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How anthocyanin mutants respond to stress: the need to distinguish between stress tolerance and maximal vigour

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ABSTRACT

Background: Anthocyanins are produced by plants in response to diverse stresses. Mutants that block the anthocyanin biosynthetic pathway (ABP) at various steps can easily be compared across numerous abiotic stresses.

Hypothesis: Anthocyanins or their precursors are required for stress tolerance. Thus, ABP loss-of-function mutants should have proportionately lower fitness than wildtype plants under stress, compared with benign conditions. In contrast, a decrease in maximal vigour – the general capacity for growth and fecundity – should be most pronounced under benign conditions that allow luxuriant growth by the most vigorous genotypes.

Tests: Determine whether, under stressful conditions, ABP loss-of-function mutants have relatively lower fitness than wildtype plants. Also, test for reduced maximal vigour by determining whether ABP mutants have comparatively decreased fitness under optimal ('benign') growing conditions.

Organism: *Arabidopsis thaliana* loss-of-function mutants (representing all steps in the ABP), as well as wildtype plants, in two genetic backgrounds.

Methods: We grew plants under near-optimal conditions and five stress treatments (UV-B, drought, cold, low Ca:Mg, high Ni). We estimated relative fitness as an individual's lifetime fertility, relative to the mean wildtype fertility in a given treatment.

Results: Stress treatments significantly reduced lifetime fertility of wildtype and mutant lines. Wildtypes outperformed anthocyanin-deficient mutants under benign conditions, but as the stress increased, the difference between wildtype and mutant fitness diminished. Fitness did not increase with a mutation's sequential position in the ABP, nor was there an effect of the ability to produce flavonols on fertility.

Conclusions: Mutations in the ABP did not reduce stress tolerance. Rather, the loss of ABP function reduced maximal vigour, most evidently in near-optimal growth conditions.

Keywords: abiotic stress tolerance, anthocyanin biosynthetic pathway, flavonols, mutants, trade-offs, vigour.

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INTRODUCTION

Abiotic and biotic stresses play significant roles in determining the abundance, distribution, and evolution of organisms (e.g. Grime, 1977; Tilman, 1988; Hoffman and Parsons, 1991; Chapin *et al.*, 1993; Westoby *et al.*, 2002). Limits of the fundamental ecological niche are generally set by physiological tolerances, whereas boundaries of the realized niche are set, to varying degrees, by dispersal, biotic interactions, physical tolerances, and the interactions among these factors (Hutchinson, 1957; Pulliam, 1988; Bertness, 1991). Tolerance of abiotic stress is considered a fundamental axis for niche evolution in plants (Grime, 1977; Southwood, 1988), and physiological and life-history adaptations to stressful conditions appear to be important drivers of plant diversification (Davies *et al.*, 2004). Consequently, understanding mechanisms of plant stress tolerance is central to understanding plant diversity and distributions.

Plants experience stress under adverse conditions that limit their ability to access or utilize available resources (Arendt, 1997). Although this broad definition overlooks important differences in how various types of stressors limit plant growth, it suggests that some general phenotypes may experience reduced negative fitness impacts over a range of stressors (Chapin *et al.*, 1993; Westoby *et al.*, 2002). Within this framework, the stress tolerance of different genotypes can be compared by measuring fitness under stressful conditions relative to that achieved under benign conditions. But, if fitness differences among lineages are also manifested in benign conditions, stress tolerance may be conflated with the ability to exploit opportune habitats (i.e. vigour *sensu* Grime (1977)]. This is an important distinction, since some models of life-history evolution assume that there is a fundamental trade-off between tolerance of adverse environmental conditions and maximal vigour under optimal conditions (Grime, 1988; Arendt, 1997; Taylor *et al.*, 1990). Comparing performances of experimental lineages under both stressful and benign conditions is necessary if one is to distinguish variation in stress tolerance from variation in the ability to exploit optimal growing conditions.

Although the existence of truly generalized stress tolerance traits has been hotly debated (Tilman, 1994; Grace, 1995; Craine, 2005; Pierce *et al.*, 2005), there are suites of stressors that cause fundamentally similar changes on a cellular level, and adaptive responses to those stresses may employ some common mechanisms. For example, drought, salinity, and cold all disrupt the osmotic balance of tissues (Chinnusamy *et al.*, 2004). Adaptive responses to these stresses might involve maintenance of osmotic balance by increasing tissue solute levels through the formation and deposition of non-toxic metabolites of low molecular weight [i.e. anthocyanins (Close and Beadle, 2003)]. Similarly, many stressors, including drought, salinity, heavy metals, ultraviolet (UV) radiation, and some pathogen attacks, cause the generation of excess reactive free radicals that can change protein conformation and damage nucleic acids (Miller *et al.*, 2010). Increased accumulation of antioxidants or chaperone proteins can limit the damage suffered from free radicals caused by a diversity of such ecological stresses (Mittler, 2002). Functional analysis of the underlying metabolic pathways and their interactions provides one means for understanding mechanisms of specific or general stress tolerance.

Metabolic pathways that putatively influence tolerance to multiple stressors provide intriguing opportunities for experimental genetic analysis. In plants, the anthocyanin biosynthetic pathway (ABP; Fig. 1) has repeatedly been suggested to play a role in ameliorating stressful conditions (Chapin *et al.*, 1993; Winkel-Shirley, 2001; recently reviewed in Strauss and Whittall, 2006; Rausher, 2008). Purple, blue, and red anthocyanins are often visually prominent in

