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QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs

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Abstract

Local adaptation, defined as higher fitness of local vs. nonlocal genotypes, is commonly identified in reciprocal transplant experiments. Reciprocally adapted populations display fitness trade-offs across environments, but little is known about the traits and genes underlying fitness trade-offs in reciprocally adapted populations. We investigated the genetic basis and adaptive significance of freezing tolerance using locally adapted populations of *Arabidopsis thaliana* from Italy and Sweden. Previous reciprocal transplant studies of these populations indicated that subfreezing temperature is a major selective agent in Sweden. We used quantitative trait locus (QTL) mapping to identify the contribution of freezing tolerance to previously demonstrated local adaptation and genetic trade-offs. First, we compared the genomic locations of freezing tolerance QTL to those for previously published QTL for survival in Sweden, and overall fitness in the field. Then, we estimated the contributions to survival and fitness across both field sites of genotypes at locally adaptive freezing tolerance QTL. In growth chamber studies, we found seven QTL for freezing tolerance, and the Swedish genotype increased freezing tolerance for five of these QTL. Three of these colocalized with locally adaptive survival QTL in Sweden and with trade-off QTL for overall fitness. Two freezing tolerance QTL contribute to genetic trade-offs across environments for both survival and overall fitness. A major regulator of freezing tolerance, *CBF2*, is implicated as a candidate gene for one of the trade-off freezing tolerance QTL. Our study provides some of the first evidence of a trait and gene that mediate a fitness trade-off in nature.

Keywords: abiotic stress, adaptation, antagonistic pleiotropy, cold acclimation, cost of resistance, C-repeat-binding factor

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Introduction

Understanding the genetic basis of adaptation is a long-standing and central goal of evolutionary biology. Major questions include how many mutations are required for adaptation, and what is the distribution of their effect sizes (e.g. Fisher 1930; Orr 1998, 2005) Recent work has focused on whether or not adaptive alleles in one environment are costly in a different environment (reviewed in Anderson *et al.* 2011). Studies of populations that are adapted to their local environments are particularly amenable to addressing the genetics of adaptation because they provide a tractable definition of adaptation, that is higher fitness of local compared with foreign genotypes, and the variation necessary to study it. Local adaptation is commonly identified in reciprocal transplant studies (Clausen *et al.* 1940; Kawecki & Ebert 2004; Leimu & Fischer 2008; Hereford 2009), and while reciprocal adaptation in all studied populations is not universal, populations that are reciprocally adapted provide evidence for fitness trade-offs across environments (Hereford 2009). Fitness trade-offs can be caused by genetic trade-offs (antagonistic

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pleiotropy), where an allele that increases fitness in one environment reduces fitness in another (reviewed in Anderson *et al.* 2011). Genetically based trade-offs provide an intuitive explanation for biological diversification (Futuyma & Moreno 1988), otherwise widely distributed multipurpose genotypes would be expected. Alternatively, unique loci may be responsible for adaptation to different environments, with alternate alleles being selectively neutral in the contrasting environment, termed conditional neutrality (reviewed in Anderson *et al.* 2011). This scenario is most likely with restricted gene flow limiting the spread of locally favoured alleles.

In studies of the genetics of adaptation using reciprocally adapted populations (Lowry *et al.* 2009; Hall *et al.* 2010; Anderson *et al.* 2013; Leinonen *et al.* 2013), conditional neutrality is found more often than genetic trade-offs (but see Ågren *et al.* 2013). Studies commonly cited as evidence for conditional neutrality include those where populations are not reciprocally adapted, that is at least one population is not locally adapted (Verhoeven *et al.* 2004; Gardner & Latta 2006). It is unclear whether genetic trade-offs would be expected in such cases. Other studies cited as evidence for conditional neutrality involve transplant experiments conducted in sites that are not the ancestral locations (Weinig *et al.* 2003; Fournier-Level *et al.* 2011). While these studies can identify geographic variation in selection for different genomic regions, they are not designed to estimate the relative contributions of conditional neutrality and genetic trade-offs in locally adapted, native populations.

We suggest that understanding the importance of fitness trade-offs in local adaptation requires research on population pairs that are reciprocally adapted, that is where both local genotypes outperform nonlocal genotypes. This provides the opportunity to estimate the number and effect sizes of genomic regions that underlie local adaptation (i.e. fitness) and identify the traits and genes that mediate such adaptation. This is not an easy task. Mapping the genetic basis of fitness in ancestral habitats is seldom attempted, and large-scale experiments replicated over several years are probably required to detect trade-offs in the face of spatial and temporal variation in selection pressures (Anderson *et al.* 2011; Ågren *et al.* 2013). In the last few decades, great strides have been made in understanding the genetic basis of quantitative traits (reviewed in Mackay *et al.* 2009; Alonso-Blanco & Méndez-Vigo 2014), but the adaptive significance of these traits is often unknown, or assumed rather than demonstrated. Identifying the traits involved in local adaptation provides insight into the life history stages that experience selection and can help identify the mechanisms of potential trade-offs (e.g. biochemical and physiological costs, or a mismatch between phenology and environment) and may focus the search for candidate genes.

Our recent work on the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) combines modern molecular genetic tools with classical ecological genetic techniques (Ågren & Schemske 2012; Ågren *et al.* 2013). Reciprocal transplant experiments conducted in the ancestral habitats combined with knowledge of the likely major selective agents at the two sites provide a unique opportunity to investigate the genetic basis of traits that confer local adaptation and fitness trade-offs. The project aims to identify the traits that underlie local adaptation, determine the location, number and effect size of QTL for those traits and ultimately uncover the genes underlying those QTL.

Two populations from close to the northern (Sweden) and southern (Italy) edges of the native range (Koornneef *et al.* 2004; Beck *et al.* 2008) were investigated, and five years of reciprocal transplants clearly demonstrate overall local adaptation (Ågren & Schemske 2012). While the relative fitness of the Swedish genotype when grown in Italy was consistently low (mean ~ 15% of that of the Italian genotype, range = 5–32%), there was substantial variation in the relative fitness of the Italian genotype when grown in Sweden (mean ~ 67%, range = 28–121%). The greater temporal variation in relative fitness of the Italian genotype in Sweden, was largely due to year-to-year variation in relative survival, which was strongly positively associated ($R^2 = 0.83$, $n = 6$ years) with minimum winter soil temperature at the Swedish site (updated from Ågren & Schemske 2012). This strongly suggests that survival via freezing tolerance is a major mechanism for adaptation at the Swedish field site.

