



A SURVEY OF WOUND- AND METHYL JASMONATE-INDUCED LEAF POLYPHENOL OXIDASE IN CROP PLANTS

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Abstract—Polyphenol oxidases (PPOs) are widespread enzymes which oxidize plant phenolic compounds. In tomato leaves, PPO is systemically wound-induced, regulated by the tomato wound signal systemin via the octadecanoid wound-signalling pathway, and appears to function as an anti-nutritive defense against folivore insect pests. In order to determine if PPO could be important for the induced defense of other crop plants, plants from five plant families were surveyed for constitutive, wound-induced, and methyl jasmonate-induced PPO activity. In addition to tomato, PPO was most strongly induced by wounding or methyl jasmonate in tobacco and hybrid poplar. In these plants the response was also shown to be systemically induced by wounding. Two species, willow and soybean, had very high constitutive PPO activities yet showed no significant PPO induction. Most of the species tested had low constitutive levels of PPO activity, and low or no induction by wounding or methyl jasmonate. Although PPO is widely distributed, only in some species does it appear to be a component of the inducible anti-herbivore defense. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The rapid accumulation of anti-herbivore phytochemicals and proteins in response to insect attacks is a defensive strategy of plants for adapting to an unpredictable environment [1–3]. Inducible proteins with anti-insect activity include inhibitors of digestive proteinases, anti-nutritive oxidative enzymes, proteases, and enzymes of secondary metabolism [2, 4, 5]. In some plants, defense activation occurs systemically, an adaptation thought to prevent herbivorous insects from avoiding locally-induced defenses by moving to unwounded leaves. In the tomato, more than 15 systemically wound-induced proteins have been identified. These are coordinately induced by the mobile wound signal systemin [4, 6, 7] acting via the octadecanoid wound-signalling pathway [8].

A major anti-nutritive protein induced in the tomato by wounding, systemin and the octadecanoid pathway is polyphenol oxidase (PPO; EC 1.10.3.1 or EC 1.14.18.1) [9, 10]. This enzyme oxidizes phenolic compounds to quinones, reactive molecules which can interact with many biological molecules. During feeding by folivore insects, the enzyme is mixed with phenolic substrates and the resulting quinones alkylate

essential amino acids of the dietary protein, making them nutritionally unavailable to the insect [5, 11]. The effectiveness of PPO as a defense has been demonstrated by experiments in which purified PPO plus a substrate inhibited the growth of tomato pests [12].

PPOs have been studied in many different plant species, and a recent survey found that leaf PPO is essentially ubiquitous in higher plants [13]. To determine if PPO functions as an inducible anti-herbivore defense in other species besides tomato, we undertook a survey of a number of agronomically important plants for wound-induced PPO. We also tested these species for PPO induction by methyl jasmonate (MeJA). This volatile compound is closely related to components of the octadecanoid wound-signalling pathway and is a strong inducer of PPO and other anti-herbivore defenses of tomato [8, 9]. Our results indicate that only tomato (*L. esculentum*), tobacco *N. tabacum*, and hybrid poplar (*P. trichocarpa* × *deltoides*) exhibited a strong induction of PPO by wounding and MeJA; the majority of the surveyed species responded with a three-fold or lower induction of leaf PPO. In addition, two species possessed high constitutive levels of PPO activity.

RESULTS

Leaves of young plants from several families were wounded with a haemostat or exposed to MeJA

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vapours, and after an induction period of 72 hr, leaf PPO activity was measured. PPO assays were performed essentially as described by Sherman *et al.* [13]; DOPA (3,4-dihydroxyphenylalanine) was used as the substrate and catalase was included to eliminate interfering H_2O_2 and peroxidase activities. As some plant PPOs exhibit latency but can be activated by detergents [14], SDS was included in the assay buffer [13]. Constitutive PPO activity was detected in all species tested, but at very variable levels, even in plants within the same family (Table 1, Control). *Salix* and *Glycine max* contained very high constitutive levels of PPO, but in neither case was this level increased further by wounding or MeJA treatment.

