Title:
Signature of co-evolution between defense and vegetative lifespan strategies in *Arabidopsis thaliana*

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ABSTRACT:

The selective impact of pathogen epidemics on host defenses can be strong but remains transient. By contrast, life-history shifts can durably and continuously modify the balance between costs and benefits of immunity, which arbitrates the evolution of host defenses. Their impact, however, has seldom been documented. Here, we show with a simple mathematical model how the optimal investment into defense is expected to increase with increasing lifespan. We further document that in natural populations of the model plant *Arabidopsis thaliana*, the expression levels of defense genes correlate positively with flowering time, a proxy for the length of vegetative lifespan. Using a genetic strategy to partition lifespan-dependent and –independent defense genes, we demonstrate that this positive co-variation can be genetically separated. It is therefore not explained by the pleiotropic action of some major regulatory genes controlling both defense and lifespan. Moreover, we find that defense genes containing variants reported to impact fitness in natural field conditions are among the genes whose expression co-varies most strongly with flowering time. In agreement with our model, this study reveals that natural selection has likely assorted alleles promoting higher expression of defense genes with alleles that increase the duration of vegetative lifespan in *A. thaliana* and *vice versa*. This is the first study documenting the pervasive impact of life history variation on the maintenance of diversity in host immunity within species.

Keywords: Co-evolution, Defense, Flowering time, Lifespan, *Arabidopsis thaliana,* Transcriptomics, Trade-off

INTRODUCTION

The ability of living organisms to defend against pathogens is a major determinant of survival in natural populations (Parker & Gilbert 2004; Chisholm *et al.* 2006; Lee & Mazmanian 2010). Pathogens have long been suspected to impose a rapid evolutionary pace on the host defense system and the "Red Queen" Hypothesis is nowadays a keystone of evolutionary biology (Van Valen 1973; Liow *et al.* 2011). Evidence that pathogens drive the molecular evolution of host defense systems has been accumulating in an array of plant and animal systems (Bergelson *et al.* 2001; de Meaux & Mitchell-Olds 2003; Moeller & Tiffin 2005; Laine *et al.* 2010; Ravensdale *et al.*

2010; Maekawa *et al.* 2011; Dybdahl *et al.* 2014; Karasov *et al.* 2014; Siddle & Quintana-Murci 2014; Parker *et al.* 2015; Metzger *et al.* 2016).

Yet, the possible impact of changes in ecology on the evolution of defense systems should also be considered as they may durably change the exposure of hosts to pathogens. Invasive species, for example, owe much of their success to the release from pathogen and pest pressures (Mitchell & Power 2003; Mitchell *et al.* 2010). Similarly, shifts in life history can alter the balance between costs and benefits of host defense systems. Shifting from perennial to annual life cycles, or evolving from a winter-annual to summer-annual cycling occurs frequently across plant phylogenies (Garnier 1992; Michaels *et al.* 2003; Franks *et al.* 2007; Tank & Olmstead 2008; Matthew Ogburn & Edwards 2015; Kiefer *et al.* 2017). The reduction in lifespan that follows such life history changes concomitantly reduces the probability to encounter a virulent pathogen. Its consequences on the optimal investment in defense can be formally described in a simple mathematical model (Fig. 1). Considering that the total energy (σ) that is available for a plant is limited by natural resources, it must be divided between defense (d) and growth (g). Evidence for costs associated with defense have been documented in both, plants and animals (Lochmiller & Deerenberg 2000; Purrington 2000). Some plant resistance genes (R genes) involved in the surveillance systems directed against pathogenic virulence factors were shown to incur substantial fitness costs (Tian *et al.* 2003; but see also MacQueen *et al.* 2016). In addition, analysis of basal defense responses triggered by Pathogen Associated Molecular Pattern (PAMPs), which are broadly conserved across pathogen taxa, revealed a negative correlation between PAMP detection and growth (Vetter *et al.* 2012). Plants thus generally face a trade-off between size at reproduction and the investment into defense. We modeled lifetime fitness (W) as the product of final size (s) by survival rate (r). Assuming that an optimal lifespan can evolve in any given environment, the investment into defense (d) and the lifespan (ft) can be linked by equation (1),

$$d = 1 - \frac{1}{2\,ft * \tau * \upsilon} \quad (1)$$

where τ is the probability of encountering a pathogen per unit of time and ν describes the virulence of the pathogen population (see Suppl. Document for details on the model). In this model, lifespan alters the so-called "effectiveness of defense", which was an important modulator of allocation into defense in previous models (Jokela *et al.* 2000). Our model now formulates the simple prediction that the allocation into defense will be attenuated where reduced lifespan evolves and incremented where extended lifespan evolves. Positive co-variation between defense and lifespan is therefore expected.

Variation in flowering time in *A. thaliana* offers an opportunity to test the impact of adaptive life history changes on defense. *Arabidopsis thaliana*, the flagship species of plant molecular genetics, has become over the last decade a powerful model system to address ecological questions at the genetic level (Mitchell-Olds & Schmitt 2006; Bergelson & Roux 2010; Roux & Bergelson 2016). Experiments in common gardens have been performed to describe the architecture of natural variation in fitness and to infer geographic distributions of locally adaptive mutations (Fournier-Level *et al.* 2011; Hancock *et al.* 2011; Fournier-Level *et al.* 2016). Analyses of mutants and recombinant inbred lines (RIL) have allowed reconstructing the contribution of phenotypes to fitness (Wilczek *et al.* 2009; Chiang *et al.* 2013; Fournier-Level *et al.* 2013). Secondary chemical compounds were shown to have evolved to deter predominant herbivores in natural populations (Brachi *et al.* 2013; Kerwin *et al.* 2015). Clinal variation along the latitudinal range of the species

reveals how phenotypes expressed along the life cycle are jointly shaped by natural selection (Lasky 2012; Debieu *et al.* 2013b; Vidigal *et al.* 2016).

A. thaliana is also an optimal model system for questioning the impact of life history changes, which modify plant vegetative lifespan, on the evolution of the defense system. It is arguably one of the species with the greatest amount of genetic and phenotypic information on both, defense reactions against pathogens and variation in the duration of the vegetative lifespan. Indeed, in annual (monocarpic) species, which grow and reproduce only once, flowering time marks the end of the vegetative growth phase. Seed production in monocarpic species is terminated by senescence and death, so that flowering time provides a good proxy for lifespan. In *A. thaliana*, it has been scored in a number of conditions (Brachi *et al.* 2010; Sasaki *et al.* 2015; Roux & Bergelson 2016) and there are convergent indications that flowering time changes are locally adaptive (Corre 2005; Toomajian *et al.* 2006; Montesinos-Navarro *et al.* 2011; Debieu *et al.* 2013a; Li *et al.* 2014; Lasky *et al.* 2016; Hu *et al.* 2017). Natural variation in flowering time can thus be used to investigate the impact of lifespan changes on host defenses.

The defense system has also been intensively studied in this species, revealing multiple layers of defenses, ranging from basal defense, which is sufficient to control most microbes, to severe reactions that actively defeat virulent pathogens (Jones & Dangl 2006). Strain-specific defense components are likely to be linked in their evolution to the virulence specificity of co-occurring pathogens (de Meaux & Mitchell-Olds 2003; Moeller & Tiffin 2005; Roux & Bergelson 2016). Recent fluctuations in the composition of the pathogen population may therefore affect the specific components of defense targeted by these epidemics and thereby mask or blur the long-term impact of lifespan modifications. To minimize this effect and to highlight the impact of lifespan variation, we took a genomics approach and examined how flowering time co-varies with expression levels of genes with a experimentally-supported function in defense. These approximately 700 genes jointly reflect a broad spectrum of defense traits (Eulgem 2005), whose effectiveness is predicted by our model to increase with lifespan.

