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Activation of Polyphenol Oxidase in Dormant Wild Oat Caryopses by a Seed-Decay Isolate of *Fusarium avenaceum*

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Incubation of dormant wild oat (*Avena fatua* L., isoline M73) caryopses for 1−3 days with *Fusarium avenaceum* seed-decay isolate *F.a.*1 induced activity of the plant defense enzyme polyphenol oxidase (PPO). Both extracts and leachates obtained from *F.a.*1-treated caryopses had decreased abundance of an ∼57 kDa antigenic PPO and increased abundance of antigenic PPOs ranging from ∼52 to 14 kDa, as compared to extracts and leachates from untreated caryopsis. Leachates from caryopsis incubated for 2 days with *F.a.*1 also had 5.1- and 7.5-fold more total PPO activity/g fwt and specific activity, respectively. Fractionation of leachate proteins by ion-exchange chromatography associated the majority of PPO activity with an ∼36 kDa protein from untreated caryopses and ∼36, 25, and 24 kDa proteins from *F.a.*1-treated caryopses. Predicted peptide sequences obtained from high-performance liquid chromatography−tandem mass spectrometry analyses indicated that the ∼57 and 36 kDa wild oat proteins had a strong similarity to wheat PPO. However, the 25 and 24 kDa proteins were most similar to a Chitinase and oxalate oxidase, respectively. Our results indicate that *F.a.*1-induced activation of latent PPO in wild oat caryopsis likely involves a cleavage mechanism allowing activated PPOs to be readily mobilized into their surrounding environment.

KEYWORDS: *Fusarium avenaceum*; polyphenol oxidase; seed decay; seed longevity; weed; wild oat

INTRODUCTION

Wild oat (*Avena fatua* L.) is a weed of economic importance in wheat and small grain production systems worldwide (*1*). Factors associated with the persistence of wild oat seeds within the soil seed bank include dormancy and resistance to biotic and abiotic stress (*2*). Thus, dormant weed seeds must possess adaptive defense mechanisms that evolved to overcome a wide range of environmental conditions including climate, predation, and attack by soil microorganisms. To date, there is relatively little information on mechanisms of resistance to seed decay organisms and no known reports of biochemical responses to pathogens in dormant weed seeds. Because microbially mediated seed decay is one approach to seed bank depletion (*3*), understanding the biochemistry associated with decay resistance mechanisms in seeds of wild oat could enhance our understanding for developing new biological control agents.

Correlations between seed longevity in the soil seed bank and seed *o*-dihydroxyphenol content (*4*) suggest that phenolic compounds likely play a role in some weed seed persistence mechanisms (*2*). Polyphenol oxidase (PPO), enzymes that catalyze either the *o*-hydroxylation of monophenols to *o*-diphenols [EC 1.14.18.1; monophenol monooxygenase (tyrosinase and cresolase)] or the dehydrogenation of *o*-dihydroxyphenols to *o*-quinones [EC 1.10.3.1; diphenol oxygen oxidoreductase (catechol oxidase)], are also proposed to play a role in the defense against pathogens and herbivores (*5*, *6*) and in wound healing (*7*). Furthermore, *o*-quinones can undergo polymerization reactions to form melanins that have antimicrobial activity (*8*) and may be involved in forming a barrier against entry and spread of pathogens in potato tubers (*9*). In many species, PPO genes are differentially induced in plant tissues in response to wounding and plant defense-related hormones such as systemin, salicylic acid, and jasmonates (*5*, *6*, *10*). Nearly all of pathogen-related studies on plant PPO induction have occurred in tissues other than seeds; however, in the case of wheat (*Triticum aestivum* L.), *Fusarium graminearum* Schwabe (head blight) is known to attack developing caryopses and increase PPO activity (*11*).

Plant PPOs belong to a nuclear-encoded, multigene family (*5*,*6*,*12*). In wheat, at least three kernel and three nonkernel PPO genes have been characterized (*13*, *14*). However, many other PPO sequences have been reported in wheat (*15*). Sequence data indicate that genes encoding plant PPOs contain two conserved copper-binding domains associated with enzymatic activity and a transit peptide required for import into the plastid lumen (*5*,*6*,*12*). In plants, the chloroplast appears to serve as a primarily storage site for PPO, while phenolic substrates are generally isolated within vacuoles. Several exceptions include an aureusidin synthase,

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a PPO homologue of snapdragon that lacks a transit peptide and is likely localized in vacuoles (*16*), and a PPO of *Taraxacum* species that contains a transit peptide but is localized in laticifers where it appears to function in latex conjugation (*7*).

Several post-translational processing steps are typically required to activate PPO (*5*, *6*, *17*). Unprocessed plant PPOs generally range from ~68 to 73 kDa and contain an N-terminal plastid transit peptide, which is usually cleaved in a two-step process to produce a ~55−68 kDa "mature" protein during transport into the chloroplast. Although the mature 55−68 kDa plant PPOs are often latent or only partially active (*5*, *17*), proteolytic cleavage of a C-terminal peptide from many eukaryotic PPO proteins activates a ~37−44 kDa PPO (*5*, *6*, *17*). Latent forms of PPO complicate in vitro measurement of total enzyme activity, but many harsh treatments including detergents, solvents, chaotropes, and proteolysis are known to activate these latent forms of PPO (*5*, *15*). These findings further strengthen observations that mature PPOs of plants have remarkable enzymatic stability, which tolerates many extreme treatments including heat (*13*, *15*, *18*). Further specifics related to plant PPOs are available from numerous reviews (*5*, *6*, *8*, *12*, *15*).

In wheat seed, PPO can exist in both water-soluble unbound and bound forms, with the bound form predominating (*19*). However, exposure to seed decay pathogens has been shown to induce PPO activity in dormant wild oat seeds (*20*), and the majority of this activity appears to be leachable as a water-soluble, unbound form (unpublished). Because PPOs are known to be induced by defense signals and pathogens attack in other plant species, we are interested in determining their potential roles in wild oat seed longevity. In this study, we characterized the effects of *F. avenaceum* isolate *F.a.*1 on induction of PPO activity and its correlation to protein processing in wild oat caryopses.

METHODS AND MATERIALS

Chemicals. Unless otherwise stated, all chemicals and reagents were from Sigma-Aldrich, Inc. (St. Louis, MO). Pefabloc was obtained from Roche Diagnostics Corp. (Indianapolis, IN). NP-40 detergent was obtained from Calbiochem, EMD4Biosciences, Inc. (La Jolla, CA). Bio-Safe Coomassie G-250 and PVDF membrane were obtained from Bio-Rad Laboratories (Hercules, CA).

