Brassicaceae) to CMV (*Bromoviridae*), which in nature is a significant parasite (Pagán et al., 2010) and, probably, a cause of selection for resistance and tolerance in its wild host populations (Montes et al., 2019). *A. thaliana* is an annual semelparous plant, the model organism for the study of a wide range of plant traits, including resistance and tolerance against parasites (Mysore & Ryu, 2004; Pagán et al., 2008; Shukla et al., 2018; Somerville & Koornneef, 2002), host–parasite co-evolution (Karasov et al., 2014; Pagán et al., 2010; Salvaudon et al., 2005), and life-history trait responses to abiotic stress (Pigliucci & Kolodynska, 2006; Salvaudon et al., 2005). Two distinct developmental phases can be differentiated in the *A. thaliana* post-embryonic life cycle: the vegetative growth period (GP) and the reproductive period (RP). The GP is marked by the production of a rosette of leaves and ends at flowering (Ausín et al., 2005). During the RP there is a continuous production of flowers that develop into siliques. The RP ends at complete senescence and plant death. Within this developmental schedule, life-history traits associated with the vegetative growth effort, total reproductive effort, and progeny production are easily differentiated. The biology of CMV has been reviewed recently (Palukaitis & García-Arenal, 2019). Briefly, CMV is a generalist virus that infects about 1200 mono- and dicotyledonous plant species. CMV is horizontally transmitted by more than 75 species of aphids in a nonpersistent manner and through the seed with efficiencies that depend on the host species and genotype, varying between 2% and 8% in *A. thaliana* (Cobos et al., 2019; Hily et al., 2014; Pagán et al., 2014). CMV has a positive-sense, single-stranded, three-segmented RNA genome encapsidated in three isometric particles.

In this plant–virus system tolerance is a quantitative host trait that depends on the host genotype by virus genotype interaction, with moderate to high broad-sense heritability (Pagán et al., 2007). Analysis of 21 accessions of *A. thaliana* challenged with three CMV genotypes showed that the effect of infection on plant fitness broadly differs among genotypes that sustain similar levels of virus multiplication, that is, *A. thaliana* genotypes differ in tolerance to CMV (Pagán et al., 2007). *A. thaliana* genotypes can be categorized into two groups: tolerant ones have a longer life cycle (Group 1) and nontolerant ones a short one (Group 2). Tolerant genotypes reprogramme their development on CMV infection, reallocating more resources to reproduction than to growth and reducing the length of the RP (Pagán et al., 2008). Tolerance of *A. thaliana* to CMV is modulated by environmental factors such as light, temperature, and plant density (Hily et al., 2016; Pagán et al., 2009), but under most conditions is associated with an increase in both lifespan and GP on infection, which does not occur in nontolerant genotypes. Life-history traits such as lifespan, flowering time, and plant size are known to have a role in the adaptation of *A. thaliana* to the abiotic environment (Manzano-Piedras et al., 2014; Méndez-Vigo et al., 2011; Tabas-Madrid et al., 2018). However, it has been shown that tolerance variation in natural populations of *A. thaliana* is not maintained by the effect of natural selection through environmental factors that modulate plant developmental architecture and phenology, but most possibly from selection due to CMV infection (Montes et al., 2019). All this evidence indicates that tolerance of *A. thaliana* to CMV involves an alteration of the transition from vegetative growth to reproduction with an associated increase in resource allocation from growth to reproduction.

The aim of the present study was to further understand the relationship between tolerance and the transition from growth to reproduction. We first identified quantitative trait loci (QTLs) associated with tolerance through the analysis of a recombinant inbred line (RIL) population derived from a cross between two accessions differing widely in that trait. Three QTLs were identified that overlap with three genes known as negative regulators of the transition to flowering, *FLOWERING LOCUS C* (*FLC*), *FRIGIDA* (*FRI*), and *ENHANCER of AG-4 2* (*HUA2*). The role of these candidate genes in the expression of tolerance was then assayed in a set of host genotypes having functional or nonfunctional alleles at these loci, which showed that the expression of tolerance requires a functional allele at *FLC* and/or *FRI* and *HUA2*, and indicated that the function of *FLC* in tolerance differs from that in inhibiting flowering.

2 | RESULTS

2.1 | Identification of *A. thaliana* genetic determinants of tolerance to CMV

To identify QTLs determining tolerance of *A. thaliana* to CMV, 129 RILs derived from a cross between accessions L*er* and Ll-0, with extreme nontolerance or tolerance responses to CMV, respectively,

(Pagán et al., 2008) were analysed. Plants were inoculated with CMV strain LS (LS-CMV). Rosette weight (RW), inflorescence weight (IW), and seed weight (SW) were measured for each infected or mock-inoculated plant, and the mean values for each line were used in QTL analyses (Table 1).

The two parent genotypes differed in RW, IW, and SW for mock-inoculated and infected plants (Wald $\chi^2_{(1,13)} \geq 3.60$, $p \leq 0.048$). They also differed in the effect of infection in these three traits because L*er* showed lower values in infected than in mock-inoculated controls (Wald $\chi^2_{(1,13)} \geq 4.46$, $p \leq 0.035$), while Ll-0 displayed comparable values (Wald $\chi^2_{(1,13)} \leq 1.19$, $p \geq 0.276$), in agreement with previous results (Pagán et al., 2008). Both parents also differed in LS-CMV multiplication in systemically infected leaves (Wald $\chi^2_{(1,13)} \geq 4.01$, $p \leq 0.045$), values being always higher in Ll-0 (Table 1). In the RIL population, bidirectional transgressive variation for RW, IW, and SW was observed for both mock-inoculated and infected plants, the range of variation being smaller for infected plants (Table 1). For all three traits, a generalized linear model (GzLM) analysis using RIL and treatment as factors showed significant interactions between the factors (Wald $\chi^2_{(28,1,334)} \geq 288.13$, $p < 10^{-5}$), which indicates L*er*/Ll-0 allelic variation for tolerance to CMV infection (Figure 1).

These data were used to identify and map QTLs associated with differences in tolerance between L*er* and Ll-0 (Figure 2 and Table S1), and loci were named according to the genetic marker nearest to the maximum logarithm of the odds ratio (LOD) value. Four QTLs were identified affecting the various traits and together explained between 24.8% (SW) and 47.2% (RW) of the trait variance. Three QTLs showed major effects (>10% of the phenotypic variance in one of the treatments) and one had a minor effect (<10% of the variance) on one or several of the six traits. The minor effect QTL (*F2103*), located on chromosome 3, only affected IW in mock-inoculated, but not in infected, plants, the Ll-0 allele reducing IW value. The three major effect QTLs (*FRI*, *FLC*, *MN5-7*), mapping on chromosomes 4 and 5, affected RW and IW in mock- and CMV-inoculated plants and SW in mock-inoculated plants. In all QTLs, the Ll-0 allele resulted in higher RW and IW, and lower SW (Figure 2). QTLs showed similar effects in infected and in mock-inoculated plants for RW and IW, and they had an effect on SW in mock-inoculated but not in infected plants.