RESULTS and DISCUSSION

Positive co-variation between expression levels of defense genes and the timing of flowering in Swedish *A. thaliana* populations

We first focused on a set of 138 genotypes originating from Sweden. For these genotypes, high quality data for both, genome-wide expression profiles and flowering time estimates were available (Dubin *et al.* 2015; Sasaki *et al.* 2015). In these two studies, flowering time and gene expression were characterized in the same experiment at both 16°C and 10°C under long day conditions. We focused on the data collected at 16°C and computed Spearman correlation coefficients between the expression level of each gene and flowering time. Of 22,686 genes, for which expression levels could be quantified, 1,374 (6%) were significantly correlated with flowering time under a 5% false discovery rate (FDR). Among those genes, we observed a significant excess of defense genes (8.6% of 691 genes at 5% FDR, hypergeometric test, $p=0.002$). The distribution of correlation coefficients was also significantly skewed towards higher correlation coefficients for defense genes (Fig. 2A, Kolmogorov-Smirnov test, $p<2.2e-16$). By contrast, genes with a demonstrated or annotated function in flowering time in the genome were not enriched among those genes (6.9% of 630 genes at FDR 0.05, Hypergeometric test, $p= 0.19$). Nevertheless, the two most strongly correlated genes were two well-known repressors of flowering time, FLOWERING LOCUS C and SUPPRESSOR OF OVEREXPRESSION OF

CONSTANS-1 (FLC and SOC1, Spearman correlation ρ=0.50 and -0.62, FDR-corrected p=2.87e-6 and p=7e-12, respectively). This first analysis revealed a specific pattern of positive co-variation between flowering time and the expression of defense genes.
However, many genotypes in *A. thaliana* show an obligate requirement for vernalization. For these genotypes, differences in natural lifespan might be overestimated (Brachi *et al.* 2010; Li *et al.* 2014). In fact, only the 51 genotypes that advanced their flowering time at 16°C compared to 10°C, showed a correlation in their flowering across temperatures (Sasaki *et al.* 2015). The latter sub-sample of genotypes, which does not require low temperatures to induce flowering, may therefore allow a more accurate classification of genotypes with increasing vegetative lifespan. Among the 507 out of 22,686 (2.2%) genes that displayed a significant positive correlation with flowering time at 10% FDR across this restricted sample of genotypes, 16/630 and 28/691 genes belonged to flowering time or defense gene categories, respectively. Logically, several known flowering time regulators were among the genes associated with flowering time, such as FLOWERING LOCUS C (FLC), GIGANTEA, FLOWERING PROMOTING FACTOR 1-LIKE PROTEIN 2 (FLP2) or even the genes PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME INTERACTING FACTOR 5 (PIF5), which had been associated with accelerated flowering (Andrés & Coupland 2012; Thines *et al.* 2014). Although flowering time genes were not significantly enriched among genes correlating positively with the timing of flowering (2.5%, hypergeometric test, p=0.24), this was the case for defense genes (4%, 1.8-fold enrichment, hypergeometric test, p=0.0009). Importantly, in this subsample of genotypes, defense genes tended to be more highly expressed in late flowering genotypes (excess of positive correlations, Kolmogorov-Smirnov test, p<2.2e-16, Fig. 2B). This stood in contrast with flowering time genes, which tended to be more highly expressed in early flowering genotypes (excess of negative correlations, Kolmogorov-Smirnov test, p=1.16e-13, Fig. 2B).

Positive co-variation of defense gene expression with flowering time is independent of population structure and is also detected in a second sample of broader geographic origin

Because flowering time in the Swedish lines is strongly associated with the demographic history of these populations and thus with their population structure (Dubin *et al.* 2015; Sasaki *et al.* 2015), we also computed correlation between gene expression and flowering time with a mixed-model that included a kinship matrix for the 51 genotypes that lacked vernalization requirement (see methods; Yu *et al.* 2006; Stich *et al.* 2008). This analysis revealed that, for defense genes, the distribution of Spearman correlation coefficient remained strongly skewed towards positive values, after population structure was controlled for (Kolmogorov-Smirnov test, p=2.2e-16, Suppl. Fig. 1). However, defense genes were no longer enriched among genes with a significant co-variation with flowering time (5.2%vs. 5%, hypergeometric test, p= 0.6).
Accounting for population structure also did not change the pattern of co-variation between gene expression of flowering genes and timing of flowering itself. They showed a coefficient distribution that was strongly skewed towards negative values (Kolmogorov-Smirnov test, p=2.2e-16) and were significantly over-represented among genes with expression significantly associated with flowering time (8% vs 5% at 5% FDR, hypergeometric test, p=0.0005).
We further investigated whether the skew towards positive co-variation between defense gene expression and flowering time is limited to the regional subset of genotypes growing in Sweden or whether it is a feature of diversity that segregates across the whole range of the species. For

this, we turned to a species-wide dataset of gene expression variation collected in young seedlings (Schmitz *et al.* 2011). For 52 of these genotypes, the duration of vegetative growth had been determined under natural conditions in the field (Brachi *et al.* 2010). Neither flowering time nor defense genes were particularly enriched among genes with significantly correlated expression and flowering time at 5% FDR (5% for both, hypergeometric test, minimum p=0.24). Nevertheless, we again observed a strong skew towards positive correlation between defense gene expression and flowering time (Kolmogorov Smirnov test, D=0.19, p<2.2e-16, Fig. 2C), as well as a skew towards negative correlation for flowering time genes (D=0.09, p=5.3e-5, Fig. 2C). Contrasting genotypes of diverse flowering time (e.g. lifespan) revealed that, in natural populations, defense genes tend to co-vary positively with this trait. The latter two analyses showed that this effect remained when population structure was controlled for and was also detectable in another gene expression dataset and with a different set of genotypes.

Defense genes that show positive co-variation with flowering time are not under the control of flowering time

The tendency of defense genes to show expression levels correlating positively with flowering may be due to the pleiotropic action of regulatory genes that co-regulate flowering time and defense. In plants, the impact of development and growth regulators on defense systems is being increasingly recognized (Alcázar *et al.* 2011). There is evidence that development and life history strategies influence defense reactions against pathogens (Korves & Bergelson 2003; Pagán *et al.* 2009; Lozano-Duran *et al.* 2013; Fan *et al.* 2014; Jiménez-Góngora *et al.* 2015; Lozano-Durán & Zipfel 2015). In crops, decoupling flowering time and defense is sometimes problematic, in that breeding for resistance disrupts the timing of flowering and vice versa (Develey-Riviere & Galiana 2007). For example, in tomato, the introduction of the resistance gene against the pathogen *Alternaria* of a wild relative caused earlier flowering (Pajerowska-Mukhtar *et al.* 2009). In potato breeding, the conflict between resistance and earliness of tuber formation is of such importance that breeders select for maturity corrected resistance (Collins *et al.* 1999; Rodriguez-Falcon *et al.* 2006; Pajerowska-Mukhtar *et al.* 2009). In addition, infections and resistance reactions themselves can induce precocious flowering, possibly via the developmental effect of defense hormones (Martinez *et al.* 2004; Whalen 2005). Effects of infection on flowering time depend on the degree of infection, the pathogen type and the developmental stage of the host at the time of infection (Korves & Bergelson 2003; Pagan *et al.* 2008). A direct molecular link between basal plant defense triggered by the PAMP flagellin and the action of growth promoting hormones has also been demonstrated (Pieterse *et al.* 2012; Lozano-Duran *et al.* 2013; Jiménez-Góngora *et al.* 2015). Such pleiotropic action could in fact prevent the independent evolution of the two trait, and explain the pattern of covariation we observed.

We therefore tested whether the co-variation of flowering time with the expression of defense genes in natural populations could result from a pleiotropic action of flowering time regulators. If such regulators control the pattern of covariation reported in Fig. 2, it should not be possible to separate variation in defense gene expression from variation in flowering time in a segregating recombinant inbred population. We used the two genotypes Col-0 and Bur-0, which differ in flowering time (Simon *et al.* 2008) and were also reported to exhibit markedly distinct sensitivities to flagellin, with the later flowering genotype Bur-0 displaying stronger basal immunity (Vetter *et al.* 2012). We analyzed the transcriptomes of these two lines at 14 and 28 days after germination (see methods) and found that the skews shown in Fig. 2 remain when the dataset was reduced to

the genes that differed in expression between these two lines (Suppl. Fig. 2). This confirmed that these two genotypes could help identify defense genes that share genetic regulators with flowering time.

We designed a cost-effective approach to identify the genes whose expression variation cannot be separated from flowering time. We used 244 recombinant inbred lines (RILs) derived from a cross between the parents Bur-0 and Col-0, followed by >8 generations of selfing (Simon *et al.* 2008). We bulked RILs by their flowering time and characterized their transcriptomes at 14 and 28 days after germinations using RNA sequencing (see methods). In RILs, the genomes of the parental genotypes are randomly shuffled by recombination. Because of this genetic property, RILs are commonly used to identify Quantitative Trait Loci (QTL), which are genomic regions underlying the genetic control of phenotypic variation. In our approach, this means that differences in gene expression between early- and late-flowering RILs reflect differences that are genetically associated with flowering time. The experimental strategy is described in Suppl. Fig. 3-4. This strategy does not allow characterizing the exact genetic architecture of gene expression variation, but it allows the identification of genes whose expression variation is controlled either by flowering-time regulators or by genes located in the genomic vicinity of these regulators. Thereafter, we named these genes FT-controlled genes.