The degree of inducibility by wounding or MeJA varied widely, again among plant species of the same family. Tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) showed very dramatic increases in PPO activity following the treatments. The two *Solanum* species demonstrated low induction of PPO, while in *Capsicum annuum* no detectable increase in PPO was detected (Table 1). In general, however, plants of this family contained relatively high constitutive and induced PPO levels. Likewise, the Salicaceae seems to be characterized by high PPO

activity, with *Salix* showing very high constitutive PPO, and hybrid poplar (*P. trichocarpa* × *deltoides*) showing strong induction of PPO. In the Poaceae, some species showed a slight induction of PPO, but both induced and constitutive levels were low. Neither *Brassica* species tested responded to wounding with significantly increased PPO activity above the low constitutive amounts (Table 1), nor did *Arabidopsis thaliana*, another member of this family (data not shown). The Fabaceae, with the exception of *G. max*, were also low in PPO.

In tomato we had previously shown that PPO is systemically wound-induced [9]. We therefore tested tobacco and hybrid poplar, the two species with a clear induction of PPO (Table 1), for a systemic wound induction of PPO. Young tobacco plants were wounded several times on lower leaves, the wound response allowed to proceed for 24 or 48 hr, and extracts assayed for PPO activity. In both wounded and unwounded leaves of wounded tobacco plants, PPO activity increased six- and four-fold, respectively, within 24 hr, and more than ten-fold for both leaves in 48 hr (Fig. 1). Similar results were obtained with two-month-old hybrid poplar plants. Wounding the lower leaves resulted in a two- and four-fold increase

Table 1. Induction of leaf PPO activity by wounding and methyl jasmonate*

Plant species	Control	Treatments	
		PPO activity ($\Delta A_{490} \text{ min}^{-1} \text{ g}^{-1} \text{ fr. wt} \times 10^3$)	
		Wounded	MeJA
Poaceae			
<i>Zea mays</i>	88 (6)	104 (18)	189 (21)
<i>Hordeum vulgare</i>	36 (4)	38 (6)	94 (9)
<i>Triticum aestivum</i>	28 (8)	55 (4)	122 (13)
<i>Oryza sativa</i>	65 (5)	139 (35)	108 (35)
Solanaceae			
<i>Lycopersicon esculentum</i>	2430 (600)	43 300 (1630)	111 500 (61800)
<i>Solanum tuberosum</i>	380 (68)	1100 (170)	1630 (380)
<i>Nicotiana tabacum</i>	850 (63)	4500 (60)	20 300 (2900)
<i>Capsicum annuum</i>	1450 (125)	1530 (95)	1680 (230)
<i>Solanum nigrum</i>	47 (9)	158 (25)	400 (29)
Brassicaceae			
<i>Brassica nigra</i>	47 (1)	58 (8)	52 (14)
<i>Brassica napus</i>	48 (33)	56 (32)	68 (37)
Fabaceae			
<i>Medicago sativa</i>	52 (5)	78 (3)	115 (20)
<i>Glycine max</i>	19 500 (1700)	16 000 (800)	19 800 (3000)
<i>Phaseolus vulgaris</i>	128 (6)	145 (18)	148 (11)
<i>Lens culinaris</i>	111 (8)	244 (6)	129 (6)
<i>Cicer arietinum</i>	158 (14)	208 (2)	242 (6)
Salicaceae			
<i>Salix</i>	38500 (7500)	44000 (6000)	43000 (2250)
<i>Populus trichocarpa</i> × <i>deltoides</i>	1400 (570)	7700 (2200)	9200 (300)

* Leaf extracts were assayed for PPO activity either for two min (species containing high PPO levels), or 60 min (species with low PPO levels). Values represent mean ($\pm$ s.e.) of three samples, each consisting of pooled tissue from at least three individual plants.

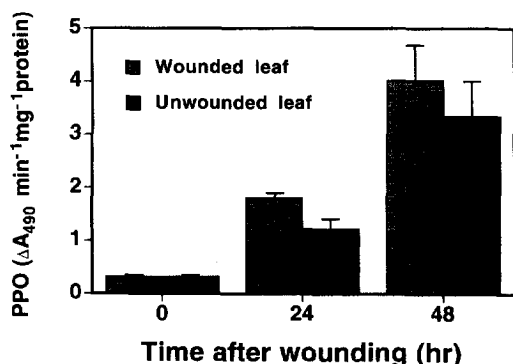


Fig. 1. Local and systemic induction of PPO in tobacco plants. Three-week-old plants were wounded on lower leaves, and both the wounded and upper, unwounded leaves assayed for PPO activity at different times. Results shown are means $\pm$ s.e. of three individually assayed plants.

