PART OF A SPECIAL ISSUE ON REACTIVE OXYGEN AND NITROGEN SPECIES

Polyphenol oxidase-mediated protection against oxidative stress is not associated with enhanced photosynthetic efficiency

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Received: 13 February 2015 Returned for revision: 16 March 2015 Accepted: 21 April 2015 Published electronically: 3 June 2015

Polyphenol oxidases (PPOs) catalyse the oxidation of monophenols and/or o-diphenols to highly reactive o-quinones, which in turn interact with oxygen and proteins to form reactive oxygen species (ROS) and typical brown-pigmented complexes. Hence PPOs can affect local levels of oxygen and ROS. Although the currently known substrates are located in the vacuole, the enzyme is targeted to the thylakoid lumen, suggesting a role for PPOs in photosynthesis. The current study was designed to investigate the potential involvement of PPOs in the photosynthetic response to oxidative stress.

Methods Photosynthesis ($A$, $F_{v}/F_{m}$, FPSII, $q_{N}$, $q_{P}$, NPQ) was measured in leaves of a wild-type and a low-PPO mutant of red clover (Trifolium pratense ‘Milvus’)$'$ under control conditions and under a stress treatment designed to induce photooxidative stress: cold/high light (2 °C/580 μmol m$^{-2}$ s$^{-1}$) or 0–10 μM methyl viologen. Foliar protein content and oxidation state were also determined.

Key Results Photosynthetic performance, and chlorophyll and protein content during 4 d of cold/high light stress and 3 d of subsequent recovery under control growth conditions showed similar susceptibility to stress in both lines. However, more extensive oxidative damage to protein in mutants than wild-types was observed after treatment of attached leaves with methyl viologen. In addition, PPO activity could be associated with an increased capacity to dissipate excess energy, but only at relatively low methyl viologen doses.

Conclusions The presence of PPO activity in leaves did not correspond to a direct role for the enzyme in the regulation or protection of photosynthesis under cold stress. However, an indication that PPO could be involved in cellular protection against low-level oxidative stress requires further investigation.

Key words: *Trifolium pratense* ‘Milvus’, red clover, polyphenol oxidase, PPO, cold, methyl viologen, photosynthesis, chlorophyll fluorescence, protein oxidation, reactive oxygen species, ROS.

INTRODUCTION

Polyphenol oxidases (PPOs) are type-3 copper enzymes which catalyse the oxidation of monophenols and/or o-diphenols to o-quinones. The cresolase or monophenolase activity (EC 1.14.18.1) of the enzyme is not recognized for all plant species with known PPO activity (Steffens et al., 1994; Solomon et al., 1996). PPOs are therefore often sub-categorized as tyrosinases, with both cresolase and catecholase activity, or as o-diphenolic-specific catecholases (EC1.10.3.1) (Steffens et al., 1994). Because of the highly reactive nature of the o-quinone end-products of PPO activity, secondary reaction products include potentially cytotoxic reactive oxygen species (ROS) and brown o-quinone-protein complexes, as is commonly observed following the wounding of fruits (Steffens et al., 1994).

Although PPO activity has been recognized since 1895, its function in plants is still debated. PPO is encoded in the nucleus with a bi-partite N-terminal transit peptide sequence for targeting to the thylakoid lumen (Somer et al., 1994; Keegstra and Cline, 1999). This targeting has been confirmed for the red clover (T. pratense ‘Milvus’) plant material used here (Webb et al., 2013). While this seems to be a prerequisite for most PPOs, recent studies have identified PPO protein without the chloroplast targeting sequence (Tran et al., 2012) which can accumulate in the cytosol (Nakayama et al., 2000, 2001) and vacuoles (Ono et al., 2006). PPO is expressed differentially in shoot, leaves, stems, roots and nodules according to expression of one or more products of the multigene family (Sullivan et al., 2004; Webb et al., 2013). In the leaves, PPO activity has most often been related to pathogen (Bashan et al., 1987; Thipyapong et al., 2004a) and arthropod defence mechanisms (Felton et al., 1989; Kowalski et al., 1992). Where substrates are localized to the vacuoles and the PPO protein to the chloroplasts (Mayer and Harel, 1979; Vaughan and Duke, 1984) the o-quinone–protein complexes formed following pathogen-induced cell damage are believed to reduce the nutritional value of the tissue (Felton et al., 1989; Thipyapong et al., 2004a). Yet it is still unclear why the nuclear-encoded PPO protein is targeted to the thylakoid lumen (Arnon, 1949; Tolbert, 1973;
The ability to manipulate PPO expression (Lee et al., 2004; Sullivan et al., 2004; Thipyapong et al., 2004b; Winters et al., 2008; Araji et al., 2014; Webb et al., 2014) provides an opportunity to test this principle. Here we have used previously characterized wild-type and a low leaf PPO mutant of *T. pratense* ‘Milvus’ (Lee et al., 2004; Winters et al., 2008) to test the hypothesis that plants containing PPO have enhanced photosynthetic performance by limiting oxidative stress through down-regulating photosynthetic electron transport during periods of environmental stress.