Of a total of 20,553 genes expressed in both the parental genotypes and RIL pools, 6,097 (29%) were differentially expressed between early and late flowering RIL pools, i.e. FT-controlled. As expected, we found a strong excess of genes annotated as flowering time genes among them (223/630 – 36%, hypergeometric test, p=3.7e-5). Defense genes were not over-represented among FT-controlled genes. More so, they were clearly under-represented among FT-controlled genes at the second time point of sampling (1.15 fold less abundant than expected by chance at day 28, hypergeometric test, p=0.01). Yet, 19% of all defense genes where FT-controlled. These genes, however, did not explain the skew towards positive co-variation with flowering time reported in Fig. 2. Defense genes, whose expression was not controlled by flowering time, indeed tended to be more skewed towards positive correlation coefficients than FT-controlled defense genes (Kolmogorov-Smirnov test, p=0.01, Fig. 3A). By contrast, FT-controlled flowering time genes did not shift significantly (Kolmogorov-Smirnov test, p=0.15, Fig. 3A). Therefore, the excess of positive expression co-variation with flowering time observed among defense genes is not due to a common genetic control of both traits, because the two traits can be easily separated by recombination. Since the correlation between flowering time and defense gene expression that we observed in natural populations is neither explained by population structure nor by genetic correlation, our results suggests the action of natural selection. Alleles attenuating the expression of defense genes were assorted with alleles promoting a shortening of flowering time and *vice-versa*. Our results thus indicate that defense and the duration of the vegetative life span co-evolve within natural *A. thaliana* populations.

Age-regulated defense genes often show positive co-variation with flowering time

Defense genes are often observed to change their activity with age and development (Barton & Boege 2017). Because we had sampled material at day 14 and day 28 after germination, we could also separate genes whose expression changed with age (here after named age-regulated genes) from genes with similar expression levels in 14- and 28-day-old plants (see methods). Age-regulated genes were markedly more frequent among annotated defense genes than among annotated flowering time genes (243/630 – 38% vs 334/691 - 48%, for flowering time and

defense genes, respectively, Chi Square test, p= 7.2e-11). In *A. thaliana,* the effectiveness of defense is indeed regulated by plant development: the onset of age-related resistance in *A. thaliana* provides a defense barrier against a broad spectrum of pathogens (Rusterucci *et al.* 2005). In agreement with our findings, the timing of age-related resistance had been reported not to stand under the direct control of flowering time (Wilson *et al.* 2013).
The subset of genes, whose expression variation in natural populations correlated with flowering time, were also enriched among age-regulated genes (hypergeometric test, p= 7.2e-11). Altogether, 4% (348/8565) and 6% (498/7935) of age-independent and age-regulated genes, respectively, were correlated with flowering time at 5% FDR. Defense genes contributed significantly to this excess, because the expression levels of defense genes that were age-regulated tended to show a strong skew towards positive correlation with flowering time in natural populations (Fig. 3B, Kolmogorov-Smirnov test, p=0.0009). Our analysis thus indicates that the tendency of defense genes to co-vary positively with flowering time in natural population is i) not explained by the genetic control of flowering time and ii) increased among genes whose expression is regulated by plant age.

Genes activated by elicitors of basal defense also show an excess of positive correlations with flowering time

In the above analysis, defense levels were represented by a set of 731 genes annotated for functions related to defense. To test whether the trend we report above was limited to a set of genes defined by Gene Ontology categories, we analyzed an independent set of defense-related genes: the 245 genes activated in *Arabidopsis* seedlings upon perception of flagellin by the PAMP receptor kinase FLAGELLIN SENSING 2 (FLS2), hereafter named FlaRe genes (Navarro *et al.* 2004). FlaRe genes coordinate cellular and developmental responses to exposure of molecular signatures of bacteria. Only 10 FlaRe genes overlapped with the defense-annotated genes used above. We observed that FlaRe genes were enriched among genes showing positive co-variation with flowering time (Fig. 2A-C, Kolmogorov-Smirnov test, p< 2.2e-16). This observation remained when controlling for population structure (Suppl. Fig. 1, Kolmogorov-Smirnov test, p<2.2e-16) and was also seen for flowering time measured in the field in a species-wide sample of genotypes (Fig. 2C, Kolmogorov-Smirnov test, p<2.2e-16). When partitioning genes according to whether or not they were FT-controlled or age-regulated, we observed that FT-control did not significantly change the distribution of correlation coefficients between FlaRe gene expression and flowering time across natural genotypes (Fig. 2A-B, Kolmogorov-Smirnov test, p=0.15 and p=0.32, for FT-controlled and age-regulated genes, respectively). Nevertheless, FlaRe genes were significantly under-represented among FT-controlled genes, especially at the second sampling time point (1.8-fold less frequent among flowering time controlled genes, hypergeometric test, p= 2.24e-05). By contrast, they were over-represented among age-regulated genes (2.1-fold more frequent among age-regulated genes, hypergeometric test, p= 2.6e-08). Thus, the positive co-variation reported in Fig. 1A-C is unlikely to result from the pleiotropic action of flowering time regulators on FlaRe genes. This suggests that, like for annotated defense genes, alleles attenuating the expression of FlaRe genes were assorted with early-flowering alleles in natural populations and vice versa.

Fitness-associated defense genes show higher correlation coefficients with flowering time

We further asked whether genes of stronger relevance for fitness have expression levels that are more strongly assorted with variation in the timing of flowering. A reciprocal transplant experiment performed in 4 locations throughout Europe identified 866 nucleotide variants in the genome of *A. thaliana* that significantly associated with fitness differences manifested in natural conditions (Fournier-Level *et al.* 2011). Of these variants, 15 mapped to defense genes and 17 to flowering genes. Association with fitness coincided with a skew towards higher correlation coefficients for defense genes only (Fig. 4, Kolmogorov-Smirnov test, D=0.46, p=0.014 and p>0.05 for defense and flowering time genes, respectively). One of the defense genes (AT3G16720), which is activated upon exposure to the fungal PAMP chitin, was FT-regulated but it did not explain this pattern (Kolmogorov-Smirnov test, p=0.028 without AT3G16720). Five of the defense genes with FT-independent immune functions were age-regulated (AT1G18150, AT1G80840, AT4G01700, AT5G19510, AT5G57220) but this did not explain the pattern either (Kolmogorov-Smirnov test, p=0.009 without these genes). Of the 245 FlaRe genes, 3 contained fitness-associated SNPs. These three genes were among the genes with highest correlation coefficients (AT1G19670: ρ=0.397, AT3G16720: ρ=0.282, AT4G38860: ρ=0.487). In agreement with our model, we thus observe that defense genes that can be most relevant for fitness in natural populations of *A. thaliana* are also genes whose expression levels were most strongly assorted with alleles determining flowering time.

Convergent indications that the defense system in *A. thaliana* co-evolves with flowering time

In agreement with our model predicting that the optimal defense level is a function of the duration of vegetative lifespan, we observe that, in *A. thaliana*, individuals with a longer vegetative lifespan tend to express defense genes at a higher level. Our bulk analysis of early- and late-flowering recombinant inbred lines (RILs) shows that this pattern of co-variation results from the combination of independent alleles controlling defense gene expression and flowering time in natural populations, because these alleles could be separated in the segregating recombinant offspring of an early- and a late-flowering genotype. Because co-variation is also i) robust to the demographic history of the populations and ii) particularly pronounced for defense-gene variants that associate with fitness, our analyses suggest that this allelic combination is assembled by natural selection. This pattern holds for both, a regional and a global sample of ecotypes and is confirmed by the examination of genes annotated with a function in defense and genes observed to respond to elicitation by the common bacterial elicitor flagellin.

In this study, expression levels of defense genes provided a large set of defense-related traits, which could be contrasted with the expression of all other genes in the genome, i.e. a set of quantitative traits with no *a priori* connection to defense. The approach we took thus allowed examining general defense levels and circumvented the signature that past epidemics may have left on some components of defense against specific pathogen strains. Our findings do not contradict evidence that a tug of war characterizes the evolution of pathogen-specific components of defense (Tellier & Brown 2007; Roux & Bergelson 2016). Nevertheless, to our knowledge, this work is the first to highlight the role that life history changes played in the global evolution of the defense system at the species level.

Further work is now warranted to determine the number of genetic modifications that allowed the adaptive tuning of the defense system to the timing of flowering. Our data suggest that much of the positive co-variation between defense gene expression and flowering depends on plant age. This factor is of recognized importance in plant immunity (Alcázar *et al.* 2011; Carella *et al.* 2015; Lozano-Durán & Zipfel 2015) and also very well documented in ecological studies (Barton & Boege 2017). Why age-dependent regulation of defense is so widespread and variable across taxa has not yet been fully explained. Based on our findings, it is tempting to speculate that it may mediate the response to changed selective forces on defense caused by life span modifications.

Adaptive evolution of flowering time is widespread in annual plant species

In monocarpic plant species, flowering terminates the vegetative growth phase and thus determines the amount of resources available for seed production; but its shortening can also enhance the probability to survive until maturity (Mitchell-Olds 1996; Metcalf & Mitchell-Olds 2009; Ashworth *et al.* 2016). In *A. thaliana*, the timing of flowering is greatly variable (Lempe *et al.* 2005; Balasubramanian *et al.* 2006) and changes in the control of flowering have probably been instrumental in the expansion of the species' climatic and geographic range (Corre 2005; Toomajian *et al.* 2006; Méndez-Vigo *et al.* 2011; Brachi *et al.* 2013; Li *et al.* 2014). Patterns of co-variation between flowering time and vegetative growth rate or seed dormancy change across broad climatic regions, suggesting that the timing of flowering has been adaptively remodeled multiple times across the species range (Debieu *et al.* 2013a; Burghardt *et al.* 2015a; Vidigal *et al.* 2016). Our study now indicates that the maintenance of diverse locally adapted flowering time alleles also promotes the maintenance of diversity in defense.