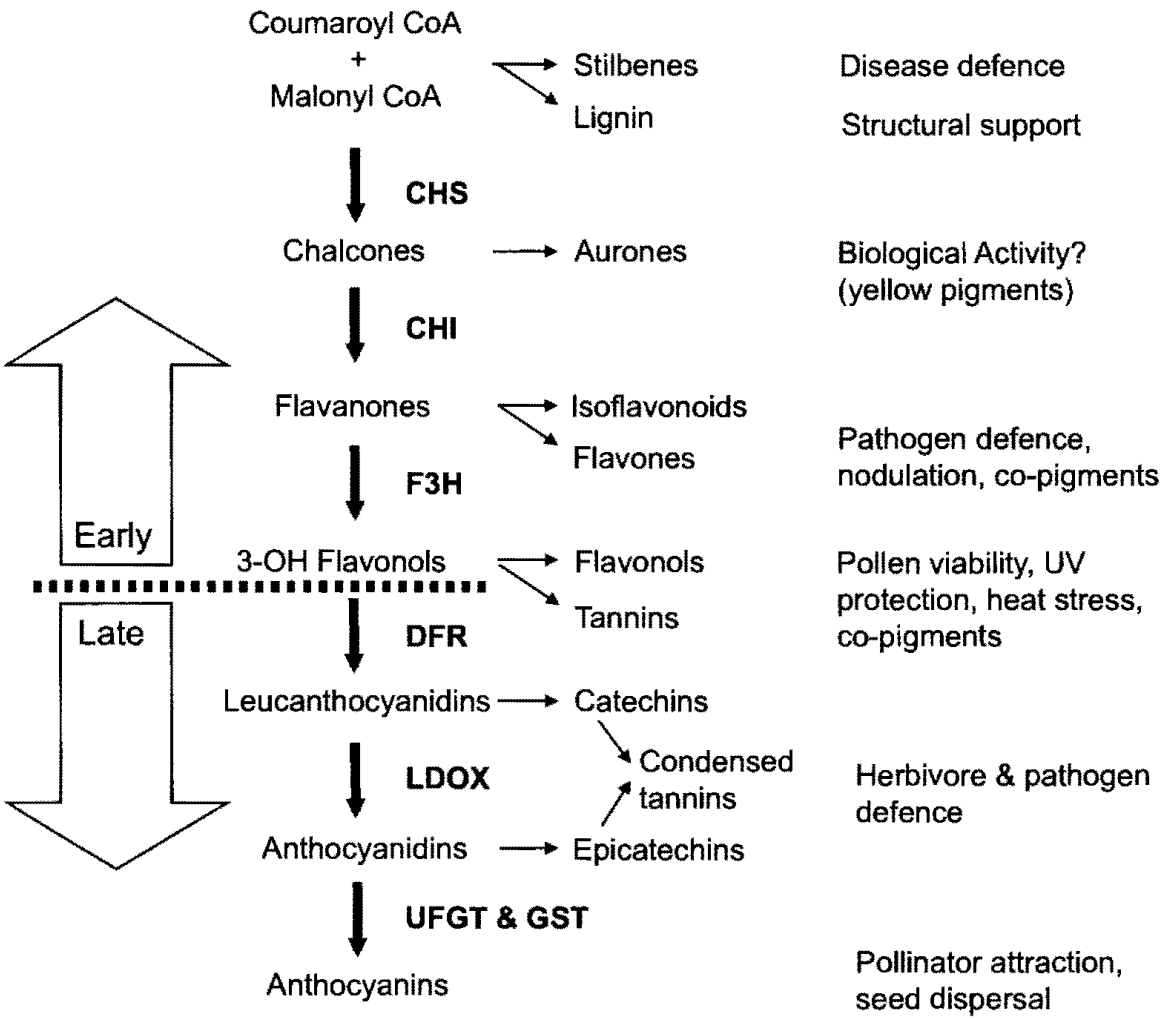

Fig. 1. The ABP is a six-step, linear pathway. The first three enzymes (early steps) produce intermediates that are involved in side-branches, most notably flavonols. Putative ecological roles of the intermediates are shown.

flowers (Grotewold, 2006) and fruits (Steyn, 2007), but also occur in vegetative tissues such as leaves, stems, and roots. Anthocyanins may be expressed constitutively, or may be induced by a range of environmental cues, including UV light, intense visible light, cold, osmotic stress, deficiencies in nitrogen and/or phosphorus, ozone exposure, heavy metal exposure, low pH, methyl jasmonate (a defence signal), wounding, pathogen infection, and many other factors (Chalker-Scott, 1999; Gould, 2004). Although anthocyanins can be expressed in response to a diversity of cues, the hypothesis that anthocyanin production directly enhances stress tolerance has not been tested in an explicit evolutionary framework. Alternatively, anthocyanins could be involved indirectly in stress tolerance due to changes in the regulation of upstream steps in the ABP, such as those genes involved in the production of flavonols (Fig. 1) (Chalker-Scott, 1999; Gould, 2004). At the other extreme, anthocyanin accumulation could be an entirely non-adaptive by-product of a more general stress response cascade involving a diversity of plant hormones and plant nutritional level (Loreti *et al.*, 2008), although this hypothesis has not been formally articulated previously.

To understand how the ABP functions during a plant's response to stress, it is important to consider not only how anthocyanins are produced, but also how the pathway interacts with the production of other potentially stress-ameliorating metabolites. Successive steps of the pathway not only produce different intermediates, but are also metabolic gateways to the production of ecologically important flavonoids, such as flavonols, tannins, and catechins (Fig. 1). In *Arabidopsis*, anthocyanins are created in a six-step pathway, each mediated by a single copy enzyme, from the starting material coumarate (Fig. 1) (Chalker-Scott, 1999; see also Lepiniec *et al.*, 2006). There is a major branching point towards flavonol production after the first three steps, and several other metabolically linked pathways are associated with non-anthocyanin pigments, defence, and structural support, especially in the early half of the ABP.

Although the ABP and its interacting pathways are becoming increasingly well characterized at the biochemical and molecular scales, the ecological and evolutionary roles of anthocyanins and their precursors remain uncertain. Floral anthocyanins are often assumed to be involved in pollinator attraction (Winkel Shirley, 2001) and vegetative anthocyanins have been repeatedly implicated in stress response (e.g. Chalker-Scott, 1999; Close and Beadle, 2003). These assumed functions are largely based on a diversity of studies attempting to discern the adaptive value of these pigments by comparing pigmented individuals with non-pigmented individuals (Meléndez-Ackerman and Campbell, 1998; Irwin *et al.*, 2003; Hodges *et al.*, 2004; Lacey and Herr, 2005; recently reviewed in Strauss and Whittall, 2006). To demonstrate unequivocally that a trait like anthocyanin production is adaptive, Rausher (2008) calls first for evidence that the plants are responding to natural selection (not drift), followed by positive identification of the agents of selection (e.g. pollinators or ecological stress). The results from a diversity of studies across angiosperms suggest that pigmented morphs perform better in stressful conditions (Strauss and Whittall, 2006), but most of these studies were done in non-model plant species, and none were able to differentiate the effect of the loss of anthocyanins from performance differences due to genetically linked loci (Rausher, 2008). Making this distinction should be possible in the model system *Arabidopsis thaliana*, since multiple, stable loss-of-function mutations are available for every step in anthocyanin biosynthesis.

If anthocyanins, or their biochemical precursors, play a role in tolerating stress, then loss-of-function mutations in the ABP should reduce their performance under stress when compared with wildtype (Fig. 2A). If anthocyanins are most essential for achieving maximal plant growth, then the fitness of mutant lines, relative to wildtype, will be reduced most under benign conditions (Fig. 2B). Alternatively, if ABP function is similarly important under both stressful and optimal conditions, then loss-of function mutants should show comparable reductions in absolute (Fig. 2C) or relative (Fig. 2D) performance, compared with wildtype, in environments of widely ranging quality. Unequivocal tests for these hypotheses rest on the assumption that mutant lines have been impaired principally at the targeted biochemical step and not elsewhere in their genome.

Mutants for individual steps of the ABP are potentially powerful but under-utilized tools for distinguishing the consequences of this pathway for stress tolerance versus for maximal vigour. In *A. thaliana*, mutants are available in both the Columbia (Col-0) and Landsberg (Ler) backgrounds, making it possible to compare the stress responses of step-specific mutants to wildtype in two different genetic contexts. We used a set of these mutants to examine the role of the ABP in tolerance to five different stress treatments: augmented UV radiation, cold, drought, low soil calcium : magnesium ratio, and high soil nickel. Our design allowed us to test for the possibility that fitness effects of these mutations could be