Plant Material and Fungal Cultures. Dormant (M73) wild oat seeds were germinated in Sunshine mix #1 (Sun Gro Horticultural Distribution Inc., Bellevue, WA), watered daily, and maintained at 25 °C under a 16:8 h day:night photoperiod. Seeds obtained from greenhouse plants were stored at −20 °C to preserve dormancy. Prior to experiments, caryopses were surface disinfected by running them under tap water for 10 min, followed by incubation with 70% ethanol on an end-over-end shaker for 3 min, followed by incubation with 0.5% sodium hypochlorite on the shaker for 5 min and five final rinses with sterile water on the shaker for a total of 10 min.

Seed viability was also tested by inducing germination in the presence of 3 mM GA_3, which produced seed viability counts of >95% (data not shown). *F.a.*1-treated caryopses were exposed to potato dextrose agar (PDA)-cultured *F.a.*1 in Petri dishes for 1−3 days by placing seeds across the growing edge of the culture; untreated (control) caryopses were on PDA alone.

Protein Extraction from Caryopses. Caryopses were incubated ±*F.a.*1 for 1, 2, or 3 days. Caryopses were washed twice for 10 s in MOPST buffer [MOPS (2-(*N*-morpholino) propane sulfonic acid) buffer with Tween 20 (0.02%)], pH 6.5. PPO was extracted from caryopses as previously described (*13*, *21*). Wild oat caryopses were ground to a fine powder in liquid N_2 using a mortar and pestle and extracted with 50 mM MOPS buffer (pH 6.5), 100 mM KCl, 0.2 mM Pefabloc, and 1.0% (w/v) polyvinylpyrrolidone (4 mL/g fwt tissue). The final solution was adjusted to 0.2% (v/v) with NP-40 detergent. After 10 min of incubation on ice, the extracts were centrifuged at ~20000*g* for 15 min at 4 °C, and the pellet was discarded. The total protein concentration was determined using the Bradford procedure according to the manufacturer's instructions, and the PPO activity of extracts was determined.

Caryopsis Leachates. To obtain sufficient protein for fast protein liquid chromatography (FPLC) and high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) characterization procedures, large-scale leachate protocols were utilized. For one biological replicate, 400 caryopses (untreated and *F.a.*1-treated) were incubated on multiple Petri dishes for 2 days. A 2 day incubation of caryopses on *F.a.*1 was chosen because significant PPO activity was induced at this time; yet, very few caryopses showed evidence of seed decay. Caryopses were removed from cultures, residual mycelia were removed with forceps, and any decayed caryopses were discarded. Caryopses were not washed to retain leachable PPO activity. Caryopses were weighed and placed in a 250 mL flask containing 40 mL of MOPST buffer with 0.2 mM Pefabloc. Caryopses were incubated on a rotary shaker at 150 rpm for 1 h at 21 °C. The leachates were decanted into a flask on ice, and caryopses were leached a second time as previously described. The sequential leachates were pooled and filtered through a Whatman GF/A (1.6 μm pore size) glass microfiber filter (Whatman, Inc., Piscataway, NJ) to remove mycelial contamination.

Leachates were also obtained from mycelia using quantities of mycelia >10-fold that are visible in *F.a.*1-treated caryopses. The mycelia were weighed, transferred, and leached as described above. There were three biological replicates of all treatments, and all leachate samples were stored at −80 °C until further analysis.

Proteins from replicate leachate samples were individually precipitated by direct addition of solid ammonium sulfate (AMS) to produce a final concentration of 70% (w/v). Following incubation on ice with stirring for 2 h, precipitated proteins were recovered by centrifugation at ~20000*g* for 30 min. Precipitated proteins were resuspended in 50 mM MOPS buffer (pH 6.5), and 200 μL of each sample was desalted on a Sephadex G-50 spin column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for monitoring PPO activity and protein content. Replicate samples were pooled for each treatment and concentrated using a Amicon Ultra P-10 size-exclusion spin column (10000 MW cutoff) to concentrate the pooled samples according to the manufacturer's (Millipore Corp., Bedford, MA) protocol. To desalt the pooled samples, concentrated samples were diluted 5-fold with 50 mM MOPS buffer (pH 6.5), and the samples were reconcentrated on P-10 spin columns; this desalting and concentrating process was then repeated.

PPO Activity Assays. The total PPO activity (both latent and activated) in leachates or whole caryopsis protein extracts was assayed spectrophotometrically following established protocols (*19*, *21*−*23*). All assays were started by the addition of assay buffer and substrate to samples to equal a final reaction mix containing 50 mM MOPS buffer (pH 6.5) and 10 mM L-DOPA (3,4-dihydroxy-L-phenylalanine), and ΔAbs_{475} was measured over a 3 min time course at 25 °C. One unit of PPO activity was defined as a change of 0.001 absorbance unit/min in a 1 cm path at 475 nm (*22*, *23*).

Protein Characterization by FPLC. Precipitated and desalted leachate proteins were fractionated on an FPLC Mono Q 5/50 GL anion-exchange column (GE Healthcare Bio-Sciences AB) equilibrated with 20 mM Tris buffer (pH 7.8) at a flow rate of 1.0 mL/min. Bound proteins were eluted from the anion exchange column with a 0−1 M gradient of NaCl, and 1 mL fractions were collected. The PPO activity of caryopsis leachate proteins was measured in each fraction, using 10 or 100 μL of each fraction from the *F.a.*1-treated or untreated samples, respectively. Each fraction was further analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots.

SDS-PAGE and Immunochemical Assays. Protein extracts were separated by either 7.5 or 10% SDS-PAGE as previously described (*13*). Proteins were either visualized by Bio-Safe Coomassie G-250 staining or were transblotted to PVDF membrane. Transblots were incubated for at least 1 h in blocking buffer consisting of 5% (w/v) BSA in TBST [25 mM Tris buffer (pH 7.5), 138 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20]. Transblots were then incubated for at least 1 h with a 1:10000 dilution of wheat PPO polyclonal antibody (*13*) in TBST containing 1% (w/v) BSA. Blots were washed 4 × 15 min with TBST containing 1% BSA prior to incubation for at least 1 h with a 1:10000 dilution of goat antirabbit conjugated alkaline phosphatase secondary antibody. Blots were then washed 4 × 15 min in TBST containing 1% BSA, followed by 2 × 5 min in dd H_2O and developed by addition of SIGMA *FAST* BCIP/NBT according to the manufacturer's protocol. Westerns probed with prebleed rabbit serum controls or with goat antirabbit conjugated alkaline phosphatase

secondary antibody alone produced negative results (data not shown) and confirmed the specificity of the wheat polyclonal PPO antibody for wild oat PPOs reported in this study. The optical density of staining for proteins antigenic toward the PPO antibody was determined with a Fluor-S imager and Quantity One imaging software (Bio-Rad Laboratories).