Our model describes how changes in the duration of vegetative growth alter the likelihood to encounter a pathogen. We note, however, that flowering time measured in common garden conditions is an imperfect proxy for vegetative lifespan. It varies greatly across the species range, along seasonal fluctuations, as well as in the genetics of the timing of flowering (Korves *et al.* 2007; Burghardt *et al.* 2015b; Hu *et al.* 2017). We also observed that the pattern of covariation between defense gene expression and flowering time measured in controlled laboratory conditions is magnified in plants whose flowering is not dependent on prior cold exposure (Fig. 2A-B). Even if the flowering time data used to compute correlation in Fig. 2C was collected in common garden conditions, it may not reflect perfectly the actual lifespan that genotypes realize in their native environment. Admittedly, our study does not rule out the possibility that flowering time could co-evolve with factors other than lifespan, that also increase the likelihood of infection, as e.g. humidity. A better characterization of local ecological conditions is surely required to confirm that the assortment of flowering time and defense alleles reported here reflects the joint optimization of defense and lifespan by natural selection.

We anticipate, however, that the observed attenuation of defense gene expression is likely to be widespread across both model and non-model plant species that have reshaped their life history to decrease overall vegetative lifespan. Early flowering is actually often favored when the favorable season is shortened (Franks *et al.* 2007; Kenney *et al.* 2014). Ongoing selection for early flowering is clearly widespread at temperate latitudes (Munguía-Rosas *et al.* 2011) and transitions from perenniality to annuality occur frequently within phylogenies (Kiefer *et al.* 2017).

The impact of life history evolution on defense systems is expected across all kingdoms

In animals, the idea that the optimal investment in defense depends on the life history of a species was also incorporated in evolutionary models (Jokela *et al.* 2000). For plants and animals alike, resources available to the organism are limited. Energetic demands on growth may compete with those required for mounting defense or counteracting the negative effects of parasites and pathogens (van Boven & Weissing 2004; Dowling & Simmons 2009; Lazzaro & Little 2009; Seppälä 2015). Several evolutionary models show that a prolonged lifespan is predicted to favor resource investment into immunity (Jokela *et al.* 2000; Medzhitov & Janeway 2000; van Boven & Weissing 2004; Miller *et al.* 2007). As a consequence, changes in life history can mold the evolution of defense systems in animals as well (Van Valen 1973; Sheldon & Verhulst 1996; Schulenburg *et al.* 2009). This theoretical prediction is supported by analyses of sexual dimorphism in the duration of effective breeding: females with increased reproductive longevity show stronger immune-competence (Rolff 2007; Nunn *et al.* 2009). In frogs, fast developing species were also shown to be more susceptible to infection by trematodes (Johnson *et al.* 2012). Yet, such studies cannot exclude that longevity and immunity are constrained in their evolution by common regulatory factors or causal inter-dependence. To the best of our knowledge, this study is the first to provide evidence that natural variation in the activity of genes that are important for defeating pathogens is assorted with alleles controlling variation in a life history trait of considerable importance for adaptation. Local adaptation for lifespan should therefore be considered as a potentially important contributor to the maintenance of genetic diversity in defense systems.

MATERIAL AND METHODS

Flowering and defense candidate genes

Gene Ontology (GO) categories were used to identify functionally related genes whose annotation was inferred from experiments, direct assays, physical interaction, mutant phenotype, genetic interactions or from expression patterns. Based on the keyword "flowering" in the TAIR database, 659 flowering time genes were selected. For defense genes, we united 17 GO categories yielding 731 genes (Suppl. Table S1). For flagellin responsive (FlaRe) genes, we took the set of 245 genes that were activated in seedlings described in (Navarro *et al.* 2004) (Suppl. Table S1). Subsets of flowering, defense and FlaRe genes containing fitness-associated single nucleotide polymorphisms (SNPs) were retrieved from Fournier-Level *et al.* 2011.

Correlation between gene expression and flowering time in a natural population

We analyzed two published sets of natural ecotypes for which both genome-wide expression profiles and flowering time estimates were available. The first dataset comprised 138 lines from Sweden scored for both flowering time (for plants grown at 16h light-8hour dark at constant 16°C) and gene expression in whole rosette collected at the 9-true-leaf stage (Dubin *et al.* 2015; Sasaki *et al.* 2015). For this first dataset, gene expression and flowering were determined in the same experiment. The second dataset combined data from two sources. RNA extracted from 7-day old seedlings of 144 genotypes grown on agar plate in long days had been sequenced (Schmitz *et al.* 2013) and expression levels quantified as quantile normalized fragment numbers per kilobases and million reads (FPKM). For 52 of these genotypes, flowering time, measured in cumulative photothermal units, had been scored in the field (Brachi *et al.* 2010). Photo-thermal units sum up the combination of temperature and day length and thus provide an estimate of the duration of the favorable season.

Expression counts were $\log_e$ +1-transformed to include null values of expression and a Spearman correlation coefficient between flowering time and expression level was computed for each gene. P-values were adjusted for false discovery rate using the p.adjust function in R (Benjamini & Hochberg 1995; Yekutieli & Benjamini 1999). A Kolmogorov-Smirnov test was used to compare the distribution of Spearman correlation coefficients ρ of flowering time and defense genes with the distribution of ρ for 22,686 genes for which gene expression was quantified. Gene enrichments were tested for using hypergeometric tests in R. To test the impact of population structure on the correlation, we ran a mixed model using the R package *lmekin* and the kinship matrix was generated with a matrix of SNPs segregating among Swedish genotypes (Dubin *et al.* 2015).

Correlation between gene expression and flowering time in bulk segregant pools

Seeds of Bur-0, Col-0 and 278 Bur-0xCol-0 Recombinant Inbred Lines (RIL) obtained after 8 generations of selfing were provided by the Arabidopsis Stock Center at INRA Versailles (France, Simon *et al.* 2008). Each line was grown individually in six replicates, each in 6cm diameter pots randomly allocated to 24 trays, each containing 35 pots. Seeds were stratified at 5°C for 3 days and grown in growth chambers (Elbanton BV, Holland, equipped with Sylvania Gro-Lux F36W /Gro (T8) fluorescent tubes and Osram 25 W 220 Lumen light bulbs) under

long-day conditions (21°C, 16h light, 18°C, 8h dark). Trays were rotated within the chamber every other day. Flowering time was scored as the day to the first open flower. Genotypes of individuals lines were retrieved from Simon *et al.* (2008) and mapping of flowering time recovered the same QTL (not shown).

We selected the 40 RIL in the 15% and 85% quantiles of flowering time for RNA sequencing. Each RIL and the two parental lines were planted in 20 replicates in the conditions described above. At days 14 and 28, the oldest true leaf was flash-frozen in liquid nitrogen. Three pools, each combining 13 RIL, were produced at each time point for early and late lines, for a total of 3 biological replicates, 2 pool types (early and late RIL) and 2 time points (14 and 28 days). For each of the two parental lines, leaves of 12 replicates were pooled for each time point.

RNA was isolated using the TRIzol extraction protocol (ThermoFisher Scientific, USA). DNA traces were removed with the Ambion DNA-free kit (ThermoFisher Scientific, USA) and purified RNA was stored in TE buffer at -80°C. RNA quality and integrity was confirmed with the 2100 Expert Software on a Bioanalyzer (Agilent Technologies, Inc. Waldbronn, Germany). Only samples with RNA integrity index (RIN) of at least 8 were sequenced. Single-read libraries were prepared with 1µg of total RNA per sample using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina Inc. San Diego, USA) based on poly-A RNA purification. Sequencing of 75bp single reads was performed on the Illumina HighScan SQ system of the Core Facility of the Department of Genetic Epidemiology, Institute of Human Genetics, University of Münster, Germany. Raw data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE97664.

