Habitat-Associated Life History and Stress-Tolerance Variation in *Arabidopsis arenosa*[1][OPEN]

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Weediness in ephemeral plants is commonly characterized by rapid cycling, prolific all-in flowering, and loss of perenniality. Many species made transitions to weediness of this sort, which can be advantageous in high-disturbance or human-associated habitats. The molecular basis of this shift, however, remains mostly mysterious. Here, we use transcriptome sequencing, genome resequencing scans for selection, and stress tolerance assays to study a weedy population of the otherwise nonweedy *Arabidopsis arenosa*, an obligately outbreeding relative of *Arabidopsis thaliana*. Although weedy *A. arenosa* is widespread, a single genetic lineage colonized railways throughout central and northern Europe. We show that railway plants, in contrast to plants from sheltered outcrops in hill/mountain regions, are rapid cycling, have lost the vernalization requirement, show prolific flowering, and do not return to vegetative growth. Comparing transcriptomes of railway and mountain plants across time courses with and without vernalization, we found that railway plants have sharply abrogated vernalization responsiveness and high constitutive expression of heat- and cold-responsive genes. Railway plants also have strong constitutive heat shock and freezing tolerance compared with mountain plants, where tolerance must be induced. We found 20 genes with good evidence of selection in the railway population. One of these, *LATE ELONGATED HYPOCOTYL*, is known in *A. thaliana* to regulate many stress-response genes that we found to be differentially regulated among the distinct habitats. Our data suggest that, beyond life history regulation, other traits like basal stress tolerance also are associated with the evolution of weediness in *A. arenosa*.

Life history traits differ between and within plant species and commonly reflect the requirements of the habitats in which they are found (Baker, 1974; Weinig et al., 2003; Grime, 2006). Depending on abiotic and biotic conditions, a variety of strategies can be favored, and accordingly, weeds are phenotypically diverse. In environments that are unpredictable, with frequent occurrences of stresses like drought, temperature fluctuations, or human-associated perturbations, rapid cycling and early flowering are common (Hall and Willis, 2006; Sherrard and Maherali, 2006; Franks et al., 2007; Wu et al., 2010). Life history adaptations can help mediate tradeoffs between resource accumulation and stress avoidance and are important for wild species as well as for crops (Jung and Müller, 2009). Comparing results among species, as well as the correlates of these traits with other fitness-related traits, promises new insights into the mechanisms of adaptation to unpredictable habitats.

A common phenotype of plants in unpredictable habitats is early and prolific flowering relative to related populations in more stable habitats (Baker, 1965; Grotkopp et al., 2002; Blair and Wolfe, 2004; Burns, 2004; Hall and Willis, 2006; Sherrard and Maherali, 2006; Franks et al., 2007). The complex genetic architecture of flowering has been well studied in the annual *Arabidopsis thaliana* (Andrés and Coupland, 2012), where independent changes in two genes in particular, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), have been repeatedly found to underlie natural variations in flowering time and vernalization responsiveness (Michaels and Amasino, 1999; Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003; Lempe et al., 2005; Shindo et al., 2005; Werner et al., 2005; Brachi et al., 2010; Méndez-Vigo et al., 2011, 2016; Salomé et al., 2011; Song et al., 2013). The same genes also are important in the closely related *Arabidopsis lyrata* (Kuittinen et al., 2008) and in other species in the Brassicaceae (Slotte et al., 2009; Wang et al., 2009; Guo et al., 2012). Active *FRI* alleles

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enhance the expression of *FLC*, which in turn represses floral activators including *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Michaels and Amasino, 1999; Searle et al., 2006). *Arabidopsis thaliana* accessions with functional alleles of both *FRI* and *FLC* are late flowering in the laboratory, but prolonged exposure to cold (vernalization) epigenetically represses *FLC* expression, allowing flowering upon the return to warm temperatures (Song et al., 2013). Many independent disruptions of *FRI* or *FLC* have been identified in *A. thaliana* (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003; Shindo et al., 2005; Werner et al., 2005; Méndez-Vigo et al., 2011) that result in reduced or abrogated *FLC* expression, leading to earlier flowering and reduced need for vernalization. However, nonfunctional *FRI* alleles are associated with negative pleiotropic effects on branching and fitness, likely limiting the adaptive potential of the *FRI* locus (Scarcelli et al., 2007). Natural variation in *FLC* and *FRI* also affects other important life history traits, including seed germination (Chiang et al., 2009), water use efficiency, which is a major dehydration avoidance mechanism (McKay et al., 2003), and even flower tolerance to heat shocks (Bac-Molenaar et al., 2015). *FLC* also plays a role in more long-lived plants. In the perennial *Arabis alpina*, an ortholog of *FLC*, *PERPETUAL FLOWERING1* (*PEP1*), contributes to late flowering and the vernalization requirement as it does in *A. thaliana* but it also promotes a return to vegetative development after each flowering episode, which is an important feature of perennial life cycles (Wang et al., 2009). Variation in PEP1 activity is associated with distinct life histories in different *A. alpina* accessions (Albani et al., 2012).

Arabidopsis arenosa is a close relative of *A. thaliana* and *A. lyrata* (O'Kane, 1997; Clauss and Koch, 2006). In contrast to *A. thaliana A. arenosa* is a perennial obligate outcrosser with high genetic diversity and both diploid and tetraploid variants (Hollister et al., 2012; Schmickl et al., 2012). The autotetraploid *A. arenosa* arose from a single diploid population closely related to populations found today in the Carpathian Mountains of Slovakia around 19,000 generations ago; by 15,000 generations ago, autotetraploid lineages had begun radiating across the landscape into the distinct types found in diverse habitats across Europe today (Arnold et al., 2015). Distinct genetic lineages correlate with geography and habitat. Rocky outcrops are generally populated by a perennial mountain form, while ruderal settings, especially railways, are colonized by an annual, flatland form (Scholz, 1962). Although the mountain form comprises at least four distinct genetic lineages associated with geography, we found previously that railway populations from geographically distant locations are extremely closely related, suggesting that this habitat was colonized just once by a single genetic lineage that subsequently spread along this habitat (Arnold et al., 2015).

Here, we study representative populations of the perennial mountain form and the flatland form of *A. arenosa*. Specifically, we use phenotypic, genomic, and transcriptomic experiments to assess flowering time, vernalization responsiveness, and stress resilience. We found that populations from ruderal sites are rapid cycling, do not require vernalization, and do not resume vegetative growth after a single flowering episode, while mountain populations remain vernalization responsive. We compared transcriptomes of early-flowering (railway) and late-flowering (mountain) plants across time series that were either vernalized or not. We found that rapid-cycling plants from railway populations have very low *FLC* expression and a sharply abrogated vernalization response, while plants from a mountain population show transient repression of *FLC* by vernalization similar to what was described in *A. alpina* (Wang et al., 2009; Albani et al., 2012). We also found constitutive differences in the expression of cold and heat stress-response genes. Consistent with the expression data, we found that railway plants had higher basal heat and cold stress tolerance than mountain plants. A genome-resequencing scan for divergence identified 20 loci with evidence of positive selection in the weedy railway lineage. Among these is the circadian clock regulator *LATE ELONGATED HYPOCOTYL* (*LHY*), which regulates many of the cold- and heat-responsive genes we found to be differentially expressed in these two *A. arenosa* types. Our data suggest that, in addition to flowering behavior, traits like flowering induction and heat and cold stress tolerance that are environmentally inducible in mountain plants became constitutive in the weedy railway plants.

RESULTS

Flowering Time and Vernalization Response in *A. arenosa*

We grew plants from seeds sampled from five mountain and four railway populations of *A. arenosa* in controlled conditions from seeds collected from wild plants (Fig. 1A). We measured flowering time (as days from germination to first open flower) for plants grown with or without an 8-week vernalization period that consisted of a cold treatment (4°C) under short-day conditions (8 h of light instead of 16 h; see "Materials and Methods"). All mountain populations flowered significantly later than all railway populations when unvernalized (Fig. 1B), but all populations flowered similarly when vernalized. This shows that all sampled railway populations are rapid cycling and have lost vernalization responses, while all mountain populations retain them and flower late without cold treatments. We previously showed that the railway plants are all extremely closely related, suggesting a single colonization event that is consistent with the genetic similarity of the different railway populations (Arnold et al., 2015).

