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Polyphenol oxidase in leaves; is there any significance to the chloroplastic localisation?

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Running title: The enigma of polyphenol oxidase in leaves.

Highlights: Why should polyphenol oxidase be located in the thylakoid lumen if it is not associated with photosynthesis? We consider whether recent evidence makes the answer any clearer.

Abstract

Polyphenol oxidase (PPO) catalyses the oxidation of monophenols and/ or *o*-diphenols to *o*-quinones with the concomitant reduction of oxygen to water which result in protein complexing and the formation of brown melanin pigments. The most frequently suggested role for PPO in plants has been in defence against herbivores and pathogens, based on the physical separation of the chloroplast localised enzyme from the vacuole localised substrates. The *o*-quinone-protein complexes, formed as a consequence of cell damage, may reduce the nutritional value of the tissue and thereby reduce predation but can also participate in the formation of structural barriers against invading pathogens. However, since a sufficient level of compartmentation-based regulation could be accomplished if PPO was targeted to the cytosol, the benefit derived by some plant species in having PPO present in the chloroplast lumen remains an intriguing question. So is there more to the chloroplastic location of PPO? An interaction between PPO activity and photosynthesis has been proposed on more than one occasion but to date evidence either for or against direct involvement has been equivocal, and the lack of identified chloroplastic substrates remains an issue. Similarly PPO has been suggested to have both pro- and anti-oxidant functions. Nevertheless, several independent lines of evidence suggest that PPO responds to environmental conditions and could be involved in the response of plants to abiotic stress. This review highlights our current understanding of the *in vivo* functions of PPO and considers the potential opportunities it presents for exploitation to increase stress tolerance in food crops.

Keywords: polyphenol oxidase, photosynthesis, abiotic stress, secondary metabolism.

Abbreviations: PPO, polyphenol oxidase; ROS, reactive oxygen species; DM, dry matter.

Introduction

Over the past five decades, yields of wheat and maize crops have decreased by an estimated 1 to 2 % per decade, affecting food supplies for both humans and livestock (IPCC, 2014). In order to feed a growing human population, global food production must be maintained or preferably increased, under increasingly unstable climatic conditions and rising temperatures (IPCC, 2014). New approaches are needed to meet this major agricultural challenge whilst using existing or even reduced tracts of agricultural land (Foresight, 2011).

A key agricultural target is to improve yields of crops growing under such periods of abiotic, as well as biotic, stress. The enzyme polyphenol oxidase (PPO) is found in most plant species and the foliar expressed gene products may have a role in either acclimation or short term response to stress, indicated by circumstantial evidence such as enzyme localisation and its response to environmental factors. PPO has been reported in all land plants surveyed to date with the exception of Arabidopsis. In contrast no PPO-like sequences have been reported in chlorophytes (green algae; Tran *et al.,* 2012). This study showed that the size of PPO gene families varied widely with numbers of PPO genes ranging from one to 13 in the 18 genomes analysed. It is postulated that the occurrence of this enzyme is strongly correlated with the emergence of land plants suggesting a role in adaption to abiotic stresses associated with a dry/non-aquatic environment. However, as yet, there is no conclusive evidence for the existence of an underlying mechanism explaining the relationship between PPO and abiotic stress; indeed it is unclear if the presence of PPO activity is beneficial or detrimental to the plant (Mayer, 2006). While PPO activity can be related to the accumulation of reactive oxygen species (ROS; Thipyapong *et al.*, 2004*b*; Mayer, 2006) and overall redox potential values (Webb *et al.*, 2014), its presence could also be beneficial as a proposed oxygen buffer (Vaughn and Duke, 1984) or by down-regulating photosynthesis (Trebst and Depka, 1995).

Here we review the current state of what is often contradictory hypothetical and experimental evidence regarding the potential of PPO in leaves to confer an advantage in crop production, particularly during periods of abiotic stress, such as drought, heat and cold.

