Temperature-sensitive alternative oxidase protein content and its relationship to floral reflectance in natural *Plantago lanceolata* populations

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Summary

• In many plant species, the alternative respiratory pathway consisting of alternative oxidase (AOX) is affected by growth temperature. The adaptive significance of this temperature-sensitivity is unresolved.

• Here, leaf and spike (flower cluster) AOX protein content and spike/floral reflectance of genotypes from European *Plantago lanceolata* populations found in regions differing in reproductive season temperatures were measured. Cloned genotypes grown at controlled warm and cool temperatures were used to assess the natural within- and between-population variation in AOX content, temperature-sensitive phenotypic plasticity in content, and the relationship between AOX and temperature-sensitive floral/spike reflectance.

• AOX content and plasticity were genetically variable. Leaf AOX content, although greater at cool temperature, was relatively low and not statistically different across populations. Spike AOX content was greater than in leaves. Spike AOX plasticity differed significantly among populations and climate-types and showed significant negative correlation with floral reflectance plasticity, which also varied among populations. Genotypes with more AOX at cool than at warm temperature had greater floral reflectance plasticity; genotypes with relatively more AOX at warm temperature had less floral reflectance plasticity.

• The data support the hypothesis that plasticity of AOX content in reproductive tissues is associated with long-term thermal acclimatization.

Key words: alternative oxidase (AOX), floral reflectance, flower, genetic variation, natural populations, phenotypic plasticity, temperature, thermal acclimatization.

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Introduction

In addition to the cytochrome pathway, plants have an alternative mitochondrial electron transport pathway consisting of a single enzyme, alternative oxidase (AOX) (McDonald *et al.*, 2002). This oxidase diverts electrons from the ubiquinone pool and reduces oxygen to water, but without any proton translocation and ATP synthesis. Although the alternative pathway appears to waste energy, the pathway can prevent accumulation of electrons in the ubiquinone pool, diminishing formation of damaging reactive oxygen species (Møller, 2001), and process excess reductant generated from photosynthesis (Raghavendra & Padmasree, 2003; Yoshida *et al.*, 2007) and the tricarboxylic acid (TCA) cycle (Lambers, 1982; Moore *et al.*, 2002; Gray *et al.*, 2004). Collectively, these functions could provide metabolic flexibility to help maintain physiological homeostasis during normal environmental conditions (Lambers, 1982; Clifton *et al.*, 2006) and/or to counteract the negative effects of

environmental stress (Juszczuk & Rychter, 2003). Both may account for the widespread presence of AOX in plants.

The relationship between AOX and thermal acclimatization (i.e. temperature-sensitive adaptive phenotypic plasticity) is unclear. For some species, predominantly aroids, controlled thermogenesis owing to AOX activity thermoregulates floral tissue in response to ambient temperature (Meeuse & Raskin, 1988; Seymour *et al.*, 1998; Seymour & Blaylock, 1999; Patiño *et al.*, 2000; Ito *et al.*, 2003; Watling *et al.*, 2006). For the preponderance of species, in which thermoregulation by AOX does not occur, studies suggest that AOX could be involved with low-temperature acclimatization (Abe *et al.*, 2002; Fiorani *et al.*, 2005) or with high-temperature acclimatization (Rizhsky *et al.*, 2002; Rachmilevitch *et al.*, 2007). Other reports show conflicting correlations between growth temperature-sensitive change in AOX protein and activity and early growth rate, suggesting an indirect, if any, involvement of AOX in thermal acclimatization (Gonzàlez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000; Kurimoto *et al.*, 2004). Consequently, although the alternative pathway is responsive to growth temperature in some species, it is unclear if AOX is part of an adaptive response to stressful temperatures (uncommon high or low extremes), and/or to typically varying temperatures.

Understanding the role of AOX in long-term thermal acclimatization will be further advanced when we learn about AOX in natural populations from environments with different temperature regimes. Genotypic variability and phenotypic plasticity in natural populations have been documented for a variety of species and traits (Dudley & Schmitt, 1996; Sultan, 2001; Gonzáles *et al.*, 2007; van Poecke *et al.*, 2007), yet such information regarding AOX is negligible. For previous studies of AOX and temperature in natural (noncrop) species, the genetic origins of experimental plants were unreported (Yoshida & Tagawa, 1979; Smakman & Hofstra, 1982; McNulty & Cummins, 1987; Weger & Guy, 1991; Collier, 1996; Watling *et al.*, 2006; Rachmilevitch *et al.*, 2007; Campbell *et al.*, 2007), or temperature was uncontrolled (Kumar *et al.*, 2007).

Plantago lanceolata (Plantaginaceae) provides an excellent system for examining the relationship between temperature and AOX in natural populations. This genetically variable pan-temperate herbaceous perennial species has a broad ecological temperature tolerance and is plastic for many fitness traits (Wolff & van Delden, 1987, Lacey *et al.*, 2003; Lacey & Herr, 2005). Salicylhydroxamic acid-sensitive AOX activity is present in its leaves (Atkin & Day, 1990) and roots (Lambers *et al.*, 1981; Smakman & Hofstra, 1982; de Visser & Blacquière, 1984), where the activity changes with growth temperature (Smakman & Hofstra, 1982).