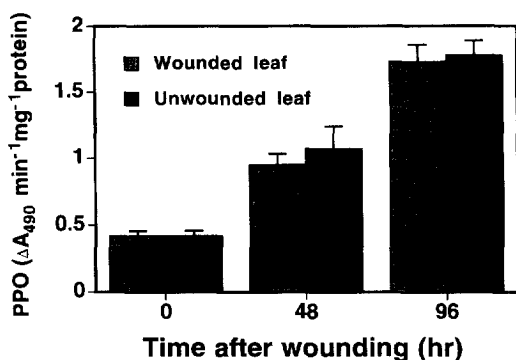


Fig. 2. Local and systemic induction of PPO in hybrid poplar plants. Eight-week-old potted plants were wounded on three leaves, and both the wounded and three adjacent, unwounded leaves were assayed for PPO activity at different times. Results shown are means $\pm$ s.e. of three individually assayed plants.

of PPO activity in 48 and 96 hr, respectively, in both the wounded and unwounded, upper leaves (Fig. 2). These experiments demonstrated that, as in tomato, tobacco and hybrid poplar plants respond to mechanical damage with a systemic increase of PPO activity.

DISCUSSION

A selection of plants was tested for induction of foliar PPO activity by wounding and MeJA. PPO activity was detected in all plants assayed, including unwounded controls. The almost ubiquitous occurrence of constitutive PPO in the plant kingdom was previously reported by Sherman *et al.* [13]. In that survey, as well as in our experiments the levels of measured PPO activity varied widely (Table 1). This may be partly due to differing substrate preferences of PPOs from different plants. Plant PPOs have broad substrate specificities and oxidize a range of di-ortho-phenolic compounds [14], but reaction rates of PPOs from different sources will vary depending on the substrate used. This makes a direct comparison of absol-

ute PPO activity levels between different species difficult. However, we observed that extracts with high PPO activity also browned rapidly, while the colour of extracts with low PPO activity remained unchanged. Since browning of plant tissues and extracts is associated with high PPO levels [15], the values obtained in Table 1 allow for at least semi-quantitative comparisons between the different species. Substrate specificities of leaf PPOs from closely related species can be expected to be similar, and possible substrate preferences will not affect comparisons of PPO activity between different treatments of the same species.

The species surveyed varied widely in their capacity to respond to wounding and MeJA with increased PPO activity, ranging from none to over 50-fold (Table 1). A clear and strong induction was observed in tomato, tobacco, and hybrid poplar, while the response of *S. nigrum* and *S. tuberosum* was significant but less dramatic. The systemic induction of PPO by wounding and MeJA in tomato has been previously reported [9], as was the systemically wound-induced accumulation of PPO protein and PPO mRNA transcripts in potato [16]. Figures 1 and 2 demonstrate the systemic induction of PPO by wounding in tobacco and hybrid poplar, suggesting that PPO is a component of the defense arsenal of these plants also. The systemic induction of protease inhibitors in tobacco, and the accumulation of protease inhibitor-like mRNA transcripts in hybrid poplar have been previously described [17, 18].

Interestingly, wound-induction of PPO generally correlated with MeJA-induction (Table 1). MeJA or closely related jasmonates are components of the tomato octadecanoid pathway proposed to transduce wound signals inside the cell [8]. The defense-activating properties of MeJA in other species may be an indicator of the presence of octadecanoid signals regulating systemic wound responses there also. PPO will be a very useful defense response marker for further analysis of signalling pathways in these plants.

A number of species showed no induction of PPO by wounding or MeJA. This was unexpected since an induction of PPO activity by wounding has been reported for a number of plants [14]. However, the absence of a response in our experiments does not preclude a potential induction under other conditions, or by different types of tissue damage. For example, a phloem-feeding insect, the three-cornered alfalfa hopper, induced foliar PPO in *G. max*, whereas the leaf-feeding bean leaf beetle failed to do so [19]. We found no induction by mechanical damage in our study (Table 1). Likewise, Stout *et al.* [20] demonstrated that oxidative defenses including PPO were induced differentially depending on the type of damage.