Data analysis of RNA-seq from bulk segregant pools

Raw sequences were demultiplexed and read quality validated with FastQC. Bad quality base calls were trimmed using the fastx-toolkit (Version 0.013, Li *et al.* 2009). Trimmed reads (FastQ, quality score 33, quality threshold 20 and minimum length 30 base pair) were mapped to the *A. thaliana* TAIR10 annotated transcriptome using Bowtie 2 (version 2-2.0.0-beta6, (Langmead & Salzberg 2012). Tophat (version-2.0.5.Linux_x86_64) was used to discover splice sites and Cufflinks for assembling the transcriptome (Trapnell *et al.* 2010). We used a custom R script to verify that coverage was uniform across transcripts and confirmed that the RNA sequenced was not degraded. Read counts were calculated by counting the number of reads that mapped uniquely to the corresponding gene (isoforms were not considered). Lowly expressed genes with less than 20 reads over all samples were excluded from the analysis. The samples clustered by time point of sampling (Suppl. Figure 4), with the exception of RNA samples from the Col-0 at 28 days, which resembled more expression levels measured at 14 days, probably because of its early shift to flowering. Differentially expressed (DE) genes were identified by running a nested analysis of sampling time effects within parental genotype (and/or early- and late-flowering leaf pools) with DESeq2 version 1.2.5 (Anders *et al.* 2013; Love *et al.* 2014). P-values were corrected for false discovery rate (Benjamini-Hochberg correction; Benjamini & Hochberg 1995). DE genes were defined as having an adjusted p-value<0.05. This analysis allowed the identification of genes showing differential expression between the parents (Suppl. Table S2) and genes showing flowering time dependent expression (differential expression between early and late flowering RIL pools, i.e. FT-regulated genes Suppl. Table S3) both at day 14 and at day 28. We performed further analyses to disentangle significant sources of gene expression variation. To test whether gene expression was significantly modified at each time point, separate tests were performed for each parental genotype and RIL pool type. Genes differentially regulated at 14- and 28-days in

Bur-0 (adjusted p-value<0.05) were defined as age-regulated genes (Suppl. Table S4). To determine whether one or both sampling time points drove significant differential expression, separate tests were performed for each time point (not shown).

Confirmation with qRT PCR

We confirmed gene expression levels for 11 selected defense genes with differential expression between Bur-0 and Col-0 or early vs. late flowering pools (log2-fold change > 1.5) using RT-PCR. We followed standard protocols and used RNA Helicase (AT1G58060), Protein Phosphatase 2A Subunit A3 (PP2AA3) and transcript AT5G12240 as control genes. Gene expression based on RNA sequencing and RT-PCR were strongly correlated (Pearson correlation, 0.58<R<0.96, max p <0.01).

Figure legends

Figure 1: Model of co-evolution between defense and the timing of flowering (f_t) which marks the end of the vegetative lifespan in annual plants. f_t influences survival via its impact on the risk of infection, as well as plant size, which is a function of the length of vegetative lifespan. Optimal defense thus maximizes survival as a function of f_t. Size at flowering determines the amount of resources available for seed production and thus fitness (W). Parameters of the model are listed in the inset.

Figure 2: Distribution of Spearman correlation coefficients between expression levels of each expressed *A. thaliana* gene and flowering time. Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes; Pink: Flagellin-responsive (FlaRe) genes (Navarro *et al.* 2004). **A.** For 138 Swedish genotypes; **B.** Analysis restricted to 51 Swedish genotypes showing correlated flowering time at 10°C and 16°C; C. Species-wide sample of 52 genotypes. Distribution for each group of genes was compared to the genome-wide distribution (black double-head arrow) with a Kolmogorov-Smirnov test. P-values are given in the color corresponding to the gene class. Spearman correlation coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin *et al.* 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki *et al.* 2015). *** p < 0.001.

Figure 3: Distribution of Spearman correlation coefficients between gene expression level and flowering time. **A.** Partition of genes controlled by flowering time (blue border) vs independent from flowering time (black border); **B.** Partition of genes controlled by development (orange border) vs independent from development (black border). Defense genes that are not controlled by flowering time but controlled by development tend to have higher correlation coefficients of natural variation for expression with natural variation for flowering time. Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes; Pink: Flagellin-responsive (FlaRe) genes (Navarro *et al.* 2004). P-values for Kolmogorov-Smirnov test comparing the distribution of genes within each category that are independent of or regulated by **A.** flowering time or **B.** age are shown when significant. Note that only 12 FlaRe genes are controlled by flowering time in our experiment. Spearman correlation

coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin *et al.* 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki *et al.* 2015). * $p < 0.05$, *** $p < 0.001$.

Figure 4: Distribution of Spearman correlation coefficients between gene expression level and flowering time. All expressed genes -black border- vs. genes with fitness-associated SNPs in Fournier-Level *et al.* (2011), - purple border-. Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes. Defense genes that carry SNPs associating with fitness tend to have higher correlation coefficients of natural variation for expression with natural variation for flowering time. P-values for Kolmogorov-Smirnov test comparing the distribution for genes within each category are shown when significant. Spearman correlation coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin *et al.* 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki *et al.* 2015). * $p < 0.05$.

Supplementary Tables

Suppl. Table 1: List of defense genes and GO categories (only gene annotations based on experimental validation were considered). FlaRe genes and flowering time genes used in the study.

Suppl. Table 2: FT-regulated genes (DE between early and late flowering RIL pools). Output of gene expression analysis includes mean read count (FPKM), log2 fold-change, Standard error of the log2 fold-change; p-value and FDR adjusted p-value. FT-regulated genes have FDR adjusted p-values <0.05.

Suppl. Table 3: Differentially expressed genes between Col-0 and Bur-0. Output of gene expression analysis includes mean read count (FPKM), log2 fold-change, Standard error of the log2 fold-change; p-value and FDR adjusted p-value. Genes differently regulated between Col-0 and Bur-0 have FDR adjusted p-values <0.05.

Suppl. Table 4: Age-regulated genes defined as differential gene expression changes in Bur-0 between 14- and 28-days. Output of gene expression analysis includes mean read count (FPKM), log2 fold-change, Standard error of the log2 fold-change; p-value and FDR adjusted p-value. FT-regulated genes have FDR adjusted p-values <0.05.

Supplementary Document

Model of co-evolution between flowering time and defense

Supplementary Figures

Suppl. Figure 1: Distribution of estimated effect of flowering time as explanatory factor of gene expression variation, taking into account population structure between Swedish genotypes of group A (Group A genotypes advance their flowering at 16°C compared to 10°C and show correlated flowering at 10°C and 16°C, Sasaki *et al.* 2015). The trend shown in Figure 1 is maintained after controlling for population structure. P-values for Kolmogorov-Smirnov test comparing estimate distribution for the gene subset compared to the genome-wide distribution are given. **A.** Density distribution, **B.** Boxplots.

Suppl. Figure 2: Distribution of correlation coefficients between gene expression and flowering time restricted to the genes showing differential expression in Col-0 vs. Bur-0. Expression differences between the genotypes Col-0 and Bur-0 recapitulate the pattern reported within natural populations in Figure 1. Distribution of Spearman correlation coefficients between gene expression level and flowering time for a set of genotypes showing consistent differences in flowering at 10°C and 16°C (Group A genotypes, Sasaki *et al.* 2015). This analysis is restricted to the 6980 genes showing differential expression between Col-0 and Bur-0. Defense genes have a stronger skew in correlation coefficients with flowering time. Spearman correlation coefficients were computed between expression level of each of 6980 expressed *A. thaliana* genes, reported in Durbin *et al.* 2015 for 9th leaf seedlings, and flowering time measured in the same condition. Genotypes originate from natural populations in Sweden (Sasaki *et al.* 2015). Black line: All expressed genes, Blue lines: Gene annotated as flowering time genes (FT genes), Red lines: Genes annotated as defense genes, Pink line: Flagellin-responsive (FlaRe) genes (Navarro *et al.* 2004).

Suppl. Figure 3: Bulk-sequencing strategy used to identify FT-controlled genes, i.e. genes whose expression is genetically controlled by flowering time regulators or by genes located closely to and therefore co-segregating with flowering time regulators.

Suppl. Figure 4: Heatmap of gene expression variation between samples for the 1000 genes showing highest expression variation between samples. Clustering of gene expression levels across sample and replicate shows that samples partition by age, and flowering time.