To test the hypothesis that AOX is involved in long-term thermal acclimatization, we surveyed the AOX protein content in whole leaf and spike (flower cluster) tissues of genotypes from seven European *P. lanceolata* populations from three climate types: cool-summer, warm-summer and intermediate. We grew the genotypes at two experimental temperatures resembling those naturally occurring during the reproductive seasons. The goal was to answer the questions:

- Do leaves and spikes differ in AOX content?
- Does AOX content of leaves and spikes vary within and among populations and with temperature?
- Are population differences associated with prevailing temperatures during the reproductive season? Information about AOX in reproductive tissue of any species is limited.

We also measured floral reflectance and the temperature-sensitive plasticity in reflectance for the same genotypes. Reproduction is critical to individual fitness, and reproductive traits in *P. lanceolata* can be temperature-sensitive (Lacey & Herr, 2005). In particular, genotypes can modify floral reflectance in response to temperature change (Lacey & Herr, 2005). Highly reflective flowers, and therefore also spikes, are produced during warm reproductive periods, while poorly reflective flowers/spikes are produced during cool periods. Available evidence suggests that this plasticity is adaptive because it helps warm reproductive structures when ambient temperatures are cool, resulting in partial thermoregulation of the spike (Lacey & Herr, 2005; E. Lacey *et al.*, unpublished).

Given the suspected adaptive advantage of temperature-mediated changes in floral reflectance, we measured the correlation between plasticities in AOX content and floral reflectance. Many physiological and morphological responses, some being functionally linked, others acting independently, can contribute to long-term thermal acclimatization. Thus, the strength of the correlation between the two plasticities should depend on whether AOX contributes to reflectance plasticity directly through metabolism, or to thermal acclimatization independent of floral reflectance. No correlation would be predicted if only floral reflectance is involved in thermal acclimatization.

Phenotypic plasticity has been defined in multiple ways (West-Eberhard, 2003; de Witt & Scheiner, 2004; Valledares *et al.*, 2006; Bell, 2008) From an evolutionary perspective, plasticity is a property of a genotype and is measured as the environmentally induced phenotypic variation in a trait for a given genotype (de Witt & Scheiner, 2004; Freeman & Herron, 2007; Bell, 2008). Because our study addresses long-term thermal acclimatization, an evolutionary process, we measure plasticity in this latter manner.

Materials and Methods

Plant material, temperature and growth conditions, tissue collection and reflectance measurements

Plantago lanceolata L. seeds were collected by maternal family from Finnish, French, and Italian populations (total of seven; Table 1). The cool-summer group, from high latitude or high altitude locations, consisted of populations F-L, FR-R and I-B. The warm-summer group, from lower latitude and

Table 1 *Plantago lanceolata* European source population characteristics

Country	Population	Latitude (°N)	Altitude (m)	AOX/Refl	FAM	Avg Min *T*	Avg Mean *T*	Avg Max *T*	Highest Max *T*
Finland	F-L (C)	61.03	86	8/8	7	10.3	15.0	19.7	20.8
France	FR-I (I)	45.55	10	7/7	7	11.8	16.0	20.2	23.8
	FR-H (W)	43.36	35	9/10	9	12.8	18.0	23.3	28.7
	FR-R (C)	45.10	1886	6/8	6	9.1	13.5	18.0	19.4
Italy	I-B (C)	45.19	1400	11/12	11	11.0	14.5	18.1	19.4
	I-A (W)	41.60	70	10/10	7	13.6	18.6	23.5	29.4
	I-CA(W)	41.03	1	12*/12	12	13.1	18.0	22.8	28.8

Shown are: country of origin; population designations used in text and figures with climate type in parentheses (W, warm; C, cool; I, intermediate); number of experimental genotypes for alternative oxidase (AOX) and reflectance analyses (AOX/Refl); maternal families (FAM); 30-yr monthly averages (Avg) during reproductive season (monthly minimum temperature (Min *T*), monthly mean temperature (Mean *T*), monthly maximum temperature (Max *T*) and highest monthly maximum (Highest Max T). The 30-yr averages were extrapolated from IPCC climatic data (http://www.ipcc-data.org/). The reproductive months were determined from guides to local floras and by biologists who contributed seeds for the experiment. *Twelve spike genotypes; eleven leaf genotypes owing to the loss of one tissue sample.

low altitude, consisted of FR-H, I-A and I-CA. Population FR-I was intermediate.

To reduce possible parental effects, one offspring was grown from each of nine to 20 maternal families per population in similar conditions for one generation. Offspring initially grew vegetatively in a glasshouse. Populations were isolated in growth chambers and glasshouse rooms set at 22°C/16 h day and 17°C/8 h night during flowering to allow for random within-population wind-pollination. All populations matured seeds in growth chambers at the same conditions.

One to two second-generation maternal half-sibs were grown from six to 12 families per population (Table 1). Initially, plants were grown in growth chambers set at 20°C/10 h day and 15°C/14 h night. After 10 wk, plants were divided to produce two genetically identical clones. One clone was randomly assigned to a location in one of three 'cool-temperature' chambers. The other was assigned to one of three 'warm-temperature' chambers. Half-sibs receiving the same temperature were placed in different chambers. All chambers were kept at 20°C/16 h day and 15°C/8 h night for 3 wk after cloning to promote vegetative growth. The temperatures were then reduced to 15°C day and 10°C night or increased to 27°C day and 20°C night. Other environmental factors were held as constant as possible. Plants were watered and fertilized with half-strength Hoagland's solution once a day. Light levels ranged from 698 to 713 μmol m^{-2} s^{-1} in cool chambers and 704 to 780 μmol m^{-2} s^{-1} in warm chambers.