Table 1. PPO Activity from Protein Extracts of Untreated or *F.a.*1-Treated Whole Caryopses after 1, 2, or 3 Days of Incubation[a]

caryopsis treatment	LSD[b] ($P < 0.05$)	day	LSD[c] ($P < 0.05$)	ave TUA/g fwt	ave spec act
untreated	a	1	a	2819	183.4
		2	b	1552	94.9
		3	b	1321	92.2
*F.a.*1	b	1	a	4338	280.8
		2	b	2837	217.0
		3	b	2692	174.5

[a] Activities are presented as either the average total units activity/g fwt (ave TUA/g fwt) of caryopses or the average specific activity (ave spec act) of three biological replicates. [b] LSD significance between treatments by day for both TUA/g fwt and spec act. [c] LSD significance between days within treatment for both TUA/g fwt and spec act.

Protein Sequence Analysis by HPLC-MS/MS. Individual protein bands were excised from Coomassie-stained gels and sent to the Laboratory for Biotechnology and Bioanalysis 2 (Washington State University, Pullman, WA) for protein sequence analysis according to the procedure of Noh et al. (*24*). Briefly, proteins were trypsin-digested in-gel and analyzed by HPLC-MS/MS as previously described. Trypsin fragments were separated on an LC Packings Ultimate Nano HPLC system with an LC Packings monolithic PS-DVB column (Dionex Corp., Sunnyvale, CA), and MS/MS was performed with an Esquire HCT electrospray ion trap (Bruker Daltonics, Inc., Billerica, MA). MS/MS fragment ion lists were compared to Viridiplantae NCBI protein sequence data using a local MASCOT server and a "semi-trypsin" search, which allowed one mismatched trypsin cleavage site. Identification of proteins was based on peptides with ion scores > 20 with a likelihood that the peptide match was not a random event (P values < 0.05).

Statistical Analysis. Statistical analysis was performed using Proc GLM of SAS v.9.1. All factors were treated as fixed effects. For quantities within Western blot bands, data were normalized by g fwt, and each band was analyzed as a separate model. Comparisons of two levels within a

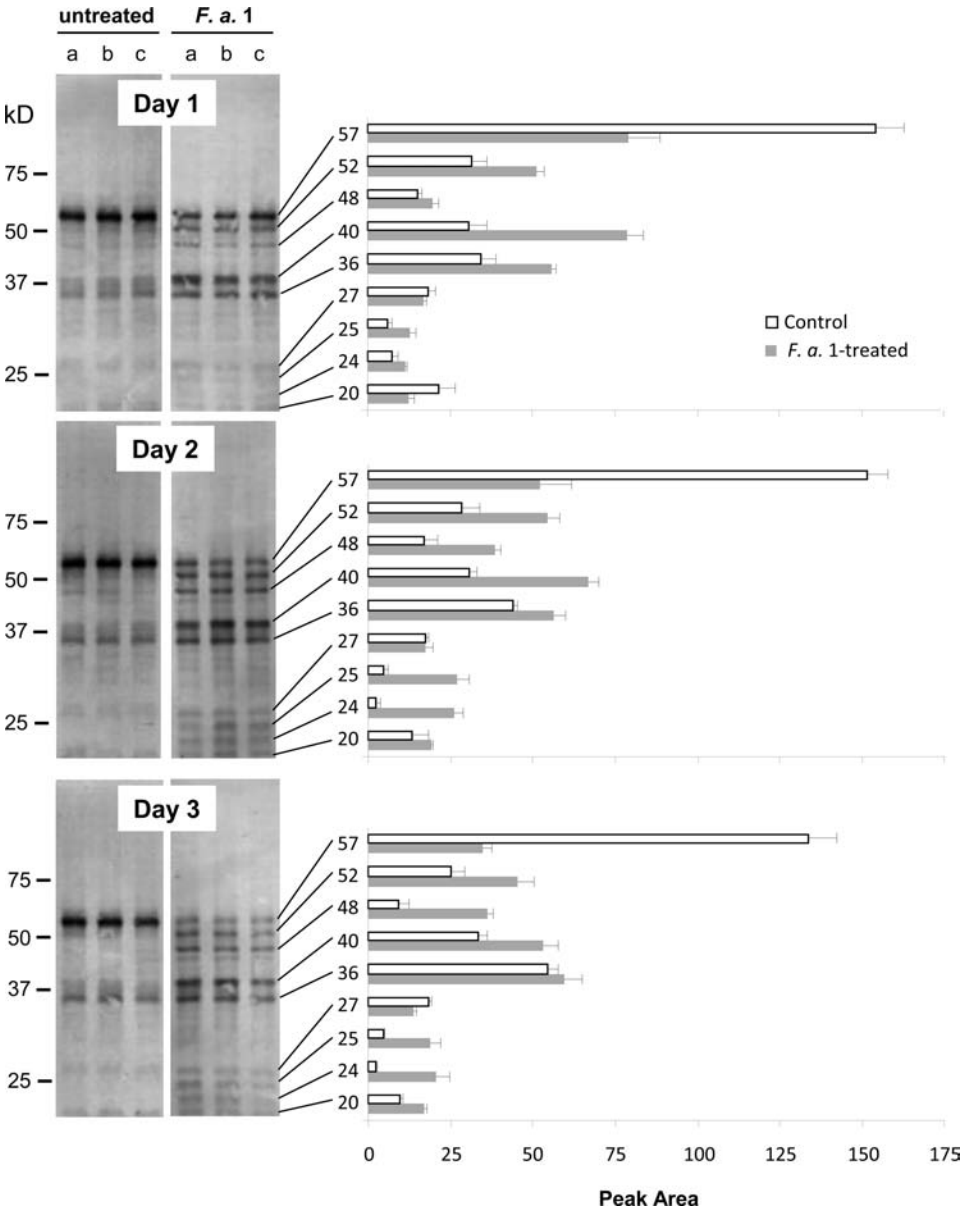

Figure 1. Western blots of protein extracts from untreated or *F.a.*1-treated wild oat (M73) whole caryopses probed with wheat PPO antibody. Molecular weight markers (left) and estimated MW of antigenic proteins (right) are indicated for each blot. The normalized peak area density (right) represents the peak area of antigenic PPO bands obtained from equal loading of 5 μg protein/lane. Error bars represent the standard error, and replicates (a, b, and c) are indicated above the lanes.

factor were conducted using contrasts and F tests, and factors with more than two levels were compared using Fisher's protected LSD.