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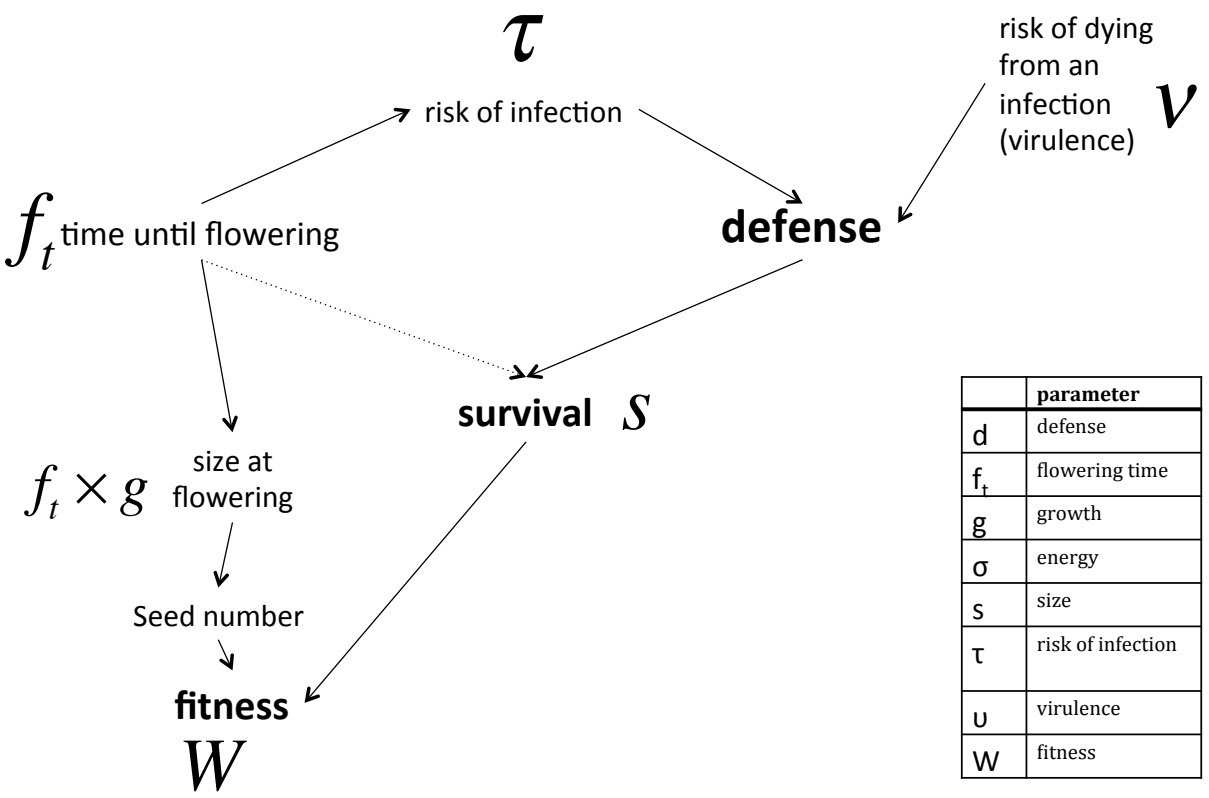
Figure 1: Model of coevolution between defense and the timing of flowering (f_t) which marks the end of the vegetative life span in annual plants. f_t influences survival via its impact on the risk of infection, as well as plant size, which is a function of the length of vegetative life span. Optimal defense thus maximizes survival as a function of f_t. Size at flowering determines the amount of resources available for seed production and thus fitness (W). Parameters of the model are listed in the inset.



	parameter
d	defense
f_t	flowering time
g	growth
σ	energy
s	size
τ	risk of infection
υ	virulence
W	fitness

Figure 2: Distribution of Spearman correlation coefficients between expression levels of each expressed *A. thaliana* gene and flowering time. Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes; Pink: Flagellin-responsive (FlaRe) genes (Navarro et al. 2004). **A.** For 138 Swedish genotypes; **B.** Analysis restricted to 51 Swedish genotypes showing correlated flowering time at 10°C and 16°C; C. Species-wide sample of 52 genotypes. Distribution for each group of genes was compared to the genome-wide distribution (black double-head arrow) with a Kolmogorov-Smirnov test. P-values are given in the color corresponding to the gene class. Spearman correlation coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin et al. 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki et al. 2015). *** p < 0.001.

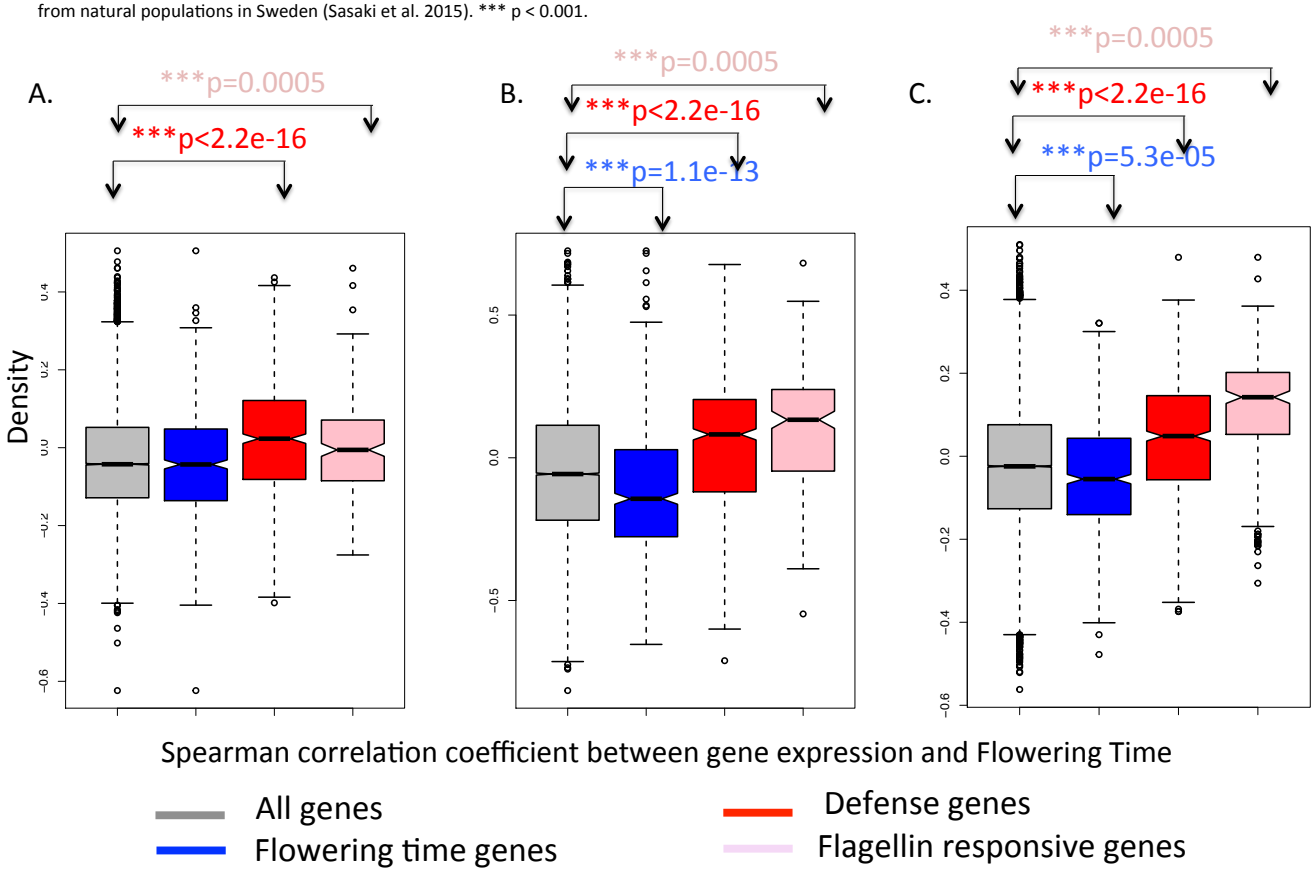


Figure 3: Distribution of Spearman correlation coefficients between gene expression level and flowering time. **A.** Partition of genes controlled by flowering time (blue border) vs independent from flowering time (black border); **B.** Partition of genes controlled by development (orange border) vs independent from development (black border). Defense genes that are not controlled by flowering time but controlled by development tend to have higher correlation coefficients between expression and flowering time. Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes; Pink: Flagellin-responsive (FlaRe) genes (Navarro et al. 2004). P-values for Kolmogorov-Smirnov test comparing the distribution of genes within each category that are independent of or regulated by **A.** flowering time or **B.** age are shown when significant. Note that only 12 FlaRe genes are controlled by flowering time in our experiment. Spearman correlation coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin et al. 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki et al. 2015). * $p < 0.05$, *** $p < 0.001$.