We selected a single railway population (TBG) and a single mountain population (KA) as representative of the two types to analyze in more depth the molecular basis of their phenotypic differences. TBG is from a railway at Triberg railway station in southwest Germany. KA is from a limestone outcrop on Kasparstein Mountain, near Loschental, Austria (Fig. 1A). These populations are members of genetically distinct

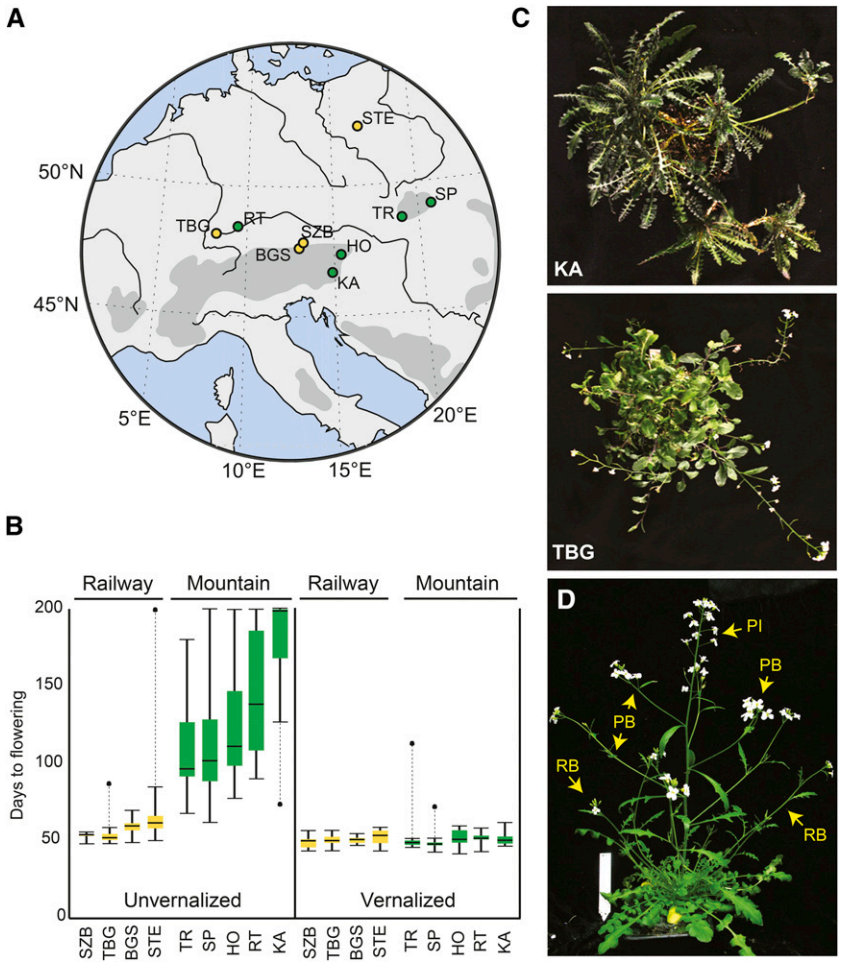

Figure 1. Phenotypes of railway and mountain *A. arenosa* plants. A, Map of central Europe with locations of *A. arenosa* populations sampled from railway (yellow) and mountain (green) sites. TBG = Triberg, Germany; RT = Upper Danube Valley, Germany; BGS = Berchtesgaden, Germany; SZB = Salzburg, Austria; HO = Hochlantsch, Austria; KA = Kasparstein, Austria; TR = Trencin, Slovakia; SP = Spisska, Slovakia. B, Box plots showing flowering phenotypes of plants grown from seeds collected from railway and mountain populations. Flowering time was quantified as the time from germination to the first open flower for vernalized (left) and nonvernalized (right) plants from both accessions. Plants that did not flower by the end of the experiment (200 d) were assigned 200 d as their flowering date. C, Images of two representative vernalized individuals taken at 38 weeks. TBG plants flower continuously, while KA plants revert to vegetative growth after an episode of flowering. The development of secondary rosettes along branched stems of KA plants can then be observed. D, Representative greenhouse-grown *A. arenosa* indicating scored phenotypes of primary inflorescence branches (PB) and rosette branches (RB). PI indicates the primary inflorescence.

mountain and railway lineages with no evidence of recent gene flow between them (Arnold et al., 2015). Among unvernalized plants, those from KA flowered much later than those from the TBG (Wilcoxon $P < 10e^{-6}$): time to open flower averaged 56 d for unvernalized TBG plants, while 67% of KA plants had not yet flowered by 200 d, at which point we ended the experiment (Fig. 1B). There was no significant difference in flowering times of cold-treated (vernalized) plants from the two populations (Fig. 1B; Wilcoxon $P > 0.08$). We confirmed the similarity of flowering behavior of the two vernalized populations using a Mann-Whitney *U* test ($P > 0.6$). Vernalization had no significant effect on the mean flowering time of TBG, although there was a reduction in the SD from 12 d (nonvernalized) to 4 d (vernalized), implying that TBG plants, although lacking a true vernalization response, still show a residual response to prolonged cold treatment. The difference in flowering behavior persists in subsequent generations in the laboratory, showing that this is not merely a maternal environmental effect resulting from differences in conditions in wild populations (data not shown).

After flowering, vernalized KA plants reverted to vegetative growth while TBG flowered continuously until senescence (Fig. 1C), paralleling the distinction between perpetual and episodic flowering described previously in *A. alpina* (Wang et al., 2009). Furthermore, from our initial phenotyping of 13 KA plants and 20 TBG plants, it was also clear that TBG plants grew more rosette inflorescence branches (RB in Fig. 1D; average rosette inflorescences longer then 5 cm at 20 d after flowering = 14.35) compared with KA, which usually had none or only one (average rosette branches longer then 5 cm at 20 d after flowering = 0.54; Student's *t* test $P = 8 \times 10^{-7}$). The number of inflorescence branches (PB in Fig. 1D) also differs similarly dramatically (0.23 branches in KA versus 18.15 branches in TBG; $P = 1.7 \times 10^{-9}$). Thus, TBG plants show common weedy phenotypic characteristics, including rapid cycling and bushy and abundant inflorescence growth (Baker, 1965; Grotkopp et al., 2002; Blair and Wolfe, 2004; Burns, 2004; Hall and Willis, 2006).

Abrogated Vernalization Responsiveness and Loss of *FLC* Expression in a Railway Accession

To compare the vernalization responses of KA and TBG, we analyzed the transcriptomes of plants from these two populations by transcriptome sequencing (RNA-seq) at four time points (3, 4, 9, and 13 weeks)

with or without a 6-week vernalization period at weeks 4 to 10 (see "Materials and Methods"). We quantified expression by read depth after aligning to the *A. lyrata* reference genome (Hu et al., 2011), which includes 32,670 annotated genes (see "Materials and Methods"). We first examined the expression of 151 *A. lyrata* homologs of 174 genes associated with flowering regulation in *A. thaliana* (Fornara et al., 2010). Among these, *FLC* was the most differentially expressed between the two vernalized time series of KA and TBG, with virtually undetectable expression in TBG. *FLC* showed initially strong expression in KA followed by a clear suppression by vernalization but a subsequent return to prevernalization levels after plants were returned to warmer conditions. We confirmed this expression profile by quantitative reverse transcription-PCR with a finer sampling resolution (Fig. 2A).

In the *A. arenosa* genome, *FLC* is present in two full-length copies and one truncated duplicate copy (Nah and Chen, 2010), but only one copy was annotated in the version of the *A. lyrata* genome we used for aligning RNA-seq reads (scaffold 6: 4,040,170–4,045,798). Since finalizing this work, a newer annotation has been published (Rawat et al., 2015) that recognizes two *FLC* genes in *A. lyrata*. Because of the very close similarity between the two genes, we could not use our coverage estimates from read alignments to *A. lyrata* or to the *A. arenosa* bacterial artificial chromosome (BAC) to differentiate the expression of the two copies. Thus, to estimate relative expression levels, we genotyped our read alignments for polymorphisms that distinguish the two full-length *FLC* copies. We found a total of nine single-nucleotide polymorphisms that differ between the paralogs in our transcriptome samples (Fig. 2B). Seven of these single-nucleotide polymorphisms had been identified previously in a BAC sequence of the *A. arenosa FLC* locus from the Care-1 strain (Nah and Chen, 2010). Only three were among the 16 polymorphisms differentiating the two *A. lyrata* paralogs. At all nine positions, the nucleotides characteristic of *AaFLC2* were at low frequency in our transcriptome alignments relative to those characteristic of *AaFLC1*, indicating low expression of *AaFLC2* relative to *AaFLC1*.

