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Costs and benefits of cold tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Cold tolerance in plants is an ecologically important trait that has been under intensive study for basic and applied reasons. Determining the fitness benefits and costs of cold tolerance has previously been difficult because cold tolerance is normally an induced trait that is not expressed in warm environments. The recent creation of transgenic plants constitutively expressing cold tolerance genes enables the investigation of the fitness consequences of cold tolerance in multiple temperature environments. We studied three genes from the CBF (*C*-repeat/dehydration responsive element *b*inding *f*actor) cold tolerance pathway, CBF1, 2 and 3, in *Arabidopsis thaliana* to test for benefits and costs of constitutive cold tolerance. We used multiple insertion lines for each transgene and grew the lines in cold and control conditions. Costs of cold tolerance, as determined by fruit number, varied by individual transgene. CBF2 and 3 overexpressers showed costs of cold tolerance, and no fitness benefits, in both environments. CBF1 overexpressing plants showed no fitness cost of cold tolerance in the control environment and showed a marginal fitness benefit in the cold environment. These results suggest that constitutive expression of traits that are normally induced in response to environmental stress will not always lead to costs in the absence of that stress, and that the ecological risks of CBF transgene escape should be assessed prior to their use in commercial agriculture.

Keywords: *Arabidopsis thaliana*, CBF, cold tolerance, costs, fitness, transgene

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Introduction

A common environmental stress faced by many plants is exposure to cold temperatures, and the ability (or lack thereof) of plants to respond to cold temperatures is an important factor in their ecological and evolutionary dynamics. For example, many tropical species lack the ability to tolerate cold temperatures, and as such cold winter temperatures may play a role in limiting their biogeographical range (Jaglo *et al*. 2001). In a similar fashion, the inability of some species of summer annual plants to tolerate cold temperatures and frosts probably limits their ability to compensate for herbivore damage, especially when early frosts reduce season lengths (e.g. Stinchcombe 2002). In contrast, many temperate species — especially winter annuals — have evolved a variety of physiological mechanisms to tolerate cold temperatures. Improving the cold tolerance of agronomically important species also remains an important applied problem (Thomashow 2001). Indeed, improving tolerance to environmental stresses such as drought, heat and cold through the insertion of genes that increase tolerance to abiotic stresses in different environments is thought to comprise the next generation of agricultural biotechnology (Rissler & Mellon 2000). Accordingly, determining the fitness consequences of cold tolerance, using known genetic pathways, across variable temperature environments, represents a major challenge for evolutionary geneticists and ecologists.

Benefits and costs of tolerance to environmental stresses such as cold are likely to be determined, in part, by whether such traits are inducible (expressed only in response to specific conditions) or constitutively expressed (Heil & Baldwin 2002). Inducible defences are employed only when cues indicate they are needed and may be less costly than constitutive defences (Bergelson & Purrington 1996; Karban & Baldwin 1997; Baldwin 1998). When naturally

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inducible genes are constitutively expressed as trangenes, they may incur greater fitness costs because of metabolic costs of producing additional compounds, autotoxicity or ecological tradeoffs (see, e.g. Purrington & Bergelson 1997, 1999). The isolation of genes — normally expressed through induction — and their insertion as constitutive overexpressers into native and non-native plant genomes allows investigators to study the effects of individual gene pathways on plant responses to cold temperatures (e.g. Sung *et al*. 2003).