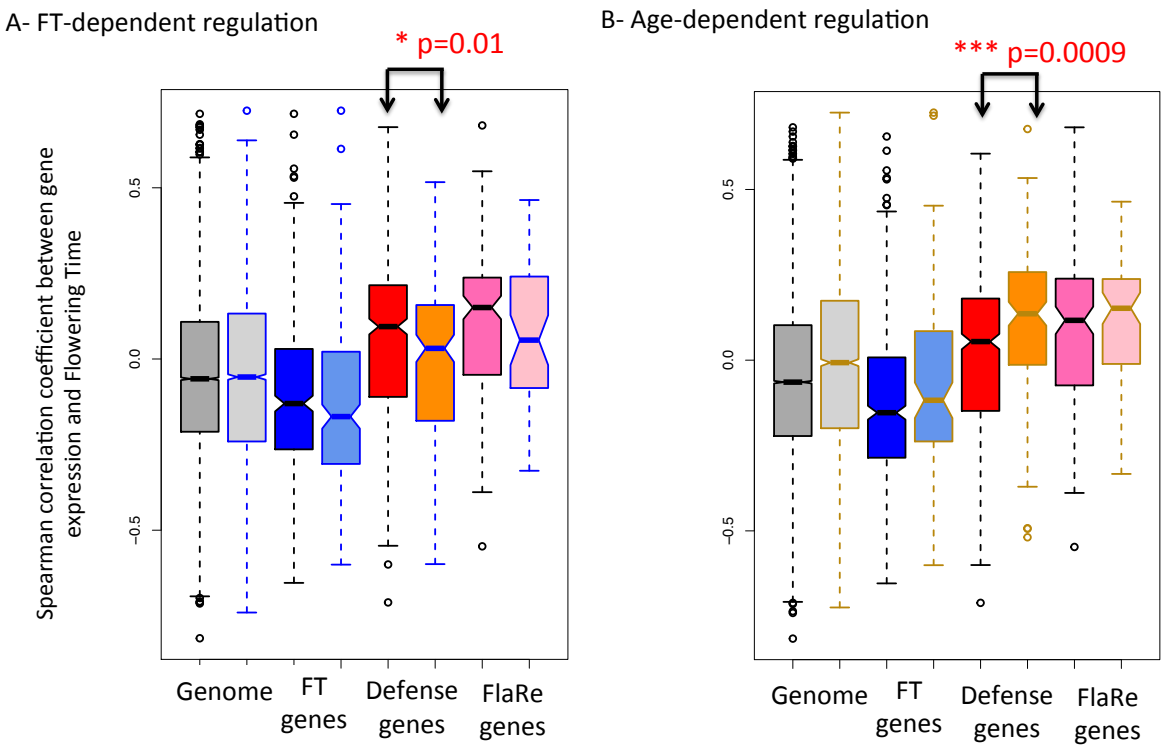
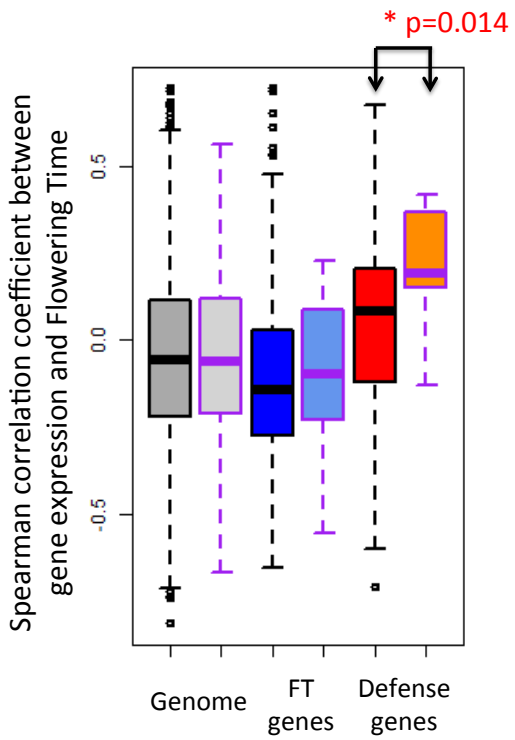
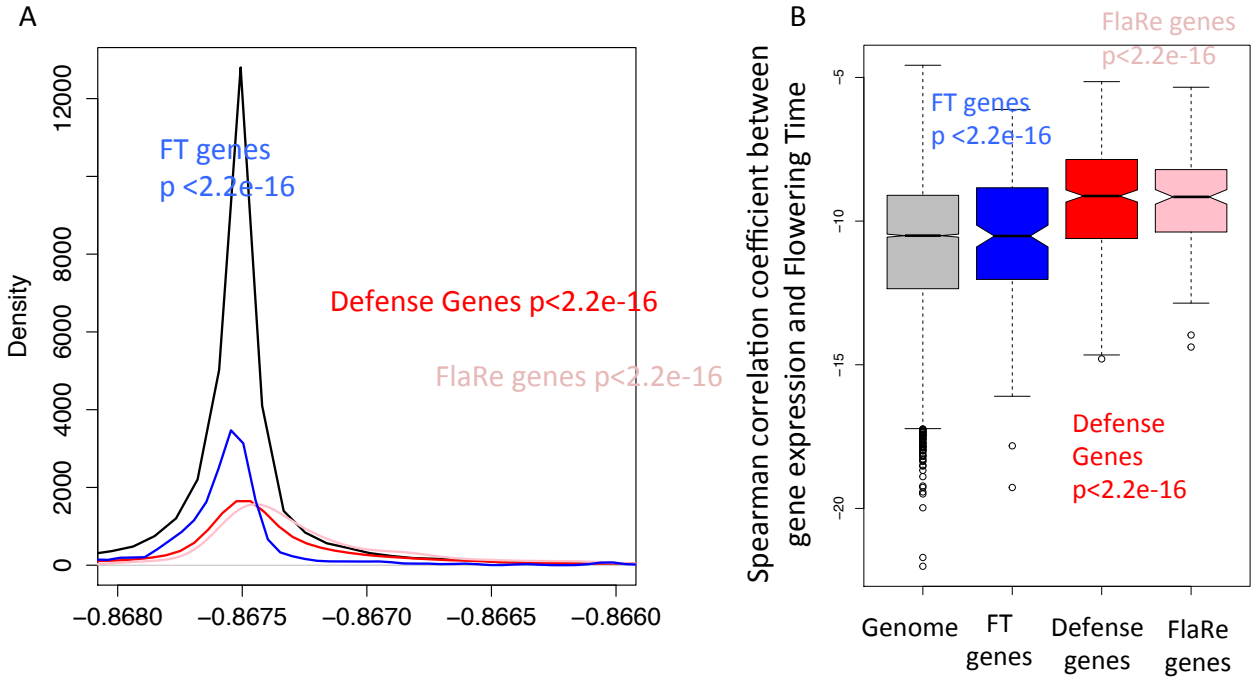


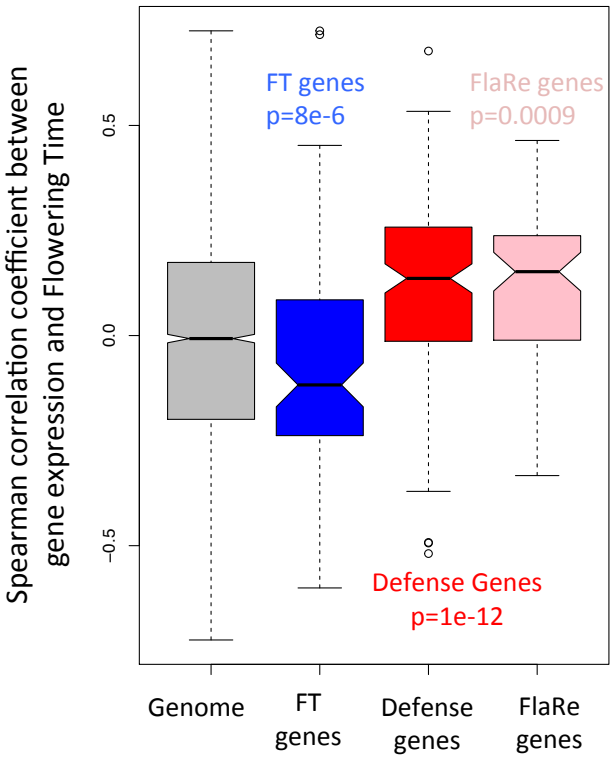
Figure 4: Distribution of Spearman correlation coefficients between gene expression level and flowering time. All expressed genes (black border) vs genes with fitness-associated SNPs in (Fournier-Level *et al.* 2011 (purple border). Grey: All expressed genes; Blue: Genes annotated as flowering time genes (FT genes); Red: Genes annotated as defense genes. Flowering time and gene expression measures were correlated for 16°C. The expression of defense genes that contain fitness-associated SNPs tend to have higher correlation coefficients with flowering time. P-values for Kolmogorov-Smirnov test comparing the distribution for genes within each category are shown when significant. Spearman correlation coefficients were computed between expression levels of each of 23,511 expressed *A. thaliana* genes, reported in Durbin *et al.* 2015 for 9th leaf seedlings, and flowering time measured in the same condition for 51 genotypes originating from natural populations in Sweden (Sasaki *et al.* 2015). * $p < 0.05$.