**MATERIALS AND METHODS**

**Plant material**

Wild-type Aa 4381 and low PPO mutant ABY-Aa 4521 red clover plants (*Trifolium pratense* L. 1753 ‘Milvus’; Lee et al., 2004) were grown from seeds in John Innes No. 3 compost in controlled environment conditions and were kept well-watered. For the cold/high light stress study, ten replicate plants were grown until 74 d old in a growth cabinet [20/16 °C day/night, 16-h photoperiod and a photosynthetic photon flux density (PPFD) of \(\sim 300 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}\) at canopy level]. For each plant, two mature trifoliate leaves were randomly selected to monitor the carbon assimilation rate and chlorophyll fluorescence (\(F_{v}/F_{m}\) (PSII), \(q_{P}\), \(q_{N}\), NPQ; see below) with GFS-3000FL and PAM-2000 instruments, respectively (Heinz Walz GmbH, Effeltrich, Germany) as described below. These leaves were then excised, flash frozen in liquid nitrogen and stored at –80 °C. At the end of day 0, five randomly selected replicates were placed in an identical controlled environment cabinet except that temperature conditions were 2 °C day/night, 580 \(\mu\text{mol} \text{m}^{-2} \text{s}^{-1}\) and a 16-h photoperiod. Control and stress-treated plants were monitored as before for a further 4 d. The entire experiment was repeated three times using new plant material on each occasion. Also, plants (three random replicates of each genotype as control and five stress-treated) were treated as above and their recovery monitored over 3 d following their return to control growth conditions at the end of day 4.

Where the effect of methyl viologen (MV) was to be tested, mature greenhouse-grown wild-type and mutant red clover plants were defoliated to ~9 cm above soil surface, clonally replicated by division of entire plants and grown for at least 1 month under controlled environment conditions (20/16 °C day/night at 400 \(\mu\text{mol} \text{m}^{-2} \text{s}^{-1}\) PPFD with a 12-h photoperiod). Clonal replicates were pre-assessed to minimize biological variation and improve the likelihood of identifying a PPO-related response to MV. Four clones of both mutant and wild-type were selected for similarity (allowing 10% error) in terms of chlorophyll fluorescence (\(F_{v}/F_{m}\), \(\Phi_{PSII}\), \(q_{P}\), NPQ; see below), total leaf protein and chlorophyll content, and total PPO activity. On the day of treatment, 1 h after the start of the photoperiod, each plant received both control and MV treatments by treating individual trifoliate leaves which were selected at random, ensuring that there was no leaf–leaf contact. Treatments included an untreated control (no spray solution was applied) as well as 0–5% Tween 20 containing 0, 1, 2, 5, 7.5 and 10 \(\mu\text{M}\) MV. The solutions were freshly prepared and applied as a saturating dosage of approx. 0.04 \(\mu\text{L} \text{cm}^{-2}\) of trifoliate leaf surface area using an airbrush model 250 (Badger Air-Brush Co.,
http://www.badgerairbrush.com). Alternating between genotypes, the plants were sequentially removed from the growth cabinet, sprayed in a contained area and immediately returned to the illuminated cabinet. Chlorophyll fluorescence measurements \( (F_{v}/F_{\infty}) \), light-adapted \( (F_{v}/F_{m} \), \( q_{N} \), NPQ) were taken with the Handy PEA fluorimeter (Hansatech Instruments, Norfolk, UK) as soon as the leaves had dried (2-5 h after treatment application) and again at 4-5 and 7-5 h after treatment application. Trifoliates from each genotype \( \times \) treatment group were harvested, flash frozen in liquid nitrogen and stored at -80 °C until analysis.