To determine the differential contribution of the QTLs in mock-inoculated and infected plants we also tested the interaction QTL × treatment. This interaction was significant for *F2103* for IW, for *MN-5.7* for RW and SW, and for *FRI* and *FLC* for RW, IW, and SW (Table S1). This indicates a different effect of QTL *MN-5*, *FRI*, and *FLC* on SW in infected and mock-inoculated plants. Because the effect of infection on SW is taken as a measure of tolerance, these three major QTLs, *FRI*, *FLC*, and *MN-5.7*, contributed strongly to the higher tolerance to CMV of Ll-0 than L*er*. Because RILs differed in virus multiplication it could be that the effect of these QTLs on the SW of infected plants was due to both resistance and tolerance. However, values of virus accumulation did not correlate with the effect of infection on seed production (SW_i/SW_m) ($\rho = -0.040$, $p = 0.661$), which suggests that this hypothesis can be discarded. These three QTLs co-located with major effect QTLs previously identified as affecting flowering time in this same RIL population, which overlap with three well-known genes, *FRI*, *FLC*, and *HUA2*, contributing to flowering time variation (Sánchez-Bermejo et al., 2012). In fact, the L*er* parent carries natural loss-of-function alleles in the three genes, whereas Ll-0 alleles are functional. Therefore, these genes are candidates for the three major effect loci contributing to the natural variation for tolerance to CMV in *A. thaliana*.

TABLE 1 Statistical parameters of virus accumulation and effect of infection on *Arabidopsis* growth and reproduction in the parental L*er* and Ll-0 accessions and in the 129 recombinant inbred lines (RILs)

	VAc[a]	RW[a]	IW[a]	SW[a]
Mock				
L*er*[b]	–	0.012 ± 0.001	0.050 ± 0.01	0.032 ± 0.003
Ll-0[b]	–	0.101 ± 0.013	0.164 ± 0.04	0.016 ± 0.004
RILs[b]	–	0.076 ± 0.003	0.118 ± 0.00	0.013 ± 0.000
Min–Max[c]	–	0.001–0.327	0.005–0.371	0.003–0.045
LSD[d]	–	0.046	0.076	0.011
Inoculated				
L*er*[b]	1.36 ± 0.04	0.006 ± 0.001	0.036 ± 0.01	0.007 ± 0.001
Ll-0[b]	8.92 ± 1.55	0.082 ± 0.019	0.117 ± 0.03	0.012 ± 0.001
RILs[b]	8.29 ± 0.33	0.048 ± 0.002	0.081 ± 0.00	0.007 ± 0.000
Min–Max[c]	0.91–27.6	0.001–0.197	0.003–0.244	0.001–0.035
LSD[d]	5.41	0.039	0.064	0.004

[a]IW, inflorescence weight (g); LSD, least significant difference test; RW, rosette weight (g); SW, seed weight (g); VAc, virus accumulation in systemically infected leaves (ng of viral RNA/μg of total plant RNA). Values are mean ± standard error of six replicated plants.

[b]Mean value of each trait.

[c]Minimum and maximum of average values across RILs.

[d]Value of the least significant difference across RILs.

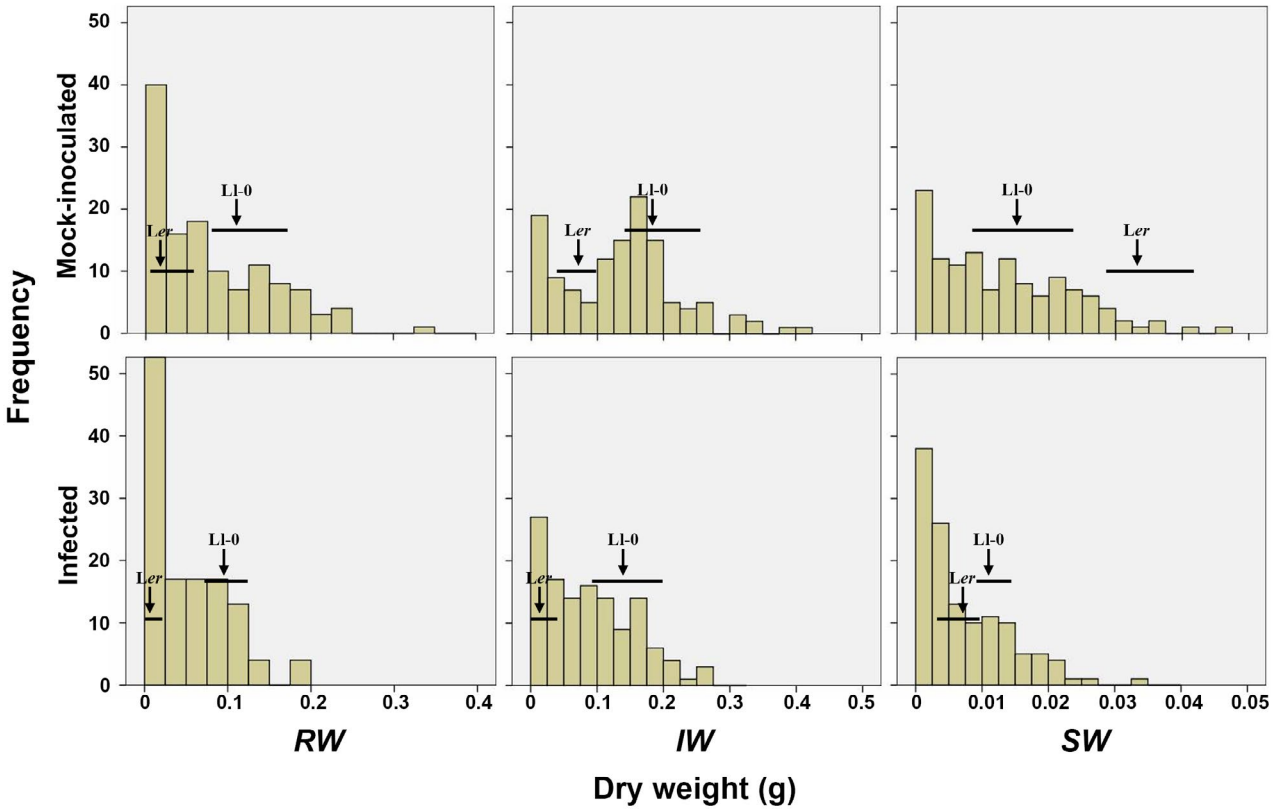

FIGURE 1 Frequency distribution of growth and reproduction traits in mock-inoculated and infected L*er* × Ll-0 recombinant inbred lines (RILs). Arrows indicate mean values for the parental genotypes and horizontal bars indicate the corresponding variance. IW, inflorescence weight; RW, rosette weight; SW, seed weight

2.2 | Analysis of the role of flowering genes in the expression of tolerance

Because the major QTLs for tolerance co-located with the flowering genes *FLC*, *FRI*, and *HUA2*, we analysed their role in the expression of tolerance to CMV in *A. thaliana*. For this, 11 genotypes of *A. thaliana* carrying functional or nonfunctional alleles of these three genes, including accessions Col-0, L*er*, and Ll-0, were analysed. These genotypes and their traits are listed in Table 2 (see also Table S2 for details). As expected from their *FRI*, *FLC*, and *HUA2* alleles, these genotypes differed in flowering time, those carrying *FRI* and *FLC* active alleles showing a late flowering phenotype, whereas the rest of lines were early flowering (Table 2).