Spike and leaf collections began 14 d earlier in the warmer chambers because warmer temperatures accelerate development (Lacey & Herr, 2005; E. Lacey & A. Umbach, unpublished). For each plant, a spike was collected just before its onset of flowering, and concurrently we collected leaf samples from two to three partially expanded leaves. Leaf development and final leaf size are highly variable during flowering owing to genetic variation, variable overall resource allocation to reproduction among individuals and heterogeneous resource allocation within a plant. Consequently, it was not possible to sample leaves all at the same developmental stage. Therefore, we chose leaves within the same size range across all genotypes and treatments (mean leaf length + SD = 7.0 ± 1.4 cm). Spike and leaf tissues had developed completely under the experimental temperature regime to allow maximal respiratory acclimatization (Atkin & Tjoelker, 2003; Campbell *et al.*, 2007).

Each spike and leaf sample was weighed, flash-frozen in liquid nitrogen, and stored at −80°C until immunoblotting. Per cent reflectance from 362 to 850 nm was measured on another spike per individual, using a spectrometer with an integrating sphere (for methodology see Lacey & Herr, 2005). The average of two scans per genotype per temperature was used for data analysis and for calculating reflectance plasticity (= warm-temperature reflectance minus cool-temperature reflectance) for each genotype (our experimental unit) before analysis. Because floral reflectance is most plastic in the visible region *c.* 550 nm and in the near infrared (NIR) region (Lacey & Herr, 2005; see the Supporting Information, Fig. S1), we examined the relationship between AOX and reflectance values at 550 nm and 850 nm. The mechanisms underlying the plasticity in the two regions likely differ (Stiles *et al.*, 2007).

Measurement of tissue AOX content

The antibody used, 'AOA' (Elthon *et al.*, 1989), recognizes a highly conserved amino acid sequence in the catalytic region of the protein from many species (Finnegan *et al.*, 1999). We prepared sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels of frozen samples using a constant tissue weight–sample buffer volume ratio in sample homogenization as previously described (Millenaar *et al.*, 2001; Umbach *et al.*, 2005). Bacterial membrane harboring *Arabidopsis* AOX1a protein as a positive control, and standard molecular mass markers (Sigma-Aldrich, St. Louis, MO, USA) were loaded on each gel. Gels were prepared in pairs: one loaded with leaf

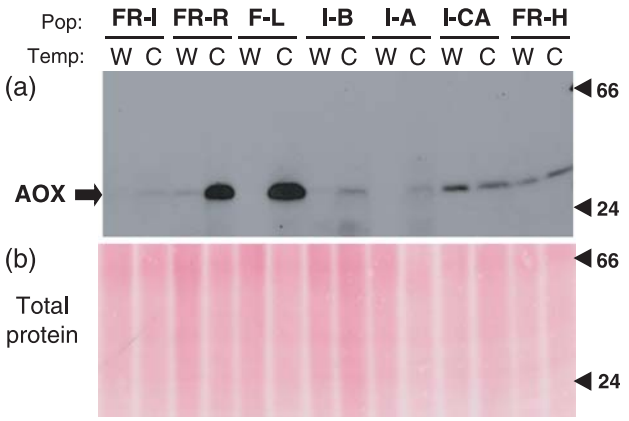

Fig. 1 Representative *Plantago lanceolata* spike tissue samples from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. (a) Spike alternative oxidase (AOX) protein detected on film by immunoblotting for one genotype from each of the seven populations. (b) The same area of the immunoblot stained for total protein. Populations (Pop) are described in Table 1. W and C, warm and cool growth temperatures (Temp), respectively. The SDS-PAGE gel molecular mass marker locations are shown on the right.

samples and the other with spike samples. When possible, a genotype from each of the seven populations was placed on a gel. For each genotype, low and high temperature tissue samples were placed side by side.

After transferring the separated proteins to nitrocellulose membranes (immunoblots), we probed for AOX using AOA at a 1 : 100 dilution and a secondary anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 10 000 dilution, and developed the immunoblots with SuperSignal (Pierce, Rockford, IL, USA) luminol reagent (Umbach *et al.*, 2005). Immunoblots prepared with secondary antibody alone confirmed that it did not react with the AOX protein, and that the AOA antibody reacted only with *P. lanceolata* AOX. We omitted dithiothreitol (DTT) from the gel sample buffer because preliminary experiments showed that AOX detection was improved without it for spike tissue samples (Fig. S2). Because without DTT we did not observe any disulfide-linked dimers (Figs 1, S2), these did not need to be considered. For some *P. lanceolata* samples, AOX was undetectable likely because of low levels of AOX protein in mitochondria which can be undetectable in whole-tissue immunoblots (Umbach *et al.*, 2005).