**Photosynthetic measurements**

Unless otherwise stated, photosynthetic parameters were measured 5 h after the onset of the photoperiod, alternating between treatment and genotype. Leaf CO\( \text{2} \) assimilation rates were measured in vivo using a GFS-3000FL system (Heinz Walz, Effeltrich, Germany) incorporating a red–blue LED light source (Walz 3055-FL). Reference [CO\( \text{2} \)] was set at 380 \( \mu \text{mol mol}^{-1} \), the leaf temperature was maintained at 20 °C and vapour pressure deficit was maintained at 6-6 kPa (Long and Bernacchi, 2003; Purdy et al., 2013). Chlorophyll fluorescence parameters were measured using a PAM-2000 portable fluorometer (Heinz Walz) which was operated via the Data Acquisition software DA-2000 or PamWin to estimate the maximum quantum yield of PSII photochemistry \( (F_{v}/F_{\infty}) \), the quantum efficiency of PSII electron transport \( (\Phi_{PSII}) \), the degree of photochemical \( (q_{P}) \) and non-photochemical quenching of chlorophyll fluorescence \( [\text{estimated both as } q_{N} (F_{m}-F_{m})/(F_{m}-F_{0}) \text{ and } NPQ (F_{m}-F_{m})/F_{m}^{-1} \text{; Genty et al.}, 1989; Baker, 2008; Murchie and Lawson, 2013] \). Randomly selected, mature and fully expanded leaves were dark adapted under treatment conditions for at least 5 min before measuring first the dark-adapted \( (F_{v}/F_{m}) \) and subsequently the light-adapted \( (A, \Phi_{PSII}, q_{P}, q_{N} \text{ and NPQ}) \) parameters. Measurements of net carbon assimilation rate and light-adapted electron transport were undertaken, first at the growth irradiance of 300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) after which PPFD was increased to 1300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (determined in preliminary experiments to be saturating for CO\( \text{2} \) assimilation). In each case measurements of light-adapted electron transport were collected once a steady-state carbon assimilation rate had been established (Wang et al., 2012). When using the Handy PEA fluorometer, measurements of light-adapted and dark-adapted fluorescence were made to obtain \( F_{v}/F_{m}^{-1}, F_{v}/F_{m}, \text{ NPQ and } q_{N}. \) Plants were kept inside the growth cabinet and light-adapted minimal \( (F_{d}) \) and maximal chlorophyll fluorescence yield parameters \( (F_{\infty}^{-1}) \) were measured first. The leaf clip shutter was then closed for dark adaptation while light-adapted measurements of the remaining trifoliates continued. Once all were measured and the leaf measuring areas had sufficiently dark adapted, \( F_{d} \) and \( F_{\infty} \) were measured. Dark-adapted \( F_{v}/F_{m}^{-1}, \text{ light-adapted } F_{v}/F_{m} \), NPQ and \( q_{N} \) were calculated as before.

**Protein extraction and quantification**

Frozen leaf samples were ground to a fine powder in liquid nitrogen. Protein was extracted by addition of 5 mL g\( ^{-1} \) f. wt of extraction buffer \( (0.2 \text{m Na}_{2}\text{HPO}_{4}, \text{ pH } 7 \) and 71 mM ascorbic acid, containing 0.1 % Triton X-100 and 0.5 % of protease inhibitor cocktail; Sigma protease cocktail plant, Sigma Aldrich, Poole, UK), which was further ground to a smooth homogenate. If the extract was to be used for measurements of PPO activity the Triton X-100 and protease inhibitor were omitted from the extraction buffer. Where required, aliquots of the homogenate were taken for chlorophyll quantification (Amon, 1949). The homogenate was then centrifuged at 10 000 g for 10 min at 4 °C. The protein content of the clarified homogenates was estimated spectrophotometrically (Bradford, 1976) with reference to a bovine serum albumin standard curve.

