A large effect genetic trade-off is caused by a single mutation in *CBF2*

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Abstract

Identifying the genetic basis of local adaptation and fitness trade-offs across environments is a central goal of evolutionary biology. Cold acclimation is an adaptive plastic response for surviving seasonal freezing, and the costs of acclimation may be a general mechanism for fitness trade-offs across environments in temperate zone species. Using locally adapted ecotypes of *Arabidopsis thaliana* from Italy and Sweden, we examined the fitness consequences of a naturally occurring functional polymorphism in *CBF2*. This gene encodes a transcription factor that is a major regulator of cold-acclimated freezing tolerance, and resides within a locus responsible for a genetic trade-off for long-term mean fitness. We estimated the consequences of alternate genotypes of *CBF2* on 5-year mean fitness and fitness components at the native field sites by comparing near isogenic lines with alternate genotypes of *CBF2* to their genetic background ecotypes. The effects of *CBF2* were validated at the nucleotide level using gene edited lines in the native genetic backgrounds grown in simulated parental environments. The foreign *CBF2* genotype in the local genetic background reduced long-term mean fitness in Sweden by greater than 10%, primarily via effects on survival. In Italy fitness was reduced by more than 20%, primarily via effects on fecundity. At both sites, the effects were temporally variable and much stronger in some years. The gene edited lines confirm that *CBF2* encodes the causal variant underlying this genetic trade-off. Additionally, we demonstrated a substantial fitness cost of cold acclimation, which has broad implications for potential maladaptive responses to climate change.

Keywords: antagonistic pleiotropy, cold acclimation, fitness trade-off, local adaptation, plasticity

Introduction

Adaptive differentiation among natural populations is driven by divergent selection. This may lead to local adaptation, where the local ecotype outperforms foreign ecotypes (1, 2). Reciprocal local adaptation in transplant experiments is direct evidence of fitness trade-offs across environments, i.e., adaptation to one environment comes at a fitness cost to adaptation to other environments (2). Such fitness trade-offs have long been thought to be important drivers of biological diversification across scales (3-5). Despite numerous studies demonstrating local adaptation (2, 6-8) the genetic and physiological mechanisms underlying local adaptation and fitness trade-offs across environments are poorly understood (9-12), as is the mechanistic basis of adaptation more generally (13-15). The identification of causal variants for adaptation bears on longstanding questions with important consequences for the rate and predictability of adaptive differentiation, including the role of large effect alleles in adaptation (16-19), and the contribution of individual loci to fitness trade-offs across environments (20, 21).

Despite a large literature on the genetic basis of adaptation, we have an incomplete understanding of the full causal chain connecting naturally occurring sequence polymorphism to traits (molecular or organismal), and ultimately to fitness in the natural environments in which the organisms have evolved. Studies have mapped genetic loci for local adaptation (22-29) but rarely identify and functionally validate causal polymorphisms. The genetic basis of natural variation in traits that are ecologically important under some contexts has been discovered and functionally validated (30-33). However, pervasive genotype by environment interactions for both traits and fitness (21, 31, 34) means that interpreting these results in the context of local adaptation and fitness trade-offs requires testing the natural alleles in the genetic backgrounds in which they occur, and in the native environments in which the organism evolved. Indeed, recent papers on the genetics of adaptation highlight the need to follow up mapping studies with functional validation of candidate genes (15), and the need to explicitly test for fitness effects (19). Answering these calls is no easy task and requires integration of field study of adaptation with experiments in realistic conditions on natural ecotypes with experimentally manipulated alleles. This is currently practical in only a few study systems.

One excellent candidate mechanism for fitness trade-offs across environments in broadly distributed temperate zone species is cold-acclimated freezing tolerance. Cold acclimation is a plastic response to low temperatures, and increases survival in climates with sub-freezing winter temperatures for both plants and animals (35-39). Cold acclimation involves dramatic changes in transcription, protein translation, and metabolism in response to cool temperatures in both animals (40-44) and plants (37, 38, 45). Cold acclimation is thought to be energetically costly (46), and could lead to broad-scale trade-offs when cool temperatures that induce this response are more geographically widespread than selection from freezing temperatures. This cost of producing the wrong phenotype for a given environment is one possible cost of plasticity, though additional costs of maintaining the machinery to be plastic have also been proposed (47). In animals, a cost of cold acclimation has been demonstrated in *Drosophila* (48-50). Cold acclimation may also lead to maladaptive responses in the face of climate change and climate variability by decreasing the predictability of the severity of winter based on cues earlier in the season.

In winter annuals and other plants, cold acclimation can increase freezing tolerance through the accumulation of soluble sugars such as raffinose (51-53) and other compounds that help decrease the freezing point of the cell, resist desiccation, and stabilize and protect cell membranes (37, 38, 45, 54). In *Arabidopsis thaliana*, C-repeat/DRE binding factor 2 (CBF2) is a member of the Apetala2 family of transcription factors and is a necessary regulator of cold acclimation in laboratory studies (45, 55). Natural variation in *CBF2* contributes to differences in freezing tolerance across Arabidopsis ecotypes (56-59). Cold responsiveness of CBF orthologues appears to be broadly conserved in plants (37, 38), and contributes to freezing tolerance in

diverse species such as canola (60), wheat (61), and poplar (62), so it seems likely that this specific mechanism of regulating cold acclimation is of general importance. Cold acclimation is common in temperate zone plants, and more information on the costs of cold acclimation can contribute to our understanding of genetic trade-offs across environments and the potential negative fitness consequences of climate change.

Testing for the costs of cold acclimation in plants is difficult and results are inconclusive thus far. Experimental tests on tree ecotypes from different geographic origins under different experimental conditions found that it is difficult to tease apart acclimation responses from phenological adaptations that are also cued by temperature and photoperiod (63). Studies in Arabidopsis have shown that overexpression of *CBF2* leads to a stunted phenotype and reduced fitness (64, 65), providing indirect evidence of a cost of *CBF2* mediated cold acclimation. Clinal and correlational associations also suggest a potential cost of cold acclimation (66-69). To our knowledge there has only been one direct test of the costs of cold acclimation in plants, which reported no cost for short term acclimation of *Arabidopsis thaliana* in the lab (65). However, the costs of cold acclimation may be expressed only under ecologically relevant conditions (c.f., 48, 49).

In our study system of locally adapted ecotypes (27, 29, 70) of *Arabidopsis thaliana* from Sweden and Italy, cold acclimation likely plays a major role in fitness trade-offs across environments. Minimum winter temperature was implicated as a primary selective agent in Sweden (57, 70). Autumn and winter temperatures are cool but typically non-freezing at the field site in Italy (57, 71), so the cost of acclimation will be expressed without the benefit. Furthermore, major freezing tolerance quantitative trait loci (QTL) co-localize with fitness trade-off QTL detected over multiple years at the native field sites (29, 57). This strongly suggests that cold acclimation mediated by these freezing tolerance loci are a mechanistic basis of genetic trade-offs. The causal variant for the largest effect freezing tolerance QTL is a loss of function mutation in the Italian allele of *CBF2* (58), which can explain 1/3 of the difference in freezing tolerance between the Swedish (SW) and Italian (IT) ecotypes (59).

The final step in conclusively demonstrating a role of *CBF2* in a fitness trade-off is to link the effects of this naturally occurring *CBF2* polymorphism to lifetime fitness in both environments. We expect that the functional Swedish allele of *CBF2* will be adaptive in Sweden, but that cold acclimation induced by *CBF2* in Italy will incur a fitness cost. As EU regulations prohibit planting GMO lines in the field, we take a two-step approach. We used Near Isogenic Lines (NILs) in the field, and engineered lines with manipulated alleles of *CBF2* in the native genetic backgrounds in growth chambers programmed to mimic temperature and photoperiod at the native sites. We addressed the following questions: 1) What are the effects of introgression segments containing alternate alleles of *CBF2* on long-term mean fitness when grown at the native sites? 2) What are the effects of this single gene on fitness and genetic trade-offs across environments? 3) Are the benefits and costs of *CBF2* in alternate environments mediated by viability selection, fecundity selection, or both?

Results

Reciprocal transplant experiments at the Swedish and Italian field sites – Overall fitness

There was a significant and strong fitness tradeoff across environments between the ecotypes for estimates of mean lifetime fitness over five years (Table S1, Fig. 1). This result is consistent with previous work in this system (29, 70), but is an important prerequisite for investigating the contribution of any polymorphism to local adaptation and fitness trade-offs across environments. In Sweden, five-year mean fitness of the Italian ecotype (IT) was 65% less than that of the local Swedish (SW) ecotype (Fig. 1A), and in Italy five-year mean fitness of the SW ecotype was 82%

less than that of the local IT ecotype (Fig. 1B). Thus, there is a pattern of strong overall selection against the foreign ecotype at both sites, though the strength of selection was somewhat stronger in Italy than in Sweden.

To quantify the effect of *CBF2* at each site, we used Near Isogenic Lines (NILs) with introgression segments containing alternate genotypes of *CBF2* in each genetic background (Fig. S1) and compared their 5-year mean fitness to that of their genetic background ecotype. There was a strong and significant signal of a genetic trade-off across environments for the foreign genotypes of CBF2 in the native genetic backgrounds (Fig. 1). At the Swedish site, the NIL containing the IT *cbf2* loss of function (LOF) genotype in the SW genetic background had 12% lower fitness than the SW ecotype. At the Italian site, the NIL containing the functional SW *CBF2* genotype in the IT genetic background had 21% lower fitness than the IT ecotype. However, NILs with the local CBF2 genotype in the foreign ecotypic background did not significantly affect fitness (Fig. 1), though on average there was a 4% and 12% increase in fitness in Sweden and Italy respectively.

Within individual years there were consistent differences between ecotypes reflecting local adaptation (Fig. 2, Table S1), but the effects of *CBF2* were temporally variable at both sites. Focusing on the foreign *CBF2* genotype in the local genetic background, we found a significant effect in Sweden in two out of five years, and significant effects in three years (with a suggestive effect in a fourth year) in Italy. On average, effects of the foreign *cbf2* in Sweden ranged from a strong reduction in fitness (25%, 71%, and 31% in 2015, 2016, and 2018 respectively) to weak increases in fitness (2-6% in the other two years). In Italy the foreign *CBF2* strongly reduced fitness on average during the last four years of the experiment (32%, 11%, 32%, and 40% respectively for 2016-2019), with a weaker negative effect in 2015 (2%).