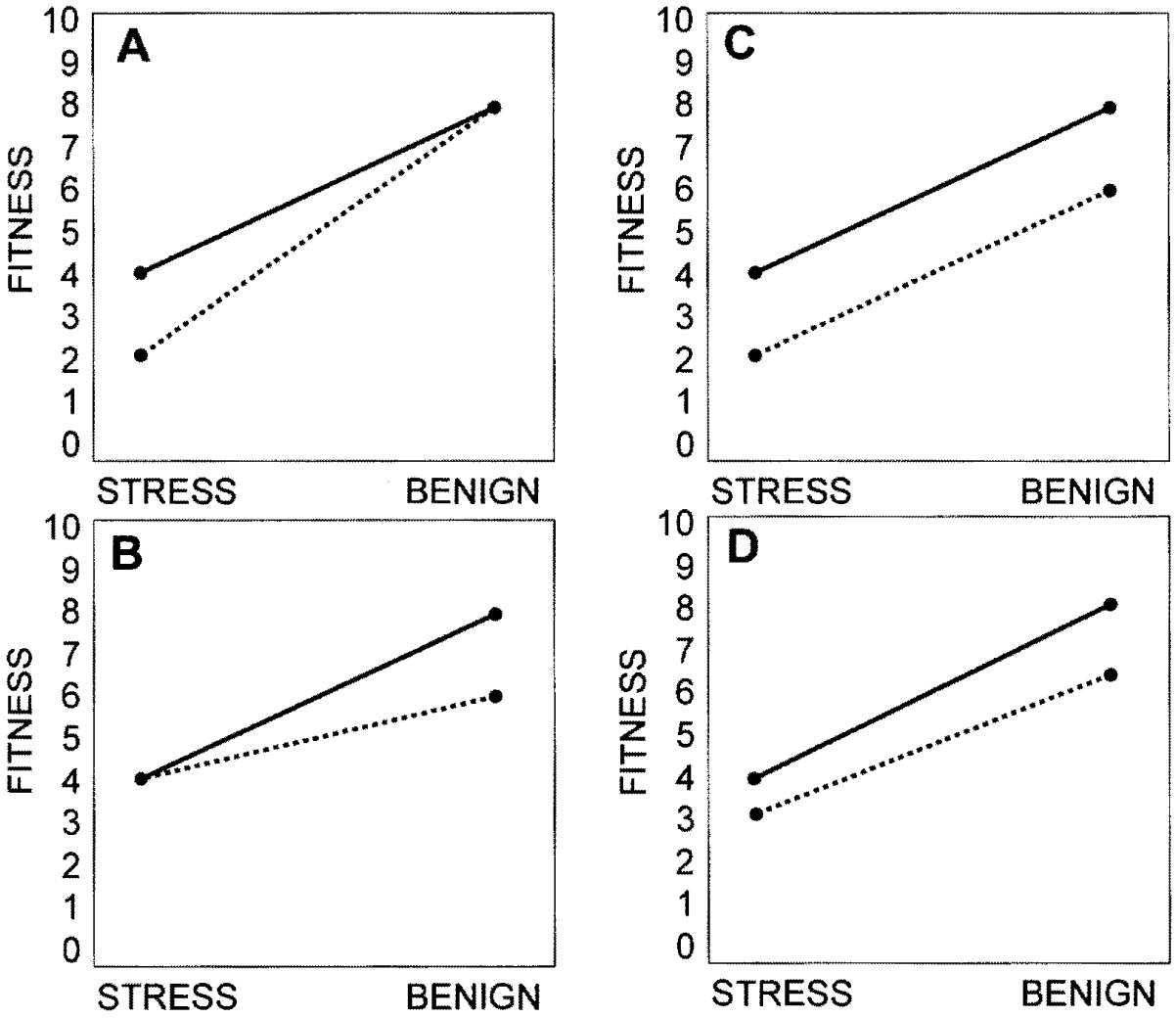

Fig. 2. Hypothetical experimental results illustrating some potential effects of genetic mutation on vigour and/or stress tolerance. The solid line depicts fitness responses by a wildtype lineage to stressful versus benign conditions; the dashed line represents a focal mutant line exposed to the same experimental treatments. In all four scenarios (A–D), the stress treatment reduces fitness of the wildtype lineage by 50%. (A) The mutant shows reduced stress tolerance compared with wildtype. (B) The mutant shows reduced vigour only under benign conditions. (C) The mutant is less vigorous overall, showing the same absolute reduction in fitness, compared with wildtype, in both experimental treatments. Note that the mutant's relative fitness is actually reduced in the stress treatment. (D) The mutant is less vigorous overall, maintaining the same fitness, relative to wildtype, in both experimental treatments.

context-dependent, for example manifested to varying degrees under different stresses or in contrasting genetic backgrounds. Moreover, as intermediates in the ABP lead to alternative pathways and may have some of the functionality of complete anthocyanins, we expected mutations earlier in the pathway to reduce fitness more severely under stressful conditions if the pathway plays either a direct or indirect adaptive role in stress tolerance. In particular, given previous work suggesting a role of flavonols in stress tolerance, we tested the hypothesis that mutations impairing steps before the flavonol branch point would be especially detrimental.

METHODS

Mutant lines

We used the Col-0 and Ler backgrounds as wildtypes, since the two lineages differ substantially in morphology and phenology (e.g. Stinchcombe *et al.*, 2004; Zhen and Ungerer, 2008). All mutants were obtained from the ABRC in Ohio, USA (www.Arabidopsis.org). In the Ler background we used the transparent testa mutants generated by chemical mutagenesis (EMS), and in the Col-0 background we used mutants from the Salk mutation set that were generated by tDNA insertion (Table 1). For the Salk tDNA insert lines, transparent testae seeds (tan) were chosen from a collection of homo- and heterozygotes for bulking, and only homozygous plants producing completely transparent testae seeds were used as seed sources for this study. Because different mutagenesis methods were used in the two *A. thaliana* backgrounds, we use the term 'genetic background' to describe both mutagenesis technique and population of origin.

To characterize our mutant lines biochemically, we grew representatives of each line under enhanced UV exposure to induce anthocyanin production (using Sun-Brella high-intensity discharge lamps, 900 $\mu E \cdot m^{-2} \cdot s^{-1}$), and then tested for the presence of flavonols and anthocyanins using high-performance liquid chromatography (HPLC). Although some flavonoid-specific stress responses have been documented, we used this test to determine the efficacy of the mutants in blocking the ABP and not as a quantitative test of flavonoid production, nor as a qualitative test for the different types of flavonoids (besides differentiating anthocyanins from flavonols). Total leaf anthocyanins and flavonoids were extracted from ~100 mg of mature leaf tissue from a single plant of each genetic line using 90% HPLC grade methanol (Sigma-Aldrich) in water. Leaf tissues were homogenized with two stainless steel ball bearings in 2-ml tubes using a paint-shaker (Sanhua S5 automatic shaker, Zhengzhou, China) set to maximum speed for 2.5 min. The resulting slurry was centrifuged for 10 min, and the supernatant was isolated and stored at −20°C until further

Table 1. ABP mutant lines of *A. thaliana* were characterized for the presence (+), absence (−) or up-regulation (++) of anthocyanins and flavonols relative to wildtype in two genetic backgrounds (Col-0 and Ler)

Ecotypic background	Step in pathway	Disabled enzyme	Mutant	Anthocyanin	Flavonols
Ler	Wildtype		Ler	+	+
Col-0	Wildtype		Col-0	+	+
Ler	1	CHS	TT4 (CS85)	−	−
Col-0	1	CHS	Salk 020583, Exon	−	−
Col-0	2	CHI	Salk 082435, UTR	+	++
Ler	2	CHI	TT5 (CS86)	−	−
Col-0	3	F3H	Salk 1133210C	−	−
Ler	3	F3H	TT6 (CS87)	−	−
Ler	4	DFR	TT3 (CS84)	−	+
Ler	4	DFR	TT3 (CS2121)	−	+
Col-0	5	LDOX	Salk 073183, Exon	−	+
Ler	6	GST	CS824348	+	++

analysis. An Agilent HPLC-DAD instrument was used to measure the amount of anthocyanins and flavonoid intermediates per 100 mg of leaf tissue.