As a model organism, much is known about the genetic pathways involved in of cold tolerance in *Arabidopsis thaliana* (Thomashow 1999, 2001; Seki *et al*. 2001). In *A. thaliana*, cold tolerance is an inducible, quantitative trait conferred through the additive effects of different genes producing physiological adaptations to cold weather (Fowler & Thomashow 2002). Low temperature stress activates the expression of ICE genes (*i*nducer of *C*BF *e*xpression; Gilmour *et al*. 1998; Chinnusamy *et al*. 2003; Zarka *et al*. 2003) which regulate the CBF, *C*-repeat/dehydration responsive element *b*inding *f*actor, family (CBF1, 2 and 3) of transcription factors. The CBF gene family is an inducible cold response pathway controlling genes with a *C-r*epea*t* (CRT) *d*ehydration *r*esponsive elemen*t* (DRT), or CRT/DRT (Jaglo-Ottosen *et al*. 1998). Fifteen minutes after exposure to low temperatures, plant cells express cold tolerance genes (Gilmour *et al*. 1998) such as the CBF family. Activation of the CBF gene family changes a plant cell's biochemistry by controlling downstream genes that alter membrane lipid composition, change solute levels in cells and in intercellular spaces, and use other cryoprotective measures to allow survival in low temperature environments (Jaglo-Ottosen *et al*. 1998; Liu *et al*. 1998; Thomashow 1999; Thomashow 2001). Microarray studies suggest that as many as 100 downstream genes may be activated by CBF1-3 (Fowler & Thomashow 2002) as a part of the cold tolerance pathway. Plants constitutively overexpressing CBF 1–3 genes enhance their freezing tolerance without cold-acclimation or prolonged exposure to lowered temperatures (see e.g. Jaglo-Ottosen *et al*. 1998; Gilmour *et al*. 2000; Fowler & Thomashow 2002; Gilmour *et al*. 2004). Other researchers have remarked qualitatively on the smaller size and reduced seed output of the CBF transgenics in comparison to the wild type (e.g. Gilmour *et al*. 2000), suggesting that fitness costs of constitutive transgene expression are present in some environments.

In this study, we examine the fitness costs and benefits of the constitutive expression of three CBF genes in *A. thaliana* that are normally cold-induced. We compare the fitness of transgenic plants overexpressing the CBF genes with control plants in both the presence and absence of a cold treatment. We use multiple insertion lines of each transgene to distinguish between effects of insertion *per se* and effects of transgene expression. We find that costs of tolerance vary by transgene; interestingly, CBF1 showed no cost of tolerance while CBF2 and CBF3 showed costs that outweighed the benefits of cold tolerance.

Materials and methods

Experimental system

A. thaliana (Brassicaceae) is a primarily selfing winter annual found in temperate regions throughout the Northern Hemisphere. We used the ecotype Wassilewskija-2 (Ws-2) as a genetic background for the insertions of the three CBF genes and four null vectors; S.J. Gilmour at Michigan State University graciously provided seeds for all lines used in this experiment. Ecotypes of *A. thaliana*, including Ws-2, naturally contain CBF1, CBF2 and CBF3 arranged in tandem on chromosome 4 (Gilmour *et al*. 1998), which are induced in response to cold temperature (e.g. Gilmour *et al*. 2000; Fowler & Thomashow 2002).

Each transgenic plant contained only one inserted CBF gene copy, which was inserted with a pGA463 transformation vector, a CaMV 35S promoter and a kanamycin resistance gene (Gilmour *et al*. 2000; Clough & Bent 1998; Gilmour pers. comm.). DNA for the CBF1 construct was cloned from a cDNA library created from the Nossen ecotype (Stockinger *et al*. 1997); CBF2 and CBF3 clones were isolated by homology to CBF1 from a cDNA library made from the Columbia ecotype (Gilmour *et al*. 1998). Details on the biology of transgenics for CBF1 (lines G5, G6, G26) and CBF2 (lines E2, E8, E24) can be found in both Fowler & Thomashow (2002) and Gilmour *et al*. (2004), while details for CBF3 (lines A28, A30, A40) have been described by Gilmour *et al*. (2000).

Constitutive expression of the inserted CBF genes was achieved with the CaMV 35S promoter (e.g. Gilmour *et al*. 2000). Thus plants transformed with CBF genes contained the normal complement of cold-inducible CBF genes (CBF1-3) *and* a single, constitutively expressed CBF gene. Three independent insertions were used per CBF gene, yielding a total of nine CBF transgenic lines. Using independent insertions of a gene takes into account position effects on gene expression and the possibility that other genes will be disrupted in particular transgenic lines (see e.g. Bergelson *et al*. 1996). In this manner, our experiment differs from many studies of the costs and benefits of transgenes that use only a single insertion line (e.g. Hails *et al*. 1997; Snow *et al*. 1999; Gueritaine *et al*. 2002; Burke & Rieseberg 2003; Snow *et al*. 2003). The four null vector lines contained a pGA463 transformation vector, the CaMV 35S promoter and a kanamycin resistance gene used for gene insertion but no CBF genes — as such, the null vectors contain the normal complement of naturally inducible CBF genes, but no constitutive overexpressers of CBF genes. There were 13 different lines in the experiment: three insertions per CBF gene for nine transgenic lines and four null vector lines.