Fundamentals of PPO biochemistry

PPO enzymes from plants are comprised of three domains including an N-terminal plastid transit peptide, a highly conserved type-three copper centre and a C-terminal region (Tran *et al.,* 2012). The family of PPO enzymes catalyse the oxidation of monophenols and/ or *o*-diphenols to *o*-quinones. PPOs are widely distributed in bacteria, animals, plants and fungi (Mayer, 2006; Tran *et al.,* 2012) but are often confused with another subclass of phenol oxidases, the laccases [benzenediol: oxygen oxidoreductase [EC 1.10.3.2] or *p*-diphenol oxidase], which oxidise a broad range of *o*-, *m*- and *p*-diphenols (Griffith, 1994). Plant laccases are mostly extracellular proteins with 22-45 % glycosylation (Solomon *et al.*, 1996) whereas PPOs are intracellular proteins and the degree of glycosylation is unclear (Steffens *et al.*, 1994). Both are multicopper oxidases, however PPOs have a coupled binuclear type-3 copper centre and laccases have a trinuclear cluster of four copper ions (Figure 1.; Solomon *et al.*, 1996).

Subclasses of PPO enzymes are tyrosinases or catecholases according to the absence or presence of cresolase or monophenolase activity (Figure 2.). Tyrosinases first catalyse the oxidation of monophenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1), and subsequently catalyse the *o*-diphenol to *o*-quinone reaction (catecholase activity; EC1.10.3.1). Catecholases on the other hand are *o*-diphenol specific, and thus are only able to catalyse the oxidation of *o*-diphenols to *o*-quinones (Steffens *et al.*, 1994). Both subclasses are however commonly referred to as PPO (Mayer and Harel, 1979; Marusek *et al.*, 2006) since the poor characterisation of these enzymes could mean that some catecholases are in fact tyrosinases in which the monophenolase activity has not yet been observed (Solomon *et al.*, 1996) as detection of monophenolase activity requires highly specific conditions in comparison with catecholase activity (Yoruk and Marshall, 2003).

The primary reaction products of PPO activity are the *o*-quinones, which are highly reactive and will covalently modify and cross-link proteins to form brown melanin pigments (Steffens *et al.*, 1994; Kroll *et al.*, 2008). An example of this is the browning of apples and potatoes, seen shortly after wounding or cutting. PPO-mediated generation of *o*-quinones has also been implicated in the indirect generation of ROS as secondary reaction products (Steffens *et al.*, 1994). Although the precise mechanism remains to be established, reverse disproportionation of *o*-quinones can

result in the formation of cytoplasmic semiquinone radicals (O'Brien, 1991; Thipyapong *et al*., 1997). Interaction between these radicals and O_2 will result in the generation of superoxide anions and the regeneration of *o*-quinone (O'Brien, 1991). Superoxide anions are very unstable and will quickly dismutate, either enzymatically via superoxide dismutase, or non-enzymatically, to form hydrogen peroxide (Grant and Loake, 2000). A Fenton reaction between divalent metal ions such as iron (II) and the relatively stable hydrogen peroxide can result in the generation of extremely reactive hydroxyl radicals. Accumulation of these cytotoxic ROS needs to be under tight control as oxidative modifications including protein cross-linking, lipid peroxidation and damage to nucleic acids may ultimately inflict cell death (Grant and Loake, 2000; Bhattacharjee, 2005; Gill and Tuteja, 2010; Foyer and Noctor, 2012). However, theoretically PPO could also contribute to decreasing the amount of oxygen locally available through the reduction of O_2 to water (Yoruk and Marshall, 2003).

Regulation of PPO is complex and the enzyme can be present in both an active and latent (an inactive, often precursor form) states in the same source material (Mayer and Harel, 1979). Following transport to the lumen and the cleavage of the N-terminal transit peptide, PPO is initially present as a two-domain protein consisting of a copper binding site and a C-terminal domain (Flurkey and Inlow, 2008). A more detailed discussion, including consideration of the 3D structure of catechol oxidases, can be found in Gerdemann *et al.* (2002). The C-terminal is linked via a highly flexible random peptide structure which is proposed to cover the active site and undergo conformational change under certain conditions (Leufken *et al.*, 2014). Flurkey and Inlow (2008) have reviewed evidence for C-terminal proteolytic processing of latent PPO to the active form which has been demonstrated for *Vicia faba*, *Vitis vinifera* and *Ipomoea batatas* PPOs. The degree of latency is not universal and differs with plant species as well as tissue type. For instance PPO activity was detected in both active and latent forms in root tissues of red clover (*Trifolium pratense*), but only in the latent form in white clover (*Trifolium repens*); this contrasts with aerial tissues where PPO activity was detected in both active and latent forms in both red and white clover (Webb *et al.*, 2013). Protein in this latent state is not only activated by proteolytic cleavage but also by chemically inducing conformational changes to the latent enzyme. *In vitro*, treatments that are effective in