To more finely quantify the differential expression of *AaFLC1* and *AaFLC2*, we mapped our RNA-seq data on the BAC sequence and followed a previously described method for the detection of allelic expression differences (Perez et al., 2015), estimating the expression of the two transcripts with MMSEQ (Turro et al., 2011). This approach confirmed the differential expression of *AaFLC1* and *AaFLC2* in the KA samples (where sufficient levels of expression were detected). In the two time series of this population, *AaFLC1* contributed to an average of 77% of the total *AaFLC* expression. The relative ratios of the duplicates remain consistent in our time series, indicating that, although *AaFLC1* dominates in terms of total expression, both paralogs respond similarly to vernalization in KA (Fig. 2C).

As expected, positive regulators of flowering showed opposite trends to the floral repressor *FLC*, including

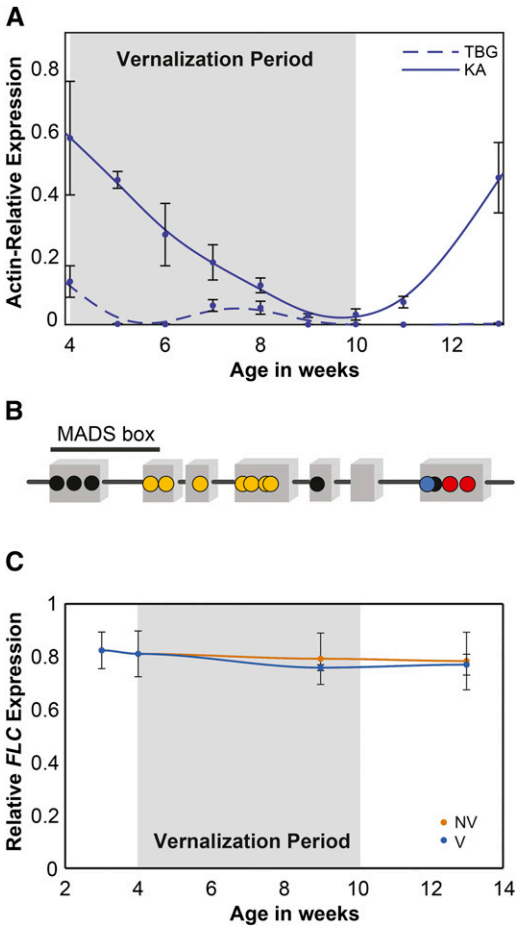

Figure 2. Differential *FLC* expression and responsiveness to vernalization. A, Quantitative reverse transcription-PCR of *FLC* expression relative to *ACTIN* (*ACT*) in vernalized KA and TBG plants. While undetectable in TBG, *FLC* is suppressed by vernalization in KA but comes back to unvernalized levels after plants are returned to warm conditions. B, Single-nucleotide differences between the coding sequences of the two *AaFLC1* and *AaFLC2* paralogs in our sample. Gray boxes are exons. Yellow indicates differences between *AaFLC1* and *AaFLC2* present in our samples as well as the published BAC sequence (Nah and Chen, 2010). Black indicates differences between *AaFLC1* and *AaFLC2* in the BAC not found in our accessions. Red indicates differences between *AaFLC1* and *AaFLC2* in our samples but not present in the BAC. Blue indicates differences between *AaFLC1* and *AaFLC2* where both paralogs differ from the BAC. C, Expression levels of *AaFLC1* as a proportion of total *AaFLC* locus expression across the vernalized (V) and nonvernalized (NV) time series, showing that the relative expression of the two *FLC* copies does not change by treatment or through the time series.

SOC1, *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE15* (*SPL15*), and *SPL4*, which were all expressed throughout the time course in TBG but only after vernalization in KA. The expression profile of *SOC1*, a flowering promoter, was especially strongly anticorrelated with *FLC* expression (Supplemental Fig. S1; Pearson correlation, −0.87). *VERNALIZATION INSENSITIVE3* (*VIN3*), which encodes a component of the POLYCOMB REPRESSIVE COMPLEX2 responsible for establishing the repression of *FLC* during vernalization, is up-regulated by vernalization

in *A. thaliana* (Sung and Amasino, 2004). *VIN3* is similarly up-regulated in both TBG and KA (Supplemental Fig. S1), but the magnitude of the response is lower in TBG than in KA, consistent with the hypothesis that TBG maintains some vernalization responsiveness, albeit in a strongly abrogated form, and that the cause of this abrogation may lie upstream of *VIN3*.

Vernalization-Response Differences

We next set out to characterize the vernalization-responsive subset of genes within the entire transcriptomes of KA and TBG with two goals: (1) to understand the vernalization response in *A. arenosa*, and (2) to compare the responsiveness of the two accessions qualitatively and quantitatively. Within each genotype, we identified vernalization-response genes as the intersection of genes that differ between growth conditions (vernalized versus nonvernalized) and those that change expression through the time course within each of the conditions. We used a nonparametric ranking test (see "Materials and Methods") to identify significantly differentially expressed vernalization-responsive genes. We considered genes to be vernalization responsive if they showed a differential expression during the vernalized time series and had a significant growth condition interaction. This category includes genes that are (Fig. 3A, a) or are not (Fig. 3A, b) differentially expressed at different time points in the nonvernalized time series.

Using our criteria, we identified 1,088 genes as vernalization responsive in KA, almost 6 times more than the 187 found in TBG (Supplemental Table S1). Only a small percentage of transcripts (representing 53 genes) are vernalization responsive in both accessions (Fig. 3B), but when we compared expression ratios between vernalized and nonvernalized time points in KA and TBG, 60% of these genes showed a significant correlation ($r^2 = 0.83$) between the two accessions. The slope of the log regression was significantly higher than 1 (1.62), suggesting that TBG still has some vernalization responsiveness, but the magnitude of the response is strongly dampened relative to that in KA (Fig. 3C). This reduced responsiveness likely explains the lower number of genes passing our thresholds of detection for vernalization responsiveness in TBG.

Gene Ontology (GO) analysis of KA vernalization-responsive genes showed a very strong representation of genes implicated in light sensitivity and abiotic stress responses. The light-related GO terms included response to UV light (GO:0009411; $P = 0.002$), response to light stimulus (GO:0009416; $P = 0.007$), and long-day photoperiod flowering (GO:0048574; $P = 2 \times 10^{-5}$); these were equally divided between the two categories (a and b) of the KA vernalization response (Fig. 3B). On the other hand, the cold stress terms, including cold response (GO:0009409; $P = 1 \times 10^{-8}$), water deprivation response (GO:0009414; $P = 0.03$), salt stress response (GO:0009651; $P = 5 \times 10^{-8}$), and hyperosmotic stress response (GO:0006972; $P = 6 \times 10^{-5}$), were mainly present in the b category, meaning that their expression shifts over the vernalized time course but not over the nonvernalized time course (Fig. 3B), suggesting that they might be coregulated with or by the vernalization response. In particular, the cold acclimation term was only enriched in the b category (GO:0009631; $P = 0.003$) and included well-known cold-regulated genes like *COLD-REGULATED47* (*COR47*), *EARLY RESPONSIVE TO DEHYDRATION*, *LOW TEMPERATURE-INDUCED*, and *KINASE1* (Maruyama et al., 2004). The b category also included other known components of the cold response of *A. thaliana* such as *COR15A*, *COR15B*, *COR78*, and *PSEUDO-RESPONSE REGULATOR9*. These genes have been shown to confer different degrees of freezing tolerance (Artus et al., 1996; Jaglo-Ottosen et al., 1998; Steponkus et al., 1998) as well as acclimation, the capacity to increase freezing tolerance after exposure to nonfreezing cold temperatures (Thomashow, 1999).

In our two *A. arenosa* populations, the magnitude of the response of most of the vernalization genes is amplified in KA relative to TBG, but the nonvernalized basal levels of expression of stress-responsive genes are higher in TBG (slope of 0.79 in nonvernalized expression levels, $r^2 = 0.75$; Fig. 4A). The core cold-responsive genes highlighted previously are outliers from this correlation, with an even stronger tendency to be highly expressed in TBG. This relationship is inverted by cold treatment, due to the stronger magnitude of the KA response to vernalization treatment (slope of 1.2 in vernalized expression levels, $r^2 = 0.95$; Fig. 4B).

The expression results led us to hypothesize that there might be a difference in the constitutive and acquired freezing tolerance of KA and TBG. To test this, we quantified the freezing tolerance of these two accessions by measuring electrolyte leakage from detached leaves of vernalized and nonvernalized plants exposed to freezing (Sukumuran and Weiser, 1972). Leaves from nonvernalized KA plants showed a very high electrolyte leakage at −6°C (72%), while TBG leaves were significantly lower, at 33% (one-tailed Student's t test $P < 0.005$). Both accessions showed significant reductions of leakage after 1 week of vernalization, to 18% and 16% for KA and TBG, respectively (Fig. 4C). This suggests that, while tolerance is constitutively higher in TBG than in KA, after prior exposure to 4°C, KA can achieve levels of freezing tolerance comparable to TBG. In addition, the tolerance of TBG also increases further with cold exposure, again consistent with the hypothesis that it retains some ability to respond to cold.