Mapping of fitness and fitness components (survival and number of fruits produced by survivors) conducted on a large number of recombinant inbred lines (RILs, n = 400) at both sites over three years identified 15 QTL for overall fitness (Ågren *et al.* 2013). Ten of these QTL were shared across environments, and six of the shared QTL yielded evidence of trade-offs, that is local genotypes were favoured at their home sites. For survival in Sweden, five QTL were detected, and in all cases, the Swedish genotype increased survival. Three of the five Swedish survival QTL colocalized with genomic regions underlying fitness trade-offs, implicating freezing tolerance as a primary candidate trait underlying genetic trade-offs in this system. A crucial next step in linking fitness trade-offs to freezing tolerance and ultimately to the genes underlying fitness trade-offs is to identify the location, number and effect size of genomic regions influencing freezing tolerance.

Molecular genetic studies of *Arabidopsis* have led to a detailed understanding of the gene regulation pathways

involved in freezing tolerance (reviewed in Thomashow 1999, 2010; Preston & Sandve 2013). Three genes (*CBF1*, *CBF2* and *CBF3*) encode major transcription factors that regulate acclimation of freezing tolerance via their effects on many downstream cold-responsive genes (Thomashow 2010). Evidence from transgenic lines suggests that induction of cold tolerance might come at a physiological cost. Expression of *CBF* genes is typically concomitant with down-regulation of photosynthesis (Preston & Sandve 2013) and constitutively expressed transgenes for *CBF2* and *CBF3* have lower fitness than null vectors under warm greenhouse conditions (Jackson *et al.* 2004). Genetic mapping studies often find a freezing tolerance QTL near the *CBF* genes (Alonso-Blanco *et al.* 2005; Kang *et al.* 2013; but see Gery *et al.* 2011; Meissner *et al.* 2013), but the role of these QTL in local adaptation is unknown.

Freezing tolerance varies considerably among *Arabidopsis* accessions and is negatively correlated with latitude (see for e.g. Zhen & Ungerer 2008a; Zuther *et al.* 2012; and references therein). This geographic pattern has been interpreted as 'relaxed selection' for freezing tolerance at southern latitudes (Zhen & Ungerer 2008b); alternatively, this could represent the costs associated with freezing tolerance in environments with mild winters (but see Zhen *et al.* 2011). Indeed, the repeated finding of independent loss of function mutations in *CBF* genes from southern latitudes (Alonso-Blanco *et al.* 2005; Kang *et al.* 2013) raises the intriguing possibility of selection for reduced freezing tolerance at lower latitudes.

Here, we map the genetic basis of freezing tolerance in an established system of reciprocally adapted populations of *Arabidopsis*, for which there is strong evidence that freezing tolerance is an adaptive trait (Ågren & Schemske 2012). We address the following questions: (i) How many QTL determine freezing tolerance (in growth chamber experiments) and what are their effect sizes? (ii) Do freezing tolerance QTL colocalize with survival and fitness QTL observed in field studies conducted on the same mapping population (Ågren *et al.* 2013)? For locally adaptive freezing tolerance QTL (where the Swedish genotype increased freezing tolerance, survival, and overall fitness in Sweden), we ask (iii) What is the potential contribution of freezing tolerance QTL to previously reported fitness trade-offs? and (iv) What are the candidate genes underlying adaptive freezing tolerance QTL?

Materials and methods

Study system and sites

Arabidopsis thaliana is a small, selfing herb and exhibits a winter-annual life history at both study sites. Seeds germinate in October and November in Italy, and in August and September in Sweden, overwinter as rosettes, flower and fruit from March to April in Italy, and from May to June in Sweden. Based on hourly temperature recordings made at each site (see Ågren & Schemske 2012) over a period of 8 years, winter soil and air temperatures differ dramatically between the sites (Table S1, Supporting information). Most notably, minimum soil temperatures lower than −6 C have occurred on up to 11 days in a single year in Sweden, and on average, soil temperatures are below freezing 132 days of the year. In Italy, by contrast, soil temperatures below freezing (−0.1 C) have only been recorded for a single day in 8 years (Table S1, Supporting information).

Freezing tolerance assay

We identified freezing tolerance QTL using a RIL mapping population derived from a cross between two lines from locally adapted parental populations from Italy and Sweden (Ågren & Schemske 2012; Ågren *et al.* 2013). The RILs used in the present study included 404 lines previously used to map fitness in the field (Ågren *et al.* 2013), plus 96 additional RILs randomly selected among the remaining 140 available. Due to chamber space limitations, we tested these 500 RILs in six batches. Each chamber (batch) could accommodate 120 petri dishes (100 × 15 mm) and specialized 4-celled petri dishes with plastic dividers (VWR Cat# 25384-308) were used, for a total of 480 cells per batch. Each batch contained 40 cells of each of two parental lines, and the maximum number of RILs that could be accommodated in a batch was therefore 400. We tested a total of four replicate cells for each RIL as follows: batches 1–4, 400 RILs with one replicate cell per RIL; and batches 5–6, 100 RILs (unique from the 400 RILs tested in batches 1–4) with two replicate cells per RIL. In each batch, RILs were fully randomized. Petri dishes were divided evenly among aluminium trays to facilitate randomization during the experiment.