Single gene effects of *CBF2* in simulated Italian and Swedish conditions – Overall fitness

Testing the effects of *CBF2* at the single gene level required us to use growth chamber experiments simulating key environmental features at the native sites (Fig. S2). The growth chamber programs (Fig. S3; Appendix S1) were constructed based on long-term site level climate data and tested and refined to recapitulate differences in relative fitness between ecotypes observed at the native sites (Fig. S4). These experiments included the parental ecotypes, two independent LOF *cbf2* lines in a SW genetic background, and two transgenic lines containing SW CBF2 and native promotor in an IT background. We omitted the IT background lines from the Swedish chamber experiment because the very low fitness of IT background lines in the field experiment (Fig. 1) and results of freezing tolerance assays with these lines (59) suggested it would not be possible to detect effects in this background in the Swedish environment.

There was a strong signature of a fitness trade-off across environments between the ecotypes in the growth chambers, and single gene estimates of the effects of *CBF2* corroborate the causality of this gene for the NIL effects estimated in the field. There was significant selection against the IT ecotype in the Swedish chamber (92%) and the SW ecotype in the Italian chamber respectively (62%; Fig. 3 & S4; Table S2). For the effects of *cbf2* in the Swedish chamber, both independent LOF *cbf2* lines in the SW background had significantly reduced fitness (23% and 22%) compared to the SW ecotype (Fig. 3A). For the effects of *CBF2* in the Italian chamber, both independent transgenic SW *CBF2* lines in the IT background had significantly reduced fitness (12% and 21%) compared to the IT ecotype (Fig. 3B). Engineered lines in the foreign genetic background were only tested in the Italian chamber, and the lines with IT *cbf2* in a SW background had significantly greater fitness (19% and 14%) than the SW ecotype (Fig. 3B).

Reciprocal transplants at the Swedish and Italian field sites – Fitness components

To determine if the costs (Italy) and benefits (Sweden) of functional CBF2 are mediated through viability selection, fecundity selection, or both, we compare survival and fecundity of the lines in

the field experiment. The ecotypes were significantly locally adapted for both fitness components at both sites except for fecundity in Sweden (Fig. 4; Table S1), IT had 67% lower survival than SW in Sweden, and SW had 33% lower survival and 73% lower fecundity than IT in Italy. The contribution of *CBF2* to fitness trade-offs was most pronounced for survival in Sweden and fecundity in Italy. For survival in Sweden, the line with LOF *cbf2* in the SW background had significantly (13%) reduced survival compared to SW, and the increase in survival (14%) of SW *CBF2* in IT compared to IT was suggestive (Fig. 4A). In Italy the effects of *CBF2* on survival (3-7%) were not significant in either background (Fig. 4B). For fecundity in Sweden, functional *CBF2* in IT decreased fecundity by 11% (Fig. 4C). Because of low survival of the IT ecotype and IT background NIL in most years, the relative fecundity of the IT background NIL reflects data from a single year (2020) with high overall survival (Fig. S5). The effect of SW *CBF2* in IT on fecundity at the Italian site was highly significant, reducing fecundity by 17% compared to the IT ecotype (Fig. 4D). The effects of IT *cbf2* in SW on fecundity were slight and non-significant at both the Swedish and Italian sites (Fig. 4C&D).

Within individual years there were consistent differences between ecotypes for fitness components reflecting local adaptation except for fecundity in Sweden (Fig. S5, Table S1). For survival in Sweden and both fitness components in Italy, the effects of *CBF2* were temporally variable. Focusing on the foreign *CBF2* genotype in the local genetic background compared to the local ecotype, we found significant reductions in survival in Sweden in 2015 (27%) and 2018 (45%) with a suggestive effect in 2016 (26%), and significant effects in Italy only in 2019 (35%). For fecundity in Italy, there were significant decreases in fecundity in 2016 (30%) and 2018 (29%).

Single gene effects of *CBF2* in simulated conditions – Fitness components

Patterns of relative fitness components for the ecotypes and the effects of *CBF2* on those components in the growth chamber experiments are consistent with the field results. There were primarily effects on survival in the Swedish environment and primarily effects on fecundity in the Italian environment (Fig. S6; Table S2). Overall, there were significant differences between the parental ecotypes for survival in the Swedish chamber (IT had 91% lower survival than SW) and fecundity in the Italian chamber (SW had 62% lower fecundity than IT). For the effects of *cbf2* on survival in the Swedish chamber, both independent LOF *cbf2* lines in the SW background had significantly reduced survival (29% and 26%) compared to the SW ecotype (Fig. S6). For the effects of *CBF2* on fecundity in the Italian chamber, both independent transgenic SW *CBF2* lines in the IT background had significantly reduced fecundity (13% and 21%) compared to the IT ecotype (Fig. 3B). The lines with IT *cbf2* in a SW background also had significantly greater fecundity (22% and 15%) than the SW ecotype (Fig. S6D).

Discussion

Identifying the genetic basis of local adaptation and fitness trade-offs across environments is a central, but often unrealized, goal in evolutionary biology. We demonstrated that a naturally occurring polymorphism linked to *CBF2* contributes to substantial differences in long-term mean fitness at the two native sites using a 5-year reciprocal transplant experiment with near isogenic lines. We then functionally validated that *CBF2* is the causal locus for this variation using single gene edited lines in the native genetic backgrounds grown in simulated parental environments. These results provide a unique confirmation of a causal polymorphism underlying local adaptation, and demonstrate a substantial cost of cold acclimation with broad implications for climate change induced maladaptation of temperate zone plants.

We found strong, but temporally variable, effects of *CBF2* at both sites which were primarily mediated by different fitness components at each site. Over five years at the native field

sites, the long-term mean fitness cost of the foreign genotype of *CBF2* in NILs was greater than 10% in Sweden and greater than 20% in Italy (Fig. 1). At both sites, the effects of *CBF2* on long-term mean fitness were even stronger in some individual years. Reductions in lifetime fitness attributable to the foreign genotype at *CBF2* in a single year were up to 71% in Sweden and up to 40% in Italy (Fig. 2). The effects of *CBF2* on both long-term mean fitness and fitness in individual years is mediated primarily via survival in Sweden, and primarily via fecundity in Italy (Fig. 4). These results confirm the strong and temporally variable effect of the genomic region around *CBF2* first reported from QTL mapping over multiple years at the same sites (26, 29). Despite the effort of replicating the present field experiment for five years, correlations between effect sizes and climate variables with five data points per site would be tenuous. However, the two years with significant effects of *CBF2* on fitness in Sweden had the coldest winter minimum soil temperatures, consistent with a role of *CBF2* in freezing tolerance (57, 59). In Italy the two years without significant effects of *CBF2* on fitness had warmer mean soil temperatures during the coldest period, suggesting greater costs for cold acclimation during cooler winters in Italy. These results indicate that the effects of a genetic trade-off can be context dependent, and the magnitude and ability to detect an effect at a locus will depend on the genetic background as well as spatial and temporal environmental variation.

Comparison of effect sizes between the current NIL experiment and previous QTL experiments is difficult, both because the experiments took place in different years, and because the effect sizes represent different contrasts. Here effect sizes represent the effect of a single segment tested against an isogenic ecotypic background, but effect sizes in the QTL study represent the effect of different alleles at a SNP averaged over heterogenous RIL backgrounds and tested as a deviation from the mean fitness of all RILs. That said, the effects of this genomic region in the NIL experiment seem to be greater than those in the QTL experiment for long-term mean fitness in Italy, and for maximum annual effects at both sites. Estimates of effect sizes previously reported for this QTL are therefore not inflated as might be expected by the “Beavis effect” (72), and our results are among a growing number of examples of large effect loci involved in adaptation and adaptive traits (18, 19, 73, 74). Transcription factors in particular may be particularly likely candidates for large effect loci involved in broad scale adaptation because they have effects on many other genes (75, 76). While the field experiments in the native environments replicated over multiple years represent the most natural test of the fitness consequences of alternate alleles of *CBF2*, an important caveat about the results from the NILs is that the introgression segments are large and contain polymorphisms in many genes in addition to *CBF2*. We were prohibited from testing single gene edited lines at the field sites due to EU regulations.

Results using ecotypes and engineered lines in growth chamber programs that mimic temperature and photoperiod changes at the two sites were a critical next step in functional validation of the *CBF2* polymorphism. Estimates from the growth chamber experiments were qualitatively similar to results from the field experiments for the two ecotypes, demonstrating that the growth chambers replicate some essential features of the field environments (Figs. 1, 3 & S4). Results for the effects of single gene edits of *CBF2* in the local genetic background were also qualitatively similar to the results from the field experiment using NILs for both cumulative fitness (Figs. 1 & 3) and fitness components (Figs. 4 & S6). This conclusively demonstrated a causal role for *CBF2* in this fitness trade-off across environments.

There were some, mostly quantitative, differences between the field experiments and growth chamber experiments. Relative to the 5-year mean fitness at the two field sites, the effects of the alternate genotype of *CBF2* in the local genetic background was nearly double in the Swedish chamber, and about 20% lower in the Italian chamber. There were similar quantitative differences for survival in the Swedish chamber and fecundity in the Italian chamber. The effects observed in the growth chambers were however well within the range of effect sizes observed in individual years in the field. The other notable difference between field and growth chamber

experiments was that in the Italian growth chamber we were able to detect significant effects of the local genotype of *CBF2* in the foreign genetic background that were undetectable in the field experiment. This seemingly greater power to detect an effect of the alternative *CBF2* allele in the foreign genetic background in the Italian environment occurred despite a lack of differential mortality in the Italian growth chamber. It is formally possible that the introgression in the SW background NIL may contain another locus or loci with opposing effects on fecundity.