Global TBG-KA Expression Differences

We next compared the overall transcriptomes of the KA and TBG time series for both the nonvernalized and vernalized samples (Fig. 5). For each comparison, we obtained a q value (false discovery rate corrected; see "Materials and Methods") for every gene annotated in the *A. lyrata* reference genome (Hu et al., 2011). Out of the 326 genes that constitute the 1% most strongly differentially expressed genes between TBG and KA, 35

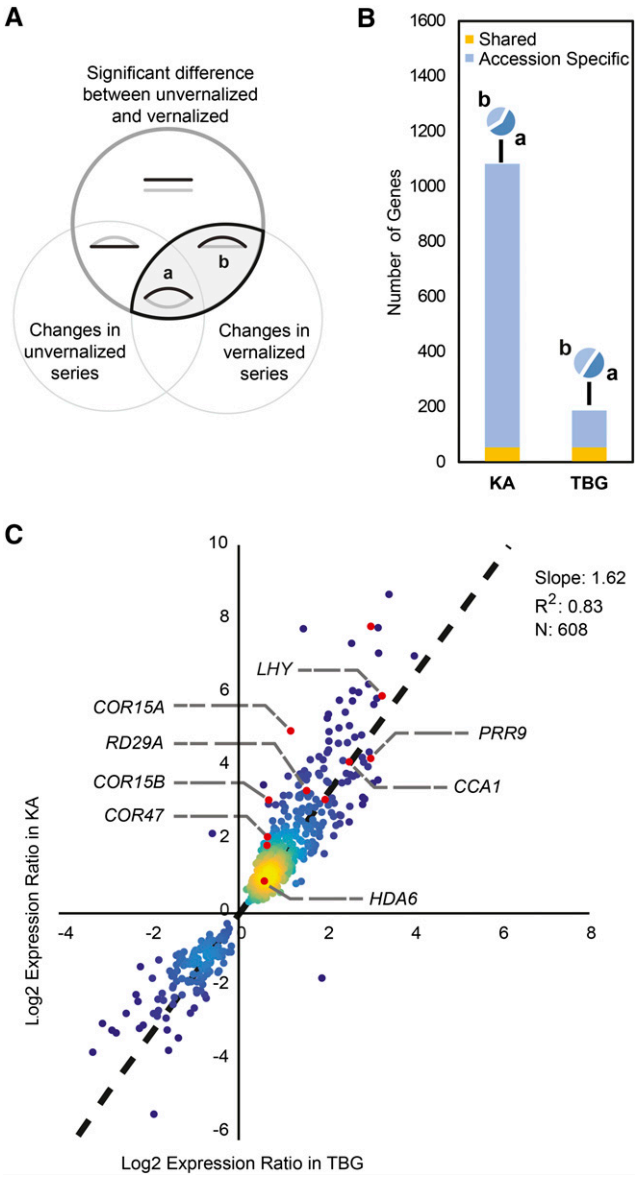

Figure 3. Vernalization response differences between KA and TBG mainly due to a reduced magnitude of response in TBG. A, Venn diagram representing the seven categories of differential responses for each accession. The top circle (dark gray) includes genes with significant differential expression when comparing the vernalized and unvernalized time series. The bottom left circle contains genes with significant differential expression among time points in the unvernalized time series. The bottom right circle contains genes with significantly different expression among time points in the vernalized time series. Cartoon curves show schematic vernalized (black) and unvernalized (gray) expression profiles of genes found in each category. Vernalization-responsive genes are found for each accession at the intersection between the top and bottom right circles. Within this pattern, a represents genes that change across the time series in both vernalized and unvernalized plants, but in distinct ways, while b includes genes that show changes in the vernalized time series but not in the unvernalized time series. B, Decomposition of each vernalization response showing which genes change in both accessions (yellow), which are accession specific (blue), and how they are partitioned between a- and b-category patterns (camembert diagrams). Almost 6 times more genes are identified as vernalization responsive in KA compared with TBG. C, Comparison of vernalization responsiveness in KA versus TBG. The responsiveness of a gene is calculated for each accession as the $\log_2$ ratio of vernalized over nonvernalized expression levels. Only ratios significantly different from 1 ($\log_2$ ratios different from 0) in both accessions are displayed. Several genes discussed here are highlighted. Colors signify plot density. The dotted line indicates the linear regression fit line based on $n = 608$ data points. The slope and r^2 values for the fit are given on the chart.

have no orthologs in *A. thaliana* (Fig. 5A; Supplemental Table S2), even though orthologless genes more frequently display null mean and variance of expression levels than genes with orthologs (Supplemental Fig. S2). Over the whole genome, the expression difference of *FLC* ranked third in comparisons of TBG and KA in both vernalized and unvernalized time series, making it the most differentially expressed gene between these accessions overall. The only genes more highly ranked for differential expression between TBG and KA in the nonvernalized series are *ALPHA TUBULIN1*, a pollen specific α-tubulin gene (Kim and An, 1992), and *BTB AND TAZ DOMAIN PROTEIN4*. In the vernalized series, the top two differentially expressed genes are *At4g10320* (which encodes a tRNA synthetase) and *g932613* (which has no *A. thaliana* homolog).

We next asked if there are any GO terms for biological processes enriched in the 1% top differentially expressed genes between KA and TBG in both time series. The significantly overrepresented categories include several stimulus-response gene categories, including heat response (GO:0009408; $P = 0.023$), protein unfolding (GO:0043335; $P = 0.015$), Golgi vesicle transport (GO:0048193; $P = 0.02$), detection of visible light (GO:0009584; $P = 0.001$), and DNA repair (GO:0006281; $P = 0.044$). Overall, these categories point to a generalized elevation of stress-associated genes in TBG. The GO category detection of visible light (GO:0009584) has the most significant P value in our analysis. Among differentially expressed genes in this category are *PHYTOCHROME C* (*PHYC*) and *PHYD*, two of the four phytochromes annotated in the *A. lyrata* genome. Both are involved in light signaling (Hu et al., 2013) and are expressed at lower levels in TBG. Mutation of *PHYC* leads to early flowering in *A. thaliana* (Monte et al., 2003), while polymorphisms in *PHYC* are thought to be involved in climatic adaptation (Balasubramanian et al., 2006; Samis et al., 2008; Méndez-Vigo et al., 2011). *PALE CRESS*, which in *A. thaliana* is important in chloroplast development and regulating levels of carotenoids and chlorophyll (Reiter et al., 1994), is more highly expressed in KA relative to TBG. Carotenoids are known to be important in the chloroplast capacity to respond to high-light stress (Havaux, 1998). In parallel, several genes implicated in DNA repair and recombination also show significant differential expression between KA and TBG in both time series, including *DNA LIGASE6*, *MMS ZWEI HOMOLOG2*, *AT3G07930*, *REPLICATION PROTEIN A1B* (*RPA1B*), *RECQ HELICASE SIM* (*RECQSIM*), and *Y-FAMILY DNA POLYMERASE H* (*POLH*). All but *RECQSIM* have lower expression in TBG