activating latent PPO include exposure to fatty acids, proteolysis (trypsin), mild heat, acid and base shocks, and detergents such as SDS and ammonium sulphate (Tolbert, 1973; Steffens *et al*., 1994; Jiménez and García-Carmona, 1996; Yoruk and Marshall, 2003). Leufken *et al.* (2014) observed that the C-terminal domain determines the pH optimum of plant PPOs in non-proteolytic activated enzyme and they postulate that non-proteolytic activation also occurs *in planta*. *In vivo*, activation of the latent PPO pool could occur as a result of direct interaction between the enzyme and its substrates. Winters *et al.* (2008) have demonstrated the potential to activate latent PPO from red clover in the presence of its endogenous *o*-diphenols substrates. It has been proposed that *o*-diphenol-mediated activation is an indirect mechanism of activation, with the resulting *o*-quinones interacting with the latent PPO pool, thereby altering their structure and exposing the active sites (Winters *et al.*, 2008). This is presumably the mechanism occurring upon tissue damage, either as a result of herbivory (Lee *et al.*, 2009), or of senescence associated cell disruption, as reported by Meyer and Biehl (1980) who observed an increase in phenolase activity and concomitant decrease of the latent form of PPO during leaf aging in spinach (*Spinacia oleracea*). More recently Molitor *et al.* (2013) identified a putative quinone binding site in the PPO enzyme, aurone synthase, from *Coreopsis grandiflora* which they propose is responsible for the observed allosteric activation of latent PPO.

The conundrum of PPO compartmentation

Arnon (1949) provided some of the earliest evidence for the intracellular location of PPO in spinach beet (*Beta vulgaris*) chloroplasts. This has been followed up by detailed investigations revealing that in leaves PPO is specifically located in the lumen or loosely attached to the luminal side of the thylakoid membrane (Tolbert, 1973; Mayer and Harel, 1979; Sommer *et al.*, 1994) in the vicinity of photosystems I and II (Lax and Vaughn, 1991). The mechanism by which this is achieved was revealed by Sommer *et al.* (1994) who demonstrated that the nuclear encoded sequences were likely to behave as characteristic thylakoid-targeted proteins which use the light-generated thylakoid pH gradient as the energy source to transport the intermediates of stromal proteins across the thylakoid membrane and into the lumen, otherwise known as the Δ pH pathway (Keegstra and Cline, 1999). More recently

PPOs have also been identified which lack the chloroplast targeting sequence (Tran *et al.*, 2012) and have been found in the cytosol (Nakayama *et al.*, 2000; 2001) and in the vacuole (Ono *et al.*, 2006).

In contrast to the mostly chloroplastic location of the PPO protein, phenolic compounds are mostly confined to the vacuoles (Mayer and Harel, 1979; Vaughn and Duke, 1984). This includes those compounds recognised as substrates for PPO. To date, potential PPO substrates have been identified within the anthocyanin, flavanol, flavone, flavonol and isoflavonoid subclasses of flavonoid polyphenols and hydroxybenzoic acid and hydroxycinnamic acid subclasses of phenolic acids based on enzyme assays or structural comparison to confirmed substrates (Parveen *et al.*, 2010). Given the physical separation of PPO enzymes from their substrates, it is commonly accepted that the PPO enzyme-substrate interaction requires the destruction of cell compartmentation, as a result of wounding for example. It is therefore not surprising that PPO enzyme activity has usually been related to arthropod (Kowalski *et al.*, 1992) or pathogen defence mechanisms (Li and Steffens, 2002; Thipyapong *et al.*, 2004*a*) as *o*-quinone protein complexes can decrease the nutritional value of the tissue (Felton *et al.*, 1989, Thipyapong *et al.*, 2004*a*) and/or ROS (secondary PPO reaction products) could trigger defence pathways (Kowalski *et al.*, 1992, Thipyapong, 2007).