Freezing tolerance assay conditions

Freezing tolerance was investigated by exposing seedlings germinated in petri dishes to subfreezing temperatures. We sowed sterilized seeds (12 per cell) onto autoclaved, no-sucrose agar prepared using Gambog's B-5 Basal Salts (Caisson Laboratories Inc.). To promote uniform germination, seeds were stratified in darkness at 4 C for 5 days. Dishes were then moved to a growth chamber for 8 days at 22 C with a 16-hour photoperiod of ~125 μmol photons m^{-2} s^{-1}. They were then acclimated for 10 days at 4 C with a 10-hour photoperiod of

~50 μmol photons m^{-2} s^{-1}. For the freezing treatment, we first dropped the chamber temperature to −2 C for 1 day, adding shaved ice to each cell to facilitate ice nucleation. We then dropped the chamber temperature to −7 C for 8 days in complete darkness. Freezing tolerance was measured at the seedling stage because plants experience freezing early in their life history in Sweden. In growth chamber experiments, older plants exhibit similar differences in freezing tolerance between parental lines (C. Garraud & D. W. Schemske, unpublished data). Temperatures were chosen to be within the range of winter mean air and soil temperatures in Sweden (Table S1, Supporting information), and plants were kept in the dark during freezing following the protocols of previous studies (Alonso-Blanco *et al.* 2005; Zhen & Ungerer 2008a; Kang *et al.* 2013). Trays were rotated twice daily, and supplemental fans were used to minimize spatial variation in temperature within a chamber. After freezing, the seedlings were thawed for 1 day at 4 C, followed by 2 days at 22 C. After thawing, we recorded mean freezing tolerance per cell as the mean per cent survival of each cell, scoring individual seedlings with white apical meristems as dead. Preliminary experiments determined that seedlings that failed to develop past the cotyledon stage prior to freezing had low freezing tolerance irrespective of genotype (plants failing to develop true leaves are unlikely to survive until winter in Sweden). Because of the large number of seedlings grown for these studies (22 726), it was not practical to record seedling size for individual seedlings for use as a covariate. Instead, we omitted seedlings without true leaves from the analysis (on average ~30% of the seedlings, leaving an average of 8.4 seedlings per cell scored for freezing tolerance). Mean freezing tolerance per batch was calculated for each parent and RIL, and overall mean freezing tolerance was taken as the mean of the batch means.

A chamber failure at low temperature during batch 4 resulted in mean freezing tolerance of the parents that was significantly different from that of the other batches, so, we omitted this batch and analysed the data from five total batches (1–3, and 5–6). An analysis of these five batches indicated no significant effect of batch ($F_{4,1970} = 1.50$, $P = 0.20$) nor a significant interaction between 'population' (Italian parental line, Swedish parental line and RI lines) and batch ($F_{8,1970} = 0.62$, $P = 0.77$). This suggests that the chamber environments were very similar across batches and that combining batches with different sets of RILs (batches 1–3 and batches 5&6) is unlikely to affect the overall QTL results. In contrast, the effect of 'population' was highly significant ($F_{2,1970} = 296.79$, $P < 0.0001$). In total, we scored freezing tolerance (survival through freezing) on nearly 16 000 individual plants consisting of more than 1900 individuals of each parent and more than 12 000 total individual RIL plants.

QTL mapping

We mapped QTL using R/qtl (Broman & Sen 2009), following the procedure employed previously for this population (Ågren *et al.* 2013). In brief, we quantile-normalized the data (Broman & Sen 2009) and determined the best multiple QTL model using Haley–Knott regression. We ran 10 000 permutations to determine LOD thresholds (experimentwise $\alpha = 0.05$) and employed automated stepwise model selection, scanning for additive and epistatic QTL at each step (Manichaikul *et al.* 2009). For each QTL, we calculated Bayesian 95% credible intervals in R/qtl (Broman & Sen 2009) as a measure of uncertainty around QTL locations. We also inspected single QTL results and pairwise marker interactions to confirm that the automated model selection did not identify spurious QTL. We used ANOVA to calculate the per cent variance explained for each QTL and then fit this model with the nonnormalized data to generate genotypic effect sizes in units of freezing tolerance.

Mean freezing tolerance of the RILs was distinctly nonnormal; 80 of the 500 RILs had zero freezing tolerance (Fig. 1). Because the stepwise model selection procedure is sensitive to departures from normality and because quantile normalization does not resolve the problem of excess zeros, we performed additional analyses to confirm the robustness of our results to

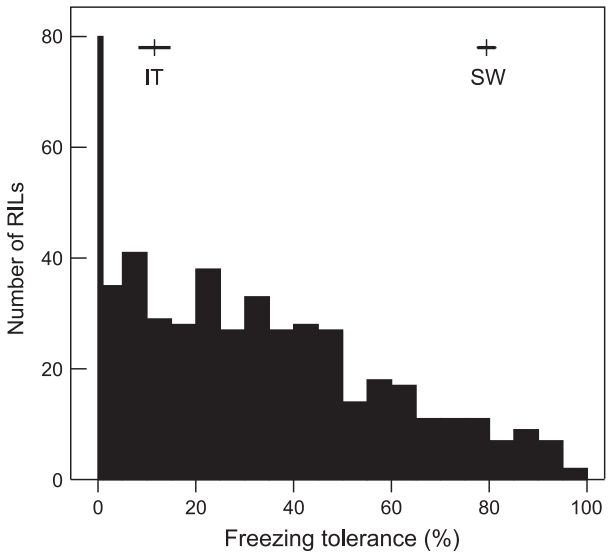


Fig. 1 Histogram of RIL mean freezing tolerance. Bin sizes are 5% except for a narrow bin to show the number of RILs with 0 freezing tolerance. Parental means (IT for Italy and SW for Sweden) are shown as vertical ticks above the histogram with horizontal error bars of 2 SEM.

violations of distributional assumptions. In brief, we ran two separate analyses as above, but with modifications. First, we scored RIL mean freezing tolerance as a binary trait (RIL mean = 0, versus RIL mean > 0) and performed a QTL analysis on untransformed data using a binary model (Broman & Sen 2009). Second, we performed QTL analysis on quantile-normalized data using all RILs with nonzero mean freezing tolerance. The validity of the standard analysis (Fig. S1c, Supporting information) described above is clearly seen by noting that it is more or less a composite picture of the LOD profile plots of the binary (Fig. S1a, Supporting information) and zero-excluded models (Fig. S1b, Supporting information). Further, manual construction of a QTL model containing all unique positions from the models of the two separate distributions, followed by manual stepwise elimination of nonsignificant QTL based on model likelihood ratio tests, yields exactly the same model (not shown) identified by the standard procedure. Thus, the standard analysis QTL results are robust to violations of distributional assumptions for this data set.