Stress treatments

We implemented stress treatments in growth chambers (Conviron E7, Winnipeg, Canada), using 16-h days and 4-cm pots across all treatments, with the exception of the UV treatment. The UV treatment was performed in a larger walk-in gas-bulb chamber to accommodate the fluorescent UV-B bulbs. For benign control conditions and all treatments not requiring modified soil media, we grew plants in standard potting soil (UC Davis mix). Four replicates of each mutant in each background and stress treatment combination were grown. For each of these replicates, we planted four seeds per pot, and thinned to a single plant 4 days after emergence. Treatments (see below) were applied after emergence of plants.

Cold stress was imposed by setting growth chamber temperatures to 10°C day/4°C night. These temperatures are sufficient both to reduce fecundity and induce expression of cold response genes (Jackson *et al.*, 2004). As flowering and seed production were greatly delayed under these conditions, the harvest was performed later than in the other treatments (see below). To impose UV light stress, we grew plants in a walk-in chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) under Sun-Brella high-intensity discharge lamps (900 $\mu E \cdot m^{-2} \cdot s^{-1}$). To this, we added supplemental UV-B with two fluorescent UVB bulbs (FUVB 40W Preheat/RapidStart ®, Phillips USA) 30 cm above the plants.

Because serpentine soils throughout the world are associated with many specialized endemic plants and ecotypes that often have anthocyanin-rich leaves and flowers [e.g. *Collinsia sparsiflora* (Wright *et al.*, 2006) and *Clarkia* (Kathleen Kay, personal communication)] we imposed two stress treatments that roughly mimicked the unusual ion balance (very low ratio of calcium to magnesium) or the heavy metal content (including high nickel) characteristic of these soils. We grew plants in sterile horticultural sand, with a modified full-strength (1X) Hoagland's solution, so that we could control ion content. To lower the Ca : Mg ratio, we replaced $CaNO_3$ with $MgNO_3$, to give a nutrient solution with a 0.04 mM Ca : Mg ratio [equivalent to treatment F in Bradshaw (2005)]. For high nickel exposure, we added $NiSO_4$ to standard (1X) Hoagland's solution to give a nickel concentration of 300 μM. Previous work with *Allysum bertolonii* and *Arabidopsis halleri* has shown that this concentration reduces growth in non-tolerant crucifers (Brooks and Radford, 1978; Persans *et al.*, 1999; Galardi *et al.*, 2007).

To subject plants to low water availability stress, we grew them in fast-draining Turface (Oil-Dri, Chicago, IL, USA) irrigated with full-strength Hoagland's solution. Beginning 4 weeks after planting, the planting medium was allowed to dry until plants wilted before reapplying water. By imposing the treatments before bolting, our aim was to reduce reproduction without causing significant mortality to small seedlings.

Fitness estimates

To estimate fitness, we counted mature fruits, immature fruits, and flowers, and collected all aboveground biomass 6 weeks after planting (except in the cold treatment, which was harvested at 15 weeks). At this point, most individuals had finished flowering, but senescence of aboveground tissues was not complete. For analyses reported here, we focus on mature fruit production as a fitness measure, which was highly correlated with two other performance indicators: total reproductive effort (the sum of mature fruits, immature fruits,

and flowers: Pearson's $r = 0.98$, $P < 0.0001$) and aboveground biomass (Pearson's $r = 0.60$, $P < 0.0001$). Although measuring seed production could have provided a more direct estimate of fitness, there are known trade-offs between seed size and seed number (e.g. Krannitz *et al.*, 1991; Aarssen and Clauss, 1992; Paul-Victor and Turnbull, 2009), and the process of capturing and weighing all seeds produced by a plant was not possible in this study. Furthermore, a range of studies has shown that fruit production is highly correlated with seed production, and fruit number is routinely used as a fitness proxy in *Arabidopsis thaliana* (e.g. Westerman and Lawrence, 1970; Mauricio *et al.*, 1997; Weinig *et al.*, 2003; Rutter and Fenster, 2007; Huang *et al.*, 2010).

Analyses

We ran three sets of analyses to investigate the effects of mutations in the ABP on stress tolerance and maximal vigour, which are detailed below and are outlined in Table 2. First, we examined whether all mutants, when pooled, had reduced overall tolerance across these five stress treatments (also pooled) (Table 2, Tests 1A–D). Second, we determined whether ABP mutants (pooled) responded differentially to specific treatments (Table 2, Tests 2A–C). Finally, we examined whether mutant performance was affected by how early or late in the ABP the mutation occurred (Table 2, Tests 3A–C). In each of these analyses, we included the benign growth environment as a treatment level to better understand how experimental lineages varied with respect to maximum vigour.

Does impairment of the ABP result in reduced generalized stress tolerance?

We pooled mutant lines and stress treatments to address this question with a three-way analysis of variance (ANOVA) (GLM procedure in SAS Version 9.1.3, SAS Institute, Cary, NC, USA), in which absolute fitness was the response variable, and predictor variables were treatment (all of the stress treatments pooled vs. benign conditions), mutation (wildtype vs. mutant), genetic background (Col-0 vs. Ler), and all interactions (Table 2, Test 1A). Because we were interested in comparing relative fitness within and among treatments, we did not transform fruit number in the ANOVA, but rather used the following procedure to test for unequal variances and its effects on hypothesis-testing. First, we conducted a Levene's test to detect sources of unequal variance by re-running the model as above, using the absolute value of its residuals as the outcome variable (e.g. Stanton *et al.*, 2004). The Levene's test detected significant heteroscedasticity between the control and pooled stress treatments ($F_{1,236} = 3.87$, $P = 0.05$), and so to account for reduced precision in estimating means for the higher variance in the benign treatment, we performed a weighted ANOVA in which each observation was weighted by the inverse of variance within that growth treatment. Results were very similar to those of the unweighted analysis, and so we report just the unweighted analysis here. We analysed wildtypes and mutants separately to examine fitness differences of wildtypes between stress and benign conditions (Table 2, Tests 1B and 1C).

The previous analyses showed that mutant lines had reduced overall fitness, compared with wildtypes, and also suggested that the relative performance of mutant lines differed between the benign treatment and the pooled stress treatments. To account for the variably reduced vigour of mutant lines, we relativized mutant performance by dividing each mutant plant's fruit production by the mean fruit production of the appropriate wildtype in that particular growth environment (stresses not pooled). Reduced stress tolerance of mutant lines would then be indicated by a decrease in the mutant-to-wildtype fitness ratio under stressful conditions, compared with benign conditions. Conversely, impaired vigour of

Table 2. Summary of statistical analyses

Model	Focal question	Samples included	Fitness measure	Factors	Treatment levels in model
1A	Does disabling the ABP pathway influence overall stress tolerance or maximal vigour?	Wildtype and mutants	Fruit production	Mutation class (yes/no), treatment, genetic background, and the interaction	Control vs. pooled stresses
1B	To what extent does stress reduce fitness in the wildtype?	Wildtype only	Fruit production	Treatment, genetic background, and the interaction	Control vs. pooled stresses
1C	To what extent does stress reduce fitness in the mutants?	Mutants only	Fruit production	Treatment, genetic background, and the interaction	Control vs. pooled stresses
1D	Does disabling the ABP pathway influence overall stress tolerance or maximal vigour?	Mutants only	Relativized mutant fitness*	Treatment	Control vs. pooled stresses
2A	Are there stress-specific responses that differ between ABP mutants and wildtype?	Wildtype and mutants	Fruit production	Mutation class (yes/no), treatment, genetic background, and the interaction	All six growth treatments
2B	Do stresses imposed in potting soil differ from those in artificial media	Wildtype and mutants	Fruit production	Mutation class (yes/no), treatment, genetic background, and the interaction	All five stress treatments
2C	Are there stress-specific responses that differ between ABP mutants	Mutants only	Relativized mutant fitness*	Treatment	All six growth treatments
3A	Does the linear order of mutation in the pathway predict overall stress response?	Mutants only	Relativized mutant fitness*	Treatment, mutation step, and the interaction	Control vs. pooled stresses
3B	Does the linear order of mutation in the pathway predict response to specific stresses?	Mutants only	Relativized mutant fitness*	Treatment, mutation step, and the interaction	All six growth treatments
3C	Does production of flavonols affect overall stress response?	Mutants only	Relativized mutant fitness*	Treatment, flavonol production (yes/no), and the interaction	Control vs. pooled stresses