**Determination of PPO activity**

Prior to each experiment, the PPO phenotype of the wild-type and mutant plants was confirmed either by a rapid PPO phenotype screening assay adapted from Winters et al. (2008) or by measuring total (active + latent) PPO enzyme pool of mature leaves under control growth conditions. For the rapid PPO phenotype screening assay, protein was extracted in a liquid nitrogen pre-cooled pestle and mortar in McIlvaine buffer (pH 7) at a ratio of 5 mL g\( ^{-1} \) f. wt. After centrifugation at 10 000 g for 10 min at room temperature, the colour of the supernatant was scored; a brown colour indicated a wild-type phenotype, while retention of the initial green colour indicated a low PPO mutant phenotype. Alternatively, PPO activity in protein extracts which had been desalted to McIlvaine buffer, pH 7, on pre-conditioned columns of Bio-Gel P6DG (Bio-Rad Laboratories, Hemel Hempstead, UK) was measured spectrophotometrically according to Winters et al. (2008), with minor modifications. Standard reactions were carried out in 1.5 mL containing 20 \( \mu \text{L} \) of desalted extract, 0.15 mM copper sulphate and 9.87 mm 4-methylcatechol in McIlvaine buffer (pH 7) alone or including 0.26 % (w/v) SDS. The reaction was initiated by addition of 4-methylcatechol and the change in absorbance at 420 nm was monitored during the initial 25 s. Rates were calculated from the linear phase of the curve as \( \Delta \text{optical density} \) with SWIFT II software (Biochrom, Cambridge, UK). One unit of PPO enzyme (1 U) was defined as the amount of enzyme that produced 1 \( \mu \text{mol} \) of quinone per minute and is expressed per mg protein in the desalted PPO enzyme extract. A conversion factor of \( U=2.717 \times \Delta \text{optical density} \) was used for the calculations (Alder and Magnussen, 1959; Lee et al., 2008).

**Protein characterization**

SDS–PAGE was performed according to Laemmli (1970) with either hand-cast 12.5 % single percentage gels or linear gradient pre-cast gels (4–20 % Mini-PROTEAN TGX gels or 10–20 % Ready Gel Tris–HCl gels; Bio-Rad). Protein was electrothermally transferred to nitrocellulose filters and PPO was detected immunologically with anti-red clover (T. pratense) PPO1 primary antiserum (a kind gift from M. L. Sullivan; Sullivan et al., 2004) used at 1 : 1000 dilution and alkaline phosphatase-conjugated swine anti-rabbit secondary antiserum (Dako, Glostrup, Denmark) used at 1 : 2000 dilution. Cross-reactions were detected as dark blue formazan bands formed by reduction of nitroblue tetrazolium and were recorded digitally with a GS-800 calibrated densitometer (Bio-Rad).
Alternatively, samples containing 4 μg protein were diluted to a final volume of 200 μL with extraction buffer (0.2 M Na₂HPO₄, 71 mm ascorbic acid, pH 7) and were transferred to nitrocellulose with the Bio-Dot SF Microfiltration Apparatus (Bio-Rad).

Oxidized proteins were detected using the Oxyblot Protein Oxidation Detection Kit according to the manufacturer’s instructions (Millipore, Watford, UK). Cross-reactions were detected by enhanced chemiluminescence and recorded by exposure to blue-light-sensitive film (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis

Statistical analyses of photosynthetic and biochemical data were performed with GenStat 13.2 (VSN International, Hemel Hempstead, UK) using restricted maximum likelihood (REML) or ANOVA functions. Where necessary data were transformed and normal distribution confirmed by testing for homogeneity (Bartlett’s test) before statistical analysis. The significance of the observations was determined using a Bonferroni multiple comparison test (adjusted $P < 0.05/n$ with $n$ number of comparisons).