At face value this physical separation of enzyme and substrate appears logical, although somewhat wasteful energetically to target mature PPO protein to the thylakoid lumen while sufficient compartmentation away from vacuolar substrates could also be achieved by targeting PPO to the cytosol. Therefore, considering the widespread occurrence of this trait in higher plants, even in the absence of detectable substrate (e.g. *Medicago sativa*; Sullivan *et al.*, 2008), this highly specific localisation of PPO could indicate that it confers a distinct advantage. However, a chloroplastic role for PPO is far from clear. For PPO activity to have a function in undamaged tissues it is necessary for the enzyme to have ready access to a suitable substrate in the chloroplast. Typical PPO substrates are *o*-diphenols because of their readily oxidisable OH-groups (Martinez and Whitaker, 1995; Parveen *et al.*, 2010). Most recognised PPO substrates are appointed to just two classes of polyphenols; the phenolic acids and the flavonoids (Parveen *et al.*, 2010). Although the presence of polyphenols and flavonoids have been reported in chloroplasts (Satô, 1966;

Halliwell, 1975; Saunders and McClure, 1976*a, b*; Agati *et al.*, 2007; Liu *et al.*, 2009), to our knowledge only catechin has so far been reported as a substrate for PPO in the mesophyll chloroplasts of tea (Subramanian *et al.*, 1999; Liu *et al.*, 2009). The identification of further potential monophenolic and/ or o-diphenolic PPO substrates in chloroplasts will be paramount in order to demonstrate an *in vivo* function of PPO in undamaged tissue.

The relationship between PPO and environment

As well as evidence in favour of involvement of PPO in plant defence against biotic stressors, several independent lines of evidence implicate the chloroplastic location of PPO in an as yet uncharacterised contribution to the response of plants to abiotic stress, potentially mediated by altering the cellular balance of ROS production/ degradation. It has been suggested that an acclimation mechanism exists whereby the oxidation of accumulated phenolics is inhibited when plants are subjected to extreme temperatures (Rivero *et al.*, 2001) or drought (Sofo *et al.*, 2005; Lee *et al.*, 2007). Indeed, experimentally-imposed conditions of cold, heat and drought have been shown to result in a significant increase in total phenolic compounds as compared to controls (Rivero *et al.*, 2001; Sofo *et al.*, 2005; Lee *et al.*, 2007). Concomitantly, oxidation of these accumulated phenolics was proposed to be inhibited by significant decreases in PPO (Rivero *et al.*, 2001; Sofo *et al.*, 2005) and peroxidase activities (Rivero *et al.*, 2001), and through the significant activation of enzymatic ROS scavengers such as ascorbate peroxidase (Sofo *et al.*, 2005; Lee *et al.*, 2007) and superoxide dismutase (Sofo *et al.*, 2005) as compared with controls. Hence the suggestion that a decrease in PPO activity following abiotic stress was associated with improved antioxidant capacity (Sofo *et al.*, 2005). This was supported by the work of Thipyapong *et al.* (2004*b*) which showed that suppression of PPO increased drought tolerance of tomato. However, a conflicting result was obtained when a drought treatment on white clover (*T. repens*) significantly increased PPO activity after 7 days (Lee *et al.*, 2007). Interestingly, PPO is implicated in the adaption of resurrection plants to desiccation and rehydration; Veljovic-Jovanovic *et al.* (2008) observed an increase in PPO of several fold when *Ramonda serbica* leaves were subjected to near-complete water loss.

Given the contrasting responses of PPO activity to environmental conditions it is not surprising that the potential impact of altered PPO activity on plant development, phenotype and yield is currently unclear. In tomato (*Solanum esculentum*), the alteration of PPO activity by silencing did not affect plant development, total leaf area or shoot and root dry weights under optimal growth conditions when compared to non-transformed controls (Thipyapong *et al.*, 2004*b*). Likewise, transgenic RNAi lines and wild-types of red clover (*T. pratense*) did not differ significantly in growth and leaf nitrogen content under optimal growth conditions (Webb *et al.*, 2013). Alternatively, a clear effect of PPO silencing was observed in walnut plants (*Juglans regia*) which developed spontaneous necrotic lesions in the leaves even when not challenged by pathogens (Araji *et al.*, 2014), suggesting increased susceptibility to oxidative stress. Furthermore, while no obvious phenotypic differences between wild-type red clover (*T. pratense* cv. Milvus) and a low PPO mutant were reported (Winters *et al.*, 2008), a field study in Aberystwyth (UK) reported a higher dry matter yield from fields seeded with wild-type red clover (5.78 tonnes DM ha^{-1}) than when seeded with the low PPO mutant (5.40 tonnes DM ha^{-1}) (R. Fychan, unpublished; 0.4 ha were sown with each of red clover cv Milvus and the PPO mutant in 2009 and the total dry matter weight of the above ground matter harvested in May 2010 was determined). Notably, both growth conditions and developmental stage influences the import of PPO into the chloroplasts (Sommer *et al.*, 1994) with corresponding effects on PPO activity (Mayer and Harel, 1979; Webb *et al.*, 2013), possibly accounting for the observed changes in PPO activity in red clover during a growing season (Figure 3; Fothergill and Rees, 2006). The latter demonstrates the impact of seasonal variation, with a peak in PPO activity in the winter months, during which the combination of high light and the relatively low demand for fixed carbon results in a high risk of photoinhibition and the associated oxidative stress.

