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SYSTEMIC WOUND INDUCTION OF POTATO (*SOLANUM TUBEROSUM*) POLYPHENOL OXIDASE

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Key Word Index *Solanum tuberosum*; Solanaceae; potato; systemic wound induction; polyphenol oxidase.

Abstract—Plant polyphenol oxidases (PPOs) have long been reported to be inducible upon biotic or abiotic wounding. However, observations of inducible PPO activity are frequently confounded by failure to distinguish PPO induction from loss of PPO latency, or by failure to distinguish PPO activity from peroxidase activity. We report the systemic induction of PPO activity, and increased steady-state levels of PPOs and PPO mRNA in potato (*Solanum tuberosum* L.) in response to wounding. During normal growth and development, PPO is present throughout potato leaf maturation, from the apical leaf node through node 11. In contrast, PPO mRNA is only detectable in apical leaf nodes 1–3. Wounding of potato leaflets at nodes 6–8 results in 1.7-fold increase in PPO activity in apical leaf nodes 1–4 within 48 hr after wounding. The increases in PPO activity are accompanied by comparable increases in PPOs and PPO-specific mRNA. No PPO induction is observed in either leaf nodes 5 or 8. These results suggest that only those tissues which are developmentally competent to express PPO mRNA are capable of responding to the systemic wounding signal by increased accumulation of PPO mRNA.

INTRODUCTION

Plant polyphenol oxidases [PPOs (EC 1.14.18.1 or EC 1.10.3.2)] catalyse the oxidation of phenols to quinones, which can covalently modify and crosslink various cellular nucleophiles, undergo melanin-forming autoxidation reactions or participate in an array of other redox reactions. In many plant species, PPO activity exists in a latent state, which may require proteolysis, detergent, acid and base shock or treatment with cations for activation [1, 2].

The role of PPOs in the biology of plants remains unclear. Because of its conspicuous reaction products, PPO has frequently been assumed to play a role in plant defence. In many *Lycopersicon* and *Solanum* species, PPOs are highly abundant in glandular trichomes, which entrap small-bodied insects through oxidative polymerization of trichome exudate [3, 4]. PPO has also been implicated as a plant defensive protein functioning to cause anti-nutritive modification of plant proteins upon disruption of cells by insect herbivores [5]. Nevertheless, other proposed roles for PPO include buffering of plastid oxygen levels, biosynthesis of phenolics and wound healing [6–9]. However, no conclusive evidence has yet been provided for such roles.

Although neither near-isogenic plants varying in PPO content nor PPO mutants have been available as a platform to test directly the defensive role of PPO, circumstantial evidence could be obtained through the demonstration that plants respond to damage by herbivores or pathogens by altering expression of PPO, as has been shown for the pathogenesis-related proteins and proteinase inhibitors suggested to have a defensive role against herbivores or pathogens [10, 11]. A large number of studies have in fact shown that PPO activity increases in response to biotic and abiotic injury [6, 12, 13]. However, these studies have not determined whether the increase in PPO activity is due to loss of PPO latency, changes in plastid or tonoplast membrane integrity, or is due to an increased amount of PPO and/or PPO-specific mRNA. We report here the kinetics of wound-inducible potato PPO expression in which increases in PPO activity are accompanied by similar increases in the amount of immunologically detectable PPO and by increased steady-state levels of PPO mRNA.

RESULTS AND DISCUSSION

When potato leaflets at nodes 6–8 were mechanically wounded by crushing at several points between major veins using a haemostat, as illustrated in Fig. 1, PPO activity in leaflets at nodes 1–4 was significantly increased ($p < 0.01$). The highest levels of induced activity were reached 48 hr after wounding and were 1.7-fold

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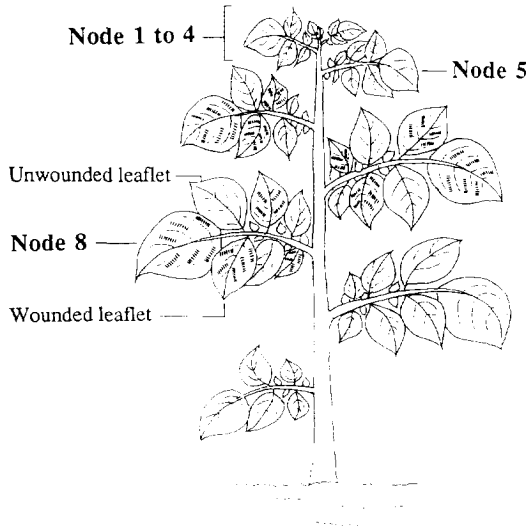

Fig. 1. Wounding of potato leaf tissues to evaluate the localized and systemic induction of PPO. Five-week-old potato plants were employed. Terminal leaflets and half of the remaining leaflets at nodes 6–8 were wounded by crushing with a haemostat at several points between major veins. Wounded sites are shown as ||||||||.