Colocalization of QTL for freezing tolerance and fitness

After identifying QTL for freezing tolerance in the growth chamber, we asked whether these QTL correspond to locally adaptive QTL for survival in Sweden, and/or overall fitness in the field and to what extent they might contribute to fitness trade-offs across the two parental environments. We only consider survival QTL in Sweden, because these provide the most direct link between freezing tolerance and fitness. We have no a priori expectation of where in the life cycle the costs (for a trade-off) in Italy, might be expressed. To address these questions, we used previously published QTL results and data for survival and cumulative fitness in both Italy and Sweden, for 3 years (Ågren *et al.* 2013). We examined colocalization of freezing tolerance QTL with fitness QTL by determining whether the point estimate of freezing tolerance QTL fell within the range of point estimates of fitness QTL observed in the field at least twice. Because fitness QTL were available for six site by year combinations, the range of point estimates across combinations provides a more conservative estimate of the location of fitness QTL than the range of credible interval(s). Because wide credible intervals around QTL positions (i.e. statistical uncertainty) have the undesirable property of making colocalization (overlap) more likely, we imposed an arbitrary maximum length of credible interval of 1/4 the length of the shortest chromosome (<15.2 cM) for examining colocalization between freezing tolerance QTL and survival QTL from Sweden. Finally, we used genotypes at the SNP markers closest to the point estimates of our locally adaptive freezing tolerance QTL as independent variables in ANOVA on survival and cumulative fitness in the field at both sites for 3 years (390 RILs × 2 sites × 3 years, Ågren *et al.* 2013). These analyses included all interactions of single QTL with site, year and site by year. In particular, we were interested in the QTL by site interactions as these are necessary (but not sufficient) for identifying genetic trade-offs. Least square mean survival and cumulative fitness, respectively, for alternative genotypes at each marker were calculated from the ANOVA model and used to quantify the potential fitness benefit of the Swedish genotype at the Swedish site, and conversely, the fitness cost of the Swedish genotype at the Italian site.

Candidate genes underlying freezing tolerance QTL

Likely candidate genes within the 95% credible intervals of our adaptive freezing tolerance QTL were identified using data sets of Gene Ontology (GO) annotations and locations (the GOSLIM, and the version 9 GFF file, respectively) downloaded from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). First, we filtered the GOSLIM file for genes containing 'cold' in the GO terms. Second, this list was filtered to include only annotations based on experimental evidence that the gene was related to response to cold (direct assay, mutant phenotypes, expression patterns, or genetic or physical interactions). Finally, using the TAIR version 9 GFF file, we filtered this list of genes to include only those in which the start position occurred within 300 Kb (~1 cM, the average distance between markers) beyond either end of the 95% credible intervals of our freezing tolerance QTL.

Results

Freezing assay

Mean freezing tolerance of the Swedish parental line (79.5%, range = 76.7–80.9) was much greater than that of the Italian parental line (11.6%, range = 6.4–14.5; Fig. 1). These values closely resemble mean per cent survival of the two parental genotypes at the Swedish field site in the coldest years (e.g. 66% vs. 22% in 2005/2006; Ågren & Schemske 2012), suggesting that our growth chamber conditions reasonably replicate differences observed in the field.

The distribution of mean freezing tolerance for the RILs was largely intermediate to that of the parental means (mean = 30.6%, median = 26.0%, Fig. 1), with the exception of the large number of RILs with freezing tolerance below that of the Italian parental mean.

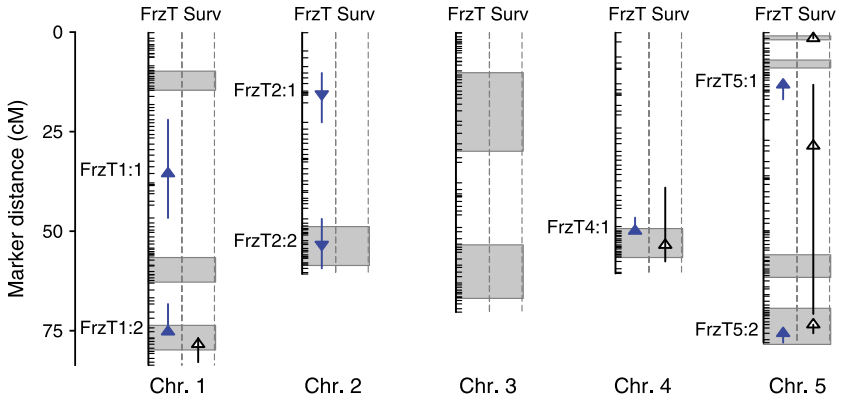


Fig. 2 QTL locations (triangles) for freezing tolerance (blue) and survival in Sweden (black). Vertical lines are Bayesian 95% credible intervals. Upward facing arrows represent QTL where the Swedish genotype increased freezing tolerance or survival, respectively. Boxes are the range of fitness QTL locations observed in more than 1 site/year. Survival and fitness data from Ågren *et al*. (2013).

Approximately 32 times as many seedlings were scored for the parental lines as for individual RIL lines, so, RIL lines with lower mean freezing tolerance than the Italian parent may in part be due to random sampling error around the true RIL mean values.

The full stepwise multiple QTL model identified a total of 7 freezing tolerance QTL (Figs. 2 & Fig. S1, Supporting information), yielded a total LOD (Logarithm of the odds) score of 79.6 and explained 51.9% of the variation in RIL mean freezing tolerance. No epistatic interactions met the model selection significance threshold, although some subtle epistatic effects between different markers on chromosome 1 are evident (Fig. S2, Supporting information). The Swedish genotype increased freezing tolerance for 5 of the 7 QTL, (Fig. 2, Table 1). These five QTL had an average proportion of variance explained (PVE) of 7.4% (range = 3.6–17.7) and explained an average increase in freezing tolerance of 14.1% (range = 10.0–24.5). Relating these genotypic effects to the difference in freezing tolerance between the parents, these QTL can on average account for 21.0% of the difference (range = 14.7–36.1). FrzT4:1 had a distinctly large effect, explaining 17.7% (PVE) of the variation in RIL freezing tolerance and having a genotypic effect of 24.5% freezing tolerance (Table 1), corresponding to 36.1% of the difference between the parental lines. The distributions of RIL mean freezing tolerance for alternate genotypes at FrzT4:1 were strikingly different; the Italian genotype accounted for 76 of the 80 RILs with 0 freezing tolerance and had a median freezing tolerance of 14.7% compared with 47.8% for the Swedish genotype. One QTL (FrzT1:1) had a wide 95% credible interval (24.8 cM) around the estimated location. The 95% credible intervals of the remaining four QTL where the Swedish genotype increased freezing tolerance were on average 4.8 cM (range = 3.7–7.0). The two QTL where the Swedish genotype decreased freezing tolerance