RESULTS AND DISCUSSION

Whole Caryopsis PPO Activity and Abundance. In this study, a time-course analysis was conducted on protein extracts obtained from *F.a.*1-treated and untreated whole caryopses of wild oat to compare correlations between PPO activity (**Table 1**) and Western blot analyses (**Figure 1**). *F.a.*1 treatment of caryopses for 1, 2, and 3 days caused a significant ($P < 0.05$) 1.5-, 1.8-, and 2-fold increase in average total units of PPO activity/g fwt (ave TUA/g fwt) and a 1.5-, 2.3-, and 1.9-fold increase in average specific activity (ave spec act), respectively, as compared to untreated wild oat caryopses (**Table 1**). Although ave TUA/g fwt and ave spec act in both untreated and *F.a.*1-treated caryopses showed an overall downward trend in PPO activity over 3 days of incubation, within each treatment, decreased activity was only significant ($P < 0.05$) between 1 and 2 days of incubation (**Table 1**). This can be explained by the known leaching of the inducible component of PPO activity (**Figure 2**), which would likely be partially removed by the brief washes performed. Therefore, the soluble leachate lost from caryopses may account for the time-dependent downward trend in PPO activity shown in **Table 1**.

Caryopsis protein extracts were further analyzed by Western blot analysis and quantified by densitometer scans, which identified multiple time-dependent protein bands antigenic toward a wheat PPO polyclonal antibody (**Figure 1**). Densitometer measurements indicated that the major PPO protein in untreated caryopses was ~57 kDa, with minor bands observed at ~52, 48, 40, and 36 kDa; little change was observed for these proteins over the 3 day incubation. The ~57 kDa PPO band was also relatively abundant in *F.a.*1-treated caryopses after 1 day of incubation but showed a time-dependent reduction in abundance over 3 days of incubation (**Figure 1**). However, the abundance of ~57 kDa PPO in *F.a.*1-treated caryopses was significantly less than observed in untreated caryopses at all time points (Supporting Information). In *F.a.*1-treated caryopses, corresponding increases in antigenic PPO protein bands of ~52, 48, 40, 36, 25, and 24 kDa (**Figure 1**) were also significant over the 3 day incubation period. These data indicate that exposure of wild oat caryopses to *F.a.*1 induces an increase in PPO activity, which, at the same time, corresponds to a shift in abundance from the ~57 kDa PPO to multiple lower MW antigenic PPO bands (**Figure 1**).

Leachate PPO Activity and Abundance. PPO activity was also evaluated in AMS-precipitated and P-10-concentrated leachate proteins. Proteins precipitated from leachates of caryopses incubated for 2 days with *F.a.*1 contained a significant 5.1-fold increase in ave TUA/g fwt as compared to leachates of untreated caryopses (**Table 2**). Although the ave TUA/g fwt of AMS-precipitated leachate proteins of untreated caryopses was twice that obtained from mycelia proteins, the difference in activity was not significant. Because > 10-fold the *F.a.*1 mycelia visible on *F.a.*1-treated caryopses was used for estimating the PPO activity contributed by mycelia, we conclude that mycelial PPO activity is a negligible component of PPO activity assayed in leachates. In addition, proteins obtained from *F.a.*1 mycelia lacked any visible antigenic PPO proteins (**Figure 2**). Therefore, mycelial samples were not analyzed further. After the precipitated leachate proteins were pooled and concentrated (**Table 2**), we observed < 1% difference in soluble protein obtained from leachates of untreated (42 μg/g fwt) as compared to *F.a.*1-treated caryopses (43 μg/g fwt). However, we did observe an overall 7.5-fold increase in spec act in leachates of *F.a.*1-treated caryopses (**Table 2**). On the basis of the increase in spec act of PPO in leachates of *F.a.*1-treated caryopsis and the shift in abundance of multiple lower MW antigenic PPO bands observed in whole protein extracts of caryopses (**Figure 1**), our data suggest that induction of PPO activity in *F.a.*1-treated caryopses may result from cleavage of the mature latent forms of PPO, similar to that observed in other plant systems (*5*).

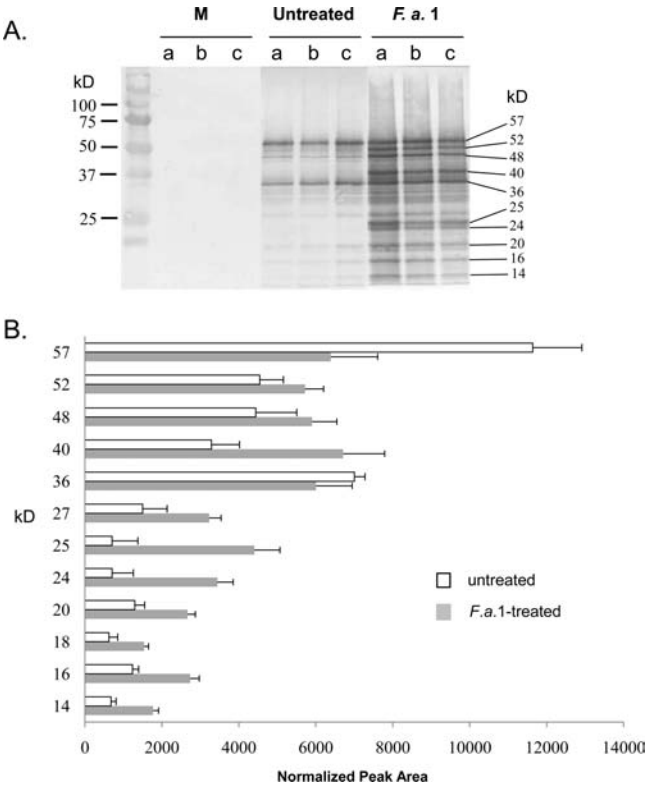

Figure 2. (**A**) Western blot of AMS-precipitated proteins of leachates obtained from 2 day untreated or *F.a.*1-treated caryopses or from mycelia alone probed with wheat PPO antibody. Replicate samples (a, b, and c) are indicated above each lane; each lane was loaded with 20 μL of a 1:1 mix of sample and 2X SDS loading buffer. (**B**) The normalized peak area density was obtained by dividing the total peak area (calculated peak area/μL leachate × total volume of leachate) by g fwt of treated caryopses. The vertical axis indicates the estimated MW of antigenic bands. Error bars indicate the standard error.