Experimental design

On 25 November 2002 we planted seeds in a 5.72 × 5.72-cm (2.25 × 2.25-inch) plastic pots using Promix soil mixture (Premier Horticulture, Red Hill, PA, USA). A total of 520 seeds (13 genotypes × two treatments × 20 replicates) were planted by line, each in a separate pot, and stratified for uniform germination at 4 °C for 7 days with no light. Approximately 88.6% of planted seeds germinated; germination rates were similar among all transgenes and the null vectors (M.W. Jackson, unpublished data). On 1 December 2002 we placed the pots into 10 blocks of 52 seeds, two pots per line per treatment; pots were positioned randomly within each block. The blocks were placed on benches in the Brown University Greenhouse, Providence, RI, USA.

On 7 January 2003, when the plants had formed a rosette but had not yet bolted, we exposed them to two treatments. Half the plants received cold treatment at 4 °C for 1 week (following Gilmour *et al*. 1988) in a cold room with a photoperiod matching the natural, short-day photoperiod in Providence, RI that week (9 hours, 18 min to 9 hours, 27 min of light). Replicate cold chambers were unavailable due to logistical and financial constraints. Thus, while we cannot exclude the possibility that differences in light intensity, water content in soil or other factors contribute to differences between the experimental treatments, we believe that the large differences in temperature should predominate. Consistent with this, the appearance of plants from the cold treatment resembled that of wild *Arabidopsis* after winter cold snaps (J.R.S., T.M.K., J.S., pers. obs, Supplementary Fig. S1), which can often impose substantial mortality selection (T.M.K. and J.S., unpublished ms.). Control plants remained in the greenhouse without cold treatment conditions. Immediately after cold treatment, we returned the plants to the greenhouse for the duration of the experiment. The plants were harvested at senescence. We used silique number to estimate fitness; seed production and silique number are correlated (Mauricio & Rausher 1997). Plants that died or failed to produce siliques were assigned a fitness value of zero.

Statistical analysis

Testing for benefits and costs. To test for benefits and costs of CBF transgene insertion, we used mean contrasts to test a priori hypotheses about the benefits and costs of transgene expression in each environment — namely that CBF transgene insertion leads to fitness benefits in the cold treatment and costs in the control treatment. Traditionally, evolutionary biologists have used this approach to study the benefits or costs of tolerance to disease and herbivores (Bergelson *et al*. 1996; Purrington & Bergelson 1999; Mauricio 2001; Tian *et al*. 2003), although the principles remain the same for testing for benefits and costs of plant responses to abiotic environmental changes.

We used mean contrasts to test for specific differences between the null vector lines and each kind of CBF transgenic line within each environment (i.e. null vector vs. CBF1, null vector vs. CBF2 and null vector vs. CBF3) to test for benefits and costs. Using STATISTICA 6.0, means contrasts were performed in the context of a general linear model that contained the predictor variables treatment, transgene (i.e. CBF1, CBF2, CBF3 and the null vector), insertion line nested within gene, block, treatment × transgene and treatment × line (transgene). Line within transgene was treated as a random effect and all other variables were treated as fixed effects. As a consequence, the denominator for hypothesis testing in the means contrasts was treatment × line (transgene). Silique number was $\log_{10}(y + 1)$ transformed to meet the requirements of the ANOVA. Because the means contrasts involve multiple, nonorthogonal comparisons to the null vectors in each treatment, the critical α was adjusted to 0.016 (= 0.05/3), although the unadjusted *P*-values for each means contrast are also reported. Additional analysis revealed that the residuals from these analyses were not significantly different from normal ($\chi^2 = 9.19$, d.f. = 12, $P = 0.79$), suggesting that conclusions based on parametric analyses are robust.

Results

Variation among insertion lines

There was considerable variation among insertion lines with the same transgene. Specifically, the insertion lines showed significant variation in fruit production [Lines(transgene) $F_{9,426} = 12.09$, $P = 0.0005$; Fig. 1]. By controlling for this variation, in our subsequent analyses we can distinguish between effects of insertion *per se* and effects of transgene expression.