All these genotypes were susceptible to LS-CMV infection resulting in mild symptoms, mostly of growth reduction. CMV accumulation ranged from 13.20 ± 2.97 to 35.26 ± 14.86 ng of viral RNA/μg of total plant RNA (Table 2). A general linear model (GLM) analysis considering host genotype as a fixed factor indicated no significant effect of host genotype on CMV accumulation ($F(9,56) = 0.774$, $p = 0.641$). In agreement, no significant correlation was found between virus accumulation and genotype lifespan or flowering time ($\rho < 0.080$, $p > 0.538$), indicating that these life-history traits are not major factors in virus accumulation.

Seed production of infected or mock-inoculated plants was estimated as seed weight (SW) (Figure 3 and Table S3). A GLM analysis considering host genotype as a fixed factor showed that SW_i/SW_m differed among host genotypes ($F(9,56) = 2.919$, $p = 0.007$), and these differences accounted for 31.94% of the trait variance. The genotypes Ll-0, *FLC*-Col L*er*, *FRI*-Sf2 Col, and *FLC*-Ll-0 *FRI*-Sf2 L*er* all showed similar seed weights whether infected or mock-inoculated. Their SW_i/SW_m varied between 0.93 ± 0.08 and 1.28 ± 0.20, not being significantly different from 1 ($t > -0.913$, $p > 0.221$) and indicating complete tolerance. The remaining genotypes had lower seed weights when infected than when mock-inoculated, with SW_i/SW_m between 0.65 ± 0.09 and 0.86 ± 0.05, values being significantly different from 1 ($t > 2.447$, $p \leq 10^{-3}$). Complete tolerance was found in genotypes with functional alleles at *FLC* and *FRI* or *HUA2*, although this was not sufficient for complete tolerance (compare Col and *FLC*-Col L*er*) (Figure 3). GLM analysis of SW_i/SW_m value distribution was performed considering the presence or absence of a functional *FLC* allele as a fixed factor, and host genotype as a nested factor. The results indicated that SW_i/SW_m was significantly higher for the genotypes carrying a functional *FLC* allele than for those carrying a nonfunctional one ($F(1,56) = 10.619$, $p = 0.002$). Note that the mutant line *flc-3 FRI*-Sf2 Col strongly differed from the isogenic line *FRI*-Sf2 Col (0.799 ± 0.023 vs. 1.230 ± 0.199), demonstrating the *FLC* effect on tolerance to CMV. Furthermore, among the genotypes that carry a functional *FLC* allele, a GLM analysis considering the presence or absence of functional *FRI/HUA2* as a fixed factor and host genotype as a nested factor showed that SW_i/SW_m was significantly higher for genotypes with functional alleles of *FRI/HUA2* than for those

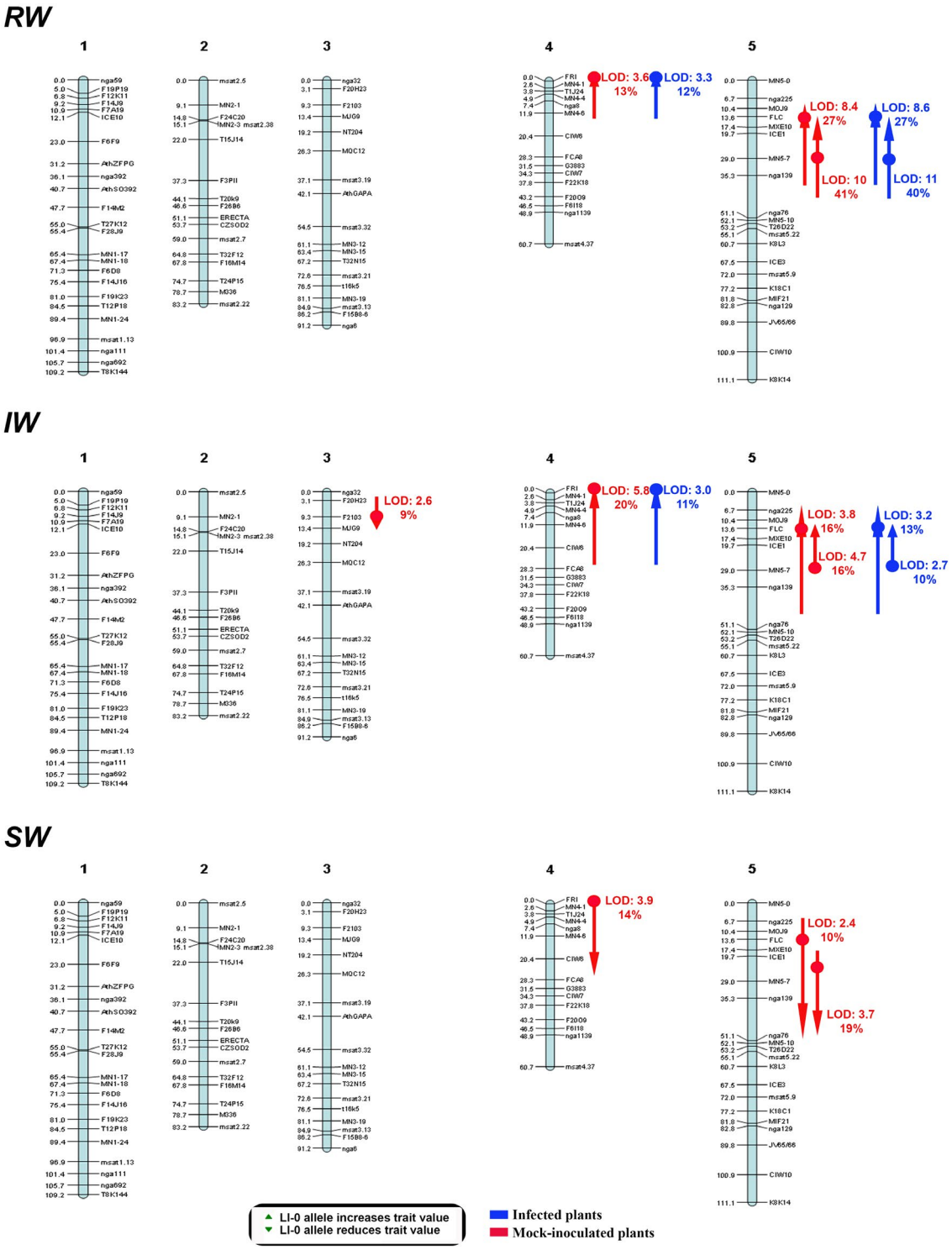

FIGURE 2 Quantitative trait locus (QTL) mapping for cucumber mosaic virus tolerance in *Arabidopsis thaliana*. Arrows indicate the QTL confidence interval. The direction of the arrow indicates whether the LI-0 allele increases (up) or decreases (down) the trait values. Dots indicate location of the maximum logarithm of the odds (LOD) ratio. IW, inflorescence weight; RW, rosette weight; SW, seed weight

with nonfunctional *FRI/HUA2* alleles ($F(1,34) = 5.716$, $p = 0.022$). Because *FRI* and *HUA2* are regulators of *FLC* expression (Amasino, 2010; Doyle et al., 2005; Srikanth & Schmid, 2011), all these results indicate that *FLC* plays a role in tolerance to CMV infection.