Table 1 QTL locations and effect sizes for freezing tolerance measured in the growth chamber and for survival measured in the field in Sweden (from Ågren *et al*. 2013)

Experiment	QTL	Chr.	Pos.	LOD	PVE	SW2a (SE)	95% CI
Chamber	FrzT1:1	1	35.5	7.7	3.6	10.00 (1.67)	21.9–46.7
Chamber	FrzT1:2	1	75.4	14.4	6.8	13.35 (1.66)	68.4–75.4
Chamber	FrzT2:1	2	15.5	4.1	1.9	−7.39 (1.66)	10.2–22.6
Chamber	FrzT2:2	2	53.4	4.0	1.8	−6.14 (1.67)	47.0–59.4
Chamber	FrzT4:1	4	50.0	34.0	17.7	24.54 (1.80)	46.7–50.2
Chamber	FrzT5:1	5	13.1	8.8	4.0	11.18 (1.75)	12.0–16.8
Chamber	FrzT5:2	5	75.8	10.2	4.7	11.53 (1.64)	74.5–78.2
Field survival		1	78.6	5.8	6.5	3.14 (0.59)	77.0–83.1
Field survival		4	53.5	5.9	4.6	7.23 (1.36)	39.1–57.7
Field survival		5	1.4	8.0	6.4	8.63 (1.39)	0–1.4
Field survival		5	28.5	2.7	2.1	4.90 (1.36)	13.1–72.9
Field survival		5	73.6	16.3	13.6	11.89 (1.31)	73.6–75.8

For all QTL, the chromosome (Chr.) and position in cM (Pos.) is given, as are the lower and upper limits of the Bayesian 95% credible intervals. For each QTL, the LOD score, proportion variance explained (PVE), and the effect (per cent freezing tolerance or survival respectively) of a substitution of the Swedish genotype (SW2a) and standard error are given. A positive value for SW2a indicates that the Swedish genotype increases freezing tolerance or field survival, respectively.

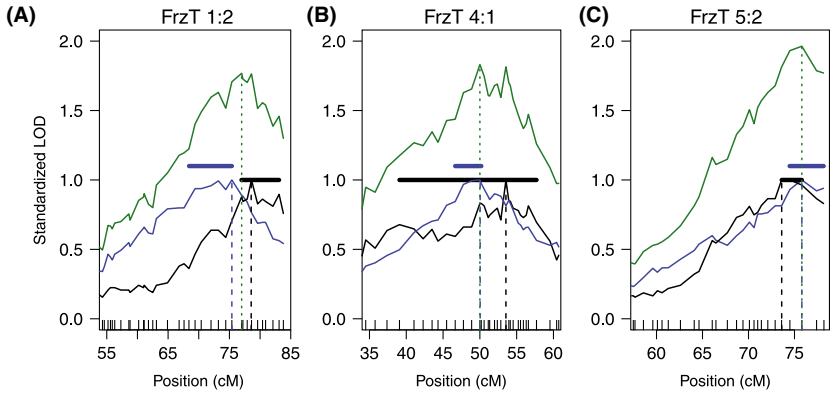
were the smallest effect QTL detected (Table 1), both having LODs around 4, PVE below 2, and average decrease in freezing tolerance of around 7%. Adding together the positive and negative effects, the 7 total QTL can explain about 84% of the difference in freezing tolerance between the parental lines.

Colocalization with field survival and fitness

Three of the seven freezing tolerance QTL were found within the range of fitness QTL estimates and near a locally adaptive QTL for survival in Sweden (Fig. 2, Table 1). In one of these cases (FrzT5:2), the freezing tolerance and survival QTL credible intervals were both extremely tight and overlapped each other (Figs. 2 & 3c, Table 1), providing strong evidence of colocalization. The other two cases do not meet the most stringent criteria for colocalization. For FrzT4:1, the point estimates of the freezing tolerance and field survival QTL are separated by 3.5 cM, and their credible intervals overlap, but the credible interval for the survival QTL is somewhat larger (18.6 cM) than our arbitrary 15.2 cM cut-off (Figs. 2 & 3b, Table 1). For FrzT1:2, the point estimates of the freezing tolerance and survival QTL are separated by 3.15 cM, and while the credible intervals do not overlap, the nonoverlapping region is just a 1.79 cM gap between two markers (Figs. 2 & 3a). FrzT2:2 overlapped with fitness (but not survival) QTL and should not contribute to fitness trade-offs because the Swedish genotype decreased freezing tolerance.

Although the Bayesian credible interval may be the best measure of QTL location available, it is not a true credible interval (Broman & Sen 2009). Credible intervals for QTL position are unreliable in the presence of multiple QTL on a chromosome (K. Broman, *pers. comm.*). Additionally, comparing credible intervals from different studies (e.g. freezing tolerance in the growth chamber vs. survival in the field) may be complicated by different numbers of lines, replicates per line and/or amounts of environmental heterogeneity. It is thus worthwhile to pursue alternative approaches for evaluating QTL colocalization.