Could PPO be involved in photosynthesis?

A potential role for PPO in photosynthesis has been speculated on previously (Mayer and Harel, 1979; Vaughn and Duke, 1984). Although theoretically plausible, evidence supporting or countering this hypothesis is still poor. Observations in support of involvement of PPO in photosynthesis include: (1) the correlation between PPO activity and chloroplasts evolving high levels of O_2 (Vaughn and Duke, 1984), (2) the association of PPO protein with the photosystems (Lax and Vaughn, 1991;

Sheptovitsky and Brudvig, 1996), (3) the inhibition of cyclic and/ or non-cyclic photophosphorylation by phenolic compounds (Neumann and Drechsler, 1967), the implication being that PPO activity could prevent such inhibition by oxidation of these potential substrates, (4) the independence of increases and decreases of substrate level and catecholase activity during growth and development (Ben-Shalom *et al.*, 1977; Winters *et al.*, 2008; Webb *et al.*, 2014), and finally (5) the modulation of PPO activity by environmental effects such as extremes of temperature, drought and time of year (Rivero *et al.*, 2001; Thipyapong *et al.*, 2004*b*; Sofo *et al.*, 2005; Fothergill and Rees, 2006; Lee *et al.*, 2007).

There have been several suggestions as to a mechanism by which PPO could directly influence photosynthesis, including it functioning as an oxygen buffer (Mayer and Harel, 1979; Vaughn and Duke, 1984) or interacting with the Mehler-peroxidase, or water-water, cycle (Tolbert, 1973) to facilitate reactive oxygen scavenging (Figure 4). The possibility that PPO modulates available oxygen is plausible given the requirement for O_2 during PPO-catalysed oxidation of phenolic compounds to *o*-quinones and H_2O (Steffens *et al.*, 1994), while the close association with the photosystems could provide a source of sufficient reducing power to regenerate *o*-diphenol by reducing *o*-quinones (Halliwell, 1975; Vaughn and Duke, 1984). The limitations to this hypothesis are the lack of definitively identified chloroplast substrates, and the relative slow rate of this catalysis compared to the speed of photosynthesis. Interaction with the Mehler-peroxidase cycle was proposed as quinones can also serve as hydrogen acceptors, with the reduced quinones being re-oxidised by PPO. In this way a pseudo-cyclic electron transport would occur without a net oxygen change (Trebst *et al.*, 1963; Tolbert, 1973), which is how the water-water cycle is indeed believed to operate (Asada, 1999). There is however no concrete evidence to support either of these hypotheses, and the water-water cycle and ROS scavenging are already well described processes (Asada, 1999; Apel and Hirt, 2004). Indeed, contrary to the above hypotheses a study of tomato plants in which PPO was suppressed by transformation showed that the transformants actually performed better under conditions designed to impose photoinhibition than did the untransformed plants (Thipyapong *et al.*, 2004*b*).

A related mechanism could potentially counter over-reduction due to accumulation of high NAD(P)H/NAD(P) ratios which can lead to inactivation of photosynthetic

electron transport (Foyer *et al.*, 2012). An *o*-dihydroxyphenol-ascorbate reduction/oxidation cycle linked with the ascorbate-glutathione cycle which involves oxidation of NAD(P)H could provide a mechanism for preventing over-reduction under conditions of decreased CO_2 fixation. This could potentially be catalysed by low pH activated latent PPO (see below) in high light conditions with elevated levels of lumen O_2. While this reduces the requirement for cyclic electron transport and quinones react readily with ascorbic acid, the extent to which ascorbate would participate in this as compared with other lumenal processes is unclear (Tóth *et al.*, 2013).