As tolerance is, at least in part, the result of resource reallocation from growth to reproduction (Pagán et al., 2008), the effect of CMV infection on this trait was estimated for the above 10 *A. thaliana* genotypes. The effect of virus infection on vegetative growth effort was quantified as the ratio of rosette weight of the infected to the mock-inoculated control plants, RW_i/RW_m, and the effect of virus infection on the development of reproductive structures was quantified as the ratio of inflorescence weight of the infected to

TABLE 2 Characteristics of *Arabdopsis thaliana* host genotypes analysed for tolerance

Host genotype	*HUA2*[a]	*FRI*[a]	*FLC*[a]	Flowering time[b]	VAc[c]
Col-0	+	−	+	15.38 ± 0.42	20.28 ± 4.66
Ler	−	−	−	12.38 ± 0.26	13.20 ± 2.97
Ll-0	+	+	++	44.50 ± 1.65	18.30 ± 1.93
FLC-Col *Ler*	+	−	+	18.25 ± 0.56	27.54 ± 8.158
FRI-Sf2 Col	+	+	+	44.13 ± 1.59	35.26 ± 14.86
flc-3 FRI-Sf2 Col	+	+	−	12.00 ± 0.00	16.93 ± 2.75
FLC-Ll-0 *Ler*	−	−	++	23.00 ± 0.38	20.25 ± 9.01
FRI-Sf2 Ler	−	+	−	17.00 ± 0.53	31.88 ± 13.39
FLC-Ll-0 *FRI*-Sf2 *Ler*	−	+	++	51.25 ± 3.78	25.20 ± 8.82
HUA2 Ler	+	−	−	15.00 ± 1.04	19.73 ± 4.56

[a]Genes have been labelled in each genotype according to the nonfunctional (−), weak (+), or strong (++) functional alleles.

[b]Flowering time of mock inoculated plants, in days. Values are mean ± standard error of eight replicates. Plants were not vernalized.

[c]VAc, virus accumulation in systemically infected leaves (ng of viral RNA/μg of total plant RNA). Values are mean ± standard error of at least six replicated plants.

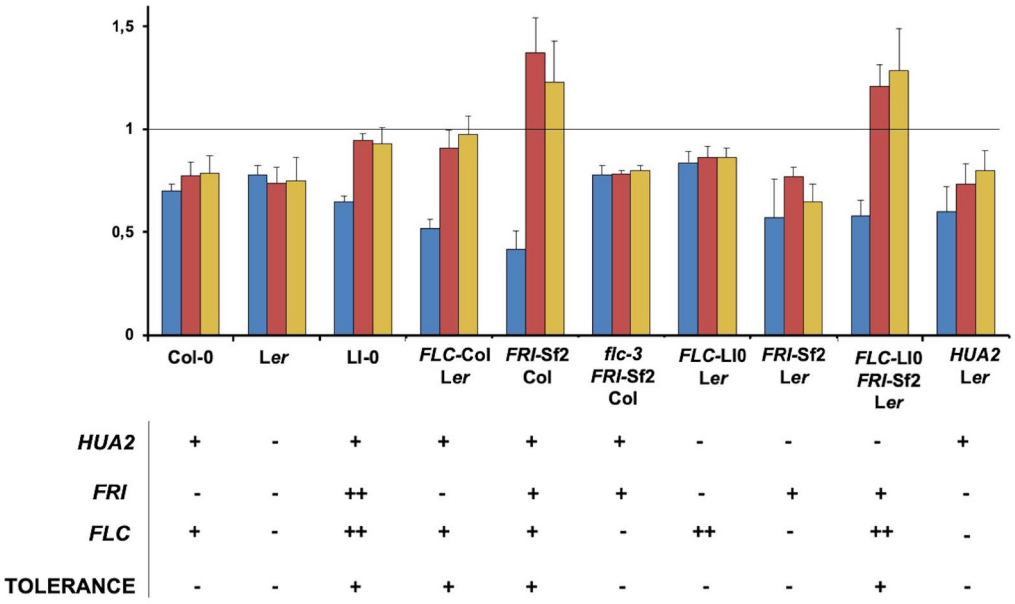

FIGURE 3 Effect of cucumber mosaic virus strain LS (LS-CMV) infection on growth and reproduction of 10 *Arabidopsis thaliana* genotypes. The effect of LS-CMV infection is shown as the ratio of infected to mock-inoculated plants of their rosette weight (RW_i/RW_m, blue bars), inflorescence weight (IW_i/IW_m, red bars), and seed weight (SW_i/SW_m, yellow bars). Data are mean ± standard error of at least six replicated plants. Genes have been labelled in each genotype according to the nonfunctional (−), weak (+), or strong (++) functional alleles

the mock-inoculated plants, IW_i/IW_m (Figure 3 and Table S3). GLM analysis with host genotype as a fixed factor showed that both RW_i/RW_m and IW_i/IW_m depended on the host genotype ($F(9,56) = 2.454$, $p = 0.020$ and $F(9,56) = 5.717$, $p \leq 10^{-3}$, respectively), which explained 28.29% and 47.89% of the variance of RW_i/RW_m and IW_i/IW_m, respectively. To evaluate the effect of LS-CMV infection on the allocation of resources to growth and reproduction, we analysed the ratio $(SW/RW)_i/(SW/RW)_m$, for which the host genotype explained 43.44% of the variance ($F(9,56) = 4.589$, $p < 10^{-3}$) (Table S4). Again, line *flc-3 FRI*-Sf2 Col differed strongly from *FRI*-Sf2 Col (1.040 ± 0.084 vs. 3.510 ± 0.895), which is evidence of *FLC* contribution to such resource reallocation. Therefore, tolerance to CMV is related to the reallocation of resources from vegetative growth to reproduction.

To analyse the effect of LS-CMV infection on the host phenology, three temporal life-history traits were considered: lifespan (LP), vegetative growth period (GP), and reproductive period (RP). The effects of LS-CMV infection on these traits were quantified as LP_i/LP_m, GP_i/GP_m, and RP_i/RP_m (Figure 4). LP_i/LP_m differed significantly among host genotypes ($F(9,56) = 2.147$, $p = 0.040$), this factor explaining 25.66% of the trait variance. In addition, a GLM analysis considering as a fixed factor whether the host genotype showed or did not show complete tolerance ($SW_i/SW_m \sim 1$), showed significant differences for the effect

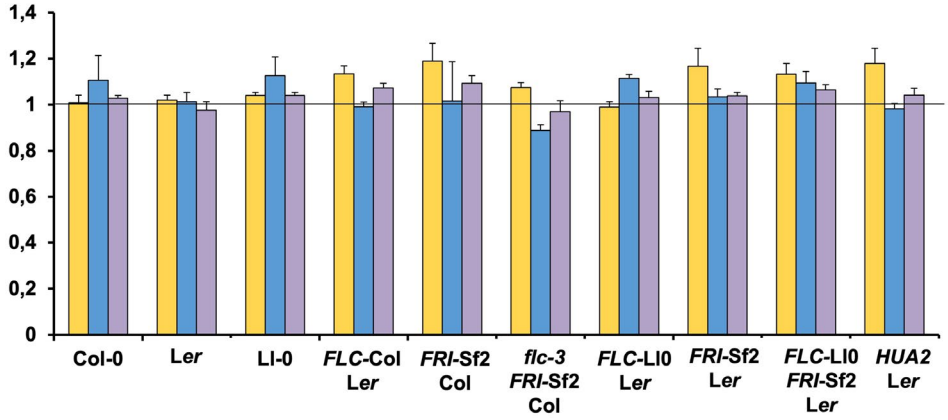

FIGURE 4 Effect of cucumber mosaic virus strain LS (LS-CMV) infection on the temporal schedule of the development of 10 *Arabidopsis thaliana* genotypes. The effects of infection by LS-CMV on the temporal schedule of development are shown as the ratio of the length in infected to mock-inoculated plants of their vegetative growth period (GP_i/GP_m, yellow bars), reproductive period (RP_i/RP_m, blue bars), and lifespan (LP_i/LP_m, purple bars). Data are mean ± standard error of at least six replicated plants