Table 2. Average Total Units of Leachate PPO Activity/g fwt (ave TUA/g fwt) of Caryopses or Mycelia in Ammonium Sulphate-Precipitated Leachates Obtained from Three Biological Replicates of Untreated or *F.a.*1-Treated Wild Oat Caryopses 2 Days Postinoculation, as Compared with Mycelia Control[a]

	A.S. precipitate		P-10 conc. leachate protein			
treatment	ave TUA/g fwt	LSD ($P < 0.05$)	TUA/g fwt	mgP total	mgP/g fwt	spec act
untreated	87.7	a	2909	1.11	0.042	2620
*F.a.*1	446.9	b	52041	2.64	0.043	19713
Mycelia	39.6	a	ND	ND	ND	ND

[a] P-10 concentrated (conc.) leachate protein represents pooled biological replicates from each treatment based on total units of activity/g fwt (TUA/g fwt) or specific activity (spec act). ND, not determined; mgP/g fwt, mg protein per g fresh weight of caryopses used in each treatment.

Western blots were used to analyze the precipitated proteins from leachates of untreated and *F.a.*1-treated caryopses to further test the hypothesis that PPO activity is induced via cleavage of larger MW latent forms into smaller MW active forms (**Figure 2**). As previously shown in caryopsis extracts (**Figure 1**), a shift in the abundance toward multiple lower MW antigenic PPO protein

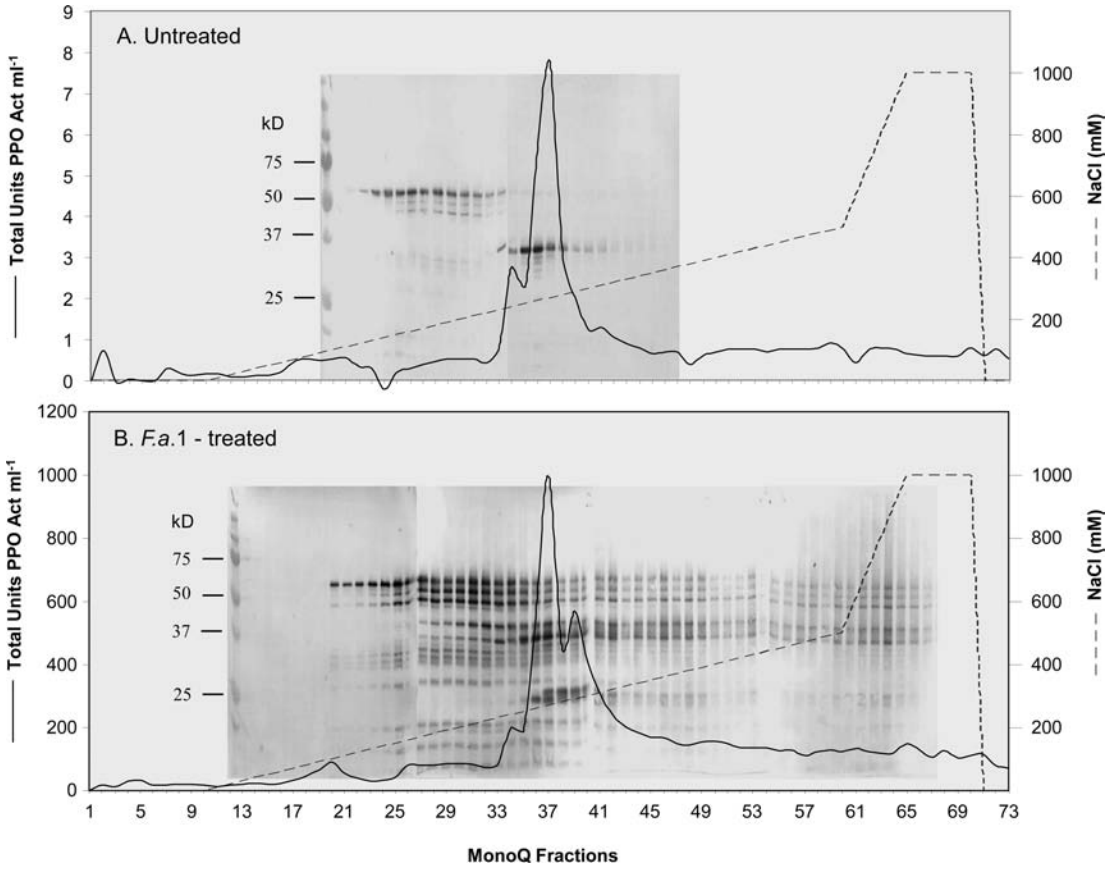

Figure 3. PPO activity and Western blot profiles of leachates obtained from 2 day untreated (**A**) or *F.a.*1-treated (**B**) wild oat caryopses. AMS precipitated leachate proteins from untreated (0.9 mg of total protein) and *F.a.*1-treated (2.4 mg of total protein) were fractionated by Mono Q anion-exchange chromatography. The PPO activity was determined using 100 (**A**) or 10 μL (**B**) of 1 mL fractions. Wheat PPO antibody was used to detect fractionated wild oat PPO protein on Western blots of each fraction. Each lane of the Western blot represents 10 μL of sample from Mono Q fractions for both untreated and *F.a.*1-treated leachate fractions.

bands was observed in leachates of *F.a.*1-treated caryopses as compared to untreated caryopses. Leachate proteins from untreated caryopses showed two major antigenic PPO bands at ~57 and 36 kDa and minor bands at ~52 and 48 kDa (**Figure 2A**,**B**). Precipitated leachate proteins obtained from *F.a.*1-treated caryopses contained major antigenic PPO proteins at ~57, 52, 48, 40, and 36 kDa and other minor bands ranging from 27 to 14 kDa (**Figure 2A**,**B**). On the basis of a normalization of peak area (g fwt of seed used in each biological replicate) for each PPO antigenic band (**Figure 2B**), the ~57 kDa PPO band observed from leachates of *F.a.*1-treated caryopses had nearly a 2-fold decrease in peak area as compared to leachates from untreated caryopses. Although the decrease in normalized peak area of the ~57 kDa PPO band was not significant in leachates of *F.a.*1-treated caryopsis, significant ($P < 0.05$) increases were observed for PPO bands of ~52, 16, and 14 kDa, while marginally significant ($P < 0.077$) increases were observed for the ~25, 24, 20, and 18 kDa bands in leachates of *F.a.*1-treated caryopses.