* Mutant fitness/wildtype fitness for a given genetic background and growth treatment.

mutant lines would be indicated by a reduced mutant-to-wildtype fitness ratio under benign conditions. We analysed relativized mutant fitness with a model in which the five stress treatments were pooled into a single 'stressed' category (to test for effects of anthocyanin impairment on generalized stress tolerance or vigour), as above. Genetic background is not included in these models because relativizing to wildtype performance already accounts for background effects. We ran our analyses by weighting each observation by the inverse of the variance in wildtype fitness for that genetic background within that environment to account for variation in wildtype fitness (following Stanton *et al.*, 2000), but found it did not differ from the unweighted analysis. We report the unweighted analysis here (Table 2, Test 1D).

Does impairment of the ABP influence performance under specific growth conditions?

We pooled mutant lines to address this general question with an ANOVA (GLM procedure in SAS), in which absolute fitness was the response variable, and predictor variables were treatment (each of the stress treatments and our benign conditions), mutation (wildtype vs. mutant), genetic background (Col-0 vs. Ler), and all interactions. As in the previous analysis, we did not transform fruit number in the ANOVA, but rather used the procedure described in more detail above to test for unequal variances and its effects on hypothesis-testing. As a Levene's test detected significant heteroscedasticity among treatments ($F_{5,236} = 7.14$, $P < 0.0001$), we performed a weighted ANOVA in which each observation was weighted by the inverse of variance within that growth treatment. Results were very similar to those of the unweighted analysis, and so we report just the unweighted analysis here (Table 2, Test 2A).

To compare the effects of soil media used (potting soil vs. artifical media), we pooled all experimental lines to address this general question with an ANOVA (GLM procedure in SAS), in which absolute fitness was the response variable, and predictor variables were media (potting soil vs. artificial media), mutation (wildtype vs. mutant), genetic background (Col-0 vs. Ler), and all interactions. We ran a weighted ANOVA as described above for other tests, but as it did not affect results we report the unweighted analyses here (Table 2, Test 2B).

To compare stress tolerance and relative vigour for mutants, as with the analysis conducted on pooled stresses, we calculated relativized mutant performance by dividing each mutant plant's fruit production by the mean fruit production of the appropriate wildtype in each of the six growth environments. We analysed relativized mutant fitness with a one-way ANOVA in which all six growth environments were included as levels of growth environment, the single explanatory factor (Table 2, Test 2C).

Does the location of the blockage in the ABP predict the fitness consequences under stressful conditions?

To address this question, we again pooled all stress treatments, but characterized each mutant by a continuous covariate representing the pathway step that is impaired in that line. We then conducted an analysis of covariance (ANCOVA) on mutant fitness (relativized to the fitness of the appropriate wildtype, as above) (Table 2, Test 3A). We removed genetic background from this analysis because it is accounted for in the relativization. An interaction between stress imposition (yes or no) and mutation step (the covariate), in which relativized fitness under stress increased with mutation step, would support the hypothesis that mutations early in the pathway have stronger negative impacts on stress tolerance than

mutations in later steps of the ABP. In a second ANCOVA, we did not pool the five stress treatments, but again included mutation step as a continuous covariate (Table 2, Test 3B).

To determine if early-step pathway mutants that do not make flavonols were more severely affected by stress than late-step pathway mutants, we lumped mutants based on whether or not they make flavonols, then conducted an ANOVA in which the response variable, relativized mutant fitness, was predicted by treatment (stressful vs. benign conditions) and mutation type (early- vs. late-step pathway mutants) (Table 2, Test 3C).

RESULTS

When exposed to high UV, most anthocyanin pathway mutants did not make any detectable anthocyanins, as assessed by HPLC under conditions that induced both wildtypes (Fig. 3, Table 1). Three mutants still produced anthocyanins: CHI (Salk 082435), GST (SAIL_572_B12/CS824348), and CHS (TT4). The first two were excluded from the subsequent analyses because they made substantial amounts of anthocyanins. Mutant TT4 was retained because its anthocyanin expression was substantially reduced.

Does impairment of the ABP result in reduced generalized stress tolerance?

When all stress treatments are pooled, we find that mutants for the ABP are less fit overall than wildtype lines (Test 1A; Fig. 4a, Table 3). The absolute fitness difference between ABP mutants and wildtypes was significantly greater in benign conditions than in the pooled stress treatments, as indicated by the significant mutant × treatment interaction (Table 3). Compared with the benign growth environment, all stress treatments (pooled) significantly reduced the fitness of *A. thaliana* wildtypes (Test 1B; $F_{1,46} = 26.71$, $P < 0.0001$). On average, wildtypes had only 32% of the fruit production under pooled stress treatments that they had under benign conditions, although specific stresses ranged broadly in their severity (98% fitness reduction in nickel, compared with 23% reduction in UV). Pooled stress treatments

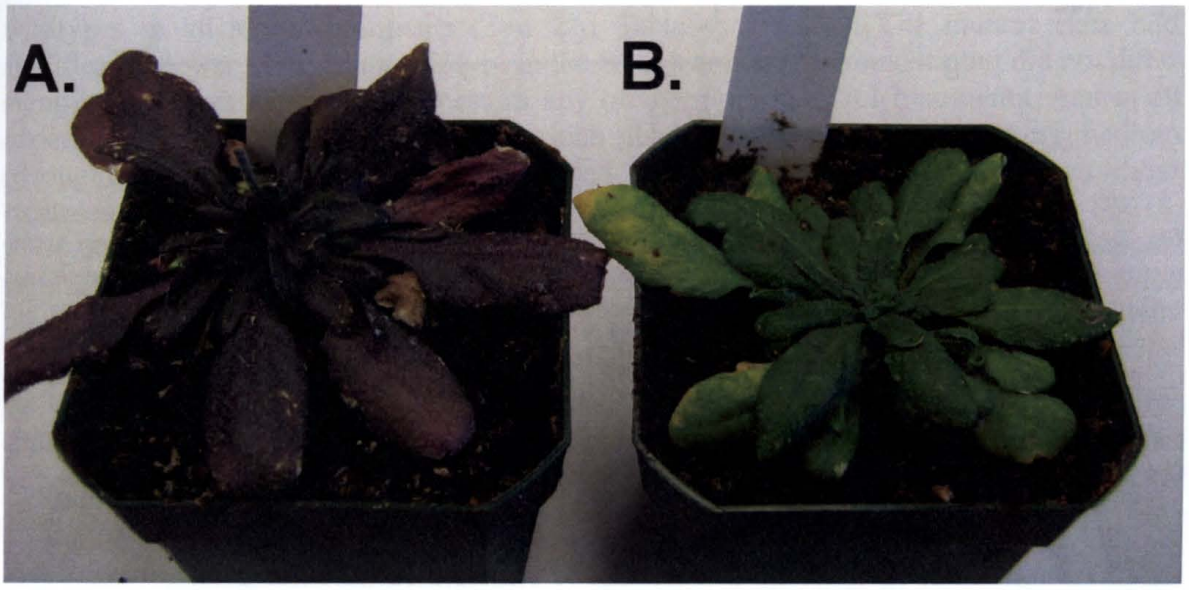

Fig. 3. After exposure to enhanced UV light, Columbia wildtype (A) expressed high levels of anthocyanin pigments in leaves, compared with ABP mutants like the one (B) with a tDNA insert in the CHS exon in the Columbia background (Salk 020583).