RESULTS

Effect of presence of PPO activity on photosynthesis under cold stress

The effect of exposure to and recovery from a cold/high light stress was determined in leaves of wild-type and mutant plants. No significant differences were observed between dark-adapted $F_v/F_m$ of wild-type and mutant red clover at any time under control growth conditions (Fig. 1A). In contrast, a progressive decrease was observed for both lines immediately after initiation of the cold/high light stress treatment at the end of day 0. The $F_v/F_m$ values of wild-types and mutants during exposure to the stress were not significantly different except for day 4 (Fig. 1A). When measured under control growth conditions, the CO₂ assimilation rate $A$ was typically greater in mutants than in wild-types although this was only significant on day 2 (Fig. 1B). As a result of the stress treatment, $A$ declined rapidly and was complete within 1 d of treatment, but there were no significant differences between the lines at any point. This effect was observed under both growth and saturating irradiances (Fig. 1B, Supplementary Data Fig. S1A). Similarly, the $\Phi$PSII of both lines was comparable under control growth conditions and declined in response to the cold and high light stress treatment (Fig. 1C). The decline in $\Phi$PSII was attributed largely to an early increase in $q_N$ (day 1; Fig. 2A) and a later (day 3) decrease in $q_p$ under growth irradiance (Fig. 2B). Differences in $q_N$ between control and treated mutants were greater than between control and treated wild-types (Fig. 2A). Treatment differences in $q_N$ were less obvious when measurements were made under saturating irradiance (Supplementary Data Fig. S2). Measurements of $\Phi$PSII, $q_N$ and $q_p$ under saturating irradiance did not amplify any potential genotypic differences regardless of treatment (Figs S1B, S2). Regardless of measurement irradiance, no significant differences between lines or treatments were observed when non-photochemical quenching was calculated as $NPQ$ (data not shown).

Effect of cold stress on PPO activity, protein and chlorophyll content

Measurements of total PPO activity levels (active + latent) confirmed the PPO phenotype of the two red clover genotypes, with significantly higher activities being recorded for wild-types (48.98 U of PPO enzyme mg⁻¹ protein) than mutants (0.25 U of PPO enzyme mg⁻¹ protein) regardless of the treatment conditions (adjusted $P < 0.0083$). No change in the PPO activities of either mutants or wild-types was observed regardless of treatment, such that these differences between the lines persisted throughout the stress treatment period, with no evidence for either loss of activity from wild-type or induction of activity in mutants (Fig. 3). In the wild-type, total activity typically exceeded the active pool by two-fold (Fig. 3).

Immunodetection of PPO confirmed that the lack of activity in the mutants was due to absence of the PPO protein, detected at molecular weights of 59.9 ± 1.3 and 46.7 ± 1.0 kDa (Fig. 4A). No changes in PPO protein abundance were observed in response to treatment or recovery (Fig. 4A) and the total protein profile was similar between the two red clover lines regardless of treatment (Fig. 4B). Similarly, apart from day 7, there were no significant differences in chlorophyll content between the lines under optimal growth conditions as a result of the stress treatment and subsequent recovery (Supplementary Data Fig. S3). Over the duration of the experiment, the chlorophyll content averaged 1.89 ± 0.03 mg chlorophyll (Chl a + Chl b) g⁻¹ f. w. regardless of line or treatment (Fig. S3) and the Chl a/Chl b ratio was similar for wild-type and low PPO mutants under control and stress conditions and in the subsequent recovery phase (data not shown).