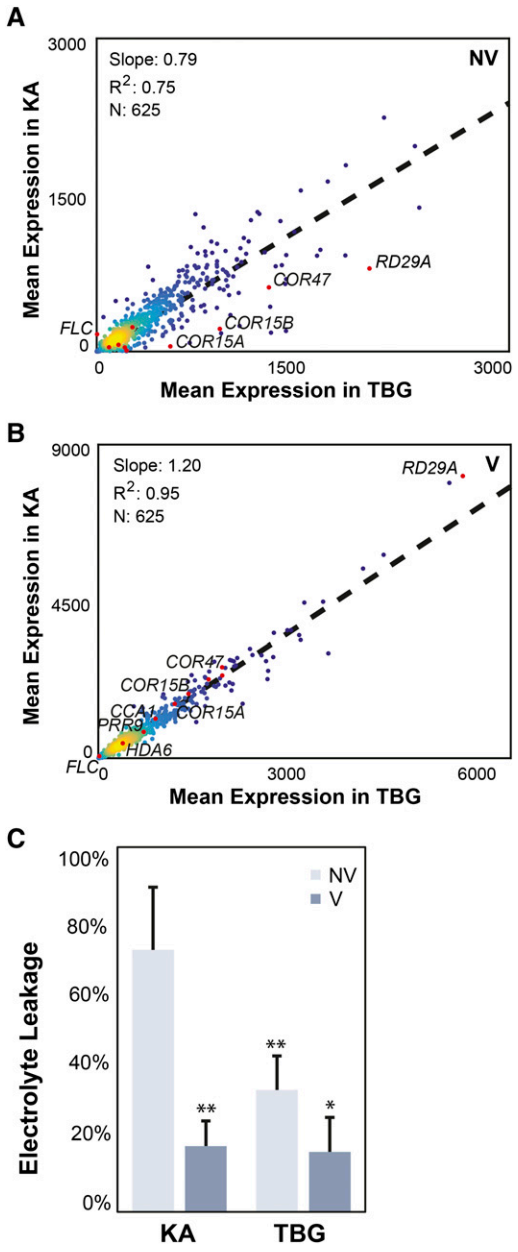

Figure 4. Expression of vernalization-responsive genes in KA versus TBG. A, Expression levels of vernalization-responsive genes in unvernalized KA plotted against their levels in unvernalized TBG. The slope of the linear regression (0.79 with $r^2 = 0.75$) indicates that vernalization-responsive genes have a higher expression in TBG in unvernalized plants. Known cold-responsive genes are highlighted in red, and in particular, *COR15A*, *COR15B*, *COR47*, and *RD29A* show even stronger bias toward higher constitutive expression in TBG. B, Expression levels of vernalization-responsive genes in vernalized KA versus vernalized TBG. Due to the stronger vernalization response in KA, the relationship is inversed compared with A (slope of 1.2 with $r^2 = 0.95$). C, Electrolyte leakage measured after freezing at −6°C of leaves from 7-week-old KA and TBG plants vernalized for 1 week (V; dark blue bars) or not vernalized (NV; light blue bars). Two asterisks indicate significant differences of vernalized KA and nonvernalized TBG compared with the high leakage of unvernalized KA plants (Student's *t* test $P < 1\%$). One asterisk denotes a significant difference of vernalized TBG from the controlled leakage of unvernalized TBG (Student's *t* test $P < 5\%$).

than in KA. *POLH* and *RPA1B*, in particular, are known to be involved in the DNA repair response to UV light damage (Ishibashi et al., 2005; Anderson et al., 2008).

A number of heat-responsive proteins are differentially expressed in TBG and KA, including *HEAT SHOCK FACTOR1* (*HSF1*) and *HEAT SHOCK PROTEIN101* (*HSP101*), which are both expressed at higher levels in TBG, and *HSFA1D*, which is expressed at lower levels in TBG than in KA (Fig. 5B). *HSF1* and *HSFA1D* are members of a four-gene family of class A heat shock factors that are regulators of the heat shock response and other abiotic stress responses (Liu et al., 2011; Yoshida et al., 2011). Overexpression of *HSF1* in *A. thaliana* induces tolerance to heat shocks (Qian et al., 2014), but its absence does not completely impede acquired thermotolerance (Liu and Charng, 2012). *HSP101* also plays a role in acquired thermotolerance (Gurley, 2000; Hong and Vierling, 2000, 2001; Queitsch et al., 2000). Additional modulators of thermotolerance, *FK506-BINDING PROTEIN62*, *GALACTINOL SYNTHASE1* (*GOLS1*), and *VPS53* (Lobstein et al., 2004; Lee et al., 2006; Wang et al., 2011), are also among our top differentially expressed genes.

The generally high constitutive expression of heat-responsive genes in TBG led us to hypothesize that the heat tolerance of TBG might be elevated relative to KA. To test this, we exposed 5-d-old KA and TBG seedlings to a 45°C heat shock for 1 h, with and without an acclimation treatment at 37°C for 3 h (see "Materials and Methods"), which allows us to assay basal and acquired tolerance, respectively. After a 5-d recovery period at 20°C, we screened seedlings for partially or totally bleached cotyledons. After heat shock without prior acclimation, fewer than 20% of TBG seedlings showed signs of bleaching after 5 d, while more than 95% of KA seedlings were partially or entirely bleached (Fig. 6). When acclimated at 37°C 2 d before heat shock, both lines performed similarly with little or no bleaching. This fits with constitutive differences in the expression of heat response genes between KA and TBG associated with higher basal tolerance to heat shock by the railway line, while KA retains a capacity for acquired tolerance.

Evidence of Selection in the Railway Population

We next looked for evidence of genetic differentiation that might be responsible for the abrogation of the vernalization response and/or constitutive induction of the stress responses in TBG relative to KA. To do so, we first complemented our previously generated genome sequence data for *A. arenosa* (Hollister et al., 2012) by sequencing a total of eight KA and seven TBG individuals, which samples 32 and 28 copies of each chromosome, respectively. To identify candidate genomic regions showing habitat-associated genetic differentiation, we used F_{ST} (a measure of differentiation; Weir, 1990), which is generally low among tetraploid *A. arenosa* populations (0.047–0.063; Hollister et al., 2012), as well as Fay and Wu's H, a statistic sensitive to an excess of high-frequency variants compared with neutral expectations (Fay and Wu, 2000).

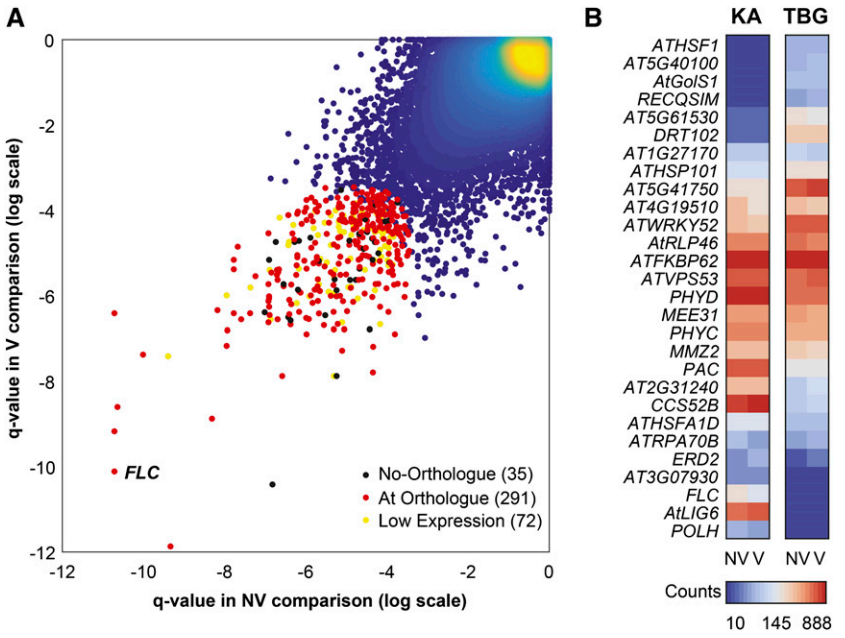

Figure 5. Overall transcriptome differential expression between KA and TBG. A, Genome-wide distribution of *q* values comparing KA and TBG nonvernalized (NV; *x* axis) and vernalized (V; *y* axis) time series. FLC is highlighted, as it is ranked third in both comparisons, which makes it the most differentiated expression pattern overall. The top 1% (326 genes) overall (sum of both *q* values) are colored in red if a homolog is known in *A. thaliana* and in black if not. Yellow dots represent genes with low expression across all time points, genotypes, and conditions and, therefore, excluded from further consideration in the top 1% subset. B, Expression heat map of genes that are both within the 1% most differentiated pattern and associated with the GO category response to stimulus (GO:0050896). For each gene, the mean expression of both vernalized and nonvernalized time series is given for KA and TBG. Expression is given in normalized gene counts.

We identified 20 genes that showed evidence of strong differentiation between TBG and KA (Table I). With respect to the expression differences in cold and heat responses, one candidate stood out: *LHY*, which plays a central role in the regulation of the plant circadian clock (Alabadí et al., 2001) but is also known to broadly affect downstream cold-response genes (Vogel et al., 2005; Bieniawska et al., 2008). *LHY* is an outlier for Fay and Wu's H in TBG but not in KA; nucleotide diversity measured by the number of pairwise differences is lower in TBG than in KA (Supplemental Fig. S3A), and 22 derived polymorphisms have significantly higher frequencies in TBG than in KA (average 82% versus 37%) in a region concentrated around exons 6 and 7 (Supplemental Fig. S3B). These patterns are consistent with the hypothesis that TBG experienced positive selection on *LHY*. Twenty of these polymorphisms fall within the coding sequence of *LHY*, and 14 are nonsynonymous. These 14 polymorphisms are all distributed along exons 6 and 7, with a dense cluster positioned at the beginning of exon 7 (Supplemental Fig. S3C). Of these 14 polymorphisms, two induce a strong change of hydrophobicity of the amino acids they encode (acidic Glu versus hydrophobic Gly) and one induces a charge change (Glu versus basic Lys; Supplemental Fig. S3C).