Film images (e.g. Fig. 1a) of the chemiluminescent immunoblots and images of the same immunoblot membranes stained for total protein with Ponceau S (e.g. Fig. 1b) were scanned using an HP Scanjet 7400c with HP PrecisionScan Pro 3.02 software (Hewlett Packard, Palo Alto, CA, USA). We performed densitometry ('Integrated Density' option of ImageJ 1.34s; Wayne Rasband, NIH; http://rsb.info.nih.gov/ij/) which calculates the sum of the grayscale values for all pixels in a designated area. The relative amounts of AOX protein from the film and total protein from the Ponceau-stained immunoblot, in intensity units, were determined for each leaf and spike sample. Next, each AOX intensity was divided by protein intensity to produce an 'adjusted' AOX intensity value, correcting for protein variation among samples within an immunoblot. Finally, we corrected for between-immunoblot variation before data analysis by using the intensities of the 29 kDa bovine carbonic anhydrase molecular mass standard to produce an immunoblot-correction factor. For each sample the adjusted AOX intensity was multiplied by its immunoblot-correction factor to produce 'AOX protein content'. The AOX protein content, a relative measure, was used as the dependent variable in the statistical analyses.

Preliminary experiments showed that protein intensity measurements were linear through the amount of sample that we used, and integrated density saturation was not detectable until the sample amount had been doubled. Standard deviations for protein intensities were within 22% of the immunoblot averages across all spike tissue immunoblots and within 30% for leaf tissue immunoblots. The AOX intensities varied widely among the samples, with standard deviations well-exceeding the immunoblot average intensities in all but two immunoblots.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS, Inc., Cary, NC, USA). The AOX data were first considered as a linear mixed model with population (seven levels), tissue (two levels) and temperature (two levels) in a factorial arrangement and considered to be in a completely randomized design. The responses protein content, phenotypic plasticity in AOX content (the phenotypic difference in content between warm and cool temperatures), and relative AOX plasticity (phenotypic plasticity divided by the mean AOX content for warm and cool temperatures for each genotype) were each modeled separately. A positive plasticity value indicates greater AOX at warm temperature; a negative value indicates greater content at cool temperature. Subsequent analyses were run for each tissue, including a two-way analysis to determine if climate type and population nested within climate type (see Table 1) influenced AOX plasticity in spike tissue. Linear mixed models were also used to determine if population and temperature affected per cent floral reflectance, reflectance plasticity, and relative reflectance plasticity of spike tissue. Given that genotype was the experimental unit, AOX and reflectance plasticities were calculated for each genotype before analyses. Brown–Forsythe tests for homogeneity of variances showed no need for transformations except for the three-way test for AOX content. The AOX data were log-transformed for this analysis. Populations were treated as fixed because we had deliberately chosen them from a larger experiment to represent the three climate types (Table 1). All pairwise comparisons were tested using the Tukey–Kramer procedure. We also performed a correlation analysis to examine the linear

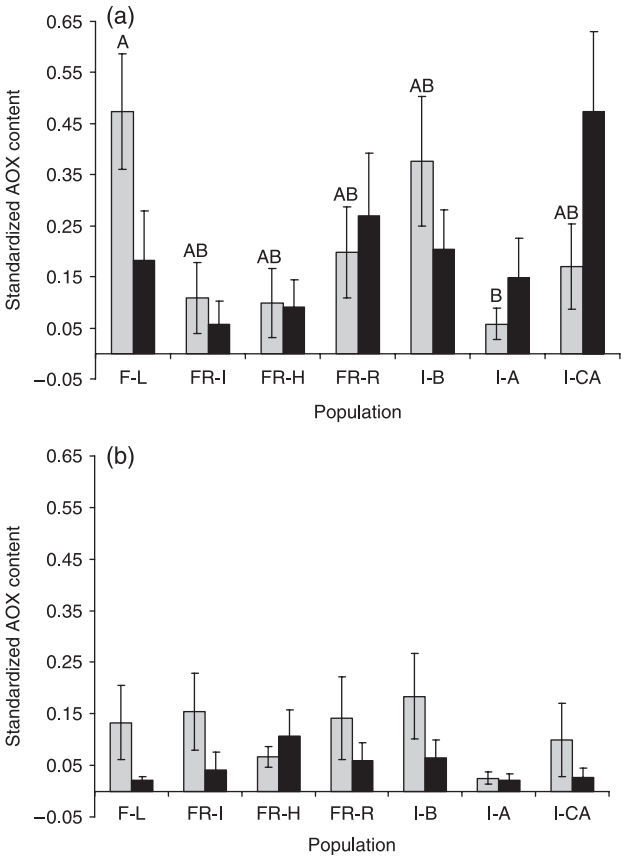

Fig. 2 Mean alternative oxidase (AOX) protein content (relative standardized 'AOX intensity' units; see the Materials and Methods section) for cool (gray bars) and warm (black bars) temperatures of (a) spikes and (b) leaves for experimental *Plantago lanceolata* populations. Table 1 identifies populations and shows sample sizes. Error bars = 1 SEM. Different letters above two means indicate populations that differed significantly in a pairwise comparison within the specified temperature. The absence of letters indicates that no population statistically differed from any other.