To this end, we used two approaches to evaluate colocalization. First, we asked how likely it was, given the size of our linkage map, marker density and average width of fitness 'boxes' (Fig. 2), that for seven freezing tolerance QTL and five survival QTL, we would observe three fitness boxes containing both a freezing tolerance QTL and a QTL for survival in Sweden (for details see Table S2, Supporting information). We estimate that observing three colocalizations is extremely unlikely due to chance alone ($P = 0.0033$, Table S2, Supporting information). Second, for each of these three cases, we employed a likelihood ratio approach, comparing the combined LOD scores of models with two distinct QTL to the peak LOD score of a model with a



	FrzT 1:2	FrzT 4:1	FrzT 5:2
FrzT LOD	14.4	34.0	10.2
Surv LOD	5.8	5.9	16.3
Sum of LOD peaks	20.2	39.9	26.5
Peak of summed LOD profiles	18.5	38.9	25.9
X^2	1.7	1.0	0.6
P	0.192	0.317	0.439

Fig. 3 Summed LOD tests of colocalization between freezing tolerance and survival QTL with point estimates within 4 cM: FrzT1:2 (A), FrzT4:1 (B), FrzT5:2 (C). Colocalization of two QTL can be rejected if the sum of the individual LOD scores is significantly greater than the single peak of the summed (combined) LOD profile (using a likelihood ratio test χ^2 with 1 d.f.). LOD profiles for freezing tolerance, survival and the summed profile are shown in blue, black, and green, respectively. Each profile has been standardized to a maximum of 1 to aid in visualizing profiles of different heights. Tick marks on the *x*-axis indicate marker positions. Vertical dashed lines are the locations of peaks of the LOD profiles corresponding to the colours above. Horizontal bars are the 95% credible intervals for the individual LOD profiles.

single QTL based on the sum of the two LOD profiles (see for e.g., Leinonen *et al.* 2013; Lovell *et al.* 2013). Colocalization of the two QTL can be rejected if the sum of the two individual peaks is significantly more likely than a single QTL model (χ^2 test with 1 d.f.) (e.g. Leinonen *et al.* 2013). In all three cases, models of two distinct QTL did not offer a significant improvement over a single QTL model, consistent with colocalization of freezing tolerance and survival QTL (Fig. 3).

Freezing tolerance as a mechanism for fitness trade-offs

For all three of the freezing tolerance QTL that colocalize with Sweden survival and overall fitness QTL, we found significant QTL by site interactions for both survival and overall fitness (Table 2), indicating different slopes of the reaction norms of these two genotypes across the two environments (Table 2, Fig. 4). Two of the three cases (FrzT4:1 and FrzT5:2) show clear evidence of fitness trade-offs across sites for both survival and fitness (Figs. 4b, c, e, & f). For both of these QTL, the relative benefit of the Swedish genotype in Sweden, is between 4 and 6% for both survival and overall fitness (Table S3, Supporting information). The relative cost of the Swedish genotype in Italy, is between 2 and 5% for survival, but a large cost is predicted for overall fitness, 11% and 18% for FrzT4:1 and FrzT5:2, respectively (Table S3, Supporting information, Fig. 4b,c). A different pattern is observed for FrzT1:2 (Fig. 4a,d). There is conditional neutrality for survival; both genotypes have similar survival in Sweden, but a 4% relative cost of the Swedish genotype in Italy (Table S3, Supporting information). For overall fitness, the Italian genotype for FrzT1:2 is favoured at both sites, but by a wider margin at the Italian site (Table S3, Supporting information, Fig. 4a). Adding other FrzT QTL to the ANOVA model did not qualitatively change the results for the 3 QTL described above (not shown).

Table 2 ANOVA on field fitness (mean fruits per RIL) and survival across 2 sites and 3 years (390 RILs common to all sites and years, from Ågren *et al.* 2013) for the three freezing tolerance QTL that colocalize with QTL for survival in Sweden, and fitness (see Fig. 2)

		Field fitness		Field survival	
Effect	d.f.	*F*	*P*	*F*	*P*
Site	1	3660.19	<0.0001	915.59	<0.0001
Year	2	752.49	<0.0001	2209.62	<0.0001
Site * Year	2	2196.59	<0.0001	350.72	<0.0001
FrzT1:2 (X1_27057077)	1	72.01	<0.0001	8.90	0.003
FrzT4:1 (X4_12547100)	1	6.30	0.012	0.75	0.386
FrzT5:2 (X5_26180944)	1	25.75	<0.0001	0.99	0.319
FrzT1:2 * Site	1	21.11	<0.0001	21.31	<0.0001
FrzT4:1 * Site	1	69.65	<0.0001	21.60	<0.0001
FrzT5:1 * Site	1	189.04	<0.0001	63.42	<0.0001
FrzT1:2 * Year	2	2.63	0.073	6.23	0.002
FrzT4:1 * Year	2	20.97	<0.0001	20.40	<0.0001
FrzT5:2 * Year	2	28.89	<0.0001	47.88	<0.0001
FrzT1:2 * Site * Year	2	0.97	0.380	0.53	0.590
FrzT4:1 * Site * Year	2	13.08	<0.0001	10.62	<0.0001
FrzT5:2 * Site * Year	2	28.26	<0.0001	16.24	<0.0001
Error	2238				

The model includes site, year, the year x site interaction and the interactions between these factors and genotypes at freezing tolerance QTL. Full model R^2 was 0.83 and 0.75 for fitness and survival, respectively.

Candidate genes

We identified 18 candidate genes underlying our three adaptive freezing tolerance QTL (Table S4, Supporting information). These were annotated for 'cold' using experimental evidence and were within 300 Kb beyond either end of the 95% credible intervals of the QTL. For FrzT1:2, 4:1, and 5:2, we found 2, 8 and 8 candidate genes, respectively. We further narrowed down this list to 1, 4 and 3 candidates, respectively (Table S4, Supporting information), based upon *in silico* predicted amino acid differences between the parents (J. K. McKay, unpublished data), with *CBF2* identified as a candidate gene underlying FrzT4:1.

Discussion

Understanding the genetic basis of adaptation and fitness trade-offs requires the study of reciprocally adapted populations and the ability to link genotype, phenotype and fitness. Leveraging previous work demonstrating reciprocal adaptation (Ågren & Schemske 2012) and identifying QTL involved in fitness trade-offs (Ågren *et al.* 2013), we investigated the genetic basis of freezing tolerance because of its likely role as a major driver of adaptation in Sweden. We found that three of the five QTL for which the Swedish genotype increased freezing tolerance were located in the same genomic regions as locally adaptive survival QTL in Sweden, and QTL involved in trade-offs for overall fitness. The marker positions for two of these freezing tolerance QTL can explain fitness trade-offs between sites. The transcriptional regulator of freezing tolerance, *CBF2*, is a likely candidate underlying one of these QTL.