A proposed alternative explanation is that PPO is more important for the dark reactions in the thylakoid lumen than those in the light, i.e. when O_2 is low (Sheptovitsky and Brudvig, 1996). This was suggested since an acidic environment is created in the light in which the PPO enzyme, with pH optima of 8, would not be expected to be active (Sheptovitsky and Brudvig, 1996). However, this simple theory seems doubtful as various previous and subsequent studies reported pH optima of between 4 and 8 for PPO (Tolbert, 1973; Rocha and Morais, 2001; Yoruk and Marshall, 2003). This variability may be explained by the work of Leufken *et al.*, (2014) who demonstrated that conformational change in C-terminus can determine pH optima. Furthermore, latent PPO is known to be activated by low pH (Steffens *et al.*, 1994; Winters *et al.*, 2003; Schmitz *et al.*, 2008), which correlates well with the decrease in lumen pH following the illumination of chloroplasts. Plus, it would appear that a source of reducing power is necessary for intra-chloroplastic PPO activity; the oxidation of *p*-coumaric acid to caffeic acid observed in illuminated isolated chloroplasts did not occur in the dark unless ascorbate or NADPH was added (Halliwell, 1975). These observations add further support for the role of PPO in high light conditions involving ascorbate and/or NAD(P)H.

In many respects however, a direct role for PPO in regulation of photosynthesis may be unrealistic given the flux through the respective pathways (Vinyard *et al.*, 2013). It is therefore more likely that PPO has an indirect role in photosynthesis. As PPO monophenolase activity could theoretically catalyse the conversion of *p*-coumaric acid to caffeic acids (Vaughn and Duke, 1984), which is one of the initial steps in the phenylpropanoid pathway of phenolic compound biosynthesis, a role via secondary metabolism is possible. This is supported by recently published work in which the

silencing of PPO gene expression in walnut plants altered the metabolite profile of the leaves, notably those involved in tryptophan and tyrosine metabolism (Araji *et al.*, 2014). It was argued that the observed endogenous increases in tyramine and decreases in DOPA in PPO silenced plants occurred because normally PPO catalysed the *o*-hydroxylation of tyrosine to DOPA and tyramine to dopamine. So far no enzymes have been characterised for these reactions in walnut. The observation that endogenous application of tyramine to wild type leaves caused the same necrotic phenotype as seen in the PPO silenced plants, supports a fundamental role for PPO in tyrosine metabolism in walnut. Also, the product of a PPO transcript from *Coreopsis grandiflora* has been demonstrated to be involved in aurone formation (Kaintz *et al.*, 2014) and further supports a potential role for PPO in secondary metabolism. Several reports have theorised that the antioxidant capacity of phenolics (Pietta, 2000; Parveen *et al.*, 2010) is indicative of their potential as radical scavengers (Neill and Gould, 2003; Agati *et al.*, 2007) and even as photochemical energy dissipaters, the focus being on the phenolic acid subclass of hydroxycinnamic acids (chlorogenic acid specifically; Grace and Logan, 2000) and the anthocyanin subclass of the flavonoids (Neill and Gould, 2003). Therefore by regulating the availability of phenolics, PPO would indirectly affect the photoprotective capacity of photosynthetic cells independently of processes such as the Mehler-peroxidase cycle. However, this is not a uniform response and upregulation of phenylpropanoid metabolism is not always accompanied by decreases in PPO activity (Rivero *et al.*, 2001; Sofo *et al.*, 2005; Lee *et al.*, 2007; Fothergill and Rees, 2006).