Table 2 Mixed-model (Type 3) ANOVA results for *Plantago lanceolata* for effects of population (Pop), temperature (Temp), and Tissue (Tis) on: (a) log-transformed standardized alternative oxidase (AOX) content, (b) standardized spike content, (c) standardized leaf content, and (d) phenotypic plasticity in AOX content; (e) effects of climate type and Pop on spike AOX phenotypic plasticity; (f) effects of Pop and Temp on spike reflectance at 550 nm and 850 nm (*, $P < 0.0001$ for all sources)

Source	Num df	*F* value	*Pr* > *F*
(a)			
Pop	6	2.16	0.0476
Temp	1	6.09	0.0143
Tis	1	14.99	0.0001
Pop × Temp	6	0.77	0.5911
Pop × Tis	6	1.63	0.1388
Temp × Tis	1	0.06	0.8011
Pop × Temp × Tis	6	0.06	0.9993
Residual	239		
(b)			
Pop	6	2.49	0.0263
Temp	1	0.02	0.8769
Pop × Temp	6	2.03	0.0663
Residual	120		
(c)			
Pop	6	0.87	0.5221
Temp	1	5.56	0.0200
Pop × Temp	6	0.72	0.6344
Residual	119		
(d)			
Pop	6	2.73	0.0162
Tis	1	1.17	0.2811
Pop×Tis	6	1.81	0.1032
Residual	119		
(e)			
Climate type	2	3.87	0.0262
Pop (Climate type)	4	2.18	0.0825
Residual	60		
		F(550) value*	*F*(850) value*
(f)			
Pop	6	7.49	9.75
Temp	1	185.62	172.51
Pop × Temp	6	5.26	5.51
Residual	112		

relationship between plasticities (including relative plasticities) in AOX content and floral reflectance.

Results

AOX content

Population, tissue, and temperature all significantly influenced AOX protein content (Fig. 2, Table 2a). Overall, spikes contained 2.6 times more AOX than did leaves (Table 2a; mean ± 1 SE: 0.215 ± 0.028 for spikes; 0.081 ± 0.014 for leaves). Temperature did not affect AOX when spikes alone were examined (Table 2b). However, twice as much AOX was detected in cool-temperature leaves when compared with warm-temperature leaves (Table 2c; leaf means ± 1 SE: 0.114 ± 0.025 at cool temperature; 0.050 ± 0.012 at warm temperature). The AOX content significantly differed among populations in spike tissue but not in leaf tissue when each tissue was examined separately (Table 2b,c). For spike tissue, populations differed significantly only at low temperature. The AOX concentration in the Finish F-L population was more than seven times greater than in the Italian lowland I-A population ($P = 0.049$, Fig. 2a).

Phenotypic plasticity values among genotypes ranged from –0.93 to +1.53, indicating that some genotypes produced more AOX at warm temperatures (Fig. 3a: positive values) whereas others produced more at cool temperatures (Fig. 3a: negative values). Populations, but not tissues, significantly differed in plasticity (Table 2d), although not in relative plasticity (data not shown: all *P* values > 0.35). For spikes, plasticities

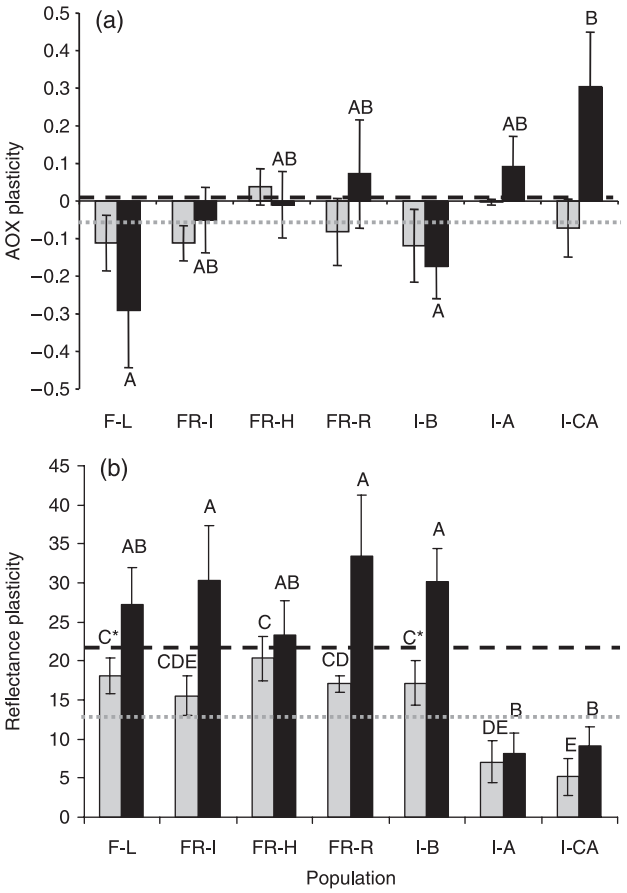

Fig. 3 Mean phenotypic plasticity (average of genotypic plasticities) for (a) alternative oxidase (AOX) content in leaves (gray bars) and spikes (black bars) and (b) spike reflectance at 550 (gray bars) and 850 nm (black bars) for experimental *Plantago lanceolata* populations. Populations identified in Table 1. Grand means for AOX plasticity in leaves (gray dotted line) and spikes (black dashed line) were –0.063 and 0.004, respectively. Grand means for reflectance plasticity at 550 nm (gray dotted line) and 850 nm (black dashed line) were 13.667 and 21.635, respectively. Error bars = 1 SEM. Different letters above two means indicate populations that differed significantly in a pairwise comparison within the specified tissue/wavelength. *, F-L and I-B differed from I-A at $P < 0.06$.