Alternatively, the lack of PPO induction in some plants may reflect specialization of plant defense mechanisms, since these plants are nonetheless capable of activating other defenses. For example, *C. annuum* and *M. sativa* accumulated significant levels

of defensive trypsin inhibitors in wounded or MeJA-treated leaves (data not shown), while little PPO induction was observed. In *B. napus*, the levels of glucosinolates increase following wounding and MeJA-treatment [21, 22], yet in our experiments PPO activity remained unaffected by these treatments. The induction of defenses by MeJA in diverse plants suggests that the octadecanoid pathway is conserved among plants, even though it may control different defense responses. This interpretation is consistent with the results of Reinbothe *et al.* [23] who demonstrated that the protein profiles of MeJA-induced leaves from different species are very distinct, and that different classes of defense proteins are induced. Conserving the signalling pathways, yet adapting responses to specific ecological conditions, may be one strategy of plants in the evolutionary arms race with insects.

Our results have demonstrated a wide variation in PPO inducibility among different species. In highly-inducible species such as tomato, tobacco, and hybrid poplar, PPO is most likely to function as a defensive anti-nutritive protein. Likewise, in plants with high constitutive PPO, the enzyme may function in defense against insects. In species with apparently little or no wound-inducible PPO, such a function is less certain; perhaps PPO plays a different role in these plants. The almost ubiquitous distribution of PPO may indicate that this enzyme has evolved for another, as yet undiscovered role, but that some plants have employed it for defense against insect herbivores.

EXPERIMENTAL

Plant material and treatments. Seeds for *Zea mays* cv Golden Cross Bantam, *Hordeum vulgare* cv Kam-iak Winter Barley, *Triticum aestivum* cv Yecoro Rojo, *Oryza sativa* M201, *Lycopersicon esculentum* cv Castlemart, *Solanum tuberosum* cv Desiree, *Solanum nigrum*, *Nicotiana tabacum* cv Xanthi, *Capsicum annum* cv Wonder Pepper, *Brassica nigra*, *Brassica napus* cv Westar, *Medicago sativa* cv Vernema, *Glycine max* cv Bragg, *Phaseolus vulgaris* cv Black Mexican, *Lens culinaris* cv Brewers, and *Cicer arietinum* cv Dwelleya were obtained from the Institute of Biological Chemistry seed collection. *Salix* cuttings were collected locally and *Populus trichocarpa* × *deltoides* (HY11-11, University of Washington/Washington State University Poplar Research Programme) were kindly provided by G. Rademaker, Department of Natural Resource Sciences, Washington State University.

Seed-grown plants were maintained in peat pellets in environmental chambers under 17 hr of light ($> 300 \mu\text{E m}^{-2} \text{s}^{-1}$) at 28° and 7 hr of dark at 18° . Leaves of two- to three-week-old plants were wounded by crushing across the midvein of each leaf or leaflet with a haemostate. This was repeated × 2 at 3 hr intervals, and plants harvested 72 hr later for PPO analyses. For systemic wound inductions, three lower leaves were

wounded, and both wounded and the upper, unwounded leaves were harvested 24 or 48 hr later. MeJA inductions were performed by placing whole plants in plexiglass boxes for 72 hr under constant light, with 20 μl of a 10% soln of MeJA in EtOH on a cotton wick. *Salix* and *Populus* were grown in greenhouses from cuttings, and assayed at *ca* eight weeks. MeJA was applied to *Salix* by spraying leaves with a 0.025% soln in $\text{H}_2\text{O}/0.1\%$ Triton X-100. For MeJA treatment of *Populus*, whole stems were excised, placed in H_2O , and incubated in plexiglass boxes as described for the other plants.

PPO Assays. Leaves were ground in a mortar in five vols of cold 100 mM NaPO_4 buffer, pH 7.0, containing 0.1% Triton X-100. The extract was clarified by centrifugation, frozen in liquid N_2 , and stored at -80° until assayed. PPO assays were performed spectrophotometrically using 3,4-dihydroxyphenylalanine (DOPA) as described by Sherman *et al.* [13] except that the reactions were carried out in 100 mM NaPO_4 buffer, pH 7.0, and the concn of SDS raised to 0.15%. The assay buffer was aerated for 5 min prior to use. Catalase (280 Units ml^{-1} ; Sigma) was added to remove any H_2O_2 present and prevent interference by peroxidases [13]. In addition, the specificity of the assay was verified by performing parallel assays with 1 mM kojic acid, a specific PPO inhibitor [24]. Assays were carried out for either 2 or 60 min depending on the amount of PPO activity present. Each data point was calcd from three samples, each consisting of pooled leaves of at least three individual plants. Protein concs were measured by the method of Bradford [25].

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