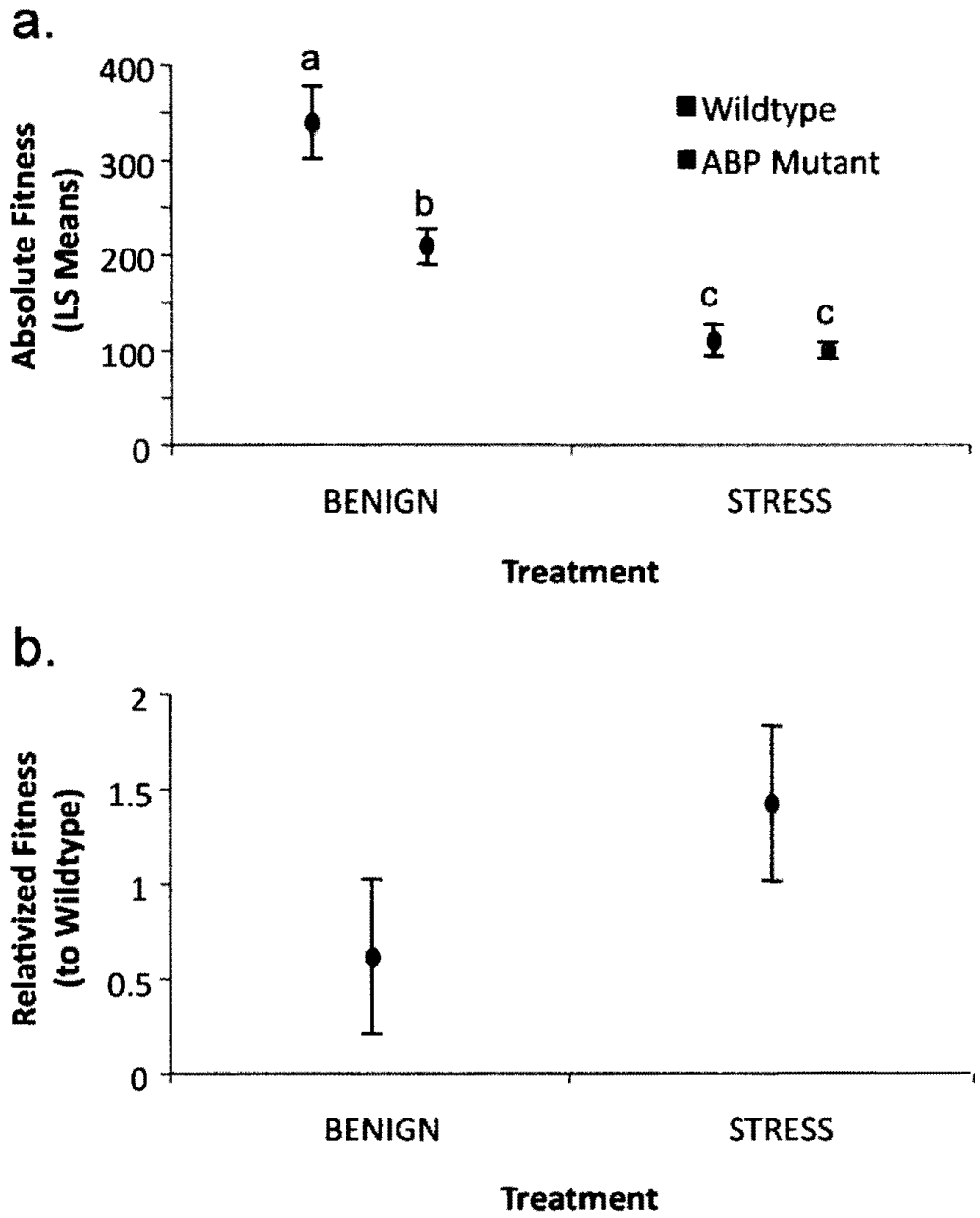

Fig. 4. Fruit production of wildtypes and pooled ABP mutants in benign and pooled stress treatments. (a) Absolute fitness. (b) Relativized fitness of ABP mutants (absolute fitness/wildtype fitness within a given genetic background and treatment). Least squared means and standard errors are based on wildtype grown in benign conditions ($N = 8$ plants), wildtype in pooled stress treatments ($N = 32$), ABP mutants in benign conditions ($N = 32$), and ABP mutants in pooled stress treatments ($N = 154$). Letters above bars indicate groups significantly different from one another with *post-hoc* least squared mean comparisons conducted using the GLM procedure in SAS v.9.13 (SAS Institute, Cary, NC, USA). LS means and standard errors from the overall model are plotted.

reduced the performance of ABP mutants by 47% compared with benign conditions (Test 1C; $F_{1,186} = 33.04$, $P < 0.0001$). Stress treatments varied widely in their fitness impacts on ABP mutants, ranging from virtually no effect in high UV to a 98% reduction of fertility in high nickel.

Table 3. Effects of stress imposition (all stress treatments pooled vs. benign), functionality of anthocyanin pathway (wildtype vs. all anthocyanin pathway mutants), and genetic background on lifetime fruit production of *Arabidopsis thaliana*

Factor	MS	*F*-value	*P*-value
Mutant (yes/no)	83120.69	7.55	0.0065
Stress (yes/no)	635366.5	57.71	<0.0001
Background (Ler/Col)	20417.13	1.85	0.1746
Mutant × Stress	65534.91	5.95	0.0155
Mutant × Background	18094.81	1.64	0.2011
Stress × Background	8284.328	0.75	0.3866
Mutant × Stress × Background	4526.564	0.41	0.522

Note: Model degrees of freedom = 7,230, MS = 113911.130, $F = 10.35$, $P < 0.0001$, $R^2 = 0.239469$. All factors have degrees of freedom = 1,236.

When we relativize mutant fitness to the appropriate wildtype within treatment to account for differences in environmental quality and the greater vigour of wildtype, we see that the pooled anthocyanin mutants tend to have a higher relative fitness under stress than in the benign growth environment, although that difference is only marginally significant (Test 1D; $F_{1,188} = 3.01$, $P = 0.08$; Fig. 4b). In some stressful treatments (see below), ABP mutants out-performed wildtypes from the same genetic background. These findings are inconsistent with the hypothesis that impairment of anthocyanin biosynthesis reduces generalized stress tolerance.

Does impairment of the ABP influence performance under specific growth conditions?

Anthocyanin-deficient mutants, pooled as a single class, did not perform less well than wildtypes in all stress treatments (Test 2A; Table 4, Fig. 5a). The mutant class had significantly lower fitness than wildtype in the benign and cold treatments, but did not differ significantly from wildtype in fitness in any of the more stressful treatments. Across all experimental lines, stress treatments in which plants were not grown in potting medium (drought, low Ca:Mg, and nickel) resulted in greater fitness reduction than those treatments grown in potting soil (Test 2B, cold and UV treatments; $F_{1,190} = 138.18$, $P < 0.0001$; Fig. 5a). Neither genetic background nor mutant status, or their interactions, significantly influenced fitness. Relativized fruit production of anthocyanin mutants varied markedly among the six treatments (Test 2C; $F_{5,184} = 4.21$, $P = 0.0012$; Fig. 5b). The average fertility of ABP mutants actually exceeded that of their matched wildtypes (although not significantly so) in two of the more stressful treatments (nickel, low Ca:Mg), and in no stress treatment was their relative fitness lower than that achieved under benign conditions.