of infection on LP ($F(1,56) = 5.342$, $p = 0.024$). Infection of LS-CMV resulted in a significant increase in LP in the four completely tolerant genotypes (Ll-0, *FLC*-Col L*er*, *FRI*-Sf2 Col, and *FLC*-Ll-0 *FRI*-Sf2 L*er*; $t \geq 2.66$, $p < 0.033$), and in none of the partially tolerant ones ($t \geq -0.45$, $p > 0.064$) as compared with mock-inoculated plants. GP_i/GP_m also depended on the host genotype ($F(9,56) = 2.461$, $p = 0.019$) (Figure 4), explaining 28.35% of the trait variance, as well as on the host genotype being completely tolerant or not ($F(1,56) = 4.747$, $p = 0.033$). In this case, infection by LS-CMV resulted in a significant increase in GP in the four completely tolerant genotypes ($t \geq 2.51$, $p < 0.045$) and in two of the others (*flc-3 FRI*-Sf2 Col, *HUA2* L*er*; $t \geq 3.42$, $p < 0.019$). RP_i/RP_m did not depend on host genotype ($F(9,55) = 1.354$, $p = 0.231$). Thus, CMV infection differently affected LP and GP, but not RP, depending on the host genotype, with the infection of completely tolerant genotypes resulting in a significant increase in LP and GP.

Together, these results show that tolerance is associated with resource reallocation on infection from vegetative growth to reproduction, as well as with a delay in flowering time and an increase in the host lifespan.

2.3 | Analysis of the effect of different functional alleles of *FLC* on the expression of tolerance

Different functional alleles of *FLC* from wild accessions have been described as differing in their repressive effect on flowering (Méndez-Vigo et al., 2016). To test if the effect of functional alleles in tolerance and in flowering repression were linked, six genotypes of *A. thaliana* with three different *FLC* functional alleles (*FLC*-Ll-0, *FLC*-Ri-0, and *FLC*-Don-0) introgressed in a L*er* background, with or without functional alleles of *FRI*, were assayed for their tolerance to LS-CMV. L*er* and Ll-0 accessions were included as controls (Table 3 and Table S2). Because *FLC* repression of flowering is suppressed by vernalization (Amasino, 2010), assays were done without and with a vernalization treatment. As expected, genotypes carrying *FRI*-Sf2 alleles were late flowering and responded to vernalization. Line *FLC*-Ri-0 *FRI*-Sf2 L*er* did not flower without vernalization, remaining as a rosette until senescence (lifespan of 111.63 ± 0.60), thus it was excluded from the analyses of nonvernalized plants.

LS-CMV accumulation ranged from 9.41 ± 2.30 to 34.53 ± 19.48 and from 6.60 ± 3.63 to 19.93 ± 6.82 ng of viral RNA/μg total plant RNA for the nonvernalized and vernalized plants, respectively (Table 3). A GLM with host genotype and vernalization treatment as factors showed no effect on virus accumulation of the host genotype ($F(7,80) = 0.669$, $p = 0.698$), treatment ($F(1,80) = 1.058$, $p = 0.307$), or the interaction of host genotype and treatment ($F(6,80) = 0.830$, $p = 0.550$). In addition, virus accumulation did not correlate with lifespan or flowering time in nonvernalized ($\rho = -0.002$, $p = 0.987$ and $\rho = 0.118$, $p = 0.436$, respectively) or vernalized plants ($\rho = -0.217$, $p = 0.167$ and $\rho = 0.225$, $p = 0.137$, respectively).

The effect of *FLC* alleles in seed production after CMV infection was estimated for vernalized and nonvernalized plants (Figure 5 and Figure S1). In nonvernalized plants, a GLM considering host genotype as fixed factor showed that SW_i/SW_m differed significantly among host genotypes ($F(6,38) = 11.707$, $p < 10^{-3}$), which explains 64.89% of the trait variance. SW_i/SW_m varied from 0.20 ± 0.06 to 1.11 ± 0.18. In Ll-0 and *FLC*-Ll-0 *FRI*-Sf2 L*er*, $SW_i/SW_m \geq 1.01 \pm 0.01$, which was not significantly different from 1 ($t > 0.06$, $p > 0.587$), indicating complete tolerance. In the absence of a functional *FRI* gene, the three genotypes were not completely tolerant, but differed significantly, ranking *FLC*-Don-0 < *FLC*-Ll-0 = *FLC*-Ri-0 for SW_i/SW_m.

After a vernalization treatment, SW_i/SW_m also depended on host genotype ($F(7,41) = 5.420$, $p < 10^{-3}$), which explained 48.01% of the trait variance, and ranged between 0.66 ± 0.09 and 1.09 ± 0.06. Vernalization reduced the effect of infection on progeny production, tolerance being complete for Ll-0, *FLC*-Ll-0 *FRI*-Sf2 L*er*, and *FLC*-Ri-0 *FRI*-Sf2 L*er* (Figure S1). Pairwise comparisons among vernalized genotypes showed that tolerance was moderate and similar for *FLC*-Don-0 L*er*, *FLC*-Ll-0 L*er*, and *FLC*-Ri-0 L*er*, and similarly high for *FLC*-Don-0 *FRI*-Sf2 L*er*, *FLC*-Ll-0 *FRI*-Sf2 L*er*, and *FLC*-Ri-0 *FRI*-Sf2 L*er*.

TABLE 3 Characteristics of *Arabidopsis thaliana* host genotypes analysed for effect of different *FLC* alleles on the expression of tolerance

Host genotype	Flowering time[a] nonvern	Flowering time[a] vern	VAc[b] nonvern	VAc[b] vern
Ler	8.88 ± 0.13	3.00 ± 0.38	17.10 ± 6.10	8.62 ± 4.40
LI-0	34.75 ± 1.40	11.50 ± 0.27	18.80 ± 4. 76	15.83 ± 2.81
FLC-Ri-0 *Ler*	16.25 ± 0.50	8.83 ± 0.79	29.69 ± 11.55	14.06 ± 7.60
FLC-Don-0 *Ler*	47.38 ± 1.12	17.50 ± 0.29	9.41 ± 2.30	10.55 ± 2.18
FLC-LI-0 *Ler*	15.00 ± 0.01	6.25 ± 0.48	22.64 ± 7.90	13.36 ± 2.82
FLC-Ri-0 *FRI*-Sf2 *Ler*	–	32.50 ± 0.43	–	16.31 ± 8.45
FLC-Don-0 *FRI*-Sf2 *Ler*	47.75 ± 1.06	20.00 ± 0.45	34.53 ± 19.48	19.92 ± 6.82
FLC-LI-0 *FRI*-Sf2 *Ler*	41.50 ± 2.30	19.00 ± 0.58	17.12 ± 5.85	6.60 ± 3.63

[a]Flowering time of mock inoculated plants, in days in nonvernalized (nonvern) or vernalized (vern) plants. Data are mean ± standard error of eight replicates.