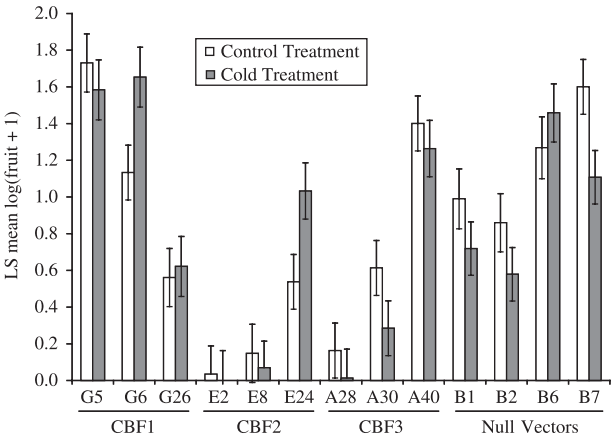

Fig. 1 Mean fruit number for CBF and null vector insertion lines. Each set of bars represents a single insertion line. Error bars indicate one standard error. The control treatment is indicated with open bars and the cold treatment is indicated with black bars. There is significant variation among insertion lines within transgenes (see text).

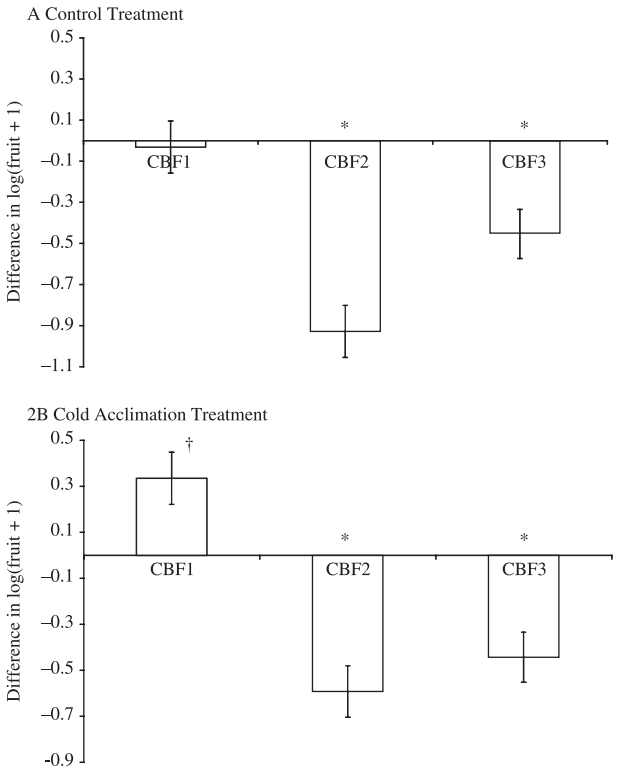

Fig. 2 Differences in fitness between the null vector and each type of CBF line in the control environment (A) and the cold acclimation treatment (B). Values above the *x*-axis indicate a fitness advantage relative to the null vector. Error bars indicate one standard error, and fruit number is $\log_{10}(y + 1)$ transformed. Symbols indicate statistical differences between null vector and CBF transgene lines as determined by means contrasts. *$P < 0.05$; †$P < 0.10$.

Costs in the control treatment

CBF1 overexpression showed no costs in the control treatment, as indicated by the absence of a significant difference in fruit number between CBF1 and the null vector lines in mean contrasts ($F_{1,9} = 0.062$, $P = 0.81$). In contrast, lines overexpressing CBF2 and CBF3 produced significantly fewer fruits than did the null vector lines (CBF2, $F_{1,9} = 38.12$, $P = 0.0002$; CBF3, $F_{1,9} = 9.18$, $P = 0.014$), indicating a cost of cold tolerance (Fig. 2a). We expected to see costs, relative to the null vector, for plants overexpressing transgenes in a normal greenhouse environment without cold treatment. Fruit production results from CBF2 and CBF3 were thus consistent with a priori expectations.