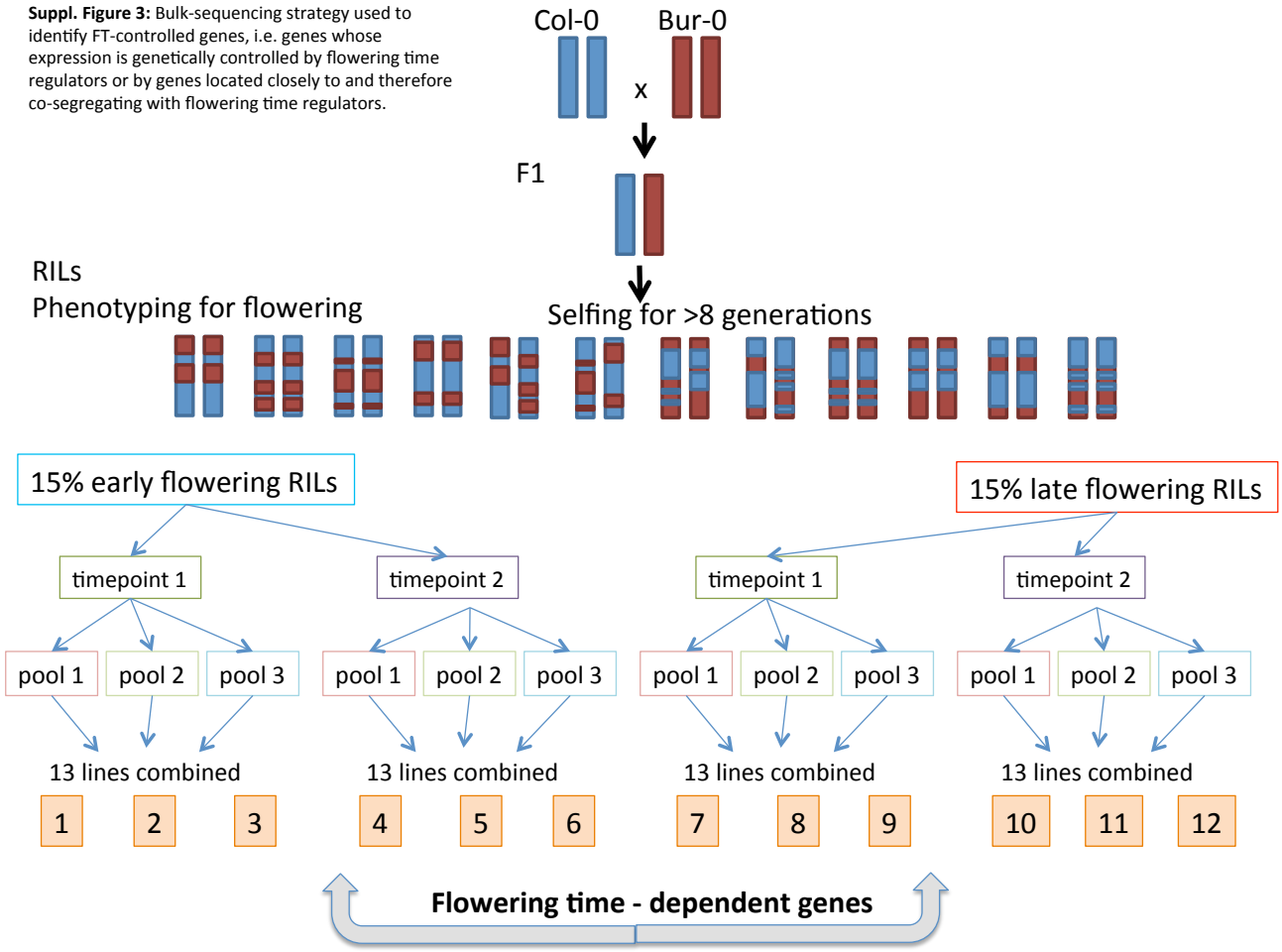
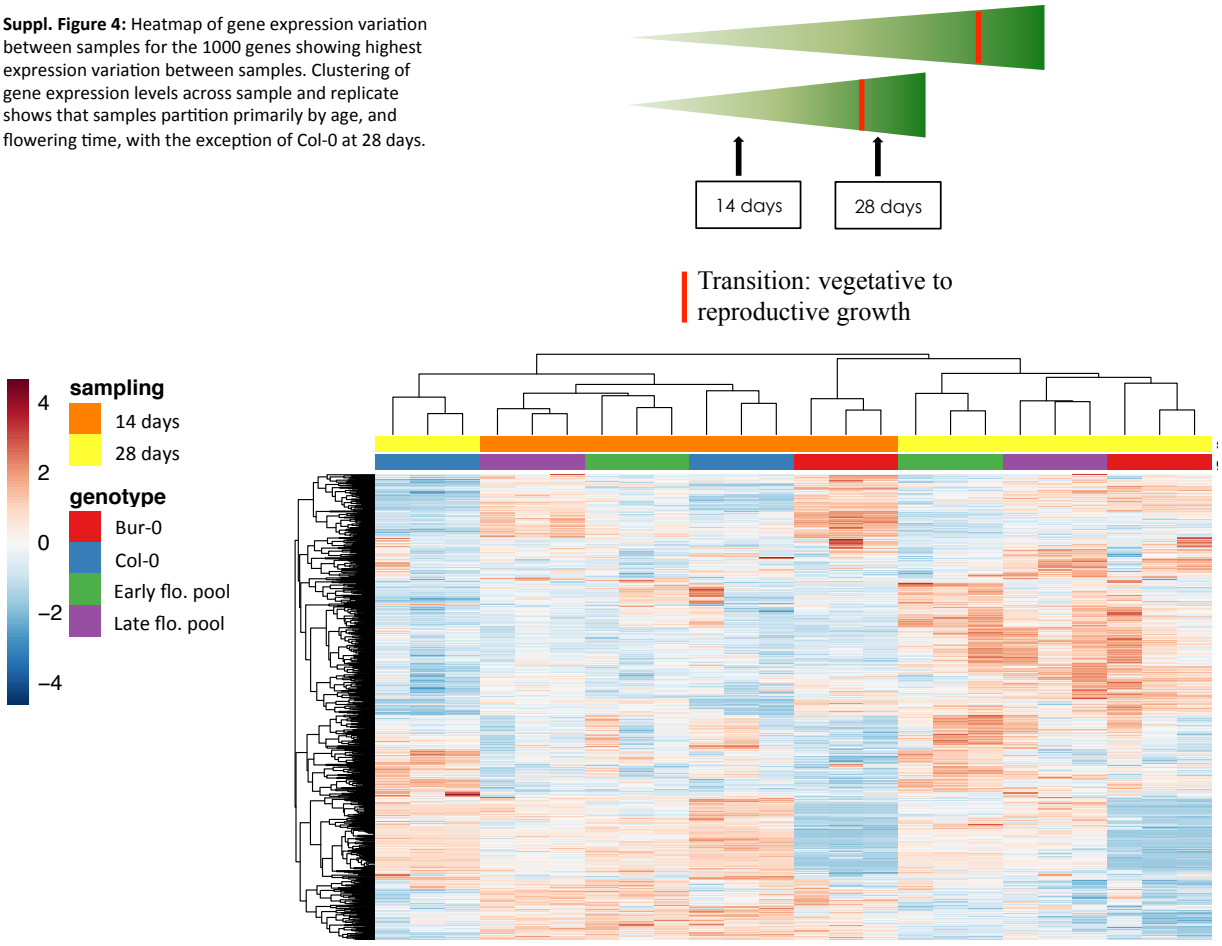
Suppl. Figure 1: Distribution of estimated effect of flowering time as explanatory factor of gene expression variation, taking into account population structure between Swedish genotypes of group A . The trend shown in Figure 1 is maintained after controlling for population structure. P-values for Kolmogorov-Smirnov test comparing estimate distribution for the gene subset compared to the genome-wide distribution are given. **A.** Density distribution, **B.** Boxplots.
What is group A?



Suppl. Figure 2: Distribution of correlation coefficients between gene expression and flowering time restricted to the genes showing differential expression in Col-0 vs. Bur-0. Expression differences between the genotypes Col-0 and Bur-0 recapitulate the pattern reported within natural populations in Figure 1. Distribution of Spearman correlation coefficients between gene expression level and flowering time for a set of genotypes showing consistent differences in flowering at 10°C and 16°C (Group A genotypes, Sasaki et al. 2015). This analysis is restricted to the 6980 genes showing differential expression between Col-0 and Bur-0. Defense genes have a stronger skew in correlation coefficients with flowering time. Spearman correlation coefficients were computed between expression level of each of 6980 expressed *A. thaliana* genes, reported in Durbin et al. 2015 for 9th leaf seedlings, and flowering time measured in the same condition. Genotypes originate from natural populations in Sweden (Sasaki et al. 2015). Black line: All expressed genes, Blue lines: Gene annotated as flowering time genes (FT genes), Red lines: Genes annotated as defense genes, Pink line: Flagellin-responsive (FlaRe) genes (Navarro et al. 2004).



Suppl. Figure 3: Bulk-sequencing strategy used to identify FT-controlled genes, i.e. genes whose expression is genetically controlled by flowering time regulators or by genes located closely to and therefore co-segregating with flowering time regulators.



Suppl. Figure 4: Heatmap of gene expression variation between samples for the 1000 genes showing highest expression variation between samples. Clustering of gene expression levels across sample and replicate shows that samples partition primarily by age, and flowering time, with the exception of Col-0 at 28 days.