[b]VAc, virus accumulation in systemically infected leaves (ng of viral RNA/μg of total plant RNA). Values are mean ± standard error of at least six replicated plants.

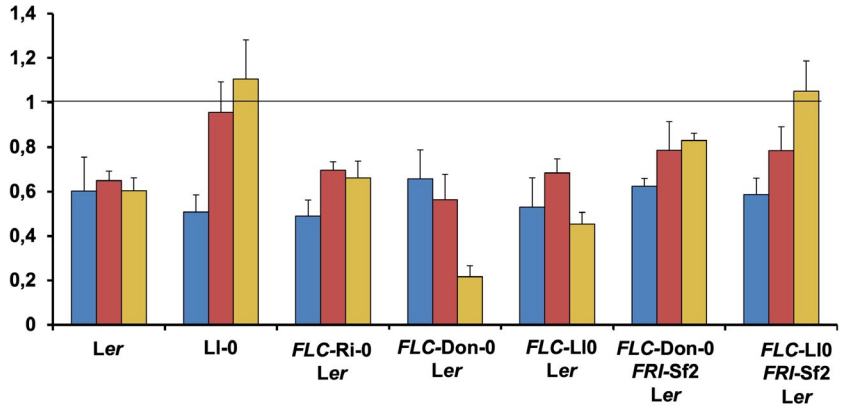

FIGURE 5 Effect of cucumber mosaic virus strain LS (LS-CMV) infection on the growth and reproduction of nonvernalized plants of *Arabidopsis thaliana* genotypes with different *FLC* alleles. The effect of LS-CMV infection is shown as the ratio of infected to mock-inoculated plants of their rosette weight (RW_i/RW_m, blue bars), inflorescence weight (IW_i/IW_m, red bars), and seed weight (SW_i/SW_m, yellow bars). Data are mean ± standard error of at least four replicated plants

Thus, genotypes carrying *FRI* functional alleles showed higher tolerance (SW_i/SW_m) than genotypes with *FRI* loss-of-function alleles under both nonvernalized and vernalized conditions.

Analyses of the effects of *FLC* alleles on the vegetative growth and reproductive efforts after LS-CMV infection (Figure 5) showed that in nonvernalized plants host genotype had no effect in either RW_i/RW_m or IW_i/IW_m ($F(6,37) = 1.795$, $p = 0.127$ and $F(6,39) = 1.475$, $p = 0.212$, respectively). However, resource reallocation from growth to progeny production, $(SW/RW)_i/(SW/RW)_m$ (Table S4), did depend on the host genotype ($F(6,36) = 3.131$, $p = 0.014$). Genotypes ranked for resource reallocation as *FLC*-Don-0 < *FLC*-LI-0 = *FLC*-Don *FRI*-Sf2 = *FLC*-Ri-0 = *FLC*-LI-0 *FRI*-Sf2 ($p < 0.009$). For vernalized plants, host genotype showed an effect on IW_i/IW_m ($F(7,41) = 3.708$, $p = 0.003$) but not on RW_i/RW_m ($F(7,41) = 0.939$, $p = 0.488$) (Figure S1).

The ratio $(SW/RW)_i/(SW/RW)_m$ also depended on host genotype ($F(7,41) = 2.891$, $p = 0.015$), which explained 33.04% of the trait variance. The ratio was similar for the three *FLC* alleles in the presence or absence of a functional *FRI* allele and higher when a functional *FRI* allele was present (Table S4). As for the tolerance to CMV, the degree of resource reallocation in both vernalized and nonvernalized genotypes did not rank according to the effect of *FLC* alleles on flowering time (Table S4).

Analyses of the effect of *FLC* alleles on host developmental timing after LS-CMV infection (Figure 6) showed that in nonvernalized plants host genotype was not a factor on the effect of infection on GP ($F(6,39) = 1.090$, $p = 0.386$), while it was a significant factor on the effect of infection on LP and RP ($F(6,39) = 4.769$, $p = 0.001$ and $F(6,39) = 5.262$, $p \leq 10^{-3}$, respectively). Host genotype explained 42.72% and 44.74% of the variance of these two traits. In vernalized plants, similar results were obtained (Table S5).

3 | DISCUSSION

Understanding the mechanisms of host defence to parasites is a central question in biology. The analysis of plant defences to parasites, including viruses, has focused on resistance, that is, on the mechanisms that decrease the rate of infection and/or the multiplication of the parasite within the infected hosts (Clarke, 1986). Other defences of plants to pathogens have received considerably less attention (Jeger et al., 2006). This is certainly the case for tolerance, which specifically decreases the negative effect of parasite infection on host fitness. The mechanisms of tolerance and its role in plant–pathogen co-evolution remain underexplored (Best et al., 2014; Little et al., 2010; Pagán & García-Arenal, 2018, 2020).

To understand the mechanisms of tolerance, genetic determinants for tolerance of *A. thaliana* to CMV were identified through the analysis of an RIL family derived from a cross of two accessions, *Ler*

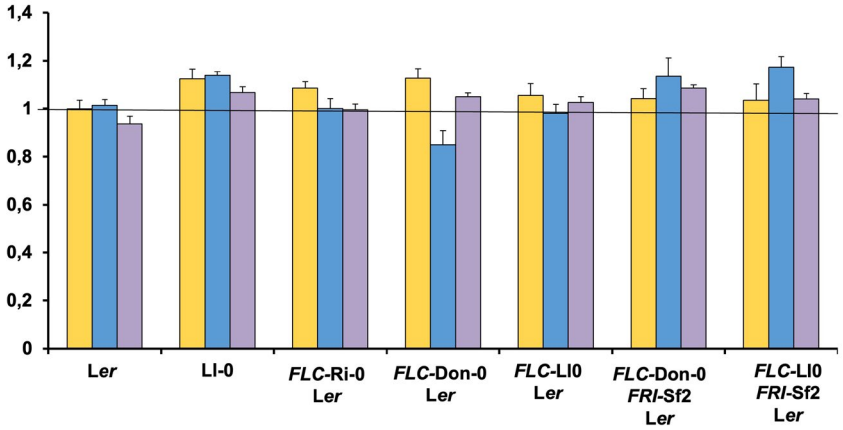

FIGURE 6 Effect of cucumber mosaic virus strain LS (LS-CMV) infection on the temporal schedule development of nonvernalized plants of *Arabidopsis thaliana* genotypes with different *FLC* alleles. The effect of LS-CMV infection on the temporal schedule of development is shown as the ratio of the length in infected to mock-inoculated plants of the vegetative growth period (GP_i/GP_m, yellow bars), reproductive period (RP_i/RP_m, blue bars), and lifespan (LP_i/LP_m, purple bars). Data are mean ± standard error of at least four replicated plants

and Ll-0, that had extreme values for this trait (Pagán et al., 2008). Three major QTLs were identified in which the allele from the tolerant parent Ll-0 resulted in a higher reproductive effort and progeny production of infected plants, that is, tolerance. These three loci co-located with major effect QTLs affecting flowering time in the L*er* × Ll-0 RIL population and with the underlying genes *FRI*, *FLC*, and *HUA2* (Sánchez-Bermejo et al., 2012), which are repressors of flowering and prolong the vegetative growth period. *FLC* functions as a flowering repressor by binding to the promoter and suppressing the expression of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), which promote flowering. *FRI* functions as a repressor of flowering by up-regulating *FLC* in nonvernalized plants and *HUA2* functions as a flowering repressor by enhancing the expression of several genes that delay flowering, including *FLC* (Amasino, 2010; Srikanth & Schmid, 2011). Tolerance to CMV is in part due to the alteration in the host developmental timing resulting in resource reallocation from growth to reproduction (Pagán et al., 2008), and QTL mapping established a link between the phenotype of tolerance and the host transition to flowering.