Elucidating the genetic basis of local adaptation is challenging because few systems allow for direct functional validation of the fitness consequences of naturally occurring sequence polymorphisms in the native genetic backgrounds grown in the native environments. Studies mapping QTL for local adaptation in field reciprocal transplants (reviewed in 21, 28, 29) identify naturally occurring sequence polymorphisms in regions of the genome associated with fitness, but these QTL are often large and contain many genes. Genome wide association mapping (GWAS) of the genetic basis of regional adaptation typically provide better resolution for the candidate polymorphisms (77-79). What both approaches have in common is that they generate hypotheses about candidate genes that need to be tested by functional validation (11, 13, 15). Such validation is limited to systems in which genetic transformation is feasible, or where one can indirectly test the effect of a gene from the focal species in a model organism (80, 81). Functional genetic studies on putatively adaptive traits provide a clear link between causal variant and phenotype (30-33), but the relationships between genotype, phenotype, and fitness will depend on the environmental context (19, 21, 31, 34). Other approaches directly test the fitness effects of mutations in field experiments (75, 82), or in laboratory studies in different environments (83) but the results are likely context dependent on the genetic background and the specific field sites or sets of laboratory conditions. All these approaches advance our understanding about the genotype-phenotype-fitness map, and each system has its own set of constraints. Our approach is relatively comprehensive in advancing the ultimate goal, though legal constraints necessitated testing single gene edits in simulated rather than actual parental environments. Such conditions cannot possibly replicate all the complex selective pressures that plants experience at the field sites. However, the concordance between field and growth chamber results suggest that we captured essential differences observed in nature.

The mechanistic basis of the fitness effects of this functional polymorphism in the Swedish environment stems from the major role of the CBF2 transcription factor in the regulation of cold acclimation. We hypothesize that investment in acclimation in response to cool, but non-freezing temperatures, results in the fitness cost in the Italian environment. This polymorphism in *CBF2* has previously been shown to explain about 1/3 of the difference between the SW and IT ecotypes in cold-acclimated freezing tolerance in the lab (57, 59), and the effects of natural variation in CBF2 on freezing tolerance has been demonstrated in other ecotypes as well (56). In our system, a short list of candidate genes that are partially regulated by CBF2 in response to short-term cold acclimation have been identified using RNAseq (59). These genes have likely roles in sugar biosynthesis, desiccation resistance, and membrane stabilization. The strongest of these candidates was *Galactinol synthase 3* (*GolS3*), which is an important enzyme in the synthesis of galactinol and raffinose. Accumulation of raffinose and other soluble sugars has previously been correlated with increased freezing tolerance (51, 52). In *A. thaliana*, accessions from greater latitudes have greater cold acclimated freezing tolerance (67, 84). Accessions from colder climates also accumulate more raffinose in response to cold, and this is negatively correlated with vegetative growth rates (53). Ongoing work in our system will characterize the role of the *CBF2* polymorphism on transcriptional and metabolic responses across the cold periods in both environments to better characterize the contribution of raffinose and other compounds to fitness trade-offs across environments.

Regardless of the specific mechanisms, our fitness results in the Italian environment provide direct evidence for a cost of cold acclimation, in agreement with several prior lines of indirect evidence. We hypothesize that such a cost is borne even in the Swedish environment,

but that in most years the benefits of freezing tolerance outweigh the costs of cold acclimation. This could have profound implications for maladaptive responses in many temperate zone plants with climate change. A decoupling between cold acclimation cues and the severity of subsequent winter could lead to reductions in fitness if autumn temperatures are insufficient to acclimate for an anomalously severe winter, or if cool autumn temperatures that induce cold acclimation are followed by a milder winter. Direct evidence for a cost of cold acclimation in plants has until now proven difficult, likely because manipulating the acclimation environment (and/or using a broad geographic sample of ecotypes) also affects phenological patterns in species where cold serves to cue phenological transitions (63). Additionally, the costs of acclimation may only be manifest under relatively realistic conditions. Accurate quantification of the potential costs of cold acclimation will likely require either using photoperiod sensitive flowering species, or as we have done here, manipulating major regulators of cold acclimation. Orthologues of CBF genes are cold responsive in many plant lineages (37). Our approach could be applied more broadly as transformation becomes feasible in more species. In the meantime, we suggest that predictions about organismal resilience in the face of climate change should carefully consider potential costs of cold acclimation.

Materials and Methods

Study system

Arabidopsis thaliana is an annual, predominantly selfing plant native to Eurasia and parts of Africa (85, 86). Our source ecotypes are from locally adapted populations from Rödåsen (hereafter "SW") in north-central Sweden (62°48′N, 18°12′E) and in Castelnuovo di Porto (hereafter "IT") in central Italy (42°07′N, 12°29′E), near the northern and southern edge of the native Eurasian range (70). At both sites, plants exhibit a winter annual life history and overwinter as vegetative rosettes. At the Italian site (42°07′N, 12°29′E), seeds germinate in autumn and plants overwinter as rosettes under cool conditions followed by flowering in March and April. At the Swedish site (62°48′N, 18°12′E), seeds germinate in late summer, and plants overwinter as rosettes and flower in May and June. These ecotypes have been used extensively to map the genetic basis of local adaptation (26, 29, 87) and ecologically important traits (27, 57, 88, 89), as well as to study physiological responses that are infeasible to map (90, 91).

Development of genetic resources

To quantify lifetime fitness effects of *CBF2* in both environments, we used a combination of Near Isogenic Lines (NILs) and genetically engineered lines. Details of NIL construction can be found in (Fig. S1) and (59). We used two NILs containing alternate introgression segments surrounding *CBF2* in each genetic background. The SW background NIL contains a segment with the IT loss of function (LOF) *cbf2* genotype. The IT background NIL contains a segment with the functional SW *CBF2* genotype (Fig. S1). These segments contain many genes in addition to *CBF2* and cannot provide single gene resolution but were used to quantify fitness effects of this genomic region in field experiments at the native sites where European Union regulations prohibit the planting of GMO organisms.

To obtain single gene resolution of the fitness effects of *CBF2* we used genetically engineered lines and the parental ecotypes in growth chamber experiments. To mimic the IT *cbf2* LOF ecotype in the SW genetic background we used two independent *cbf2* LOF lines (produced using CRISPR/Cas9), previously shown to explain over 1/3 of ecotypic differences in cold acclimated freezing tolerance (59). For IT background lines with a functional copy of *CBF2*, we used two independent transgenic lines with SW *CBF2* and native promoter inserted into the IT background, previously used in electrolyte leakage assays of freezing tolerance (58).

Reciprocal transplant experiments at the Swedish and Italian field sites

To obtain estimates of fitness and fitness components for the effects of introgression segments containing alternate genotypes of *CBF2* in both genetic backgrounds, we performed field reciprocal transplant experiments. We transplanted seedlings of the two ecotypes and two NILs (R_38 and C_19; Fig. S1) at both the native Swedish and Italian field sites in each of five years. Seed germination, transplanting, and field planting protocols closely follow previous field studies of these ecotypes (26, 29). In brief, in each individual year we plated seeds out on agar filled Petri dishes, and cold-stratified the dishes in the dark at 4°C for 1 w to break seed dormancy and synchronize germination. The plates were then transferred to a growth room set to 22°C, 16-h day (16L:8D) with photosynthetically active radiation (PAR) of 150 µmol photons m^{-2} s^{-1} for eight to ten days for germination. Seedlings were transplanted into a randomized block design in plug trays with individual cells of 20 x 20 x 40 mm filled with an equal mixture of local sand, gravel, and unfertilized peat in Sweden and with local soil in Italy. In each of five years, trays with seedlings were then set into the ground in early September at the Swedish site, and in early November at the Italian site. Sample sizes per genotype per year were variable across years but ranged from 400-600 seedlings each for IT (mean=438) and SW (mean=440), 100-150 seedlings for C_19 (mean=109), and 100-200 seedlings for R_38 (mean=150). For each individual plant we scored cumulative fitness as the total number of fruits produced, which incorporates both survival and fecundity (fruit number per surviving plant).

Within each site, we examined the effects of genotype, year and the genotype*year interaction on cumulative fitness, survival, and fecundity using ANOVA. Both genotype (two ecotypes and two NILs) and year were treated as fixed effects. Survival was analyzed with a binomial error distribution. Inspection of residuals for fecundity indicated that a normal error distribution was suitable. For cumulative fitness there were some departures from normality and equal variances, however results using a normal error distribution were qualitatively similar to both non-parametric and Poisson error distribution models (not shown), so we present the normal model results for simplicity. For any model with a significant effect of genotype or genotype*year, we used *a priori* linear contrasts to compare the IT and SW ecotypes. We also tested for the effect of the *CBF2* region on differential adaptation by contrasting NILs to their genetic background ecotypes. All statistical analyses were conducted using JMP version 16.

Single gene effects of CBF2 in simulated Italian and Swedish conditions

To quantify the fitness effects of the single *CBF2* polymorphism, we developed growth chamber programs to simulate seasonal changes in temperature and photoperiod at the Swedish and Italian sites. For details of field climate data see (Fig. S2) and (71). Daily mean high and low temperature values across years were used to guide construction and optimization of the growth chamber programs (Fig. S3, Appendix S1). Although it is impossible to completely mimic field conditions in a growth chamber, the general concordance in ecotypic differences in relative fitness between field and growth chamber experiments (Fig. S4) gives us confidence that we were able to recreate some of the essential features driving local adaptation in this system.

The optimized growth chamber programs were used to estimate fitness, survival, and fecundity for the IT and SW ecotypes, and engineered lines manipulating *CBF2* alleles in the native genetic backgrounds. In both environments, we included two independent *cbf2* loss of function lines in a SW background. In the Italian environment, we included two additional transgenic lines containing the SW CBF2 allele and native promotor in an IT background. The transgenic lines were omitted from the Swedish chamber assay because preliminary trials indicated that fitness of IT was so low as to preclude detection of an effect in an IT background line.

Methods for seed sterilization and germination closely follow those of previous freezing tolerance assays in this system (57, 59). In brief, sterilized seeds were sown on autoclaved media

containing Gamborg's B-5 and agar in Petri dishes. The seeds were cold-stratified in the dark at 4°C for 5 d to synchronize germination. The plates were then transferred to a growth chamber set to 22°C, 16-h day (16L:8D) with PAR of 125 µmol photons m^{-2} s^{-1} for germination for 12 d. Seedlings were then transplanted into a randomized design in plug trays with individual cells of 20 x 20 x 40 mm filled with propagation mix soil. The outer two rows and columns with border rows around the perimeter of each of two wire racks holding the plants in the chamber were planted with "extra" plants (no data collected) to reduce edge effects within the experiment. The trays were then transferred to a growth chamber, LTCB-19 (BioChambers, Winnipeg, MB, Canada) programmed as described above for either the "Italian" or "Swedish" environment (Fig. S3). In the Swedish chamber, we added a thin layer of shaved ice onto the plants as soon as chamber temperatures first dropped to freezing conditions (from 4°C to -2°C) to facilitate ice nucleation (58). In each chamber environment, we scored cumulative fitness for each individual plant as the total number of fruits produced, including zeros for plants that did not survive or reproduce.