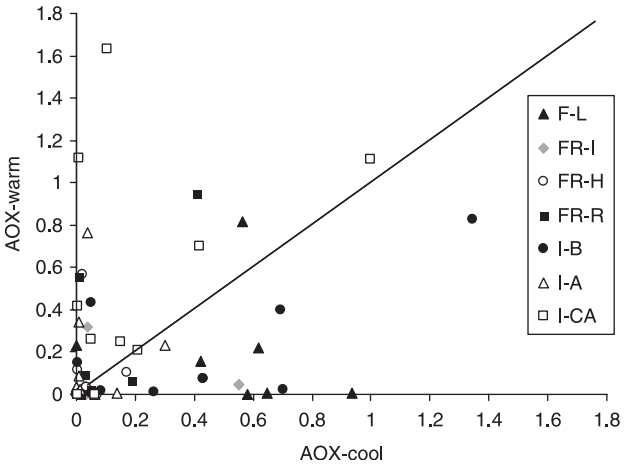

Fig. 4 Relationship of spike alternative oxidase (AOX) protein content at warm and cool growth temperatures for *Plantago lanceolata* genotypes. Genotypes identified by population (populations identified in Table 1): FR-H, I-A, I-CA, warm-summer populations; F-L, FR-R, I-B, cool-summer populations; FR-I, intermediate population. The diagonal line indicates equivalence of the two values, slope = 1.

for two cool-summer populations, F-L and I-B, significantly differed from that of the warm-summer population I-CA ($P = 0.030$ for F-L vs I-CA; $P = 0.010$ for I-B vs I-CA). The F-L and I-B populations showed more AOX at cool temperature; I-CA showed the opposite pattern. A significant difference in plasticity was found among the three summer climate groups (Table 2e). The cool-summer group showed greater AOX content at cool temperature than at warm temperature, whereas the warm-summer group showed the opposite ($P = 0.021$ for warm climate vs cool climate type). This point is visualized in Fig. 4 where cool- and warm-temperature AOX content is plotted by genotype. Although genotypes having plasticities of different sign and magnitude were present in most populations, larger negative plasticities were almost exclusively a characteristic of cool-summer genotypes (Fig. 4, closed symbols below the diagonal) whereas positive plasticities predominated in warm-summer genotypes (Fig. 4, open symbols above the diagonal).

Floral reflectance and association with AOX

Population and temperature significantly influenced floral reflectance at 550 nm and 850 nm, in the visible and NIR spectral regions, respectively (Table 2f). At warm temperature the population differences in reflectance were statistically significant but probably not biologically meaningful (e.g. < 5% between the two Italian lowland populations and the others at 850 nm), or they were absent (at 550 nm; Fig. 5). At cool temperature, however, spikes from the warm-summer Italian lowland populations (I-A and I-CA) were significantly more reflective than were spikes from other populations, approx. 80% and 39% greater at 550 nm and 850 nm, respectively (Fig. 5). These Italian populations show little response to cool temperature. Thus, phenotypic plasticity in spike reflectance was significantly lower in the Italian lowland populations (Fig. 3b; one-way ANOVA population effect: df = 6, $F(550) = 5.83$, $F(850) = 5.80$, $P < 0.0001$ for both). Relative reflectance plasticity showed the same pattern (df = 6, $F(550) = 7.23$, $F(850) = 5.50$, $P < 0.001$ for both).

At the genotypic level, plasticities in AOX content and floral reflectance were significantly negatively correlated in the visible and NIR spectral regions (Fig. 6; Spearman correlation coefficients: $r = -0.445$, $P = 0.0003$ for 550 nm; $r = -0.402$, $P = 0.001$ for 850 nm). Analyses of relative plasticities showed the same pattern (Spearman correlation coefficients: $r = -0.293$,

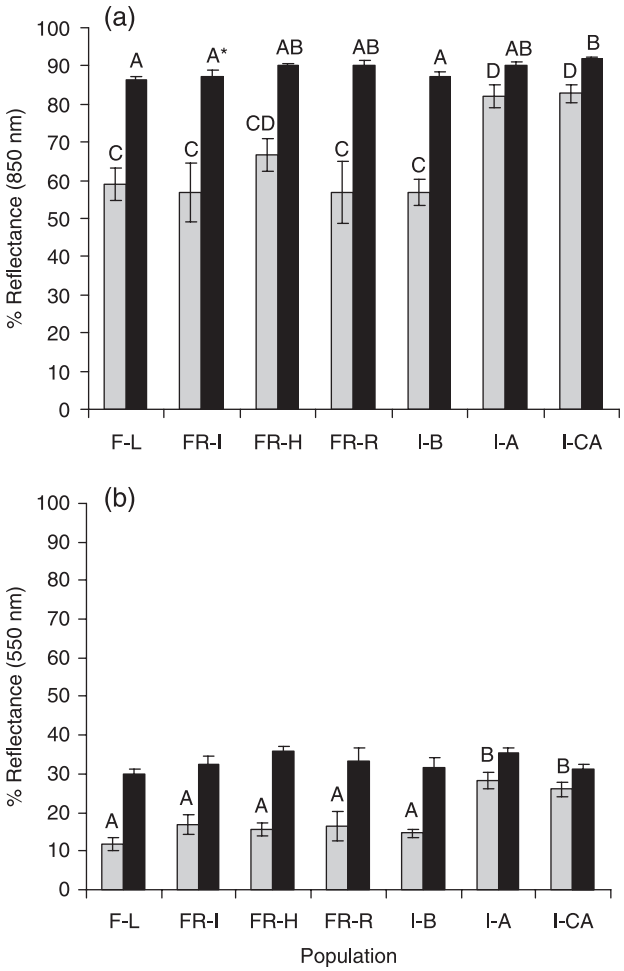

Fig. 5 Mean percent spike reflectance under cool (gray bars) and warm (black bars) temperature conditions at (a) 850 nm and (b) 550 nm for seven *Plantago lanceolata* populations. Populations identified in Table 1. Error bars = 1 SEM. Different letters above two means indicate populations that differed significantly in a pairwise comparison within the specified temperature. *, FR-I and I-CA differed at $P < 0.06$.