Other studies have shown a link between pathways for stress defence and flowering (Kazan & Lyons, 2016). However, the role of flowering genes in plant–pathogen interactions remains largely unclear. Genes have been identified in *A. thaliana* that affect both flowering time and defence to fungi, bacteria, or viruses. For example, late-flowering mutants at the clock-associated gene *GIGANTEA* (*GI*) and at genes of the autonomous pathway showed enhanced resistance to *Fusarium oxysporum* (Lyons et al., 2015). The autonomous pathway genes *FLD* and *FPA*, which repress *FLC* expression (He et al., 2003), promote susceptibility to *Pseudomonas syringae* (Lyons et al., 2013; Singh et al., 2013) and a null mutant of *LEAFY* (*LFY*), which promotes flowering, shows increased resistance to *P. syringae* (Winter et al., 2011). A positive correlation between flowering time and resistance to fungi or bacteria has also been reported in crops (e.g., Mizobuchi et al., 2013; Pinson et al., 2010; Van Inghelandt et al., 2012). In *A. thaliana*, a relationship between flowering time and resistance did not occur in four out of five assayed virus species (Shukla et al., 2018). Regarding tolerance, accession C24, tolerant to *V. dahliae*, delayed flowering on infection and had a longer lifespan than nontolerant Col. The *V. dahliae-tolerance* (*VET1*) locus was likely to function as a negative regulator of flowering (Veronese et al., 2003). In the only study of a plant–virus interaction we know, it was shown that *GI* and *FCA* control cauliflower mosaic virus symptom severity in *A. thaliana* without affecting virus titre and distribution (Cecchini et al., 2002). These two studies rated tolerance by symptom severity and not by progeny production, but they show a link between flowering gene pathways and tolerance. Note that in some plant–pathogen systems tolerance is associated with accelerated, rather than delayed, flowering, as in the interaction of *A. thaliana* with *P. viridiflava*, *H. parasitica*, or highly virulent strains of TuMV (Goss & Bergelson, 2006; Montes et al., 2020; Salvaudon & Shykof, 2013). Taken together, these reports suggest that a positive correlation between delayed flowering and resistance to fungi and bacteria is a general pattern, while the relationship between flowering time and tolerance seems to vary according to the system. Theory predicts a trade-off between resistance and tolerance as it would be redundant to divert host resources in the simultaneous expression of two different defences (Fineblum & Rausher, 1995; Mauricio, 2000; Tiffin, 2000). However, our results do not show a negative correlation between resistance and tolerance ($\rho = -0.110$, $p = 0.111$), consistent with previous reports of the *A. thaliana*–CMV (Pagán et al., 2008) and *Mimulus guttatus*–CMV systems (Carr et al., 2006), which can be explained by resistance and/or tolerance not having a cost, or because tolerance is linked to other host traits (Mauricio et al., 1997; Mauricio, 2000).

Examination of *A. thaliana* genotypes carrying functional or nonfunctional alleles of *FLC*, *FRI*, and *HUA2* showed that functional alleles of *FLC* together with *FRI* and/or *HUA2* are required for both tolerance to CMV and resource reallocation from growth to reproduction. As *FLC* expression is regulated by *FRI*, these results

indicate that *FLC* is involved in the regulation of tolerance to CMV. Moreover, infected plants of the tolerant genotypes had a longer lifespan and vegetative growth period than mock-inoculated ones. However, functional alleles at *FLC* and *FRI* or *HUA2* may not be sufficient for complete tolerance: Col and *FLC*-Col L*er* both have the same functional alleles at *FLC* and HUA2, but only the second was completely tolerant (Figure 3), which indicates an effect of the genetic background on the role in tolerance of the weak *FLC* allele from Col. Delayed flowering and resource reallocation resulting in tolerance is not a general response of *A. thaliana* to the stress of virus infection, but a plant genotype-virus-specific reaction (Shukla et al., 2018). This response is also shown by genotypes of *A. thaliana* tolerant to TuMV when challenged by mild TuMV strains (Montes et al., 2020).

FLC is an important regulator of the transition from vegetative growth to reproduction, which integrates multiple endogenous and environmental cues in its regulation (Crevillén & Dean, 2011). *FLC* is expressed in most plant organs (Sheldon et al., 2008). A Gene Ontology analysis of the transcription factor families enriched in the FLC protein target genes implied that *FLC* is likely to modulate the activity of a number of transcription factors that regulate processes other than flowering initiation, such as genes involved in response to stress, or reproductive and embryonic development (Deng et al., 2011). In fact, *FLC* expression has been associated with responses to different abiotic stresses (He et al., 2004; Kant et al., 2011; Xu et al., 2014), but not to biotic ones. This led us to examine the relationship of *FLC* function in tolerance to CMV and in flowering time regulation. For this, we analysed the effectiveness in tolerance to CMV of three alleles of *FLC* that modulate differentially the flowering timing depending on the environment (Méndez-Vigo et al., 2016). Results showed that *FLC* regulates both biological processes through different mechanisms, which is best illustrated with the *FLC*-Don-0 allele. This allele has been shown to display a constitutive high expression insensitive to *FRI* activation, producing a late-flowering phenotype (Méndez-Vigo et al., 2016). However, the *FLC*-Don-0 line was the least tolerant to CMV when plants either were or were not vernalized. By contrast, line *FLC*-Ri-0 L*er*, showing much lower *FLC* expression (Méndez-Vigo et al., 2016), was significantly more tolerant in the absence of *FRI* functional alleles. In addition, increasing expression of *FLC*-Ri-0 and *FLC*-Ll-0 by combination with *FRI*-Sf2 alleles also increased tolerance to CMV. These results indicate that the effect of *FLC* in tolerance is not, or not only, through its effect in repressing flowering and increasing the vegetative growth period, but rather through a different pathway.

A major finding of the present study is the role of *FLC* in the expression of *A. thaliana* tolerance to CMV. This is a novel result, as to date this gene has not been reported to be involved in plant resistance or tolerance to parasites. Although tolerance is due, at least in part, to a delay in flowering that allows the plant to allocate resources from growth to reproduction, our results also indicate that *FLC* regulates flowering time and tolerance to CMV in different ways, thus opening a new avenue in the study of the interplay of defence and development in plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Virus and plant genotypes

CMV virus strain LS (LS-CMV) was multiplied in *Nicotiana clevelandii* plants inoculated with transcripts from biologically active cDNA clones (Zhang et al., 1994).

A population of 129 RILs derived from the cross between *A. thaliana* Landsberg *erecta* (L*er*) and Llagostera (Ll-0) accessions, previously described (Sánchez-Bermejo et al., 2012), was analysed.