Costs and benefits in the cold treatment

With the cold treatment, there was a trend for CBF1 lines to produce more fruits than the null vector lines (Fig. 2b; $F_{1,9} = 4.39$, $P = 0.066$); however, CBF2 and CBF3 lines produced significantly fewer mean fruits than the null vector lines ($F_{1,9} = 15.92$, $P = 0.0032$ and $F_{1,9} = 9.12$, $P = 0.015$, respectively). In the cold treatment, we expected that plants overexpressing cold tolerance genes should have increased mean fitness relative to the null vector, as suggested in the case of CBF1. The trend for benefits of CBF1 overexpression suggests that the cold treatment was a physiologically stressful environment. The reduced fruit production of CBF2 and CBF3 lines relative to the null vector lines in the cold treatment was inconsistent with a priori expectations and suggests that costs of expressing these genes outweigh any benefits in the cold treatment.

Discussion

Our results suggest that the fitness effects of cold tolerance overexpression vary substantially among different transgenes and environments (Fig. 2). We found no cost of cold tolerance from overexpressed CBF1, in contrast to our expectation of finding costs, but pervasive costs of overexpression of CBF2 and CBF3. CBF1 overexpression conferred a marginally significant fitness benefit, as estimated by fruit number, in the cold treatment relative to the null vector lines. However, in the control environment there was no cost of CBF1 overexpression. In contrast, CBF2 and CBF3 expression had no fitness benefit in cold and showed costs in both environments.

Transgenes can affect fitness through the effects of the genes themselves, insertion effects (where insertion of the transgene disrupts other genes), and position effects (where the insertion position affects the transgene expression pattern) (see e.g. Bergelson *et al*. 1996). Although we cannot rule out completely that, by chance, insertion and position effects affected our results, several factors suggest that the fitness effects we observed are due to the constitutive expression of the CBF genes. First, our use of multiple transgenic insertion lines, which is rare in this context (see Bergelson *et al*. 1996 for an exception), prevents a single insertion or position effect from determining the conclusion. The comparison with null vector lines, which should be just as susceptible to insertion effects as CBF transgenic lines, also helps to control for insertion effects. Second, analysing insertion line as a random effect means that variation between transgenes is assessed relative to the variation among different lines within the same transgene and comes with a significant loss of statistical power, suggesting that our results should be conservative. Nevertheless, the appreciable variation we found among insertion lines for the same transgene (Fig. 1) suggests that results of experiments using transgenes with single or relatively few insertion lines should be viewed with caution. Clearly, inferences from experiments such as ours would be strengthened if large populations of null vector and transgenic insertion lines were available to assess the prevalence and magnitude of insertion and position effects. Such populations

would also allow an assessment of the sampling error introduced by using a small number of insertion lines.

Fitness costs of cold tolerance in the control environment were dependent upon the gene, although the reasons for this are unclear. Although each gene in the CBF family activates largely the same downstream genes, their effects on cold tolerance and other phenotypic traits are not quantitatively identical (Gilmour *et al*. 2004). Generally, it is known that CBF genes activate the COR (*co*ld *r*egulated) pathway, which produces cryoprotective polypeptides that stabilize membranes in freezing temperatures (Stockinger *et al*. 1997) and change sugar and proline concentrations (Gilmour *et al*. 2000; Shinozaki & Yamaguchi-Shinozaki 2000). One admittedly speculative possibility is that the parts of the COR pathway activated by CBF3 and CBF2 are more metabolically demanding than those activated by CBF1, and that the resources required by the CBF3 activated pathways consume resources that would otherwise be allocated to reproduction. Further progress on determining the physiological changes induced by CBF1, CBF2 and CBF3 offers the possibility of describing the physiological mechanisms of cold tolerance fitness costs.

Our results for CBF1 were similar to previous work in tomatoes with respect to benefits, but differed with respect to costs. Specifically, Hsieh *et al*. (2002b) found benefits of overexpression of CBF1 in tomatoes when exposed to cold environments, similar to our detection of marginally significant benefits. However, we found no costs of CBF1 cold tolerance, unlike Hsieh *et al*. (2002a, 2002b), who observed costs of cold tolerance in a control environment. There are several possible reasons for these differences, including interspecific differences in effects of the gene and differences in the control treatments (e.g. photoperiod, greenhouse temperatures, etc.) between these two experiments. For CBF3 we found costs of constitutive overexpression, similar to Kasuga *et al*.'s (1999) observation of a survival cost in *Arabidopsis*. However, in some lines, Kasuga *et al*. (1999) found a benefit to constitutive overexpression of CBF3 in cold environments, unlike our results. It is worth noting that the two experiments used different genetic backgrounds (e.g. Columbia vs. Ws). In addition, Kasuga *et al*. (1999) used a more severe cold treatment (−6 °C vs. 4 °C) and continuous illumination. For both CBF1 and CBF3, researchers eliminated costs of cold tolerance by using stress-induced promoters for the transgenes instead of constitutive promoters (Kasuga *et al*. 1999; Lee *et al*. 2003).