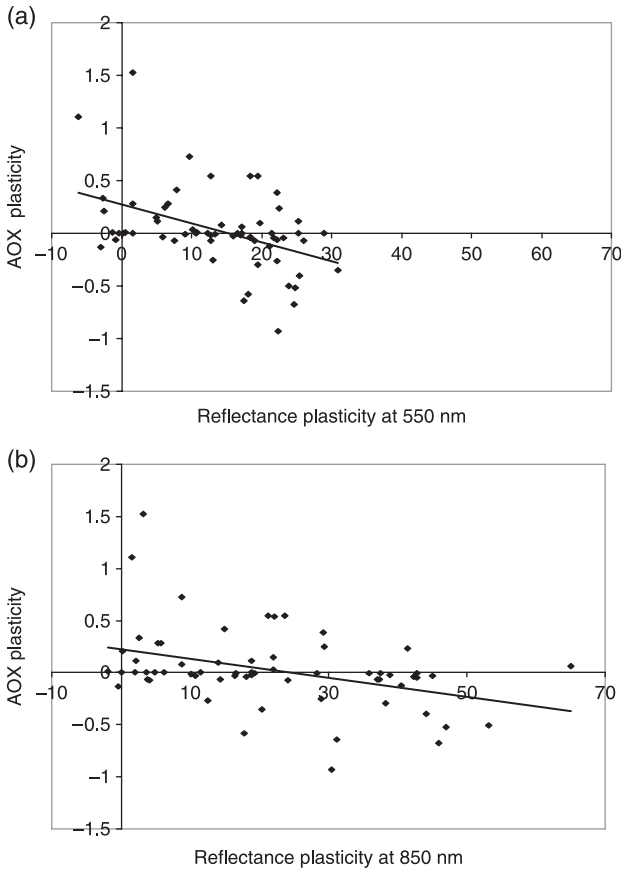

Fig. 6 Relationship between spike alternative oxidase (AOX) and reflectance plasticities (a) at 550 nm and (b) 850 nm for *Plantago lanceolata* genotypes. Spearman correlation coefficient associated with trendline: $r = -0.445$ ($P = 0.0003$) at 550 nm; $r = -0.402$ ($P = 0.001$) at 850 nm.

$P = 0.02$ for 550 nm; $r = -0.287$, $P = 0.02$ for 850 nm). High AOX content at cool relative to warm temperature (negative plasticity) was associated with high reflectance plasticity, while higher AOX at warm temperature (positive plasticity) was associated with negligible reflectance plasticity.

Discussion

By assessing AOX content of reproductive and vegetative structures in multiple natural populations of the same species, we have taken a different approach from past studies in examining the relationship between AOX and temperature. Our approach shows that temperature sensitivity of AOX content is genetically variable, differs among populations and tissues, and is correlated with a trait involved in reproductive thermoregulation and likely fitness.

Plantago lanceolata leaf AOX content was low compared with spikes, and showed no significant differences among the populations, although it was greater at cool growth temperatures. The amount of AOX protein increases in mitochondria as leaves develop (Lennon *et al.*, 1995; Svensson & Rasmusson, 2001), and developmental differences induced by the two temperatures might account for the treatment effect. However, our results are consistent with a recent study (Campbell *et al.*, 2007) showing more AOX protein content in mature leaf tissue developed at low temperature in several species, including two other *Plantago* species. The constraints placed on leaf metabolism by the interaction of photosynthesis, respiration, and temperature (Atkin *et al.*, 2006; Campbell *et al.*, 2007) may explain the relatively uniform response of AOX content across populations. In contrast to leaves, the reproductive tissue of *P. lanceolata* contained significantly higher AOX levels, and genotypes and populations showed large differences in temperature sensitivity (i.e. plasticity). Two types of floral AOX plasticity were observed: greater AOX content at cool temperature (negative plasticity) and greater content at warm temperature (positive plasticity).

AOX has been hypothesized to be involved with thermal acclimatization, either to help stabilize metabolism during typical environmental fluctuations (long-term acclimatization) (Lambers, 1982; Clifton *et al.*, 2006) or to help counteract the effects of environmental stress (i.e. extremes; Juszczuk & Rychter, 2003). Our data for reproductive tissue provide support for the former. The two AOX plasticity types were significantly associated with climate type (prevailing natural summer temperatures), and the negative plasticities for the two cool-summer populations F-L and I-B significantly differed from the positive plasticity of the warm-summer I-CA population. Thus, AOX may be involved in long-term acclimatization to both cool and warm temperatures.

Because our experimental temperatures represented those to which the source populations are commonly exposed during the reproductive season and because AOX was measured in tissue developed at these temperatures, our study does not address whether AOX is involved in a response to uncommon temperature extremes, or to rapid changes in temperature. Different experimental methodologies are needed to address properly these subjects (Atkin & Tjoelker, 2003; Arnholdt-Schmitt *et al.*, 2006; Armstrong *et al.*, 2008). However, if AOX production were stimulated mainly by uncommon temperatures, then one would predict for our experimental temperatures, that negative plasticity would predominate for the warm-summer populations and genotypes and positive plasticity would predominate for the cool-summer populations and genotypes. We observed the opposite result in reproductive tissue and a uniform response to cool temperature in leaves.