Future challenges

PPO is an enigmatic enzyme with many possibilities but few certainties. Confusingly the data suggest that PPO activity can confer both a productive advantage, and be associated with increased risk of oxidative damage. While PPO activity can be associated with non-enzymatic ROS scavenging involving flavonoid and phenolic acid substrates (Apel and Hirt, 2004; Parveen *et al.*, 2010), a role for PPO in plant function may also be associated with its pro-antioxidant activity through the generation of secondary reaction products, including ROS (O'Brien, 1991; Thipyapong *et al.*, 1997), or may even involve localised effects in cellular differentiation/ death, where concentrations may be locally high and critical but not

easily measureable e.g. in nodules Webb *et al.* (2014) and walnut Araji *et al.* (2014). The evidence indicates that PPO performs different roles in different plant species and possibly multiple roles in plants with large PPO gene families. It is therefore important that fundamental questions such as whether the *in vivo* role involves a pro- or anti-oxidant would greatly increase our understanding of this enzyme and clarify future opportunities to exploit its function for increased and sustainable crop production. Consideration of possible relationships between PPO activity and photosynthesis are clearly relevant to current issues of food security. Global population is increasing rapidly and is due to reach 9 billion in 2050; creates a pressing need to optimise sustainable food production, with careful attention on crops for both human consumption and animal feed (Kingston-Smith *et al.*, 2013). To date results of research into a possible role for PPO in photosynthesis has yielded equivocal results, possibly because the focus has been on looking for a direct effect on regulation or mitigation of photochemistry. Here we propose that a re-examination of possible indirect effects of PPO on photosynthetic performance under abiotic stress is appropriate to current global challenges. Tools such as the identification of mutants (Lee *et al.*, 2004; Winters *et al.*, 2008), plant material genetically altered in PPO composition (Sullivan *et al.*, 2004; Thipyapong *et al.*, 2004*b*; Araji *et al.*, 2014; Webb *et al.*, 2014) and *in vitro* cloning to continue the identification of still unknown catalyst of phenolic compound biosynthesis (Sullivan and Zarnowski, 2010) plus increased use of unbiased analytical techniques for metabolic analysis holds the promise of finding explanations to some of the unexplained aspects of PPO biochemistry. These analyses should determine the extent to which endogenous PPO activity has the potential to improve photosynthetic performance under abiotic stress conditions and indicate whether increased activities of this enzyme should be included as a desirable leaf trait in plant breeding programmes designed to increase yield of food crops.

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Figure legends

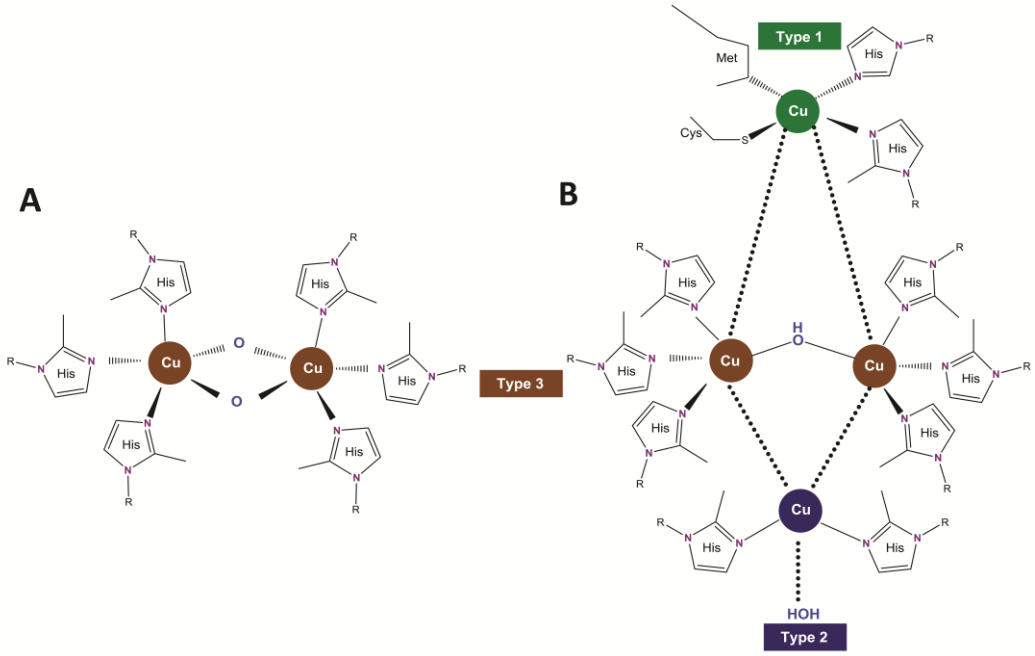
Figure 1. Active sites of (A) tyrosinase/ catecholase and (B) laccase enzymes. Both A and B contain a binuclear type 3 copper centre and B also includes mononuclear type 1 and 2 copper centres.