Effect of PPO on photosynthetic response to oxidative stress induced by MV

Rapid phenotype screening of the two mother plants prior to cloning was used to confirm their wild-type and low PPO mutant phenotype. Individual trifoliate leaves from the clonal replicates were exposed to MV and the effect on photosynthesis was assessed. In the wild-type an effect of MV on photosynthetic parameters was observed at doses between 2.5 and 10 μM and was most apparent 7.5 h after treatment (Fig. 5) (data for 2.5 and 4.5 h not shown). In contrast, the mutant was largely unaffected by MV treatment (Fig. 5). At MV doses of 2.5 μM and above, wild-types exhibited lower light-adapted $F_v/F_m$ and higher NPQ than the mutant. The high variability seen in the data could have been a result of high genetic diversity due to complexity of the PPO gene family and the fact that red clover is an outbreeder. MV treatment had no effect on measurements of active PPO (mean of 3.18 ± 0.63 and 0.24 ± 0.03 U mg⁻¹ protein for the wild-type and mutant, respectively).
respectively) despite a linear increase in total (active + latent) PPO activity levels in leaves of wild-type plants (Fig. 6A). The total PPO activity of the wild-type leaf used for 0 μM treatment (spray treatment control) appears to be an outlier, but it was included in the interest of clarity. It is possible that in this case despite selection of leaves at a similar developmental stage (mature fully expanded) for treatment, the measurement made on the trifoliate leaves treated with 0 μM MV could have been of a different chronological age to the others and hence contained PPO activity inconsistent with the other samples (Lanker et al., 1987; Goud and Kachole, 2012).

The presence of PPO was associated with protein quality, as a higher degree of protein oxidation was consistently observed in leaf extracts from mutants than was present in samples from wild-type leaves (Fig. 7A). A comparison of the protein oxidation profiles in wild-type and mutant leaves indicated that the increased intensity of protein oxidation in mutants was not the result of additionally oxidized protein species, but rather...
because of an increase in oxidation of those proteins targeted in the wild-type. These proteins had estimated molecular weights of $124 \pm 6.2$, $115 \pm 6.2$, $106 \pm 1.12$, $51.3 \pm 0.94$, $46.8 \pm 0.84$ and $27.5 \pm 1.07$ kDa (Fig. 7B). Specificity of the detection was confirmed by lack of signal from comparable negative controls (data not shown).

**DISCUSSION**

PPO protein and activity have previously been associated with oxygen-evolving chloroplasts (Arnon, 1949; Tolbert, 1973; Vaughn and Duke, 1984; Sommer et al., 1994; Webb et al., 2013). Given the close proximity of the PPO protein to PSII machinery (Lax and Vaughn, 1991), it has been argued that PPO could protect photosynthetic electron transport from over-reduction during periods of environmental stress, such as drought, chilling and nutrient limitation, when electron sink capacity is reduced compared with the rate of electron capture (Tolbert, 1973; Vaughn and Duke, 1984; Thipyapong et al., 2004b; Boeckx et al., 2015). Here we have used wild-type and a low PPO activity mutant to explore the potential role of PPO in enhancing photosynthetic activity under stress.

Changes in PPO activity in response to abiotic stress have been reported previously for several plant species but responses are not consistent between them. For instance, cold stress caused a significant decrease in total PPO activity in watermelon plants when transferred from 35 to 15 °C (Rivero et al., 2001) while exposure of olive trees to winter temperatures of below −7 °C resulted in a significant increase in the active PPO pool (Ortega-Gracia and Peragón, 2009). In the experiments reported here, the active PPO enzyme pool was remarkably stable during periods of exposure to either cold or MV treatments and subsequent recovery (Fig. 3A), with no evidence found for stress-induced loss of the enzyme from wild-type or induced expression in mutants (Fig. 4A). Two polypeptide bands (of 59.9 and 46.7 kDa) were found to cross-react with the anti-PPO1 antiserum (Fig. 4A) and corresponded with previous reports of two PPO active immunodetected protein products of 60–65 and 42–45 kDa (Robinson and Dry, 1992; Schmitz et al., 2008). Detection of multiple polypeptides is not unexpected as
The red clover PPO gene family is thought to include at least six PPO genes (Winters et al., 2009) encoding precursor molecules with a molecular weight range between 68 and 71 kDa (Sullivan et al., 2004; Winters et al., 2009), requiring proteolytic processing to the mature forms (Sommer et al., 1994; Schmitz et al., 2008; Winters et al., 2008). The anti-PPO1 antiserum is also known to cross-react with red clover PPO2 and 3 (Sullivan et al., 2004), and PPO1, PPO4 and PPO5 gene expression has been demonstrated in mature leaves of wild-type red clover (Webb et al., 2013).