Does the location of the blockage in the ABP predict the fitness consequences of stressful conditions?

When we ordered the mutants from early to late steps of the ABP, we found no significant relationship between step in the pathway and relative fitness across all treatments (Test 3A, ANCOVA: $F_{1,186} = 0.05$, $P = 0.83$). Moreover, the interaction between mutant step and treatment (benign vs. all stresses pooled) also was not statistically significant ($F_{4,186} = 0.02$,

Table 4. Effects of six specific growth treatments, functionality of anthocyanin pathway (wildtype vs. all anthocyanin pathway mutants), and genetic background on lifetime fruit production of *Arabidopsis thaliana*

Factor	d.f.	MS	*F*-value	*P*-value
Background (Ler/Col)	1, 214	11141.410	2.07	0.1520
Mutant (yes/no)	1, 214	25778.049	4.78	0.0299
Stress treatment	5, 214	322745.861	59.86	<0.0001
Background × Mutant	1, 214	13350.129	2.48	0.1171
Mutant × Treatment	5, 214	18488.591	3.43	0.0053
Background × Treatment	5, 214	4322.887	0.80	0.5495
Background × Mutant × Treatment	5, 214	3207.868	0.59	0.7039

Note: Model degrees of freedom = 23,214, MS = 94603.947, $F = 17.55$, $P < 0.0001$, $R^2 = 0.653465$.

$P = 0.89$). In another ANCOVA, we included all six growth environments as separate levels of 'treatment' and found no effect of mutant step on fitness overall (Test 3B, for the main effect of step in the pathway: $F_{1,178} = 0.331$, $P = 0.57$), but the test did identify a significant interaction between mutant step and treatment ($F_{5,178} = 8.79$, $P < 0.0001$). The apparent slope of the relationship between mutational step and relative fitness varied from 4.5 ($P = 0.0088$) in the low Ca : Mg treatment (suggestive of greater fitness deficits associated with mutations at earlier steps) to −4.6 ($P = 0.0076$) in the nickel treatment. The effects of step were inconsistent among growth environments, and accordingly inconsistent with an overarching role of the ABP in tolerance of stresses. Finally, there was no significant difference in relativized fruit production between mutants that make flavonols versus those that do not (Test 3C; $F_{1,186} = 0.05$, $P = 0.83$), and the performance of these two mutant categories did not differ between stressful and benign conditions (for the interaction between treatment and mutation type: $F_{1,186} = 0.00$, $P = 0.97$).

DISCUSSION

Our results do not support the hypothesis that the ABP plays a direct role in general plant stress tolerance. Instead, we find that a variety of mutant lineages with impaired anthocyanin production experience their greatest fitness disadvantage under the *least* stressful conditions, an indication that they experience a disproportionate reduction in maximal vigour, compared with wildtypes.

The interpretation of our experimental results must account for the fact that ABP mutants as a class had lower average fitness than wildtypes in the least stressful growth environments, an indication of reduced maximal vigour of the mutant lines. In contradiction to the hypothesis that the ABP plays a direct or indirect role in enhancing stress tolerance, we found that mutant lines tended to display greater relative fitness when challenged by five experimental stress treatments than when grown under near-optimal, benign conditions. In fact, the relative fitness disadvantage of mutants diminished with the severity of specific stress treatments, and ABP mutants even out-performed wildtype in two of the most stressful environments tested. Under these controlled conditions, we find no evidence that anthocyanins enhance tolerance to stresses, but rather we find patterns consistent with ABP mutants lacking the vigour to fully exploit favourable growth

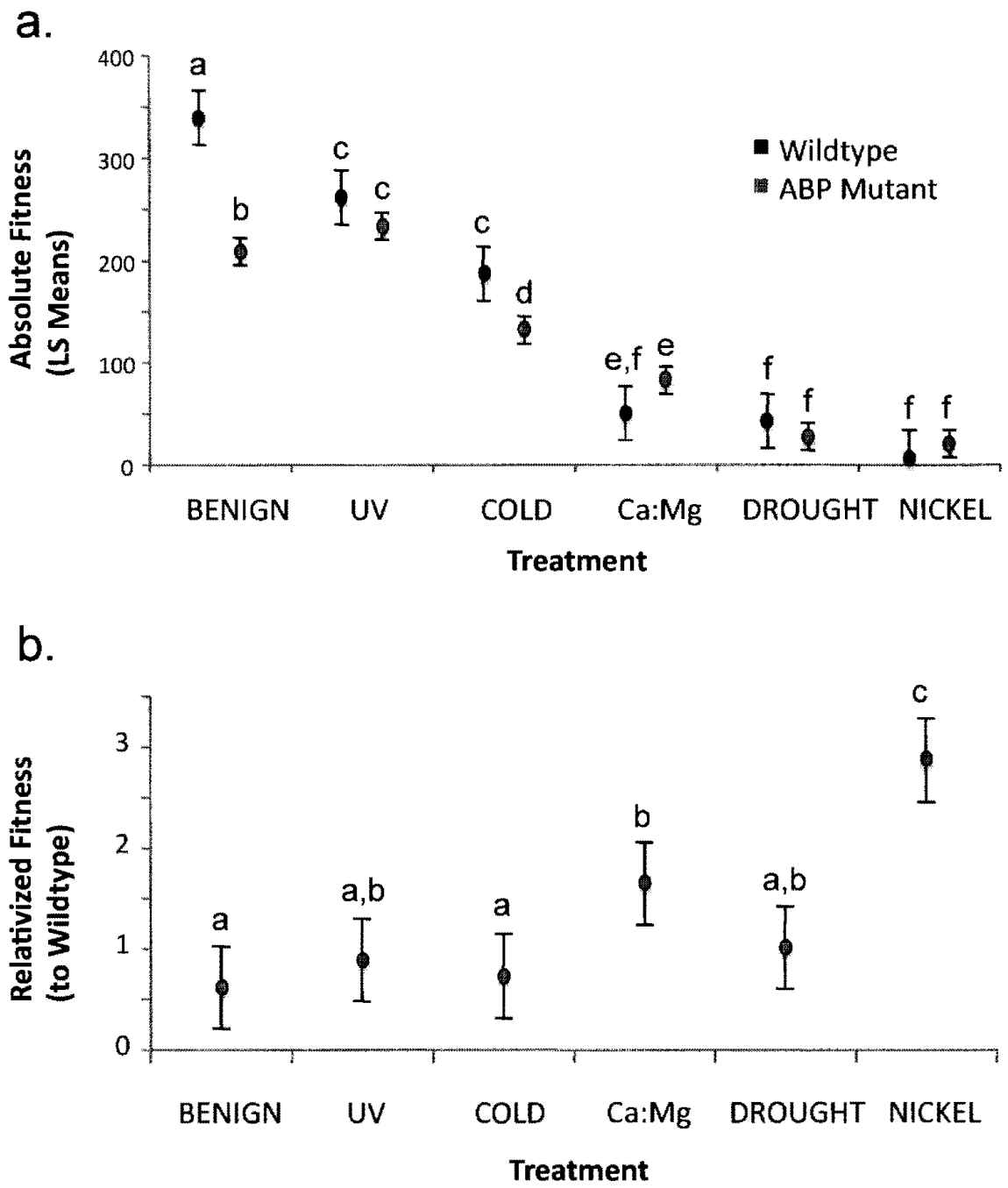

Fig. 5. Fruit production of wildtypes and ABP mutants in benign and individual stress treatments. (a) Absolute fitness. (b) Relativized fitness of ABP mutants (absolute fitness/wildtype fitness within treatment). Least squared means and standard errors are based on wildtype ($N = 8$ plants) and ABP mutant ($N = 32$) in each treatment. Letters above bars indicate groups significantly different from one another with *post-hoc* least squared mean comparisons conducted using the GLM procedure in SAS v.9.13 (SAS Institute, Cary, NC, USA). LS means and standard errors from the overall model are plotted.

conditions. Additional experiments across varying degrees of stress within a given stress regime could help elucidate when selection for greater maximal vigour changes to selection for stress tolerance.