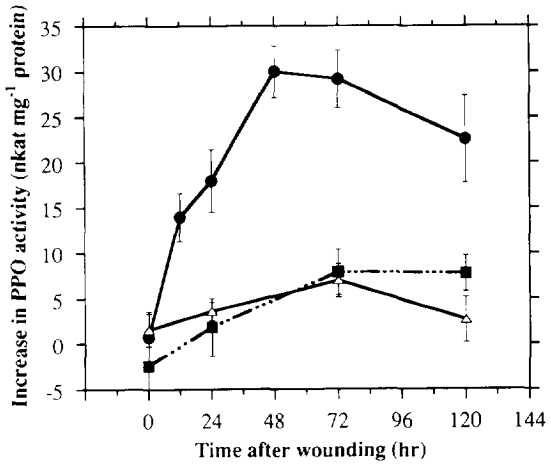

Fig. 2. Increase in PPO activity of leaflets at nodes 1–4, node 5 and unwounded leaflets at node 8 in response to wounding: (●) nodes 1–4; (■) node 5; (△) node 8. Assay was repeated 3 times with 3 replicates. Results are presented as mean ± s.e.

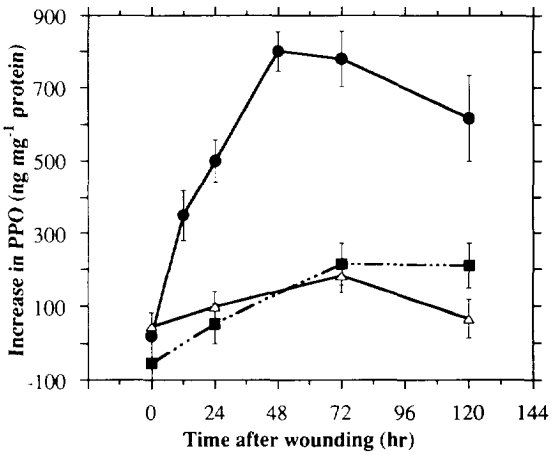

Fig. 3. Increase in PPO of leaflets at nodes 1–4, node 5 and unwounded leaflets at node 8 in response to wounding: (●) nodes 1–4; (■) node 5; (△) node 8. ELISA was repeated 3 times with 3 replicates. Results are presented as mean ± s.e.

higher than control (unwounded) plants. Neither leaf node 5 nor the unwounded leaflets adjacent to the wounded leaflets at node 8 exhibited statistically significant increases ($p > 0.05$) in PPO activity (Fig. 2). The PPO activity of wounded leaflets at node 8 was comparable to that of the unwounded leaflets (data not shown). Similarly, feeding of third instar Colorado potato beetle larvae on leaf nodes 8 and 9 resulted in a 1.8-fold induction of PPO activity only at leaf nodes 1–4 after 48 hr (data not shown).

The inducible PPO activity was accompanied by comparable increases in PPO as quantified by enzyme-linked immunosorbent assay (ELISA) (Fig. 3). PPO activity and PPO level of leaflets at nodes 1–4, 5 and 8 were highly positively correlated with correlation coefficients (r) of 0.92, 0.94 and 0.89, respectively, indicating that increases in enzymic activity are reflected by comparable increases in the amount of PPO. Hence, changes in PPO activity in this species do not appear to result from simple loss of latency nor to changes in plastid or tonoplast membrane integrity.