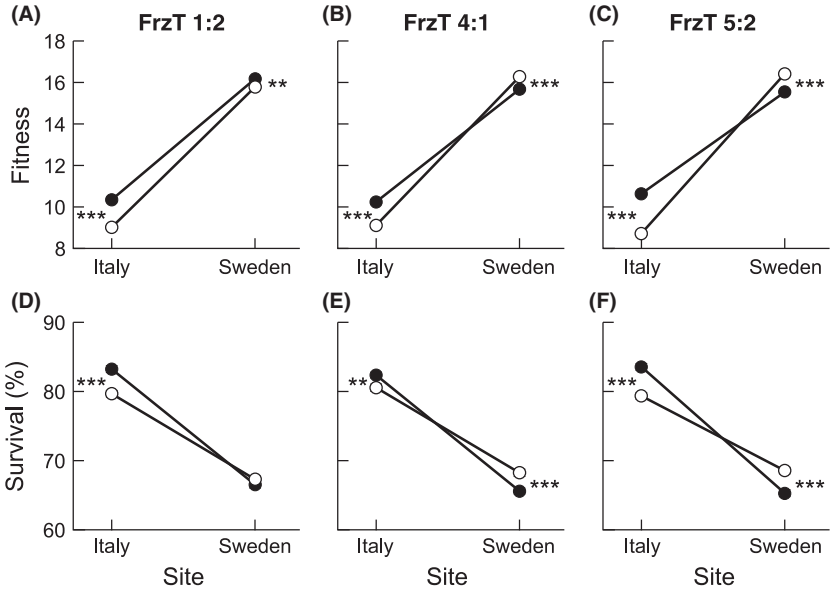


Fig. 4 Least square mean fitness (mean fruits per RIL; A–C) and per cent survival (D–F), at each of the parental sites, of alternate genotypes for (•, Italian; ○, Swedish) each freezing tolerance QTL that colocalized with a fitness region and a QTL for survival in Sweden (see Fig. 2). Survival and fitness data from Ågren *et al.* (2013). All QTL by site interactions were significant at $P < 0.0001$ (Table 2). Significance of within-site least square mean comparisons is indicated with asterisks; $^{**}P < 0.01$; and $^{***}P < 0.001$.

Freezing tolerance QTL

We identified seven freezing tolerance QTL in total, the Swedish genotype increased freezing tolerance for five of the seven. These five QTL each explained on average a 14% difference in freezing tolerance between alternate homozygous genotypes, with a single large effect QTL (FrzT4:1) explaining nearly 25%. Our study used the largest mapping population (500 RILs), and largest number of markers (348 SNPs) to date of any study mapping freezing tolerance in *Arabidopsis*. Most importantly, however, our study system of reciprocally adapted natural populations with evidence of fitness trade-off QTL provides a novel and important context for interpreting the genetic basis of freezing tolerance.

QTL mapping of freezing tolerance in *Arabidopsis* has been investigated in five other mapping populations (Alonso-Blanco *et al.* 2005; Gery *et al.* 2011; Kang *et al.* 2013; Meissner *et al.* 2013; see Table S5, Supporting information for details), typically involving one or more laboratory accessions (but see, Kang *et al.* 2013). We briefly compare our results to two studies with large differences between parental lines in freezing tolerance (as measured by per cent survival after freezing). In a cross between accessions Cvi (Cape Verde Islands) and Ler (Landsberg erecta), Alonso-Blanco *et al.* (2005) identified five QTL under temperature and photoperiod conditions similar to ours, four of which roughly correspond to the genomic positions of QTL found in the present study (FrzT1:1, FrzT1:2, FrzT4:1 and FrzT5:2). The genotypic effects of the QTL they observed were similar to ours (Ler genotype increasing freezing tolerance by an average of 19% per QTL), and the largest effect QTL (27%) also mapped to the end of chromosome 4. This QTL was associated with a predicted loss of function deletion in the Cvi allele of the *CBF2* coding sequence. Increased freezing tolerance of Cvi transgenic lines with a Ler *CBF2* construct provides strong additional support for *CBF2* as a candidate gene underlying freezing tolerance (Alonso-Blanco *et al.* 2005). In a study of two natural populations from China, Kang *et al.* (2013) found two QTL for freezing tolerance, roughly corresponding to our FrzT4:1 and FrzT5:2. The small number of QTL detected in that study compared with ours may reflect the limited power of their mapping population which consisted of 78 F2 lines and 58 markers (Kang *et al.* 2013). Kang *et al.* (2013) did identify a mutation predicted to result in a nonfunctional *CBF2* protein in the nonfreezing tolerant population. Thus, these two studies identified *CBF2* as a candidate gene underlying freezing tolerance, but the extent to which *CBF2* and other genes involved in freezing tolerance contribute to local adaptation and fitness trade-offs is unknown.

Colocalization with field survival and fitness

To determine the potential contribution of freezing tolerance QTL identified in our growth chamber assays to local adaptation in the field, we compared the genomic position of freezing tolerance QTL to that of previously published survival QTL at the native Swedish site and to overall fitness QTL at both parental sites (Ågren *et al.* 2013). We found three QTL that influenced freezing tolerance in the laboratory assay that colocalized with QTL where the Swedish genotype increased survival and overall fitness in Sweden. We then quantified the

potential contribution of genotypes at these three freezing tolerance QTL to fitness trade-offs across native field sites. Marker positions of two freezing tolerance QTL (FrzT4:1 and FrzT5:2) explain a pattern of reciprocally higher survival and overall fitness of the local compared with the nonlocal genotype, that is these QTL are potential genetic tradeoffs. Combined with previous work showing that the relative survival of the Italian genotype in Sweden, is positively correlated with minimum soil temperature (Ågren & Schemske 2012), this provides strong evidence that these freezing tolerance QTL contribute to local adaptation in Sweden. Moreover, the reduced fitness of the Swedish genotype at these loci in Italy, is consistent with the hypothesis that freezing tolerance is costly in Italy.

In contrast, FrzT1:2 shows a different pattern. Genotypes for this QTL exhibit conditional neutrality for survival. For overall fitness, the Italian genotype is significantly globally favoured, but by a wider margin at the Italian site. Such a pattern cannot contribute to local adaptation and is difficult to explain based on freezing tolerance alone. One possibility is that a nearby fitness QTL (FIT1:2, Ågren *et al.* 2013) where the Italian genotype was shown to be strongly globally favoured, is swamping the signal of this freezing tolerance QTL. Alternatively, epistasis between fitness QTL in some years in Sweden (Ågren *et al.* 2013), may make it difficult to determine the effect of this individual freezing tolerance QTL.