A. thaliana accessions, introgression lines (ILs), and mutants used in this work are listed in Table S2. ILs are named as the accession from which the alleles of *FRI* and *FLC* come from indicated with the gene name, dash, and the original accession, and the genetic background of the line indicated with the accession name at the end (e.g., *FRI*-Sf2 L*er* corresponds to an IL with *FRI* alleles from Sf2 accession in a L*er* genetic background). Briefly, Ll-0 carries functional alleles of *FRI*, *FLC*, and *HUA2*. Col-0 carries a null allele of *FRI* and functional alleles of *FLC* and *HUA2*. In the Col-0 background, *FRI*-Sf2 IL was chosen carrying a functional allele of *FRI* from accession Sf2. Line *flc-3 FRI*-Sf2 Col carries a null mutant *FLC* allele but a functional *FRI* allele in the Col-0 background. L*er* carries natural weak or null loss-of-function alleles of *FRI*, *FLC*, and *HUA2*, which here are considered as nonfunctional (Doyle et al., 2005; Méndez-Vigo et al., 2010; Michaels et al., 2003). In this background, we selected ILs *FRI*-Sf2 L*er*, with a functional *FRI-Sf2* allele, and *FLC*-Col L*er*, with the weak *FLC*-Col allele introgressed together with the *HUA2* allele from Col. Also, six genotypes of *A. thaliana* with different *FLC* functional alleles (*FLC*-Ll-0, *FLC*-Ri-0, and *FLC*-Don-0) introgressed in a L*er* background, with or without the functional allele of *FRI* from accession Sf2, were assayed. In addition, we selected line *HUA2* L*er* carrying a functional *HUA2* allele (Table S2), which has been previously described (Doyle et al., 2005) and is referred to as *HUA2* L*er*.

All genotypes were initially multiplied simultaneously under the same greenhouse conditions to reduce maternal effects. For plant growth, seeds were stratified at 4°C for 4 days and then transferred to a growth chamber at 21°C, 16 h photoperiod 220–250 μmol·s^{-1}·m^{-2} and 65%–70% relative humidity. Ten-day-old seedlings were transplanted into individual 10 cm diameter pots containing 3:1 peat:vermiculite to minimize space and resource limitation. Plants were grown in the same chamber until the end of their life cycle.

4.2 | Inoculation and quantification of virus multiplication

N. clevelandii young leaves systemically infected with LS-CMV were ground in 0.1 M phosphate buffer pH 7.0 + 0.2% sodium diethyldithiocarbamate and the sap used to inoculate *A. thaliana* plants at the four-leaf stage (stage 1.04; Boyes et al., 2001). Phosphate buffer was applied to mock-inoculated controls. RIL experiments involved six, and experiments with introgression lines and mutants eight, replicated plants per treatment or mock-inoculated controls in a fully randomized design. Inoculation success was always above 80%.

Virus multiplication was estimated as viral RNA accumulation 15 days postinoculation (dpi) in systemically infected leaves using quantitative real-time reverse transcription-PCR (RT-qPCR) as described in Shukla et al. (2018).

4.3 | Quantification of *A. thaliana* tolerance, resource allocation, and temporal life-history traits

Tolerance was estimated as the effect of virus infection on host progeny production, quantified as the weight of viable seeds (SW), as a proxy to plant fitness. Accordingly, the ratio of seed weight of infected (i) to mock-inoculated controls (m), SW_i/SW_m, is an estimation of tolerance. Previous work using different CMV strains and *A. thaliana* genotypes did not find an effect of CMV infection on seed viability or the weight of individual seeds (Hily et al., 2014; Pagán et al., 2008, 2009; Shukla et al., 2018).

Life-history traits related to resource allocation were quantified in plants harvested on complete senescence and maintained at 65°C in an oven until constant weight. The rosette weight (RW) was taken as a measure of vegetative growth effort, the weight of the inflorescence (IW) was taken as a measure of reproductive effort, and the weight of viable seeds (SW) was taken as a measure of progeny production (Pagán et al., 2007, 2008). All weights are in grams. The effect of infection on the allocation of resources to growth and reproduction was estimated by the ratio $(SW/RW)_i/(SW/RW)_m$. The temporal schedule of development was quantified following Boyes et al. (2001): the vegetative growth period (GP) was from the end of stratification to the opening of the first flower; the reproductive period (RP) was from the opening of the first flower until the first silique shatters, and the lifespan (LP) was from seed germination until complete senescence. These periods were measured in days.

4.4 | Mapping *A. thaliana* QTLs for tolerance to CMV

QTLs associated with *A. thaliana* tolerance to CMV were identified using the 129 L*er* × LI-0 RILs described in Sánchez-Bermejo et al. (2012). Plants of each RIL were CMV- or mock-inoculated, and virus multiplication, RW, IW, and SW were quantified. Mean values of the replicas of each RIL were used for QTL mapping. QTLs were located using the multiple-QTL-model method implemented in MapQTL v. 4.0 software (Van Ooijen, 2000). QTLs were detected with a LOD threshold of 2.4, which corresponds to a genome-wide significance $\alpha = 0.05$, as estimated with MapQTL permutation test. The additive allele effects, the percentage of variance explained by each QTL, and the total variance explained by the additive effects of all detected QTLs were obtained from multiple-QTL-mapping models. Additive allele effects correspond to half the differences between the estimated means of the two RIL genotypic groups. The QTL × treatment interaction was analysed by two-way analysis of variance using the marker linked to each QTL and treatment as factors.

4.5 | Statistical analysis

For each trait (variable), data normality and homocedasticity were evaluated by Shapiro–Wilk and Levene's test for equality of error variances. If data were normally distributed, analyses of the studied variables were done using full factorial general linear models (GLMs). If variables were not normally distributed, analyses were done via full factorial generalized linear models (GzLM). Still, as GLM is robust to the violation of the assumption of normality, all non-normal variables were also analysed by GLMs. In all experiments involving genotypes with different alleles of the flowering genes, the results of GLM and GzLM analyses were similar, and for simplicity we present here those from GLMs. Significance of differences among classes within each factor was determined by Fisher's least significant difference test. To assess if tolerance values in different genotypes were significantly different from 1, one-sample *t* test was performed. Spearman regression analyses were performed after checking for no autocorrelation between regressors according to the Durbin–Watson test. Analyses were performed with SPSS v. 22 (SPSS Inc.).

ACKNOWLEDGEMENTS

We thank Caroline Dean for providing seeds of *flc-3 FRI*-Sf2 Col. This research was funded by Plan Estatal de I+D+i, Ministerio de Ciencia, Innovación y Universidades, Spain (grant RTI2018-094302-B-I00 to F.G-A.). C.A.B. was funded by grant PID2019-104249GB-I00/AEI/10.13039/501100011033. P.C. was funded by grants RTI2018-097749-B-I00/AEI and RYC-2013-14689/MINECO. A.S. was supported by a scholarship of an Erasmus Mundus EU programme (BRAVE, agreement 2013-2536/001-001). Antolín López Quirós provided excellent technical support.

DATA AVAILABILITY STATEMENT

Data are available in the supplementary material files.

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How to cite this article: Shukla, A., Pagán, I., Crevillén, P., Alonso-Blanco, C.&García-Arenal, F. (2022) A role of flowering genes in the tolerance of *Arabidopsis thaliana* to cucumber mosaic virus. *Molecular Plant Pathology*, 23, 175–187. https://doi.org/10.1111/mpp.13151