Partial FPLC Purification of Wild Oat Leachate Proteins with PPO Activity. To further identify specific MW PPO bands corresponding to PPO activity in leachate proteins isolated from untreated and *F.a.*1-treated wild oat caryopses, samples were fractionated by Mono Q anion-exchange chromatography and analyzed for PPO activity corresponding to specific antigenic PPO proteins. Since ANOVA analysis indicated that replication was considered insignificant (Supporting Information), leachate proteins obtained from biological replicates of untreated or *F.a.*1-treated caryopses were individually pooled for further analysis. Comparisons of Mono Q fractionated leachate protein from untreated and *F.a.*1-treated caryopses are shown in **Figure 3A**,**B**, respectively. In both the untreated and the *F.a.*1-treated samples, a major peak of PPO activity was observed between fractions 36 and 38. However, it is important to note that the magnitude of PPO activity in Mono Q-eluted fractions obtained from leachates of untreated caryopses (**Figure 3A**) was approximately 100-fold less as compared to the PPO activity in fractions obtained from leachates of *F.a.*1-treated caryopses (**Figure 3B**). Although 2.4-fold more total protein was loaded onto the Mono Q column for *F.a.*1-treated as compared to untreated sample, this explains a relatively small component of the 100-fold difference in total PPO activity in untreated as compared to *F.a.*1-treated fractions (untreated sample was limited by availability of total leachate protein). The major peak of PPO activity in fractionated leachate proteins from untreated caryopses most closely corresponds to an antigenic PPO protein band of ~36 kDa. While the major peak of PPO activity from leachates of *F.a.*1-treated caryopses appears also to be associated with an antigenic PPO band of ~36 kDa, both the major peak (fractions 36−38) and the minor (fractions 39−40) peaks more closely parallel the abundance of antigenic PPO bands of ~25 and 24 kDa (**Figure 3B**). On the basis of this information, Mono Q fractions 21−32 and 35−37 from the untreated sample (**Figure 3A**) and fractions 21−32, 33−34, 35−37, and 38−40 from the *F.a.*1-treated sample (**Figure 3B**) were individually pooled and concentrated for further analysis.

A Predicted Model for Processing and Activation of Wild Oat PPO. To help dissect the dynamic relationships among pooled

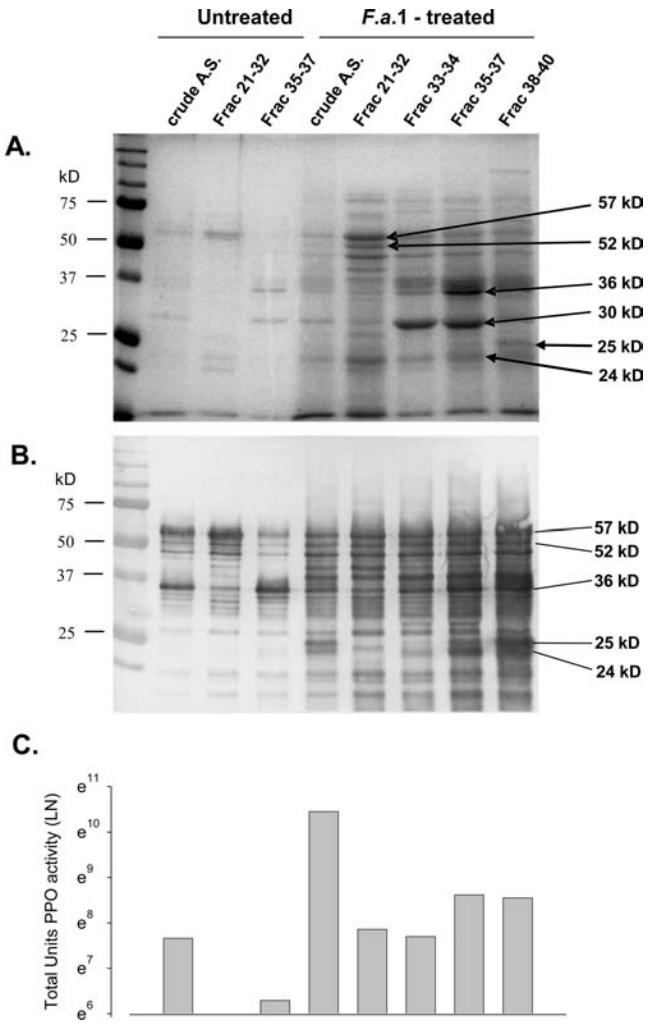

Figure 4. Comparison of Mono Q fractionated leachate proteins obtained from 2 days untreated or *F.a.*1-treated wild oat caryopses. Proteins were visualized by Coomassie staining (**A**) or reactivity to wheat PPO antibody (**B**). Each lane represents 5 μL of P-10 concentrated protein. Protein bands (*F.a.*1-57 kDa, *F.a.*1-52 kDa, *F.a.*1-36 kDa, *F.a.*1-30 kDa, *F.a.*1-25 kDa, and *F.a.*1-24 kDa) were cut from the Coomassie-stained gel for sequence analysis (**Table 3**). (**C**) Total units PPO activity on logarithmic (LN) axis.

Mono Q fractions with PPO activity, individual Coomassie-stained proteins (**Figure 4A**) of ~57, 52, 36, 30, 25, and 24 kDa were excised and subjected to HPLC-MS/MS sequencing. Note that although the ~30 kDa Coomassie-stained protein was highly induced (**Figure 4A**), it did not show an antigenic reaction with polyclonal PPO on the corresponding Western blot (**Figure 4B**) or any other Western blots presented (**Figures 1**−**3**). Predicted sequences (**Table 3**) from the ~57, 52, and 36 kDa peptides produced sequence homology matches to wheat (*T. aestivum*) PPO accessions AAT06525 (*14*) and ABK62802 (*25*). The ~57, 52, and 36 kDa PPOs bands all returned a predicted peptide match of YTYQDVGLPWLNAR, but only the ~57 kDa PPO band returned additional predicted peptide matches of FGVCD-LMDDIGADGDQ and AGSFAHTPHMVRPEETRK (**Table 3**). The ~30, 25, and 24 kDa peptides did not show any high-quality matches to known plant PPOs. However, the ~30 and 25 kDa proteins did return high-quality matches (**Table 3**) for a putative Chitinase 1 precursor (accession NP_001064608) from rice (*Oryza sativa*). The ~24 kDa protein returned a high-quality match for oxalate oxidase (accession CAD43309) from ryegrass (*Lolium perenne*), which is induced in response to wounding in ryegrass (*26*). Interestingly, oxalate oxidase is also known to be a pathogen-response enzyme (*27*).

On the basis of the predicted PPO sequences obtained for wild oat and data from previously published plant PPOs (*5*), a predicted model for wild oat PPO protein processing was established (**Figure 5**). The full-length wheat PPO accession ABK62802 (*25*) was used in this model because wild oat predicted that peptide sequences (bold and underlined amino acid sequence) had 100% sequence similarity to this full-length wheat PPO protein (**Figure 5A**). The molecular mass of this full-length, unprocessed wheat PPO is estimated to be 63.8 kDa (**Figure 5B**). On the basis of reported processing sites among other plant PPOs (*5*), **Figure 5A**,**B** shows the predicted processing sites for N-terminal cleavage to generate the estimated 55.2 kD mature PPO, and the predicted C-terminal cleavage site required for activation. The estimated molecular mass of the putative activated wheat PPO, minus both the N-terminal transit peptide (8.6 kDa) and the C-terminal peptide (15.3 kDa), is predicted to be 39.9 kDa. Amino acids within the solid arrows of the active wheat PPO region containing copper-A and -B binding sites (**Figure 5A**) are estimated to be 32.5 kDa and represent the predicted minimum core molecular mass reported by Flurkey and Inlow (*5*) to be required for retaining folding properties essential for plant PPO activity.