In order to determine whether steady-state levels of PPO mRNA increase concomitantly with increased PPO levels, total RNA was isolated from leaf nodes 1–4, and unwounded leaflets of node 8 wounded and unwounded plants at 0, 24, 48 and 72 hr after wounding. In unwounded plants, RNA blot analysis revealed a 2 Kb PPO transcript class at apical leaf nodes 1–4. PPO mRNA was not detectable at later stages of leaf development (node 8; Fig. 4) in agreement with Hunt *et al.* [14]. Wounding did not result in appearance of PPO mRNA in older leaves. However, PPO-specific mRNA increased *ca* 2-fold 24 hr after wounding in nodes 1–4. This induction was transient and subsequently decreased to control levels within 48 and 72 hr after wounding (Fig. 4). The peak of steady-state mRNA abundance *ca* 24 hr prior to the maximal accumulation of PPOs and PPO activity suggests that translation of the additional PPO mRNA may be responsible for increased PPOs at this stage. Therefore, a primary effect of wounding is to increase the availability of PPO mRNA in developing leaves, either through alteration of mRNA stability or via increased transcription. This result is in agreement with the observation of increased steady-state levels of PPO mRNA in wounded apple fruits and leaves [15].

In unwounded potato plants, the developmental programme of PPO expression is such that PPO mRNA is

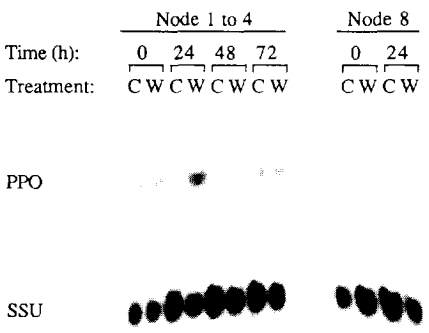

Fig. 4. Induction of PPO-specific mRNA upon wounding. Total RNA was extracted from leaflets at nodes 1–4, and unwounded leaflets at node 8 of wounded (W) and unwounded control (C) plants 0, 24, 48 and 72 hr after wounding. Total RNA (20 μg) was gel-fractionated, blotted and hybridized with ^{32}P-labelled PPO-P1 (2 Kb [14]). To indicate equivalent loading of RNA samples, a probe specific for small subunit of Rubisco (SSU) was used, showing a single band of 880 bp.

typically only detectable at apical leaf nodes 1–3. However, immunoblot analyses indicated that PPO is a stable protein whose levels do not change significantly from the apical leaf node to node 11 [14]. While our results with respect to PPO mRNA are in agreement with Hunt *et al.* [14], in this study we found that the level of PPOs and PPO activity decreased basipetally from the apex. For example, in leaf nodes 1–4, PPO was *ca* 1.3 and 2.3 times higher than that in nodes 5 and 8, respectively. This discrepancy probably arises from the greater sensitivity of ELISA and PPO activity assays relative to that of immunoblots.

Although potato PPO expression is regulated by wounding, the failure of mechanical wounding to induce PPO in tissues which do not normally express PPO mRNA suggests that the developmental pattern of PPO expression–at least in this species–exercises the primary control over expression of this gene family.

Similarly to potato, tomato PPO mRNA is found primarily in the apical leaf tissues (Hunt *et al.*, unpublished data). In contrast to our finding that potato PPO is only systemically induced in tissues which express steady-state PPO mRNA normally, tomato leaves are reported to respond to *Pseudomonas syringae* inoculation with both systemic and localized induction of PPO activity [12]. In addition, an increase in PPO activity following pathogen infection is reported in older tomato leaves [12]. Mechanical wounding of two-week old tomato seedlings grown in continuous light also resulted in both localized and systemic wound induction [16]. These differing results in tomato and potato may reflect developmental and environmental differences between experiments, differential PPO expression in response to qualitatively or quantitatively different signals, or differential wound responsive regulation between the two species. Proposed systemic signalling molecules include methyl jasmonate and jasmonic acid, chitin and chitosan, electrical signals, and systemin [17–19]. Methyl jasmonate is known to induce tomato PPO activity by 2 to 12-fold [20]. Recently, Constabel *et al.* [16] demonstrated that tomato plants overexpressing systemin exhibit elevated PPO levels, in addition to proteinase inhibitors. The finding that PPO accumulation is induced by wounding, systemin and methyl jasmonate suggests that tomato seedling PPO is regulated via the octadecanoid signalling pathway [16].