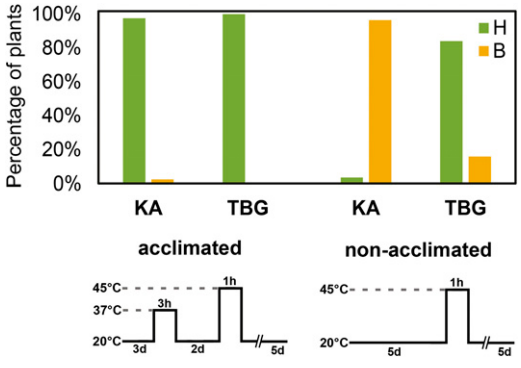

Figure 6. Constitutive heat shock tolerance in railway plants. The percentage of seedlings exhibiting partial or total bleaching of their cotyledons and leaves after heat shock treatment is shown. H indicates healthy after treatment (green), and B indicates bleached after treatment (orange). Acclimated seedlings were subjected to a 3-h 37°C pretreatment 2 d prior to a 1-h heat shock at 45°C, while nonacclimated seedlings were incubated directly for 1 h at 45°C. Bleaching was often nonlethal even for KA plants.

To determine whether the amino acid changes in TBG all lie on the same haplotype, we first cloned and sequenced a fosmid of the *LHY* locus from a TBG individual. The fosmid clone carried the derived alleles at all 22 positions, showing that the derived polymorphisms are all found together on a single haplotype that seems to have been the target of selection in TBG. We then cloned and sequenced additional PCR products from another TBG plant and found the same to be true for the exon 6/7 region in this individual as well. Since the derived polymorphisms were all present also in KA, albeit at low frequency, we asked if they were also all found on a single haplotype in KA identical to the haplotype found in KA. We cloned and sequenced PCR products from the exon 6/7 region and confirmed, first, that the derived polymorphisms were rare in KA but, also, that no KA haplotype within our sampling had all of the derived polymorphisms together. The rare derived polymorphisms in KA are in blocks of up to 18 of the 22 derived polymorphisms (Supplemental Fig. S4). In each case, a single recombination event between two haplotypes would suffice to toggle between the haplotype found in TBG and the rare haplotypes present in KA.

Table I. *List of 1% genomic outliers for F_{ST} and Fay and Wu's H*

TAIR Identifier	Common Name
AT3G03510	
AT3G56670	
AT1G01060	LHY
AT1G08135	AtCHX6B
AT1G48090	
AT1G72300	PSY1R
AT1G78770	APC6
AT3G14980	IDM1
AT2G17140	
AT2G31260	APG9
AT2G41700	ABCA1
AT3G56900	
AT5G10560	
AT5G21160	AtLARP1a
AT4G32410	ANY1
AT4G25970	PSD3
AT2G24680	
AT3G20660	AtOCT4
AT2G15620	AtHNIR
AT3G57590	

DISCUSSION

Here, we investigated the molecular basis of phenotypic differences between two *A. arenosa* populations, one of which has weedy traits associated with the colonization of railways in flatland Europe. The ancestral form usually inhabits shaded sites on hills or mountains, often in forests. These two distinct types correspond to a previously recognized distinction between a flatland and a mountain type within *A. arenosa* (Scholz, 1962). We previously showed that all railway plants we have sampled are members of a single genetically distinct lineage within tetraploid *A. arenosa* that diverged from mountain lineages fewer than 15,000 generations ago (Arnold et al., 2015). This suggests that the weedy phenotype of railway plants evolved once within *A. arenosa*, followed by the spread of this lineage throughout flatland Europe. We show here that the weedy phenotype of *A. arenosa* includes rapid cycling, abundant inflorescence growth, loss of the vernalization requirement, loss of other traits associated with perenniality, and constitutive stress tolerance. These are hallmarks of an all-in phenotype commonly observed in weedy colonizers (Baker, 1965; Grotkopp et al., 2002; Blair and Wolfe, 2004; Burns, 2004; Hall and Willis, 2006). The phenotypes observed in weedy *A. arenosa* likely reflect that railway sites are sunnier and more exposed to other abiotic stresses, including more rapid temperature fluctuations than would generally be experienced in the usually forested outcrop sites where most other *A. arenosa* populations are found. Although these types of adaptations are not uncommon for plants found in unpredictable and often human-associated habitats, their molecular basis has remained largely unknown.

Using transcriptome and phenotypic analyses, we found evidence that a major feature of the colonization of ruderal railway sites in *A. arenosa* is that responses to heat and cold that are inducible in mountain accessions became constitutive in railway accessions. These features include reproductive initiation, heat shock resistance, and freezing tolerance. Railway plants also exhibit perpetual flowering and are heavily branched, while mountain plants flower more modestly and return to vegetative growth after flowering, paralleling a distinction described previously in *A. alpina* of episodic versus perpetual flowering (Wang et al., 2009). This fits with the description of mountain accessions as perennial and ruderal railway accessions as rapid-cycling annuals, suggesting that the loss of perennial life history traits also accompanied the transition to weediness in *A. arenosa*.

One of the most differentially expressed genes when comparing railway and mountain plants was *FLC*, which is almost undetectable at any time point in the rapid-cycling railway plants. This gene has been identified frequently as a cause for independent transitions to rapid cycling and loss of vernalization sensitivity in *A. thaliana* (Méndez-Vigo et al., 2011; Guo et al., 2012). In *A. alpina*, an ortholog of *FLC*, *PEP1*, has been linked to a switch between late-flowering perennial life habits and rapid and perpetual flowering (Wang et al., 2009; Albani et al., 2012), but whether this *FLC* homolog is causal in *A. arenosa* remains to be tested. The low expression of *FLC* in the railway form, or its down-regulation in the mountain form upon vernalization, is strongly correlated with an up-regulation of floral promoters, including *SOC1*. This is also consistent with the known repression exerted by the *FLC-SVP* complex on *SOC1* in *A. thaliana* (Michaels and Amasino, 2001; Li et al., 2008). Similarly, *SPL* transcription factors, which redundantly promote both vegetative phase change and flowering in *A. thaliana* (Schwarz et al., 2008; Wang et al., 2008), are constitutively expressed in railway plants and are initially absent but up-regulated during the vernalization period in mountain plants. Thus, in general, floral promoters are constitutively expressed in the railway accession and induced by vernalization in the mountain form, where their expression is anticorrelated with *FLC* expression. This suggests that the vernalization and flowering responses in *A. arenosa*, as well as the implication of *FLC* in life history changes, are consistent with findings in *A. thaliana* and related species.

In plants from the KA mountain site, *FLC* is initially expressed at very high levels and rapidly repressed during exposure to prolonged cold, just as it is in *A. thaliana* (Sung and Amasino, 2004; Coustham et al., 2012). However, as the plants begin to flower after a return to warm conditions, *FLC* expression returns to prevernalization levels within about 3 weeks. This suggests a mechanism similar to what is found in *A. alpina*, where meristems switch from vegetative to reproductive fate during vernalization, and any meristem arising during or after vernalization remains vegetative, leading to episodic flowering cycles characteristic of perennials (Wang et al., 2009). This cyclical *FLC* expression could explain the formation of secondary rosettes as branches from basal rosettes in *A. arenosa* (Fig. 1).

We also observed this phenotype in diploid *A. arenosa*, suggesting that it is ancestral (data not shown). Paralleling phenotypes reported in *A. alpina* (Albani et al., 2012), these secondary rosettes require an additional vernalization treatment to flower. These secondary rosettes can form roots and allow the plants to make use of vegetative reproduction. In *A. alpina*, there are two *PEP1* transcripts, and these show different expression patterns thought to be associated with the perennial life cycle of this species (Albani et al., 2012). In *A. arenosa*, there are also two full-length *FLC* genes (Nah and Chen, 2010), but we detected no significant difference in the response of the two paralogs to vernalization: *AaFLC1* was expressed at higher levels than *AaFLC2*, but this difference remains consistent over our time course.

From our transcriptome analyses, we found that the railway plants have a generally strongly dampened vernalization response. Almost 6 times more genes were vernalization responsive in the mountain accession KA than in the railway accession TBG, but for 60% of them, the stronger magnitude of response in KA is nonetheless log-linearly correlated with a weaker response in railway plants, suggesting that the vernalization response is strongly abrogated but not completely absent in the railway plants.