Figure 2. Schematic illustration of the mechanism of PPO including the structures of the *o*-diphenol and monophenol substrates for the catecholase and monophenolase reactions.

Figure 3. Seasonal total PPO activity for July 2004 – June 2005. Mean of six red clover (*Trifolim pratense*) cultivars are shown ± SE (original data courtesy of M. Fothergill; Fothergill and Rees, 2006).

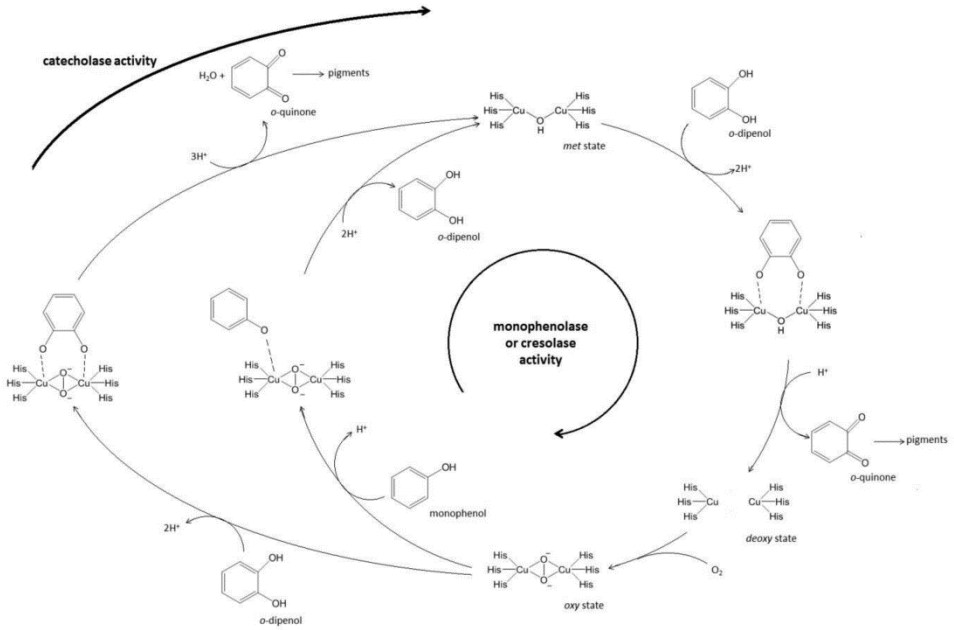
Figure 4. The potential interaction of PPO with photosynthesis (1) by acting as an oxygen buffer in the lumen to prevent O_2^- formation, and (2) to buffer NADPH accumulation in the stroma to prevent over-reduction of the photosystems. GSH (GSSG) reduced (oxidised) glutathione, DHA dehydroascorbate, GR glutatjione reductase, DHAR dehydroascorbate reductase.

Figure 1 Boeckx et al



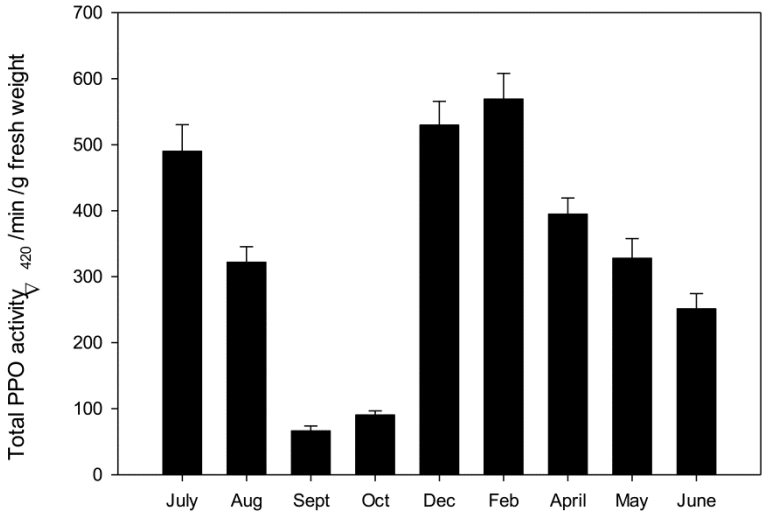
432

Figure 2 Boeckx et al



433

Figure 3 Boeckx et al



434

Figure 4 Boeckx et al