Correlations between PPO Activity and Antigenic PPOs. The predicted molecular mass for the mature and active forms of wheat PPO shown in **Figure 5** is similar to the estimated MW of the ~57 and 36 kDa wild oat PPO proteins, respectively, observed in **Figures 1**−**4**. On the basis of the antigenic PPO bands that returned predicted peptide matches to other known plant PPOs, our data suggest that the ~57 kDa wild oat PPO observed in both caryopses and leachates of caryopses likely represents a mature but latent PPO minus the N-terminal plastid transit peptide. Also, because the major peak of PPO activity in leachates obtained from both untreated (**Figure 3A**) and *F.a.*1-treated (**Figure 3B**) caryopses corresponded to the presence of an ~36 kDa antigenic PPO band, as well as ~25 and 24 kDa bands in *F.a.*1-treated, we assume these processed PPOs likely represent wild oat PPO(s) minus both the N- and the C-terminal peptides. Although our data do not conclusively implicate the ~36 kDa PPO as the only activated form corresponding to increased specific activity of PPO in *F.a.*1-treated caryopses, our data would suggest that it likely plays an important role in the overall process.

Unfortunately, sequence analysis of the ~25 and 24 kDa wild oat proteins from leachates of *F.a.*1-treated caryopses is confounded by the fact that it did not generate a predicted peptide sequence with homology to known plant PPOs. Several factors could be involved and include the possibility that too little protein was available for detection or that multiple peptides were overlapping and interfered with detection of PPOs. The fact that sequence data obtained from antigenic PPOs with peptides tentatively identified as oxalate oxidase and Chitinase may just be a coincidence and we have not ruled out that these particular MW protein bands may represent overlapping peptides representing both the above predicted enzyme and the PPO.

The results presented so far do not account for the abundance of the ~52, 48, 40, 25, and 24 kDa PPO bands in leachates of *F.a.*1-treated caryopsis observed in **Figures 1**−**4**. The existence of a PPO multigene family in cereal grains (*13*−*15*, *25*) could help explain the additional observed PPO bands in this study. Although no antigenic bands for PPO were observed above ~57 kDa in protein extracts of whole caryopses (**Figure 1**) or soluble proteins from leachates of caryopses (**Figure 2**), antigenic PPO proteins of ~61, 57, 52, 48, and 40 kDa have previously been observed in mature wild oat (M73) hull tissue (*28*). These observations indicate the potential for the existence of a multigene family of PPOs in wild

Table 3. Predicted Best Match Peptide Sequence of Mono Q-Fractionated Soluble Leachate Proteins from Wild Oat Caryopses Incubated with *F.a.*1 for 2 Days[a]

sample	MW	δ	ion score	expect	peptide sequence
*F.a.*1- 57 kDa	1694.84	0.51	63	0.04	R.YTYQDVGLPWLNAR.P
	1742.68	−0.72	31	39	R.FGVCDLMDDIGADGDQ.T
	2050.02	2.25	24	1500	C.AGSFAHTPHMVRPEETRK.G
*F.a.*1- 52 kDa	1694.84	−0.35	57	0.093	R.YTYQDVGLPWLNAR.P
*F.a.*1- 36 kDa	1862.93	−1.97	56	0.1	R.YTYQDVGLPWLNARPA.K
*F.a.*1−30 kDa	1239.62	0.26	74	0.0002	R.EYIGAQFTGVR.F
*F.a.*1- 25 kDa	1239.62	1.43	68	0.0008	R.EYIGAQFTGVR.F
*F.a.*1- 24 kDa	1328.76	−0.09	74	0.0003	K.VARAGETFLIPR.G
	1696.84	−0.83	48	0.041	R.VDFAPGGTNPPHVHPR.A

[a] Description of terms: sample, proteins excised from Coumassie-stained gel (**Figure 4A**); MW, the calculated MW of the peptide indicated; δ, the deviation of calculated MW from the MW observed by MS; ion score, a calculated indicator of the quality of match; expect, inversely correlated with the quality of the match; and peptide sequence, the best match to known protein sequences based on Viridiplantae NCBI protein sequence data. First and last amino acids, separated by a period, are putative cleavage sites. The ∼57, 52, and 36 kDa peptides best matched a wheat PPO (**Figure 5**); the ∼30 and 25 kDa peptides best matched a rice Chitinase; and the ∼24 kDa peptide best matched oxalate oxidase from *Lolium perenne*.