Statistical analyses of the growth chamber experiments closely follow that of the field experiments. In the models for the chamber analyses, we also included the effect of flat to account for microspatial variation within the chamber. This was treated as a fixed effect because of the small number of flats. Error distributions for fitness, survival, and fecundity were the same as for the analyses of the field data. In models with a significant effect of genotype, we examined *a priori* contrasts of the IT and SW ecotypes, and contrasted all engineered lines to the genetic background ecotype. All statistical analyses for both field and chamber experiments were conducted using JMP version 16.

References

1. Kawecki TJ & Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters* 7:1225-1241.
2. Hereford J (2009) A quantitative survey of local adaptation and fitness trade-offs. *The American Naturalist* 173:579-588.
3. MacArthur RH (1972) *Geographical Ecology: Patterns in the Distribution of Species.* (Princeton University Press, Princeton, New Jersey).
4. Futuyma DJ & Moreno G (1988) The evolution of ecological specialization. *Annual Review of Ecology and Systematics* 19:207-233.
5. Agrawal AA (2020) A scale-dependent framework for trade-offs, syndromes, and specialization in organismal biology. *Ecology* 101:e02924.
6. Leimu R & Fischer M (2008) A meta-analysis of local adaptation in plants. *Public Library of Science ONE* 3:10.1371/journal.pone.0004010.t0004001.
7. Johnson LC*, et al.* (2022) Reciprocal transplant gardens as gold standard to detect local adaptation in grassland species: New opportunities moving into the 21st century. *Journal of Ecology* 110:1054-1071.
8. VanWallendael A, Lowry DB, & Hamilton JA (2022) One hundred years into the study of ecotypes, new advances are being made through large-scale field experiments in perennial plant systems. *Current Opinion in Plant Biology* 66:102152.
9. Savolainen O, Lascoux M, & Merilä J (2013) Ecological genomics of local adaptation. *Nature Reviews Genetics* 14(11):807-820.
10. Tiffin P & Ross-Ibarra J (2014) Advances and limits of using population genetics to understand local adaptation. *Trends in Ecology & Evolution* 29(12):673-680.

11. VanWallendael A*, et al.* (2019) A Molecular View of Plant Local Adaptation: Incorporating Stress-Response Networks. *Annual Review of Plant Biology* 70:14.11-14.25.
12. Wadgymar SM, DeMarche ML, Josephs EB, Sheth SN, & Anderson JT (2022) Local adaptation: Causal agents of selection and adaptive trait divergence. *Annual Review of Ecology, Evolution, and Systematics* 53:87-111.
13. Stinchcombe JR & Hoekstra HE (2008) Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. *Heredity* 100:158-170.
14. Barrett RDH & Hoekstra HE (2011) Molecular spandrels: tests of adaptation at the genetic level. *Nature Reviews Genetics* 12:767-780.
15. Bomblies K & Peichel CL (2022) Genetics of adaptation. *Proceedings of the National Academy of Sciences U S A* 119(30):e2122152119.
16. Rockman MV (2012) The QTN program and the alleles that matter for evolution: all that's gold does not glitter. *Evolution* 66:1-17.
17. Rausher MD & Delph LF (2015) Commentary: When does understanding phenotypic evolution require identification of the underlying genes? *Evolution* 69:1655-1664.
18. Dittmar EL, Oakley CG, Conner JK, Gould BA, & Schemske DW (2016) Factors influencing the effect size distribution of adaptive substitutions. *Proceedings of the Royal Society B-Biological Sciences* 283:20153065.
19. Schluter D*, et al.* (2021) Fitness maps to a large-effect locus in introduced stickleback populations. *Proceedings of the National Academy of Sciences* 118:e1914889118.
20. Anderson JT, Willis JH, & Mitchell-Olds T (2011) Evolutionary genetics of plant adaptation. *Trends in Genetics* 27:258-266.
21. Wadgymar SM*, et al.* (2017) Identifying targets and agents of selection: innovative methods to evaluate the processes that contribute to local adaptation. *Methods in Ecology and Evolution* 8:738-749.
22. Lowry DB, Hall MC, Salt DE, & Willis JH (2009) Genetic and physiological basis of adaptive salt tolerance divergence between coastal and inland *Mimulus guttatus*. *New Phytologist* 183(3):776-788.
23. Hall MC, Lowry DB, & Willis JH (2010) Is local adaptation in *Mimulus guttatus* caused by trade-offs at individual loci? *Molecular Ecology* 19(13):2739-2753.
24. Anderson JT, Lee C-R, Rushworth CA, Colautti RI, & Mitchell-Olds T (2013) Genetic trade-offs and conditional neutrality contribute to local adaptation. *Molecular Ecology* 22(3):699-708.
25. Leinonen PH, Remmington DL, Leppälä J, & Savolainen O (2013) Genetic basis of local adaptation and flowering time variation in *Arabidopsis lyrata*. *Molecular Ecology* 22(3):709-723.
26. Ågren J, Oakley CG, McKay JK, Lovell JT, & Schemske DW (2013) Genetic mapping of adaptation reveals fitness tradeoffs in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 110:21077-21082.
27. Postma FM & Ågren J (2016) Early life stages contribute strongly to local adaptation in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 113:7590-7595.

28. Wright SJ, Goad DM, Gross BL, Muñoz PR, & Olsen KM (2021) Genetic trade-offs underlie divergent life history strategies for local adaptation in white clover. *Molecular Ecology*:10.1111/mec.16180.
29. Oakley CG, Schemske DW, McKay JK, & Ågren J (2023) Ecological genetics of local adaptation in Arabidopsis: An 8-year field experiment. *Molecular Ecology*:10.1111/mec.17045.
30. Mackay TFC, Stone EA, & Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. *Nature Reviews Genetics* 10:565-577.
31. Des Marais DL, Hernandez KM, & Juenger TE (2013) Genotype-by-environment interaction and plasticity: exploring genomic responses of plants to the abiotic environment. *Annual Review of Ecology, Evolution, and Systematics* 44:5-29.
32. Alonso-Blanco C & Méndez-Vigo B (2014) Genetic architecture of naturally occuring quantitative traits in plants: an updated synthesis. *Current Opinion in Plant Biology* 18:37-43.
33. Des Marais DL*, et al.* (2014) Variation in *MPK12* affects water use efficiency in *Arabidopsis* and reveals a pleiotropic link between guard cell size and ABA response. *Proceedings of the National Academy of Sciences of the United States of America* 111(7):2836-2841.
34. Josephs EB (2018) Determining the evolutionary forces shaping G x E. *New Phytologist* 219:31-36.
35. Storey KB & Storey JM (1996) Natural freezing survival in animals. *Annual Review of Ecology, Evolution, and Systematics* 27:365-386.
36. Addo-Bediako A, Chown SL, & Gaston KJ (2000) Thermal tolerance, climatic variability and latitude. *Proceedings of the Royal Society B-Biological Sciences* 267:739-745.
37. Preston JC & Sandve SR (2013) Adaptation to seasonality and the winter freeze. *Frontiers in Plant Science* 4:10.3389/fpls.2013.00167.
38. Chang CY, Bräutigam K, Hüner NPA, & Ensminger I (2021) Champions of winter survival: cold acclimation and molecular regulation of cold hardiness in evergreen conifers. *New Phytologist* 229:675-691.
39. Levitt J (1980) *Chilling, freezing, and high temperature stresses* (Academic press, New York).
40. Colinet H, Larvor V, Laparie M, & Renault D (2012) Exploring the plastic response to cold acclimation through metabolomics. *Functional Ecology* 26:711-722.
41. Long Y*, et al.* (2013) Transcriptomic characterization of cold acclimation in larval zebrafish. *BMC Genomics* 14:612.
42. Kristensen TN, Kjeldal H, Schou MF, & Nielsen JL (2016) Proteomic data reveal a physiological basis for costs and benefits associated with thermal acclimation. *Journal of Experimental Biology* 219:969-976.
43. MacMillan HA*, et al.* (2016) Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports* 6:28999.
44. Noer NK*, et al.* (2022) Rapid adjustments in thermal tolerance and the metabolome to daily environmental changes - A field study on the arctic seed bug *Nysius groenlandicus*. *Frontiers in Physiology* 13:818485.
45. Thomashow MF (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiology* 154:571-577.