The adjustment of metabolism to withstand severe cold often includes an increase in soluble protein content and a subtle qualitative shift in protein content (Siminovitch and Briggs, 1952; Li et al., 1996; Strand et al., 1999; Liu et al., 2012). This process can be rapid, as in the case of alfalfa, where 2 d at 4 °C resulted in a three-fold increase in whole-plant soluble protein content and de novo synthesis of cold acclimation-specific proteins (Mohaptra et al., 1987a, b). In winter wheat cold treatment resulted in increased chlorophyll content (Atici et al., 2003). In contrast, there was no effect of cold treatment on the foliar protein (Fig. 4B) or chlorophyll (Supplementary Data Fig. S3) contents of wild-type and mutant red clover, even after 4 d at 4 °C, a similar response to that of cabbage (Atici et al., 2003).

The mutant and wild-type red clover lines used here have previously shown differential productivity in the field with the dry-matter yield per hectare of the wild-types (57.88 tonnes ha⁻¹) exceeding that of the mutants (54.40 tonnes ha⁻¹) over the same growing season (R. Fychan, Aberystwyth University, Aberystwyth, UK, unpubl. res.). One possible explanation for this is that PPO has an effect on photosynthetic efficiency, in particular under stresses frequently encountered in the field. Previously suggested roles for PPO have included pseudocyclic photophosphorylation (Tolbert, 1973), and as a modulator of the Mehler peroxidase cycle (Vaughn and Duke, 1984). However, the flux through these pathway(s) where PPO accepts electrons via its copper moiety would have to be extremely high to cope with the electron flux generated during photosynthesis, regardless of the physical limitations of sub-cellular localization of PPO (thylakoid) and the Mehler reaction (stroma). It is therefore doubtful that flux through PPO would be sufficient to make a significant impact on photosynthetic efficiency, which is in agreement with the observed similarity of operation of carbon assimilation and the photosystems in mutant and wild-type leaves under stress (Figs 1 and 2 and Supplementary Data Figs S1 and S2), and with previous data (Thipyapong et al., 2004b).

Regardless of the presence of PPO, red clover carbon metabolism was susceptible to cold with CO₂ assimilation rates of cold-treated plants decreasing to less than half that observed in control plants after 1 d (Fig. 1B). This is likely to have been due to stomatal closure (Sonoike, 1998) and a decrease in carboxylation activity (Brüggemann, 1995; Byrd et al., 1995).
Similarly, the rapid decrease in maximum potential efficiency of PSII, 

\[ \frac{F_v}{F_m} \]

in both genotypes on exposure to the stress treatment (Fig. 1A) is a common response of non-acclimated plants to cold and high light (Krause et al., 1989; Gray et al., 2003; Zhou et al., 2004). This was almost exclusively the result of a decline in 

\[ F_m \]

with an almost negligible increase in the dark-adapted 

\[ F_o \]

(data not shown).

Cold and high light stress has been previously reported to affect both these parameters to different extents depending on conditions. For instance in spinach, exposure to 4°C and 550 μmol m⁻² s⁻¹ PPFD caused an increase in 

\[ F_o \]

with no change to 

\[ F_m \]

(Krause et al., 1989) whereas sub-zero conditions and 900 μmol m⁻² s⁻¹ PPFD decreased 

\[ F_m \]

with no effect on 

\[ F_o \]

(Somersalo and Krause, 1990). Although such data have to be interpreted carefully because of potential changes in leaf water status (Baker, 2008), in general a decrease in 

\[ F_m \]

would suggest that regardless of the presence of PPO, red clover can induce mechanisms to dissipate excess energy when under cold/high light stress (Muller et al., 2001; Baker, 2008). The increase in 

\[ q_N \]

following cold and high light stress treatment agrees with this deduction (Fig. 2D). As a result, maximum efficiency of PSII electron transport was affected during the stress treatment,
as indicated by the strong decrease in ΦPSII in both mutant and wild-type (Fig. 1C). Also, the rate and extent of recovery from the stress treatment on return to optimal growth conditions was comparable between the two lines and was achieved rapidly (Figs 1 and 2 and Supplementary Data Figs S1 and S2). A similarly rapid recovery of photosynthesis has been observed previously after cold treatment of cucumber (Zhou et al., 2004).