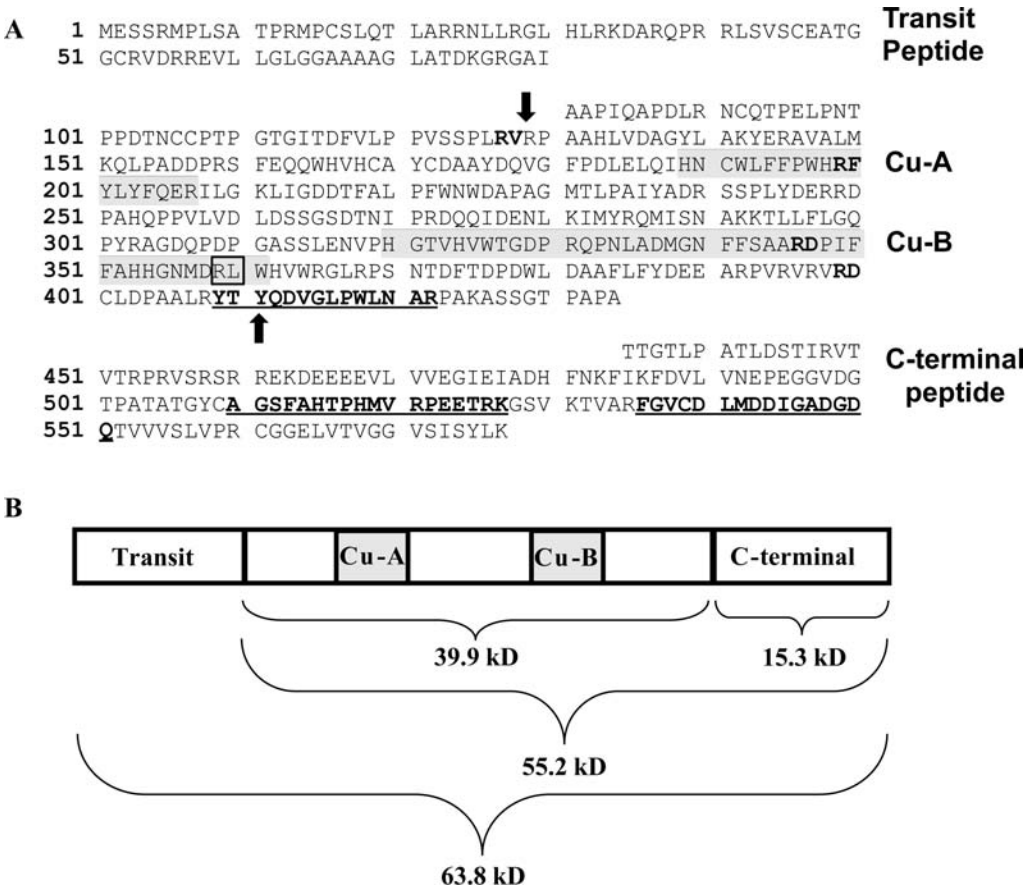

Figure 5. Predicted model for wild oat PPO protein processing. Wheat PPO (accession ABK62802 (*25*)) was used in this model since predicted wild oat peptide sequences (bold and underlined amino acid sequence) produced high-quality matches to this full-length PPO. (**A**) Predicted amino acid processing sites for the N-terminal transit peptide (AA) and C-terminal cleavage site (PA/TT). Amino acids between the solid arrows are considered the core molecular mass essential for maintaining enzyme activity of plant PPOs (*5*), which includes both copper-binding (Cu-A and Cu-B) sites (shaded). Amino acids in bold, but not underlined, represent known in vitro proteolytic cleavage sites within purified wheat PPO (*21*), and the box around amino acids within the Cu-B binding site represents a predicted C-terminal processing site in grape PPO (*29*). The predicted model (**B**) shows the estimated molecular mass of the mature (55.2 kDa) form of PPO, which lacks the N-terminal transit peptide (∼8.6 kDa), the 39.9 kDa active PPO minus the 15.3 kDa C terminus, the Cu-A and Cu-B binding sites (shaded), and the predicted C-terminal cleavage site.

oat, similar to that reported for other plant species (*6*, *13*−*15*, *25*). The existence of multiple PPO isoforms with potentially differing cleavage sites could help to account for the multiple antigentic PPO peptides observed at different MW ranges in this study. However, other post-translational modifications such as glycosylation and phenolic cross-linking of PPOs are known to occur (*5*) and could also play a potential role. Because the number of wild oat PPO genes was not the focus of this study and to date has not been investigated and the mechanism by which these wild oat PPOs are processed into smaller peptides is currently unknown, further research will be needed to fully account for the multiple antigenic PPO bands observed in this study, including those responsible for PPO activation.

On the basis of the possible estimated MW combinations that can be calculated from the observed data presented in this study (**Figures 1**−**4**), it seems unlikely that abundance of the ∼25 and

24 kDa wild oat PPO peptides occurs from a single processing event of the larger ~57 kDa wild oat PPO observed in caryopses. For example, cleaving the C terminus from the ~57 kDa wild oat PPO, to generate a ~36 kDa PPO, should produce a peptide no larger than ~21 kDa. It is also unlikely that the ~25 and 24 kDa wild oat PPOs represent processing of the N-terminal plastid transit peptide, since the process usually requires two cleavage steps (*5*). Another more plausible explanation for the ~25 and 24 kDa PPO bands is that the ~36 kDa wild oat PPO is further processed. Recent studies on grape (*Vitis vinifera*) PPO have suggested that C-terminal processing may occur at more than one site to produce multiple activated isoforms (*29*). Sites that were cleaved by trypsin in vitro within purified wheat PPO (46) are shown in **Figure 5A**. Considering the possibility that one or more of these sites corresponds to a site of in vivo proteolysis, the first known cleavage site (bold but not underlined) within the activated oat PPO is directly adjacent to the N-terminal amino acid (first solid arrow in **Figure 5A**) predicted for the minimum core molecular mass needed to retain proper folding for enzyme activity (*5*). Interestingly, the estimated MW from this first known cleavage site to the cleavage site shown in bold but not underlined within the C-terminal portion of the copper-B binding site (**Figure 5A**) is ~24.9 kDa. Although this explanation is possible, Flurkey and Inlow (*11*) concluded the minimum core molecular mass needed to retain proper folding for enzyme activity ranges from ~32 to 35 kDa, and it remains to be unambiguously determined whether the 24 and 25 kDa bands contain enzymatically active PPO. Furthermore, cleavage at the previously proposed site would also interfere with the conserved, downstream histidine residue required for copper binding; it seems unlikely that a form of PPO missing this histidine residue could be enzymatically active.

Although data from grape (*29*) indicate that C-terminal processing likely occurs near the end of the copper-B binding site (see box within copper-B binding site of **Figure 5A**), which would not disrupt the conserved histidine residue needed for copper binding, no information about the potential for enzymatic activity of grape PPO cleaved at this predicted site was reported. Because the ~25 or 24 kDa wild oat PPO peptides tightly paralleled the major peak of PPO activity observed in leachates of *F.a.*1-treated caryopses (**Figure 3B**), and no significant difference (Supporting Information) was observed in the ~36 kDa PPO band in leachates of untreated and *F.a.*1-treated caryopses, we should not rule out the possibility that further processing of the ~36 kDa wild oat PPO could account for the increased PPO activity observed in this study.

Potential Mechanism for *F.a.*1-Induced Defense Response in Caryopses. On the basis of data presented in this study, we propose that exposure of dormant wild oat to *F.a.*1 induces activity of endogenous latent PPO, which allows the multiple PPO proteins to be more freely soluble and readily exuded into the surrounding environment. Such a mechanism could provide a barrier to pathogen attack in the soil seed bank. Particularly, activation of PPO in the presence of the hull barrier would allow a more concentrated form of the activated PPO protein. Finally, to the best of our knowledge, this is the first documented case of a defense response in dormant weed seed or maybe dormant seed of any plant.

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Supporting Information Available: ANOVA analysis of antigenic PPO proteins from three biological replicates of extracts (**Figure 1**) and leachates (**Figure 2**) of untreated or *F.a.*1-treated caryopses for 1, 2, or 3 (extracts) or 2 days (leachates); leachate samples only analyzed at 2 days of incubation, thus day and treatment × day not applicable (NA). This material is available free of charge via the Internet at http://pubs.acs.org.

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