46. Loehle C (1998) Height growth tradeoffs determine northern and southern range limits for trees. *Journal of Biogeography* 25:735-742.
47. Auld JR, Agrawal AA, & Reylea RA (2010) Re-evauluating the costs and limits of adaptive pehotypic plasticity. *Proceedings of the Royal Society B-Biological Sciences* 277:503-511.
48. Kristensen TN*, et al.* (2008) Costs and benefits of cold acclimation in field-released Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* 105:216-221.
49. Schou MF, Loeschcke V, & Kristensen TN (2015) Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLos ONE* 10:e0130307.
50. Everman ER, Delzeit JL, Hunter FK, Gleason JM, & Morgan TJ (2018) Costs of cold acclimation on survival and reproductive behavior in *Drosophila melanogaster*. *PLos ONE* 13:e0197822.
51. Taji T*, et al.* (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *The Plant Journal* 29:417-426.
52. Kaplan F*, et al.* (2007) Transcript and metabolite profiling during cold acclimation of *Arabidopsis* reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. *The Plant Journal* 50:967-981.
53. Weiszmann J*, et al.* (2023) Metabolome plasticity in 241 Arabisopsis thaliana accessions reveals evolutionary cold adaptation process. *Plant Physiology*:https://doi.org/10.1093/plphys/kiad1298.
54. Barrero-Gil J & Salinas J (2018) Gene regulatory networks mediating cold acclimation: the CBF pathway. *Survival Strageies in Extreme Cold and Desiccation*, eds Iwaya-Inoue M, Sakurai M, & Uemura M (Springer Singapore, Singapore).
55. Park S*, et al.* (2015) Regulation of the *Arabidopsis* CBF regulon by a complex low-temperature regulatory network. *The Plant Journal* 82:193-207.
56. Alonso-Blanco C*, et al.* (2005) Genetic and molecular analyses of natural variation indicate *CBF2* as a candidate gene for underlying a freezing tolerance quantitative trait locus in *Arabidopsis*. *Plant Physiology* 139:1304-1312.
57. Oakley CG, Ågren J, Atchison RA, & Schemske DW (2014) QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs. *Molecular Ecology* 23:4304-4315.
58. Gehan MA*, et al.* (2015) Natural variation in the C-repeat binding factor cold response pathway correlates with local adaptation of *Arabidopsis* ecotypes. *The Plant Journal* 84:682-693.
59. Sanderson BJ*, et al.* (2020) Genetic and physiological mechanisms of freezing tolerance in locally adapted populations of a winter annual. *American Journal of Botany* 107(2):250-261.
60. Savitch LV*, et al.* (2005) The effect of overexpression of two *Brassica CBF/DREB1*-like transcription factors on photosynthetic capacity and freezing tolerance in Brassica napus. *Plant Cell Physiolology* 46:1525-1539.
61. Vágújfalvi A, Galiba G, Cattivelli L, & Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. *Molecular Genetics and Genomics* 269:60-67.
62. Benedict C*, et al.* (2006) The *CBF1*-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp. *Plant, Cell and Environment* 29:1259-1272.

63. Savage JA & Cavender-Bares J (2013) Phenological cues drive an apparent trade-off between freezing tolerance and growth in the family Salicaceae. *Ecology* 94:1708-1717.
64. Jackson MW, Stinchcombe JR, Korves TM, & Schmitt J (2004) Costs and benefits of cold tolerance in transgenic *Arabidopsis thaliana*. *Molecular Ecology* 13:3609-3615.
65. Zhen Y, Dhakal P, & Ungerer MC (2011) Fitness benefits and costs of cold acclimation in *Arabidopsis thaliana*. *American Naturalist* 178(1):44-52.
66. Zhen Y & Ungerer MC (2008) Relaxed selection on the *CBF*/*DREB1* regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*. *Molecular Biology and Evolution* 25(12):2547-2555.
67. Zuther E, Schulz E, Childs LH, & Hincha DK (2012) Clinal variation in the non-acclimated and cold-acclimated freezing tolerance of *Arabidopsis thaliana* accessions. *Plant, Cell and Environment* 35(10):1860-1878.
68. Monroe JG*, et al.* (2016) Adaptation to warmer climates by parallel functional evolution of CBF genes in *Arabidopsis thaliana*. *Molecular Ecology* 25:3632-3644.
69. Boinot M, Karakas E, Koehl K, Pagter M, & Zuther E (2022) Cold stress and freezing tolerance negatively affect the fitness of *Arabidopsis thaliana* accessions under field and controlled conditions. *Planta* 255:39.
70. Ågren J & Schemske DW (2012) Reciprocal transplants demonstrate strong adaptive differentiation of the model organism *Arabidopsis thaliana* in its native range. *New Phytologist* 194:1112-1122.
71. Zacchello G, Vinyeta M, & Ågren J (2020) Strong stabilizing selection on timing of germination in a Mediterranean population of *Arabidopsis thaliana*. *American Journal of Botany*.
72. Beavis WD (1994) The power and deceit of QTL experiments: lessons from comparative QTL studies. *Proceedings of the Corn and Sorghum Industry Research Conference, American Seed Trade Association, Washington DC*:250-266.
73. Martínez-Berdeja A*, et al.* (2020) Functional variants of *DOG1* control seed chilling responses and variation in seasonal life-history strategies in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 117:2526-2534.
74. Wang J*, et al.* (2018) A major locus controls local adaptation and adaptive life history variation in a perennial plant. *Genome Biology* 19:72.
75. Taylor MA*, et al.* (2019) Large-effect flowering time mutations reveal conditionally adaptive paths through fitness landscapes in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 116:17890-17899.
76. Lopez-Arboleda WA, Reinert S, Nordborg M, & Korte A (2021) Global genetic heterogeneity in adaptive traits. *Molecular Biology and Evolution* 38:4822-4831.
77. Fournier-Level A*, et al.* (2011) A map of local adaptation in *Arabidopsis thaliana*. *Science* 334(6052):86-89.
78. Hancock AM*, et al.* (2011) Adaptation to climate across the *Arabidopsis thaliana* genome. *Science* 334(6052):83-86.
79. Exposito-Alonso M*, et al.* (2019) Natural selection on the *Arabidopsis thaliana* genome in present and future climates. *Nature* 573:126-129.
80. Prasad KV*, et al.* (2012) A gain-of-function polymorphism controlling complex traits and fitness in nature. *Science* 337:1081-1084.
81. Barrett RDH*, et al.* (2019) Linking a mutation to survival in wild mice. *Science* 363:499-504.

82. Kerwin R*, et al.* (2015) Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. *Elife* 4:10.7554/eLife.05604.
83. Bono LM, Smith LB, Jr., Pfennig DW, & Burch CL (2017) The emergence of performance trade-offs during local adaptation: insights from experimental evolution. *Molecular Ecology* 26:1720-1733.
84. Zhen Y & Ungerer MC (2008) Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana*. *New Phytologist* 177(2):419-427.
85. Koornneef M, Alonso-Blanco C, & Vreugdenhil D (2004) Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annual Review of Plant Biology* 55:141-172.
86. Durvasula A*, et al.* (2017) African genomes illuminate the early history and transition to selfing in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* 114:5213-5218.
87. Price N*, et al.* (2018) Combining population genomics and fitness QTLs to identify the genetics of local adaptation in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 115(19):5028-5033.
88. Ågren J, Oakley CG, Lundemo S, & Schemske DW (2017) Adaptive divergence in flowering time among natural populations of *Arabidopsis thaliana*: estimates of selection and QTL mapping. *Evolution* 71:550-564.
89. Oakley CG*, et al.* (2018) Genetic basis of photosynthetic responses to cold in two locally adapted populations of *Arabidopsis thaliana*. *Journal of Experimental Botany* 69:699-709.
90. Cohu CM, Muller O, Stewart JJ, Demmig-Adams B, & Adams WWI (2013) Association between minor loading vein architecture and light- and CO2-saturated rates of photosynthetic oxygen evolution among *Arabidopsis thaliana* ecotypes from different latitudes. *Frontiers in Plant Science* 4:240.
91. Stewart JJ*, et al.* (2015) Differences in light-harvesting, acclimation to growth-light environment, and leaf structural development between Swedish and Italian ecotypes of *Arabidopsis thaliana*. *Planta* 242(6):1277-1290.

Figures and Tables

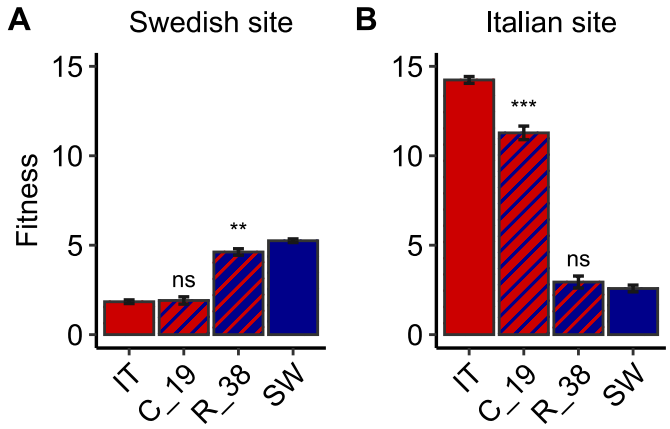

Figure 1. LSM estimates of fitness of the SW and IT ecotypes and NILs over five individual one-year experiments at the Swedish (A) and Italian (B) field sites. Error bars are 1 SE. The predominant color indicates the genetic background (SW ecotype = blue; IT ecotype = red). Hatching indicates the genotype of the introgression segment containing *CBF2* in the NILs. Asterisks represent statistically significant contrasts between a NIL and its genetic background (***, $P < 0.001$; **, $P < 0.01$; ns, not significant).

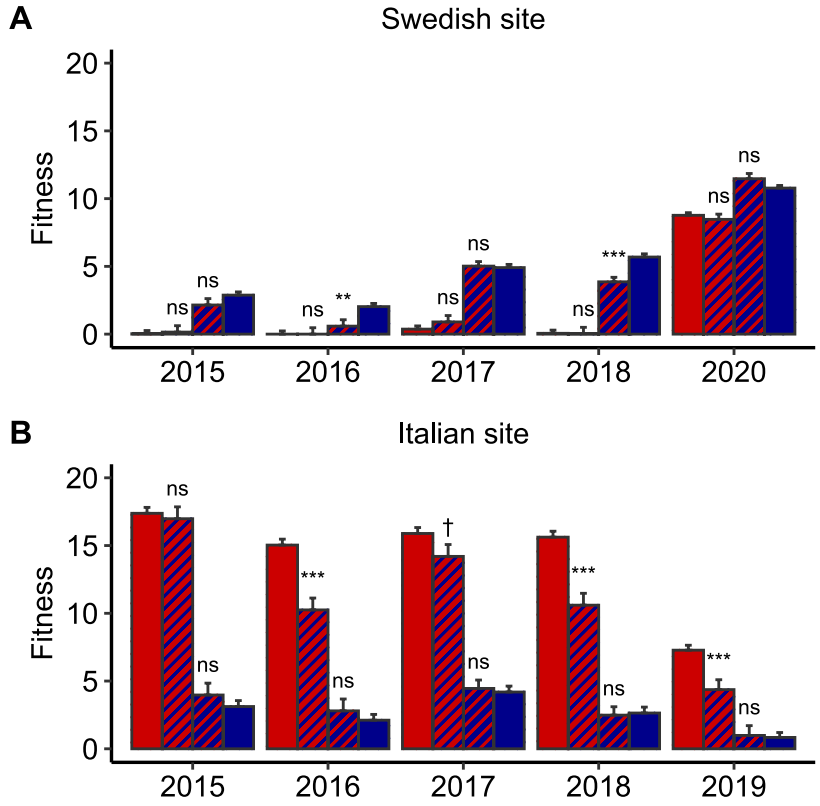

Figure 2. LSM estimates of fitness of the ecotypes and NILs in each individual year at the two field sites. Error bars are 1 SE. The predominant color indicates the genetic background (SW ecotype = blue; IT ecotype = red). Hatching indicates the genotype of the introgression segment containing *CBF2* in the NILs as shown in Fig 1. Asterisks denote significant contrasts between a NIL and its genetic background (***, $P < 0.001$; **, $P < 0.01$; †, $0.05 < P < 0.1$; ns, not significant).