No other studies have explored previously the fitness consequences of CBF2 overexpression. We found no benefits of constitutive overexpression, but we detected costs in both environments. Our finding of fitness costs of CBF2 overexpression in the cold environment is less puzzling in light of the recent results described by Novillo *et al*. (2004). Novillo *et al*. (2004) showed that at normal growth temperatures, CBF2 negatively regulates CBF1 and CBF3 expression, although these results have not been found in previous investigations (Fowler & Thomashow 2002). If CBF2 acts as a repressor to CBF1 and CBF3, its overexpression could have minimized or delayed the cold-induced activation of the native CBF1 and CBF3 genes in those plants, leading to lower fitness in the cold environment. However, in addition to its potential role repressing CBF1 and CBF3, CBF2 also plays a role in leading to cold acclimation (Gilmour *et al*. 2004; Novillo *et al*. 2004), as its constitutive overexpression leads to the upregulation of at least 30 genes that are normally induced by cold temperatures in wild-type plants (Fowler & Thomashow 2002) and increases in freezing tolerance (Gilmour *et al*. 2004). The lower fitness of CBF2 plants relative to the null vectors suggests that any beneficial cold acclimation effects that CBF2 may have due to its effects on downstream genes are outweighed by the costs of repressing CBF1 and CBF3 expression. Costs of CBF2 in the control environment are probably due more to the role of CBF2 in activating numerous other downstream cold responsive genes in an inappropriate environment than to its role in downregulating CBF1 and CBF3 expression, which are usually minimal or undetectable under control conditions (Novillo *et al*. 2004).

If there is no cost of tolerance from the CBF1 gene, why does it remain an induced gene instead of a constitutively expressed gene? Perhaps the regulation of the CBF1 gene is tied intimately to the regulation of other genes within the CBF family, and extricating the regulation of the CBF1 gene from CBF2 and CBF3 may be evolutionarily constrained. Alternatively, it is possible there are costs of CBF1 overexpression in other environments, such as natural populations with more severe biotic and abiotic conditions than those found in a greenhouse (e.g. Bergelson 1994) or in fitness components that we did not measure (e.g. seed mass, germination percentage of fruits). For instance, tradeoffs between cold tolerance and defences against herbivores and pathogens could lead to costs of cold tolerance in environments with these natural enemies. In a similar manner, costs of cold tolerance could be detected in the presence of greater intraspecific competition (Bergelson & Purrington 1996; Korves & Bergelson 2004).

The CBF gene family is well conserved throughout flowering plants, including crop plants such as *Brassica napa*, wheat, rye and tomatoes, suggesting that the CBF gene family is a promising candidate for use in agriculture (Jaglo *et al*. 2001). The introduction of CBF transgenes into agricultural crops, however, is not without ecological risk, as hybridization with wild relatives and establishment of transgenes are both real possibilities (Ellstrand *et al*. 1999; Ellstrand 2001; Spencer & Snow 2001). Increased cold tolerance could increase the natural ranges of hybrid progeny producing an aggressive weed that might displace plants in colder habitats (Schmitt & Linder 1994). Our results indicate that the constitutive expression of one cold tolerance gene

may have benefits and no costs in the absence of cold, suggesting that investigation of fitness consequences of CBF genes in crop–wild species hybrids is warranted. More generally, these results suggest that constitutive expression of normally inducible genes will not always produce fitness costs.

The conservation of the CBF gene family across plant lineages offers the possibility of studying the molecular ecology of cold tolerance in a wide array of ecological systems and temperature environments. Further progress in unravelling the biology of cold tolerance pathways should aid not only our understanding of potential benefits and risks of crop improvement, but also the ecological role of cold tolerance and the relative fitness benefits and costs of induced vs. constitutive traits.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2343/MEC2343sm.htm

Figure S1. *Arabidopsis thaliana* growing and flowering under snow cover during a cold snap in New England.

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