We also found that the reduced vernalization responsiveness in railway plants was coupled with a constitutively high expression of a number of cold- and heat-response genes. A cross talk between flowering and the cold response has been described in *A. thaliana* and is associated with the flowering regulators *SOC1*, *FLC*, and *FVE* (Kim et al., 2004; Seo et al., 2009; Richter et al., 2013). Several *COR* genes are induced during vernalization in mountain *A. arenosa* plants, including *COR15A*, *COR15B*, *COR47*, *COR314*, and *COR78*, and reach higher levels after vernalization in mountain than in railway plants. *COR* genes are an essential component of the cold-acclimation response (Thomashow, 1999). The same genes, however, show higher expression before vernalization in railway plants than in mountain plants. The ability to cold acclimate increases the freezing tolerance of a plant after exposure to low temperature, and freezing tolerance has been shown to correlate with winter temperatures in natural accessions of *A. thaliana* (Hannah et al., 2006). For *A. arenosa*, we found that (fitting with *COR* gene expression trends) the mountain accession showed a much greater capacity to cold acclimate than the railway accession, but among unacclimated plants, those from railways had a higher basal tolerance to freezing than mountain plants. This is consistent with results from a *CBF1*-overexpressing *A. thaliana* mutant, which has high expression of the *COR* genes as well as enhanced freezing tolerance (Jaglo-Ottosen et al., 1998), as well as findings that naturally increased nonacclimated freezing tolerance can result from the constitutive activation of the CBF pathway (Hannah et al., 2006).

The *COR* genes are also implicated in the response to dehydration (Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). *AtGOLS1*, which is constitutively overexpressed in railway plants, is involved in the accumulation of galactinol and raffinose (Panikulangara et al., 2004), two compounds known for their protective properties against oxidative stress (Nishizawa et al., 2008), and is involved in drought, cold, and high-salinity stresses (Taji et al., 2002). Therefore, constitutive overexpression of the *COR* genes, as well as *AtGOLS1*, could possibly also reflect selection for higher drought tolerance in the railway environment, where soil is drier and more exposed to water loss in the heat of summer. Perhaps constitutive freezing and drought tolerance could both have arisen as pleiotropic effects of alterations of *COR* gene expression during adaptation to railway habitats.

In addition to cold tolerance genes, we saw a similar shift toward constitutive expression in railway plants of heat response-associated genes. This includes *HSF1*, known in *A. thaliana* to be involved in thermotolerance (Qian et al., 2014), and *HSP101*, a key component of acquired thermotolerance (Gurley, 2000; Hong and Vierling, 2000, 2001; Queitsch et al., 2000). HSPs are molecular chaperones rapidly activated by the binding of HSFs in response to environmental stresses such as heat stress or other proteotoxic stresses such as drought and freezing (Schöffl et al., 1998). Twenty-one *HSF* genes have been annotated in *A. thaliana* (Nover et al., 2001), but the *HSFA1s* (a–d) constitute the main transcriptional activators in response to heat shock (Yoshida et al., 2011). Overexpression of *HSF1* leads to stronger induction of *HSPs* by heat shock and increased stress tolerance but not to the acquisition of basal thermotolerance, as observed in *HSFA1b*-overexpressing plants (Prändl et al., 1998; Qian et al., 2014). Indeed, HSF1 requires the stress-induced formation of homotrimers to be activated and accumulate in the nucleus (Yoshida et al., 2011; Liu et al., 2013). Here, we observed in the railway accession constitutively high expression of *HSF1* and *HSP101* correlated with an increased basal thermotolerance. Together, these patterns of overexpression of heat shock genes suggest a constitutive activation of the heat shock response pathway in the railway accession, which was corroborated by the high basal heat shock tolerance of these plants: seedlings of railway plants show stronger heat shock tolerance than those of mountain plants, but we detected no difference in the acquired thermotolerance of the two types. The acquisition of constitutive thermotolerance, thus bypassing the requirement for a priming event, may be a significant advantage for populations colonizing habitats like railways, where they are more likely to be exposed to abrupt temperature fluctuations (Zerebecki and Sorte, 2011).

Given that the mountain and railway *A. arenosa* plants show clearly distinct environmental response phenotypes that are likely adaptive to their respective habitats, we used a genome-scanning approach to determine if any genomic regions show evidence of selection. By our criteria, 20 genes in the genome showed evidence of having been under selection in the railway population. The encoded proteins are important for a variety of processes, and a few have possible implications for the

differences between railway and mountain types: *CATION/H+ EXCHANGER6B* (*AtCHX6B*) and *ORGANIC CATION TRANSPORTER4* (*AtOCT4*) are both members of gene families involved in cation transmembrane transport (Remy et al., 2012; Ye et al., 2013), and *NITRITE REDUCTASE1* (*AtHNIR*) is involved in the nitrate response (Konishi and Yanagisawa, 2010), which together hint at possible adaptation to substrate differences. This may be relevant, as KA is from a high-pH limestone site (which is likely the ancestral habitat for *A. arenosa*) and TBG is from railway ballast in the Black Forest, an acidic silicaceous region. *LA-RELATED PROTEIN1A* (*AtLARP1A*) is required for the heat-dependent degradation of mRNA involved in the thermotolerance of *A. thaliana* with consequences for its acclimation capacity (Merret et al., 2013). *AUTOPHAGY9* (*APG9*) mutants have accelerated seed set and senescence (Hanaoka et al., 2002), which may be related to the senescence difference between the rapid-cycling TBG and the perennial mountain type KA.

For the overall control of stress responsiveness, one candidate target of selection stood out: the circadian clock regulator *LHY*. In *A. thaliana LHY* is known to affect flowering, albeit indirectly (Schaffer et al., 1998; Mizoguchi et al., 2002), but also cold responsiveness (Vogel et al., 2005; Bieniawska et al., 2008) and freezing tolerance (Bieniawska et al., 2008; Espinoza et al., 2008; Nakamichi et al., 2009; Dong et al., 2011). Double mutants for *LHY* and another circadian regulator, *CCA1*, show a greatly diminished induction of cold responses and the COR genes, including *COR15A*, *COR47*, and *COR78* (Dong et al., 2011), that were differentially expressed among our *A. arenosa* strains. Thus, it is enticing to hypothesize that the derived variant of LHY that seems to have been under selection pressure in railway plants may somehow lead to a change in the regulation of downstream stress tolerance as well as possibly flowering responses. Whether LHY variation is actually responsible for the phenotypic differences in stress tolerance or flowering we observe between these mountain and railway populations remains to be tested.

Overall, we found that an *A. arenosa* lineage that adapted to railway sites in flatland Europe evolved features commonly observed in weedy plants found in human-associated habitats, including rapid cycling, perpetual flowering, extensive inflorescence growth, and constitutive heat and cold tolerance (Baker, 1965; Grotkopp et al., 2002; Blair and Wolfe, 2004; Burns, 2004; Hall and Willis, 2006). These phenotypes are all inducible in the mountain accession. Thus, there is a general trend that normally inducible phenotypes have become constitutive in the weedy *A. arenosa* form, which was likely important in the colonization of a volatile and risky human-associated habitat. The fact that these *A. arenosa* populations are autotetraploids appears to be in line with a strong potential for habitat colonization and the transition to weediness by polyploids (Soltis and Soltis, 2000; Pandit et al., 2006; Prentis et al., 2008). However, it is clear that, in this case, neither weediness nor other traits associated with the ruderal railway habitats were immediate consequences of genome duplication. Among five tetraploid *A. arenosa* lineages we have sampled, only one successfully colonized ruderal habitats (Arnold et al., 2015), and we show here that it has distinct phenotypic traits consistent with specific adaptations to that habitat not shared by other tetraploids. Thus, while colonization of the railway habitat was perhaps facilitated by the increased genetic diversity available in tetraploids (Otto et al., 2007; Hollister et al., 2012; Arnold et al., 2015), the autotetraploids as a group are not globally tolerant of ruderal habitats, and adaptation was required post-polyploidy for this colonization.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We collected seeds from natural populations in June and July, 2009 to 2011. All populations are autotetraploid; all originate from regions where only autotetraploids occur (Jørgensen et al., 2011), and ploidy of a subset of individuals from each population was confirmed using flow cytometry (Hollister et al., 2012). For flowering time phenotyping, we grew 48 single progeny from each of several individuals per population. Plants were grown as described previously (Hollister et al., 2012) in Conviron MTPC-144 chambers with 8 h of dark at 12°C, 4 h of light (Cool-White fluorescent bulbs) at 18°C, 8 h of light at 20°C, and 4 h of light at 18°C. A subset (24) of 4-week-old plants were transferred to a chamber with a constant temperature of 4°C and short days (8 h of light) for 8 weeks and then returned to warm and long-day conditions.