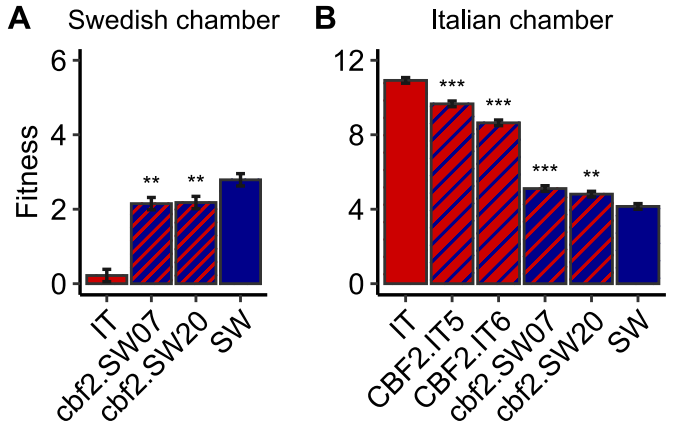

Figure 3. LSM fitness of the ecotypes and engineered lines in the "Swedish" (A) and "Italian" (B) growth chambers. Error bars are 1 SE. The predominant color indicates the genetic background (SW ecotype = blue; IT ecotype = red). Hatching indicates the genotype of *CBF2* (capitals = functional copy, lower case = loss of function) in engineered lines. Asterisks represent statistically significant contrasts between an engineered line and its genetic background (***, $P < 0.001$; **, $P < 0.01$). Note that the scale of the Y axis of fitness for the Italian chamber is double that of the Swedish chamber.

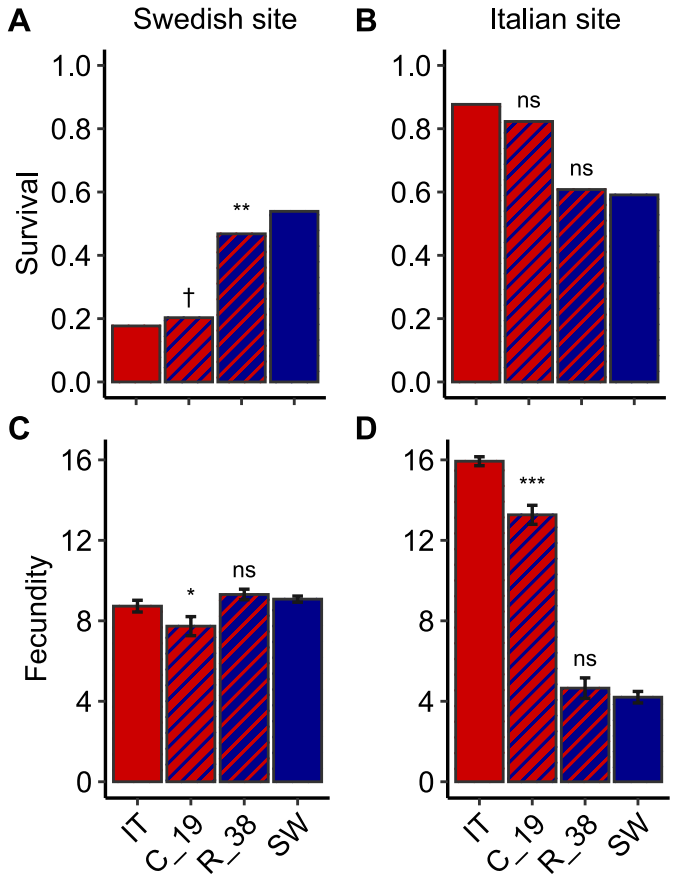

Figure 4. Fitness components of the SW and IT ecotypes and NILs over five individual one-year experiments at the Swedish and Italian field sites. Mean proportion survival (averaged over years) at the Swedish (A) and Italian (B) field sites. LSM fecundity at the same sites (C and D). Error bars for fecundity are 1 SE, no bars are given for survival because it was analyzed with a binomial error distribution. Asterisks represent statistically significant contrasts between a NIL and its genetic background (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; †, $0.05 < P < 0.1$; ns, not significant).

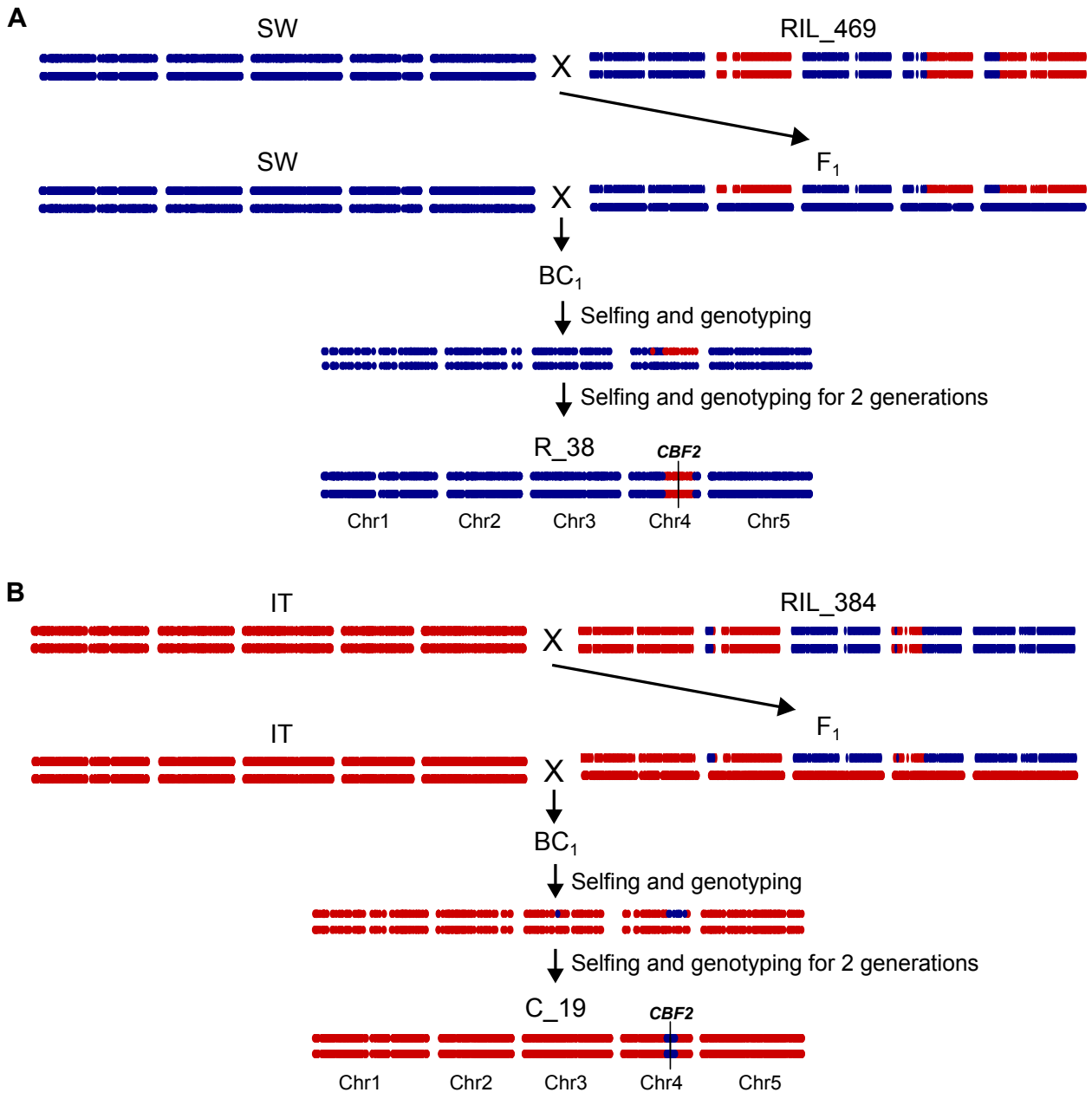

Figure S1. Construction of the SW background NIL (R_38) containing an IT fragment including the *cbf2* LOF mutation (A) and the IT background NIL (C_19) containing a SW fragment including functional *CBF2* (B). Blue points indicate SW marker genotypes and red points are IT marker genotypes (approximately 2000 SNPs). Position of *CBF2* is marked with a black vertical line. NIL construction was initiated by crossing individual recombinant inbred lines to the parental ecotypes, and genotyping to confirm formation of an F_1. The F_1 was then backcrossed (BC_1), and resultant progeny genotyped, to isolate alternate introgression segments surrounding *CBF2* on chromosome 4 in otherwise isogenic parental genetic backgrounds. Repeated selfing and genotyping for several generations produced NILs with a single homozygous introgression segment. Genotyping was performed between generations using a combination of 2b-restriction site associated DNA (2b-RAD) sequencing and cleaved amplified polymorphic sequence (CAPS) markers. The SW background NIL (R_38) contains an approximately 8.0-Mb introgression segment. The IT background NIL (C_19) has an approximately 2.5-Mb introgression segment.