Overall these results provide no evidence that increased PPO activity in red clover leaves could protect the photosynthetic machinery from damage or down-regulation under low temperature stress.

It was possible that the lack of differential response in cold-treated mutant and wild-type red clover was due to treatment conditions being too harsh for the capacity of any possible PPO-mediated protective mechanism. Therefore, a proof of principle study was used to explore the impact of PPO activity on the dose-dependent effect of oxidative damage, generated by foliar application of increasing doses of the pro-oxidant MV. Notably, increasing the concentration of MV above 2.5 μM resulted in a tendency for increased non-photochemical quenching in the wild-type but not in the mutant leaves, which were unchanged in comparison with control values (Fig. 5C, D). The increased non-photochemical quenching in the wild-type in the absence of changes in \( F_{v}/F_{m} \) suggested that a currently unidentified non-photochemical quenching-based adaptation could have occurred in the absence of photoinhibition (Björkman and Demmig, 1987) but if so is subject to considerable phenotypic variability and even so may be only indirectly linked to the presence of PPO. Furthermore, the induction of non-photochemical quenching in wild-type leaves appears to be a specific response to a mild-to-medium range of oxidative stress; increased non-photochemical quenching capacity was not observed after a single application of a concentration greater than 10–15 μM MV to wild-type leaves and differences...
in chlorophyll fluorescence between wild-types and mutants became much less apparent than those seen in Fig. 5 (data not shown).

Although apparently operational over a limited stress range, PPO activity was found to have implications for susceptibility of cellular components to oxidative damage, as evidenced by the relative increase in protein oxidation in mutant as compared with wild-type leaves, even when maintained under control conditions (Fig. 7). Candidate proteins subject to oxidation include the large subunit of Rubisco at 58-5 kDa and the D1 polypeptides of PSI and PSII reaction centres, respectively (Kingston-Smith and Foyer, 2000). Differences in the apparent impact of PPO on the responses of red clover to cold and MV could be because cold tolerance is a complex trait, for instance also being associated with membrane composition (Chen et al., 2014). Red clover is also relatively more cold tolerant than many other PPO-containing species (e.g. tomato), which could explain differences in responses across species.

In summary, the work described here is in agreement with the argument that despite its chloroplast location, the presence of PPO activity in leaves does not correspond with a direct role for the enzyme in regulation or protection of photosynthesis. However, limited evidence for the involvement of PPO in protection against low-level, chloroplastically derived oxidative stress was found and further studies to determine which component of the NPQ parameter (qN, qT or qL) is involved in the response could be helpful in elucidating the mechanism of PPO in chloroplasts. It is possible that PPO may have an indirect role via secondary metabolism, for instance as a participant in phenylpropanoid metabolism, which can promote energy dissipation under excess light conditions (Grace and Logan, 2000).

Identification of chloroplastic substrates for PPO participation under excess light conditions (Grace and Logan, 2000). Phenylpropanoid metabolism, which can promote energy dissipation in chloroplasts. It is possible that PPO may have an indirect response could be helpful in elucidating the mechanism of PPO.

SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: effect of chilling and return to optimal growth conditions on photosynthesis in leaves of wild-type and mutant red clover. Figure S2: changes in non-photochemical quenching and photosynthetic quenching measured at saturating irradiances in response to control and stress conditions. Figure S3: total leaf chlorophyll content of wild-type and low-PPO mutant under control and stress conditions. Figure S4: change in Fv/Fm values of wild-type and low-PPO mutant under control and stress conditions.

ACKNOWLEDGEMENTS
We thank and acknowledge Teri Davies for her assistance in the lab, Alan Cookson for his help in operating the controlled environment growth cabinets and Sylvia Lutkin for her advice on aspects of the statistical analysis. This work was supported by an IBERS postgraduate studentship (T.B.), BBSRC Institute Strategic Programme Grant BBS/E/W/10964A01 (A.K-S.), and the European Regional Development Fund through funding provided for the BEACON project by the Welsh European Funding Office (A.W.).
Trifolium pratense L. (red clover) using a large insert BAC library. BMC Plant Biology 9: 94.