For the transcriptomic time series, we grew arrays of 48 siblings from seeds harvested from single individuals growing in nature. Plants were grown under similar growth conditions in three flats in which we mixed TBG and KA plants in order to avoid flat effects. Half of the plants from each flat were vernalized for 6 weeks before being returned to their flat of origin. For our RNA extractions, we used tissue from young rosette leaves from both vernalized and unvernalized plants using one biological replicate from each flat. Leaves were harvested every 7 d after 9 to 10 h from Zeitgeber time 0 in long-day conditions and after 5 to 6 h in short days. Zeitgeber time 0 is defined as the time of lights on.

Phenotyping of Flowering Time

Using germination date recorded as the first date when root tip emergence was evident on agar plates, we measured flowering time as the time to first open flower. We calculated flowering time for vernalized plants based on the total number of growing days excluding the cold treatment. For plants that had not flowered by experiment end (200 d), we assigned these cutoff values. Phenotypic values for time to first open flower were not normally distributed even after transformation (Shapiro-Wilk W test $P < 0.0001$), so we used nonparametric Wilcoxon tests to assess the significance of differences among populations.

RNA Isolation, Sequencing, and Expression Analysis

We extracted RNA using the RNeasy Plant Mini Kit (Qiagen). We synthesized single-stranded complementary DNA (cDNA) from 500 ng of total RNA using VN-anchored poly-T(23) primers with MuLV Reverse Transcriptase (Enzymatics) according to the manufacturer's recommendations. Quantitative PCR was carried out on a Stratagene Mx3005P machine with an annealing temperature of 55°C using Taq DNA polymerase (New England Biolabs). Reactions were carried out in triplicate, and we normalized *FLC* expression against the expression of *ACT* using the $2^{-\Delta\Delta CT}$ method taking into account each primer's efficiency as described in the Bio-Rad Real-Time PCR Applications Guide. The SD of each biological replicate was calculated using a first-order propagation of error formula on the variance of the technical replicates. We used cDNA-specific primers 5′-CAGCTTCTCCTCCGGCGA-TAACCTGG-3′ and 5′-GGCTCTGGTTACGGAGAGGGCA-3′ for *FLC* (87% efficiency) and 5′-CGTACAACCGGTATTGTGCTGGAT-3′ and 5′-ACAATTTCCCGCTCTGCTGTTGTG-3′ for *ACT* (91% efficiency).

We prepared RNA-seq libraries using the TruSeq RNA Sample Prep Kit version 2 (Illumina) on RNA samples from both vernalized and unvernalized

plants at four time points (3, 4, 9, and 13 weeks). Libraries were sequenced on an Illumina HiSeq 2000 with 50-bp single-end reads. We sequenced between 9.8 and 18.8 million reads (average, 13.6 million). We aligned reads to the *Arabidopsis lyrata* genome (Hu et al., 2011) using TopHat2 (Kim et al., 2013) and realigned unmapped reads using Stampy (Lunter and Goodson, 2011). We acquired read counts for each of the 32,670 genes using HTSeq-count (Anders et al., 2015) with *A. lyrata* gene models (Hu et al., 2011). We normalized for sequencing depth using DEseq2 in R (Anders and Huber, 2010). Further analyses were performed in MATLAB (MathWorks) except GO enrichment analyses, realized with the Bioconductor package GOstats (Falcon and Gentleman, 2007) in RStudio. RNA-seq read data have been deposited in the National Center for Biotechnology Information Short-Read Archive database under accession number SRP070489 within the National Center for Biotechnology Information BioProject PRJNA312410.

We obtained estimates of differential expression through the time series using a combination of two tests, a Kruskal-Wallis test and a two-way ANOVA coded on MATLAB. We used the first as a nonparametric ranking test within each condition and accession, and this allowed us to detect significant effects of time on the expression level of each gene within a time series without assuming a normal distribution of gene counts. We then used a two-way ANOVA to account for paired data, screen for time series effects, and generate comparisons of gene expression profiles between time series. We combined the results of these tests into seven categories of gene expression profiles for each pairwise comparison between time series, of which two reflect the response to vernalization (Fig. 3A, a and b).

Global expression differences were estimated using a Kruskal-Wallis test between TBG and KA within each condition (vernalized and nonvernalized). The *P* values obtained were then corrected for false discovery rate using the linear step-up procedure originally introduced by Benjamini and Hochberg (1995). For each gene, the two *q* values thus obtained for each condition were then summed in order to establish the 1% most differentiated genes with the lowest sum (QQ50 in Supplemental Table S2). Genes with a normalized gene count below 50 across all time points, genotypes, and conditions were excluded in order to filter from this 1% subset genes with high relative but low absolute differences.

Heat Stress

We adapted a protocol by Meiri and Breiman (2009) to *Arabidopsis arenosa*. Seedlings were germinated on plates containing Murashige and Skoog medium for 5 to 14 d. After 1 week at 4°C in the dark for stratification, seeds were grown on plates at 20°C under long-day conditions (16 h of light and 8 h of dark). Three-day-old seedlings were incubated for 3 h at 37°C in an incubator and then returned to recovery conditions of 22°C under long days for 2 d (see figure legends). For thermotolerance bioassays, the plates were then incubated for 60 min at 45°C under light. After the 45°C treatment, the plates were incubated at 20°C for recovery under long-day conditions for another 5 d.

Freezing Tolerance

Cold tolerance was assessed after 1 week of acclimation at 4°C. Fully expanded leaves of 7-week-old plants were harvested and placed in glass test tubes containing 0.4 mL of deionized water. The tubes were placed on ice, and extracellular freezing of the leaf tissues was initiated by the addition of deionized ice chips to each tube. After transfer to the controlled freezing bath set at 0°C and a 1-h equilibration period, the samples were cooled at 2°C h^{-1} to −6°C. The tubes were withdrawn after 30 min at −6°C, placed on ice, and thawed overnight at 4°C. After thawing, 12 mL of deionized water was added to each tube, and tubes were shaken gently (200 rpm) at room temperature for 3 h. The conductivity of the extraction solution was measured with an Orion conductimeter (model 105), and the leaves were frozen at −80°C for 1.5 h. The same extraction solution was readded to each tube after 30 min of reequilibration at room temperature and shaken for 2.5 h, and the conductivity of the solution was measured once again to normalize by the total amount of electrolytes.

Fosmid Libraries

We extracted DNA from 3-week-old TBG plants using a large-scale cetyltrimethyl-ammonium bromide protocol (Porebski et al., 1997) including treatment with pectinase (Rogstad et al., 2001). We constructed a fosmid library using the Copy Control Fosmid Library Production Kit (Epicentre) and screened it as described previously (Bomblies et al., 2007) using digoxigenin-labeled (Roche) PCR probes to *LHY* (primers 5′-ACGCGGTTCAAGATGCTCCCA-3′ and 5′-GCTGCAGCATGAGCAGCAGGA-3′). We bar coded positive clones as described (Peterson et al., 2012) and sequenced 100-bp paired-end reads on an Illumina HiSeq 2000. We assembled reads de novo using Velvet (Zerbino and Birney, 2008).

Differentiation Analysis

To test for genetic differentiation, we used our previously published genomic short-read sequences for *A. arenosa* (Hollister et al., 2012; Yant et al., 2013) that we complemented with similarly processed genomes to reach a total of eight KA and seven TBG individuals. We aligned reads to the *A. lyrata* genome (Hu et al., 2011) using BWA (Li and Durbin, 2009) and realigned unmapped reads using Stampy (Lunter and Goodson, 2011). We calculated F_{ST} (Weir, 1990) and Fay and Wu's H (Fay and Wu, 2000) after genotyping the alignments with GATK (McKenna et al., 2010). *LHY* haplotypes were sequenced from one TBG fosmid (see "Fosmid Libraries") and PCR clones obtained from cDNA of three KA and one TBG individuals. The PCR products amplified with primers 5′-TTTCCACGCGGGTATTGTGA-3′ (forward) and 5′-TGTGTTCCCAACTTGGCTCT-3′ (reverse) were then ligated in pBluescript and sequenced from both ends with M13 forward and reverse primers. Where more than four clones were sequenced per individual, only four different haplotypes were reported.

Sequence data from this article can be found in the NCBI SRA data libraries under accession number SRP070489 within the NCBI BioProject PRJNA312410.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. RNA-seq expression of *SOC1* and *VIN3*.

Supplemental Figure S2. Marks of selection on a derived haplotype of *LHY* in the railway accession.

Supplemental Figure S3. High frequency of null expression levels and/or variance among genes without orthologs.

Supplemental Figure S4. *LHY* haplotypes in KA and TBG.

Supplemental Table S1. Vernalization response genes (a) + (b).

Supplemental Table S2. The 1% most differentially expressed genes.

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