The involvement of AOX in long-term thermal acclimatization of reproductive tissue is also supported by its relationship to floral reflectance. The two warm-summer populations I-A and I-CA, which showed little reflectance plasticity, consisted of genotypes that displayed primarily positive AOX plasticity. Further, a significant negative correlation between AOX content plasticity and floral reflectance plasticity was found among all genotypes. One would not predict these associations if only floral reflectance was involved in thermal acclimatization.

Evidence supports independent functions for AOX and reflectance plasticities in thermal acclimatization rather than a direct functional link between the traits. Part of this evidence is the lack of absolute correspondence between the two traits at either the population or genotype level. Other considerations also argue against a direct functional link. Increased AOX content at cool temperatures could help to support the metabolism that leads to increased anthocyanin production, which underlies the decreased reflectance at 550 nm (cf. Stiles *et al.*, 2007). Alternative oxidase has been associated with anthocyanin production at low temperature in *Arabidopsis thaliana* (Fiorani *et al.*, 2005). However, AOX protein was undetectable in floral bracts where anthocyanins occur in *P. lanceolata* spikes (A. Umbach, unpublished). Alternative oxidase activity contributes to the warming of floral tissues in a few specialized thermoregulatory plant species (Watling *et al.*, 2006) and, theoretically, AOX could add independently to the warming brought about by decreased spike reflectance. However, the amount of AOX protein and a spike's morphology probably could not support thermogenesis (Breidenbach *et al.*, 1997). Also, the greater AOX content in warm-summer genotypes is contradictory to only a thermogenic role. Therefore, more likely AOX has a thermal acclimatization function different from the thermoregulatory function of floral reflectance.

A few studies suggest that AOX may be critical for normal pollen development (Conley & Hanson, 1994; Kitashiba *et al.*, 1999; Abe *et al.*, 2002) and AOX, which is detectable in *P. lanceolata* anthers (A. Umbach, unpublished), has been linked to low temperature tolerance of pollen formation (Abe *et al.*, 2002). These observations, together with the results of our study are consistent with a role of AOX in long-term acclimatization to both warm and cool temperature by providing flexibility to metabolic processes in the anther through, for example, reductant recycling and facilitation of TCA cycle operation (Moore *et al.*, 2002; Fiorani *et al.*, 2005; Clifton *et al.*, 2006). Further work is needed to address these possibilities.

It is not yet known if our observed changes in AOX at the whole-tissue level reflect changes in AOX content within mitochondria (Stewart *et al.*, 1990; Gonzàlez-Meler *et al.*, 1999; Kurimoto *et al.*, 2004) or are the result of changes in mitochondrial number (Armstrong *et al.*, 2006). Also, although AOX protein amounts provide a measure of steady-state metabolism that gene transcripts do not (Gibon *et al.*, 2004), and although AOX protein concentration correlates well with AOX capacity (Stewart *et al.*, 1990; Kurimoto *et al.*, 2004; Rachmilevitch *et al.*, 2007), it is not yet known if *in vivo* alternative pathway activity (Gonzàlez-Meler *et al.*, 1999; Millenaar *et al.*, 2001; Rachmilevitch *et al.*, 2007) in reproductive spike tissues is affected by growth temperature.

These unanswered questions do not diminish the importance of our results. Many studies have demonstrated that leaves acclimatize to low or elevated temperatures by modifying respiration (Billings *et al.*, 1971; Larigauderie & Körner, 1995; Atkin *et al.*, 2005; Campbell *et al.*, 2007). However, it is still unclear if plants from cold climates or more variable climates have greater acclimatization ability (Larigauderie & Körner, 1995; Atkin & Tjoelker, 2003). Even less is known about respiratory acclimatization in reproductive structures. Published studies examining temperature effects on floral respiration have focused on thermogenic species, which are predominantly aroids (Seymour & Blaylock, 1999; Watling *et al.*, 2006). When viewed in the larger context of respiration, our study suggests that respiration in natural populations of *P. lanceolata* is genetically variable within and among populations, and is more variable in reproductive structures than in leaves.

Because we used populations of plants originating from several climates, we were able to exploit differences in long-term thermal adaptation within a species, rather than comparing across species. The observed variability in AOX content and plasticity may help to reconcile the results of previous studies.

For example, intraspecific tissue-dependent genetic variability in AOX content or plasticity could underlie the conflicting findings regarding the relationship of the alternative pathway to cold tolerance in inbred lines of *Zea mays* and *Triticum aestivum* (McCaig & Hill, 1977; Stewart *et al.*, 1990; Ribas-Carbo *et al.*, 2000, Kurimoto *et al.*, 2004). While at first, genetic variability in AOX parameters may appear to be an impediment to understanding its relationship to plant physiology, if used appropriately, this variation can help clarify the function of the alternative respiratory pathway in nature.

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Supporting Information

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

Fig. S1 Spectral scans showing mean spike reflectances for the experimental *Plantago lanceolata* populations (symbols identified in Table 1) grown at warm ('W', thin lines) and cool ('C', thick lines) temperatures.

Fig. S2 Detection of alternative oxidase (AOX) on immunoblots prepared with (+) and without (–) dithiothreitol (DTT) in the gel sample buffer for the same tissue samples.

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