Stress-induced alteration of plant defence gene mRNAs is well established [21, 22]. Activation of genes encoding phenylalanine ammonia lyase, chalcone synthase and chitinase by fungal elicitor is primarily regulated at the transcriptional level [23, 24]. In contrast, fungal elicitor-induced proline-rich protein mRNA down-regulation is due to destabilization [25]. Our finding that PPO gene expression is modified in response to wounding, resulting in increased levels of PPOs and PPO activity, supports the defensive role of PPOs in plant–pest interactions. The disruption of plant cells by injury not only causes the release of PPOs from thylakoid association, facilitating interaction with phenolic substrates, but also systemically induces *de novo* synthesis of PPOs in young leaf tissues, presumably to protect the growing part of the plant. Hence, PPO can be considered both an inducible and a constitutive component of plant defences. Further studies are necessary to determine whether such increases in PPOs afford a significantly protective effect against herbivores or pathogens.

The apparent wound inducibility of PPO activity has been studied intensively (e.g. *ca* 20 refs cited in ref. [6]). However, these studies have frequently been confounded by the failure to distinguish inducibility from release of latent PPO activity and by the failure to distinguish PPO from peroxidase activity [6]. We report here direct evidence that wound responsive expression of PPOs in potato is regulated at the level of transcriptional activity or mRNA stability, and results in increased accumulation of PPOs and PPO activity.

EXPERIMENTAL

Plant material. Potato (*Solanum tuberosum* L. cv. Superior) plants were grown under greenhouse conditions with a 16 hr photo-period. Selected leaflets at nodes 6–8 of 5-week-old potato plants were mechanically wounded by crushing with a haemostat at several points between major veins (Fig. 1). At 0, 12, 24, 48, 72 and 120 hr after initial wounding, leaflets from wounded and unwounded (control) plants were harvested and immediately frozen in liquid N_2. Leaflets from apical nodes 1–4 were pooled, and leaflets from nodes 5 and 8 were harvested separately. For node 8, wounded and unwounded leaflets were harvested separately to evaluate the possibility of enzyme inactivation as a result of wounding.

Protein isolation and quantification. Frozen leaves were ground to a fine powder with a pestle and extracted at a ratio of 150 mg fr. wt to 1 ml extraction buffer

(100 mM Tris–HCl, pH 7.0, 100 mM KCl, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1% (v/v) Triton X-100 and 3% (w/v) PVPP. The homogenates were centrifuged at 12 000 g for 15 min, and the supernatant protein concn was determined by the method of Bradford [26] using BSA as standard.

PPO activity assay. PPO activity was assayed spectrophotometrically by measuring the decrease in absorbance at 412 nm of 2-nitro-5-thiobenzoic acid after reacting with quinones generated through enzymic oxidation of 3,4-dihydroxyphenylalanine (DOPA) to form colourless adducts [27]. The reaction mixt. contained 1 ml 96 μM 2-nitro-5-thiobenzoic acid and 25 mM DOPA in 100 mM Tris–HCl, pH 7.0, which had been aerated for 5 min prior to assay. Catalase (560 units in 20 μl 100 mM Tris–HCl, pH 7.0) was added to eliminate peroxidase contribution to DOPA oxidation. Sp. act. was expressed as nkat mg^{-1} protein.

ELISA. Leaf homogenates (1–20 μg total protein) were incubated overnight at 4° in polystyrene plates. ELISA was performed according to ref. [28]. Both primary antibody (polyclonal rabbit anti-*S. berthaultii* 59 kDa trichome PPO) and secondary antibody (goat anti-rabbit alkaline phosphatase conjugate) were used at 1:2000 dilution. PPO (10–100 ng) from *S. berthaultii* leaf wipes [3, 28] was used as a standard for PPO quantification.

RNA isolation and analysis. Total RNA was isolated from frozen leaf tissue as described in ref. [29]. The contaminating polysaccharides were then removed by dissolving RNA samples in high-salt (2 M NaCl) soln followed by *iso*-PrOH precipitation [30]. Total RNA was gel-fractionated and transferred according to ref. [14]. The filter was hybridized with 1.5×10^6 dpm ml^{-1} [^{32}P]-labelled potato leaf PPO cDNA (PPO-P1 [14]) and washed under the following conditions: two 15 min washes in 2 × SSC and 0.5% SDS at 45° and two 15 min washes in 1 × SSC and 0.5% SDS at 65° (1 × SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0).

Statistical analysis. A two-way analysis of variance model was used to evaluate the difference in PPO activity between wounded and control plants at nodes 1–4, 5 and 8, respectively. Each treatment was replicated × 3, and each replication consisted of two potato plants.

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