Further evidence against either a direct or indirect role of the ABP in stress tolerance was provided by the fact that plant performance was not influenced by whether a specific mutation disables early or late pathway steps, or whether or not a mutant is able to produce flavonols. These results indicate that neither anthocyanins nor their precursors play a direct role in providing *A. thaliana* with general stress tolerance under these experimental conditions. Our results caution against the use of mutant lines to relate function to fitness, and point to the need to compare mutant and wildtype fitness in both challenging and benign environments.

Finding a role for the ABP in vigour, but not in stress tolerance, is contrary to a litany of studies that provide direct evidence that anthocyanins are up-regulated in response to a range of stresses (e.g. Gould, 2004) and more generally that anthocyanin production frequently correlates with increased performance under stressful ecological conditions (Strauss and Whittall, 2006). Collectively, these studies argue strongly for a role of the ABP in stress tolerance, yet we do not detect any evidence for this role in our study. After accounting for the dramatic differences in vigour between wildtype and mutant fitness, our study identified no consequence of the inability to produce flavonols, nor any relationship with the order of the mutant in the ABP. Not finding a role for anthocyanins, or their precursors, across a diverse array of stresses suggests that the ABP may not be as essential to stress tolerance in *A. thaliana* as some have thought. Our results are reminiscent of those reported in nearly 25% of published studies of inbreeding depression, in which the most severe effects of inbreeding depression are detected under benign conditions (reviewed in Armbruster and Reed, 2005). In these cases, it is possible that the largest effect of inbreeding is an impaired ability to capitalize on abundant resources under benign conditions. We suspect that our results have a similar underlying cause: the mutant lineages we tested apparently lack the vigour to exploit highly favourable environments.

We envision two possible explanations for the reduced relative fitness of ABP mutants under benign conditions, compared with that seen under a range of stresses: first, direct effects of completely blocking the ABP and, second, background effects of the mutagenesis process. If a base level of anthocyanin production (or other metabolic products of the ABP genes) is essential for plant growth, then knockout mutants may not be the most appropriate tool for determining the role of anthocyanins in plant stress tolerance. If the most important factor during stress response is a plant's ability to induce anthocyanins above some basal level, then we would have overlooked the role of an induced anthocyanin response. Future studies investigating natural variation in quantitative production of anthocyanins and variation in inducibility above basal levels such as those of Lacey and Herr (2005) would certainly complement our study. Furthermore, direct effects of blocking the ABP could be magnified under benign conditions if the fitness benefits of producing anthocyanins are only fully realized under opportune conditions – contradicting the hypothesis that anthocyanins enhance stress tolerance. Under stressful conditions, it is possible that other factors overwhelm the benefits of anthocyanin production, thereby reducing the fitness differences between mutants and wildtype.

Alternatively, background effects of the mutagenesis process itself could decrease fitness if mutants carry increased genetic loads due to mutations elsewhere in the genome. If these background mutations affect traits such as resource uptake or metabolic efficiency, their effects may be most apparent under high resource conditions. However, if the mutagenesis process explains the difference in vigour, we would then expect mutants generated with traditional genome-wide mutagens (the TT mutants in the Ler background) to have displayed

lower fitness than the tDNA insert mutants in the Col background, each having only one insertion event per genome on average. Although we found a trend towards decreased fitness of the TT mutants in the Ler background compared with the tDNA-generated Col-0 mutants, the overall performance of the two genetic backgrounds did not differ significantly, and there were no significant interactions of background with growth environment (Tables 2 and 3). To untangle the roles of direct and background effects, the next step would be to isolate the mutation of interest through backcrosses of mutants to wildtype to develop near-isogenic lines.

If the ABP is not essential to stress tolerance, why is it so widely reported to be associated with stress tolerance?

There are several possibilities. It is possible that previously observed correlations between anthocyanin production and stress tolerance are caused by genetic linkage, rather than by direct effects of anthocyanins or biochemically related pathways on performance under stress. Physical linkage between ABP genes and other loci under direct, stress-based selection is one possible type of association, but we know of no evidence for such linkage from any model or emerging model organism (e.g. from high-density QTL or association mapping studies of stress tolerance). Furthermore, we would not expect such tight linkage to persist across such a wide diversity of angiosperm lineages, as recombination would break down an association of ABP loci and other stress loci in any lineage in a few generations. A more likely possibility is that anthocyanin biosynthesis is up-regulated as a by-product of a more general stress response cascade (Hemm *et al.*, 2003; Loreti *et al.*, 2008; Daniel Kliebenstein, personal communication). For example, the ABP begins with the core metabolite coumarate, which is a starting point for the production of phenyl-propanoid secondary metabolites such as flavonols, and is itself recycled into 'primary' metabolism (Gould, 2004). If another coumarate-derived phenyl-propanoid metabolite plays a direct role in stress tolerance, stress-induced up-regulation of coumarate could also result in enhanced anthocyanin production as a by-product. Third, anthocyanins could simply be a biomarker of a broader stress response if they are acting to squelch oxidative bursts related to stress responses. As anti-oxidants and photostabilizers (Chalker-Scott, 1999; Gould, 2004), anthocyanins may be expressed as a means of suppressing the oxidative bursts that are part of metabolic responses to stress (Apel and Hirt, 2004). But, they may not actually be essential to tolerating the stress, and could just occur as a by-product of up-regulated pathways that share precursors and intermediates. Ultimately, ongoing progress in understanding the genetics and biochemistry of the ABP (e.g. Yanekure-Sakakibara *et al.*, 2008) will clarify more of its biochemical functions, but future studies should consider the possibility that the ABP in *A. thaliana* primarily enhances resource utilization under opportune conditions.

Anthocyanins are present throughout vascular plants and bryophytes, suggesting that anthocyanin biosynthesis is an ancient pathway that has been broadly conserved. Even in lineages where anthocyanins have been lost, they appear to have been replaced by similar molecules, such as the betalains of the Caryophyllales. The preservation of the pathway across such a broad phylogenetic spectrum begs for a functional explanation, but from the stresses we tested, we cannot conclude that the ABP is directly involved in *A. thaliana* stress tolerance. Instead, the mutants used here appear most compromised in their ability to grow under benign conditions.

Our results demonstrate the importance of distinguishing vigour under benign conditions from stress tolerance. Although mutants or ecotypes may differ in their fitness

compared with wildtypes when challenged by stress, that difference is not always attributed to tolerance. It is the relative difference of their performance under both stress and benign conditions that allows us to distinguish these two outcomes. Conflating the two has the potential to misinform our interpretation of patterns and mechanisms of stress tolerance.

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