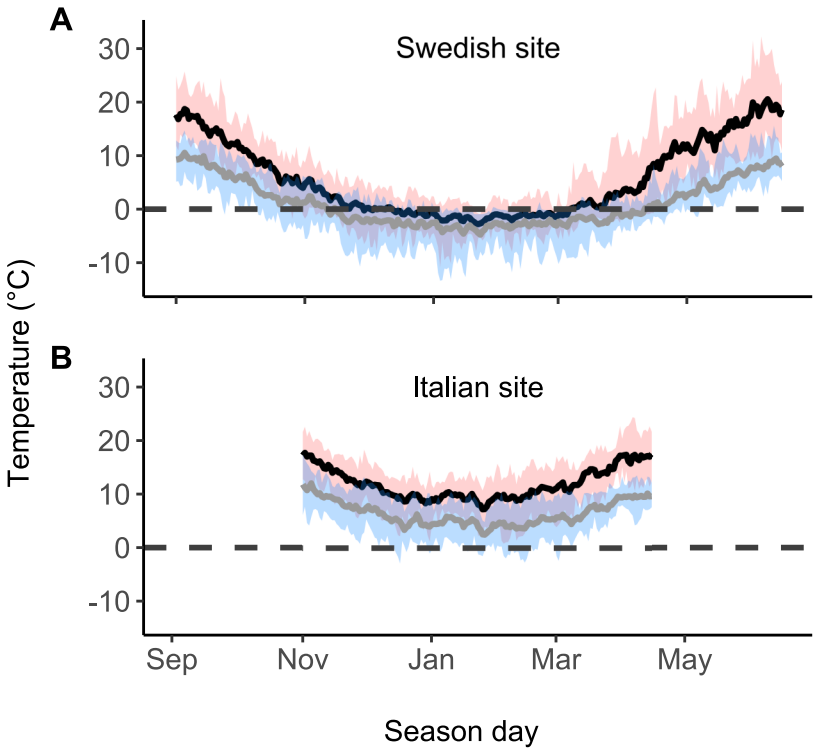

Figure S2. Temperature profiles for the Swedish (A) and Italian (B) field sites approximately from seed emergence to seed production. The solid black and gray line represent the mean daily maximum and minimum temperatures, respectively across 13 years (2003-2015). The pink and blue shaded regions represent the absolute range of daily maximum and minimum temperatures (averaged over soil and air temperatures) observed across the years. For each day at each site, we averaged over multiple time periods for each sensor, and then averaged over sensors for both air and soil temperature.

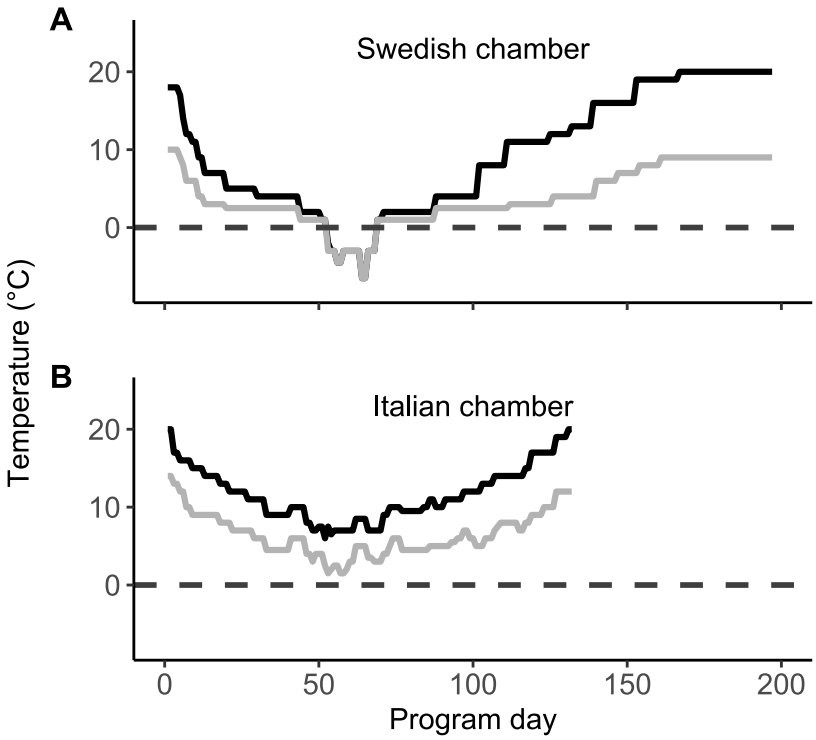

Figure S3. Temperature profiles for the growth chamber programs to mimic important aspects of the parental environments (Fig. S1). The programs were used for the Swedish chamber (A) and the Italian chamber (B) assays, respectively. Black lines indicate daily high temperatures and grey lines are daily low temperatures. Program day of the Italian chamber is shorter than the Swedish chamber because of the shorter life cycle at the native field site in Italy (see Fig. S2).

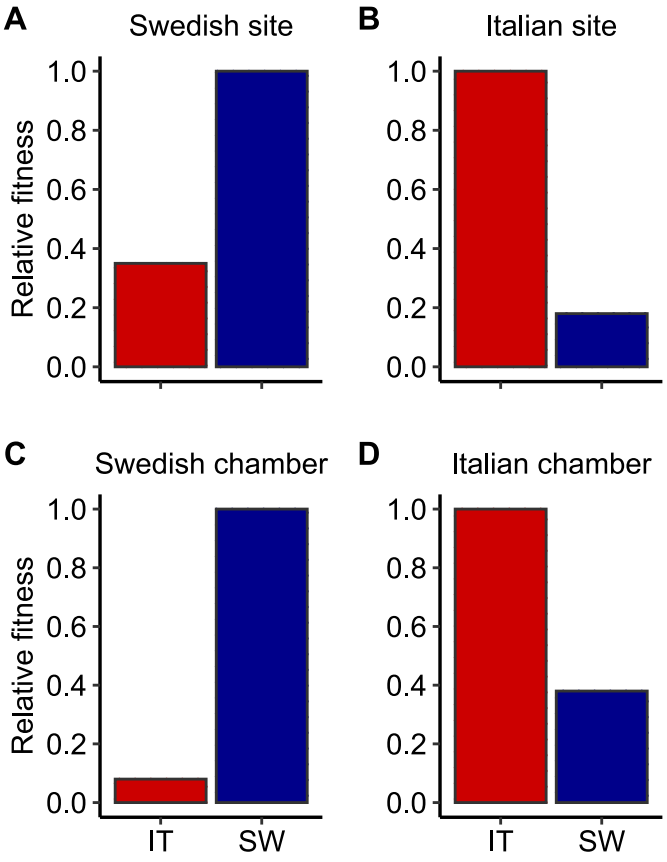

Figure S4. Comparison of relative fitness of ecotypes between the field (A & B) and growth chamber (C & D) experiments.

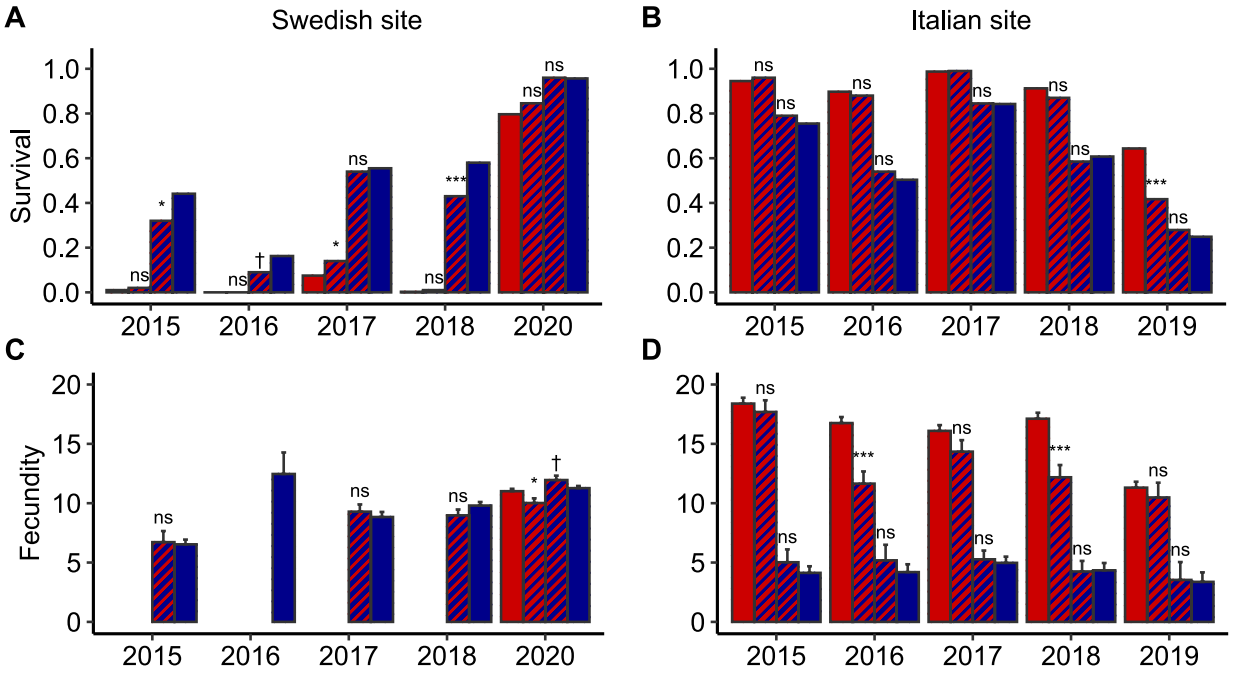

Figure S5. Fitness components of the SW and IT ecotypes and NILs separately for each of the five individual one-year experiments at the Swedish and Italian field sites. Mean proportion survival (averaged over years) at the Swedish (A) and Italian (B) field sites. LSM fecundity at the same sites (C and D). Error bars for fecundity are 1 SE, no bars are given for survival because it was analyzed with a binomial error distribution. At the Swedish site, lines with 30 or fewer surviving individuals were omitted from analyses of fecundity, and these are represented by missing bars in C. Asterisks represent statistically significant contrasts between a NIL and its genetic background (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; †, $0.05 < P < 0.1$; ns, not significant).