Nature of fitness costs in milder environments

Future work is needed to verify causality of FrzT4:1 and FrzT5:2 for fitness trade-offs, both at the level of QTL and of the gene(s), and also to determine the mechanism of the cost at cold, but nonfreezing, temperatures typical of Italian winters. It is likely that the cost relates to the physiology of acclimated freezing tolerance. In our study populations, at temperatures experienced in nature, there is no constitutive freezing tolerance (C. Garraud & D. W. Schemske, unpublished data), but rather freezing tolerance is induced by acclimation at cold, nonfreezing temperatures. In fact, with acclimation, even the Italian line exhibits some freezing tolerance at less severe freezing temperatures (C. Garraud & D. W. Schemske, unpublished data). Induction of freezing tolerance by a period of acclimation is generally observed in *Arabidopsis* (Thomashow 1999, 2010; Zhen & Ungerer 2008a). Induced rather than constitutive resistance typically implies a cost (c.f. Heil & Baldwin 2002). The observation of two minor QTL where the Swedish genotype decreased freezing tolerance further suggests the possibility that freezing tolerance is costly even in Sweden, and that there may be selective fine-tuning of the balance between costs and benefits of tolerance, as has been suggested for the evolution of plant defences against herbivores (c.f. Mauricio & Rausher 1997). The exact mechanism of the cost of acclimated freezing tolerance is unknown, but may stem from the large-scale changes in gene regulation that follow acclimation, and the production of metabolites thought to be helpful in withstanding freezing temperatures (Thomashow 2010), and/or suppression of the photosynthetic gene pathway that occurs concomitantly with cold acclimation (Preston & Sandve 2013).

Zhen *et al.* (2011) investigated the potential fitness cost of induced freezing tolerance in *Arabidopsis* in growth chamber studies with six accessions each from northern and southern latitudes. Despite finding significant differences in freezing tolerance (northern > southern), they found no evidence for a cost in acclimated plants that did not experience freezing. It is possible that their short acclimation period (9 days), while enough to confer freezing tolerance, did not provide the sustained induction of *CBF* genes over longer periods needed for the costs to be manifest. Additionally, post-acclimation growth conditions in their controlled environments might not be stressful enough to manifest the costs. Measures of the costs of freezing tolerance may require conditions that more closely mimic the parental environments, and/or combining growth chamber and field studies as we do here. Based on our results, we expect that having the Swedish genotype at both FrzT4:1 and FrzT5:2 would come at an average cost in Italy of a 29% reduction in overall fitness.

Other studies of reciprocally adapted populations have investigated whether QTL for traits colocalize with fitness QTL and whether there is evidence of fitness trade-offs. Studies of *Mimulus guttatus* have thus far found no evidence for fitness trade-off QTL (Lowry *et al.* 2009), or only evidence of trade-offs between different fitness components (Hall *et al.* 2010). In *Arabidopsis lyrata*, two QTL that exhibited trade-offs for fitness components were found (Leinonen *et al.* 2013), but the mechanism of these trade-offs are unknown. In field studies of *Boechera stricta*, Anderson *et al.* (2013) found a trade-off QTL (probability of reproduction), which mapped to the same genomic region as a QTL for flowering time found in growth chamber studies, but not detected in the field (Anderson *et al.* 2010). Our study represents an important contribution to efforts to understand the traits and genes underlying genetic trade-offs.

Candidate genes

We searched for candidate genes underlying our three adaptive freezing tolerance QTL, as these are strong candidates contributing to local adaptation via freezing

tolerance. Using the criterion of gene annotation for cold acclimation, or response to cold based on experimental evidence, we narrowed down a list of 1417 total genes to just 2–8 genes for each of these three QTL. Of these, we have *in silico* prediction of amino acid differences between the parents for 1–4 genes per QTL (J. K. McKay, unpublished data). Not surprisingly, we identify *CBF2* on this short list of candidate genes for FrzT4:1. Comparison of our parental sequences of *CBF2* has identified a novel deletion in the Italian allele that probably results in a nonfunctional protein, as is supported by reduced gene expression of cold regulated genes in the Italian line following acclimation (Dong 2012). Additional research is needed to identify which, if any, of the short list of candidate genes in fact underlie differences in freezing tolerance at FrzT5:2, the other QTL associated with a fitness trade-off. There are limitations to the candidate gene approach. For example, a total of 405 genes are within the credible interval of FrzT5:2, and in principle, any of these could be causal. Additionally, it is possible that variation in cis-regulatory elements could be responsible for some of the freezing tolerance QTL.

Future work

Future research will use a combination of approaches to validate the effects of individual freezing tolerance QTL and identify the causal genes. As mentioned above, QTL regions may contain hundreds or even thousands of genes, thus distinguishing true antagonistic pleiotropy of a single locus from conditional neutrality at tightly linked loci with opposite effects on fitness in contrasting environments requires a finer scale of resolution than QTL mapping can provide. We are therefore developing transgenic lines for candidate genes to investigate causality, and near-isogenic lines (NILs) for freezing tolerance QTL to aid in gene discovery by fine mapping.

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C.G.O. and D.W.S. designed the study. R.A.A. and C.G.O. carried out the study. C.G.O. analysed the data and wrote the manuscript with contributions from all other authors.

Data accessibility

RIL seeds and genotype data available at ABRC (CS98760).

Annotated R/qtl analysis script and formatted genotype and phenotype files, and data for LOD profile comparisons and fitness trade-offs available at Dryad: http://dx.doi.org/10.5061/dryad.p26bp.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Number of days per growing season with freezing temperatures.

Table S2 Frequencies of the number of simulated colocalizations found.

Table S3 Benefits/costs at field sites for the Swedish genotype at adaptive freezing tolerance QTL.

Table S4 Candidate genes underlying adaptive freezing tolerance QTL.

Table S5 Summary of studies mapping the genetic basis of freezing tolerance in *A. thaliana*.

Fig. S1 QTL LOD profile plots using different approaches for analysing freezing tolerance.

Fig. S2 Heatmaps illustrating all pairwise (Scan two) additive and epistatic LOD scores.