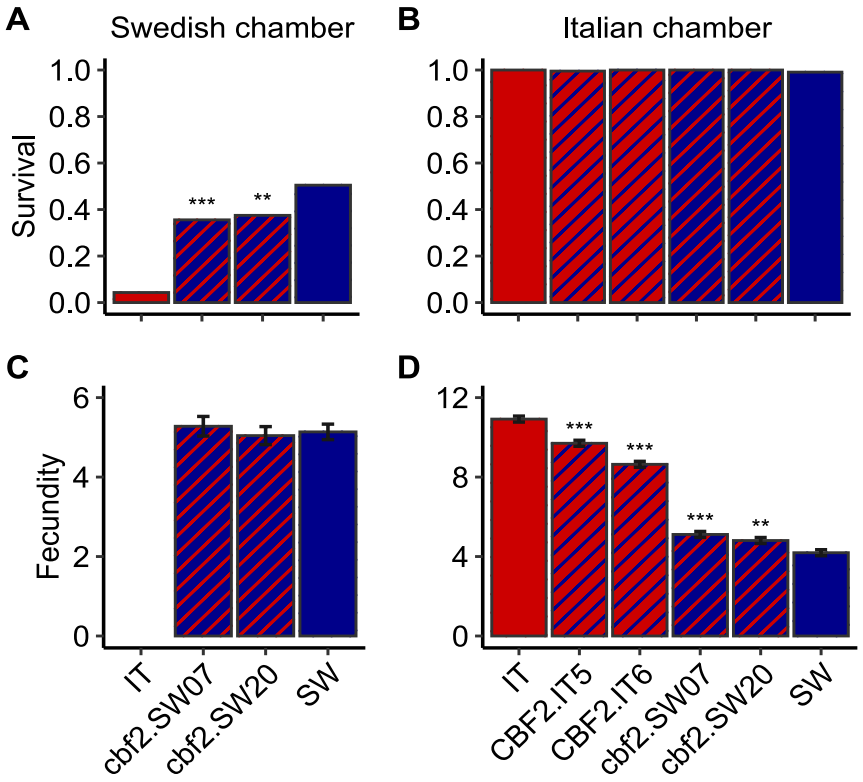

Figure S6. Fitness components of the SW and IT ecotypes and engineered lines in the Swedish and Italian chambers. Proportion survival (averaged over flats) in the Swedish (A) and Italian (B) chambers. LSM fecundity in the same chambers (C and D). Error bars for fecundity are 1 SE, no bars are given for survival because it was analyzed with a binomial error distribution. The Italian ecotype had fewer than 30 surviving individuals and it was excluded from fecundity analyses (missing bar in C). Asterisks represent statistically significant contrasts between an engineered line and its genetic background (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; †, $0.05 < P < 0.1$; ns, not significant). No contrasts were performed for C because there was no significant main effect of genotype.

Table S1. ANOVA results for estimates of fitness and fitness components for the two field sites over 5 individual year experiments. For each dependent variable, the effects of genotype (2 ecotypes and 2 NILs), year, and the interaction between genotype and year were tested. Linear contrasts are also given between the IT and SW ecotypes over years and for each individual year. Because of near complete mortality in Sweden in 2019, results are presented for this year in Italy only. Data from an additional year (2020) were collected in Sweden to provide 5 years of data at each site. For fitness and fecundity, numerator degrees of freedom, F ratios, and P values are given as well as the t statistic and P values for the contrasts. For survival (binomially distributed), DF and χ^2 test statistics are given. The genotype by year interaction could not be included in the model for fecundity at the Swedish site because of high mortality of some lines in some years. In this case, contrasts between IT and SW are given for 2020 from a model run separately by year.

		Swedish field site			**Italian field site**		
Fitness							
	Effect	Num. DF	F	P	Num. DF	F	P
	Genotype	3	219.7	**<0.001**	3	736.8	**<0.001**
	Year	4	538.8	**<0.001**	4	81.6	**<0.001**
	Genotype*Year	12	11.2	**<0.001**	12	14.8	**<0.001**
	IT vs. SW contrasts		t	P		t	P
	Overall		23.7	**<0.001**		43.6	**<0.001**
	2015		8.5	**<0.001**		23.0	**<0.001**
	2016		6.1	**<0.001**		21.1	**<0.001**
	2017		13.6	**<0.001**		18.9	**<0.001**
	2018		16.9	**<0.001**		21.0	**<0.001**
	2019		-	-		12.5	**<0.001**
	2020		7.4	**<0.001**		-	-
Survival							
	Effect	DF	χ^2	P	DF	χ^2	P
	Genotype	3	1004.4	**<0.001**	3	554.3	**<0.001**
	Year	4	1957.3	**<0.001**	4	761.7	**<0.001**
	Genotype*Year	12	99.7	**<0.001**	12	28.1	**0.005**
	IT vs. SW contrasts		χ^2	P		χ^2	P
	Overall		126.8	**<0.001**		461.7	**<0.001**
	2015		76.3	**<0.001**		47.7	**<0.001**
	2016		12.6	**<0.001**		126.2	**<0.001**
	2017		161.6	**<0.001**		33.3	**<0.001**
	2018		51.2	**<0.001**		86.7	**<0.001**
	2019		-	-		188.5	**<0.001**
	2020		75.4	**<0.001**		-	-
Fecundity							
	Effect	Num. DF	F	P	Num. DF	F	P
	Genotype	3	3.3	**0.021**	3	410.4	**<0.001**
	Year	3	59.5	**<0.001**	4	9.8	**<0.001**
	Genotype*Year	-	-	-	12	4.3	**<0.001**
	IT vs. SW contrasts		t	P		t	P
	Overall		1.2	0.237		32.3	**<0.001**
	2015		-	-		19.2	**<0.001**
	2016		-	-		15.0	**<0.001**
	2017		-	-		15.6	**<0.001**
	2018		-	-		60.0	**<0.001**
	2019		-	-		8.4	**<0.001**
	2020		0.9	0.349		-	-

Table S2. ANOVA results for estimates of fitness and fitness components for the growth chamber experiments simulating the Swedish and Italian environments. For each dependent variable the effects of genotype (2 ecotypes and 2 CRISPR lines in the Swedish chamber, and an additional 2 transgenic lines in the Italian chamber) was tested. We also include a term for flat to account for spatial variation within the chamber. Linear contrasts are also given between the IT and SW ecotypes. For fitness and fecundity, numerator degrees of freedom, F ratios, and P values are given as well as the t statistic and P values for the contrasts. For survival (binomially distributed), DF and χ^2 test statistics are given. In cases where there was not a significant effect of genotype (survival in the Italian chamber, fecundity in the Swedish chamber), no contrasts were performed.

		Swedish chamber			**Italian chamber**		
Fitness							
	Effect	Num. DF	F	P	Num. DF	F	P
	Genotype	3	45.3	**<0.001**	5	356.2	**<0.001**
	Flat	7	37.0	**<0.001**	7	25.8	**<0.001**
	IT vs. SW contrast		t	P		t	P
			11.0	**<0.001**		31.5	**<0.001**
Survival							
	Effect	DF	χ^2	P	DF	χ^2	P
	Genotype	3	161.2	**<0.001**	5	1.4	0.923
	Flat	7	166.7	**<0.001**	7	1.4	0.987
	IT vs. SW contrast		χ^2	P		χ^2	P
			151.4	<0.001		-	-
Fecundity							
	Effect	Num. DF	F	P	Num. DF	F	P
	Genotype	2	0.3	0.756	5	358.9	**<0.001**
	Flat	7	5.4	**<0.001**	7	25.4	**<0.001**
	IT vs. SW contrast		t	P		t	P
			-	-		31.4	**<0.001**

Appendix S1. Construction and optimization of growth chamber programs to simulate the Swedish and Italian environments.

Chamber programs were initially created based on daily mean high and low temperature profiles at the two sites (Fig. S2) and then optimized to satisfy logistical constraints and to recreate ecotypic differences in relative fitness observed at the native field sites. We shortened the overall length of the programs to minimize strain on growth chambers running low temperatures for extended periods. For the Swedish chamber program (Fig. S3A), we shortened the total freezing period and added two cold spikes representative of temperatures in a cold year. For the Italian chamber program (Fig. S3B), this shortening was applied approximately evenly across the program. However, we ensured at least eight weeks of daily high temperatures below 10°C to satisfy the vernalization requirement of the SW ecotype as would occur at the Italian field site. Selection against the foreign ecotype in Sweden has been shown to be positively correlated with winter minimum temperature (1, 2). Photoperiod in the chamber programs was set to reflect seasonal changes at the two sites as described in (3). It is not practical to mimic fluctuation in light intensities observed in the field, so we used a maximum value of photosynthetically active radiation (PAR: $\mu mol\ m^{-2}\ s^{-1}$) of 300 during high temperatures of 19°C or greater, and reduced PAR by approximately 50 units for every reduction in high temperature of ~3.5°C, similar our previous observations and protocols (3). During freezing periods in the Swedish chamber, PAR was reduced to zero to minimize circadian effects on freezing tolerance (4, 5) and because plants are typically covered in snow during these temperatures. Finally, we applied a sequential dry down by gradually reducing the amount of water given at the end of each chamber program to mimic the decreasing soil water potentials observed in the field (6).

Growth chamber programs (Fig. S3) were then fine-tuned to reproduce, as closely as possible, ecotypic differences in relative fitness in field experiments, and ensure that absolute fitness values of the parental ecotypes were within the range observed in the field across all years (1, 7). Optimization was done by qualitatively assigning greater weight to temperature profiles of individual years with stronger selection against the foreign ecotype.

References for Appendix S1

1. Ågren J & Schemske DW (2012) Reciprocal transplants demonstrate strong adaptive differentiation of the model organism *Arabidopsis thaliana* in its native range. *New Phytologist* 194:1112-1122.
2. Oakley CG, Ågren J, Atchison RA, & Schemske DW (2014) QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs. *Molecular Ecology* 23:4304-4315.
3. Dittmar EL, Oakley CG, Ågren J, & Schemske DW (2014) Flowering time QTL in natural populations of *Arabidopsis thaliana* and implications for their adaptive value. *Molecular Ecology* 23:4291-4303.
4. Gehan MA, Greenham K, Mockler TC, & McClung CR (2015) Transcriptional networks — crops, clocks, and abiotic stress. *Current Opinion in Plant Biology* 24:39-46.
5. Dong MA, Farre EM, & Thomashow MF (2011) CIRCADIAN CLOCK-ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL regulate expression of the C-REPEAT BINDING FACTOR (CBF) pathway in *Arabidopsis*. *PNAS* 108:7241-7246.
6. Zacchello G, Vinyeta M, & Ågren J (2020) Strong stabilizing selection on timing of germination in a Mediterranean population of *Arabidopsis thaliana*. *American Journal of Botany*.
7. Oakley CG, Schemske DW, McKay JK, & Ågren J (2023) Ecological genetics of local adaptation in Arabidopsis: An 8-year field experiment. *Molecular Ecology*.