Integrated Metabolite and Transcript Profiling Identify a Biosynthetic Mechanism for Hispidol in Medicago truncatula Cell Cultures

Mohamed A. Farag, Bettina E. Deavours, Ângelo de Fátima, Marina Naoumkina, Richard A. Dixon, and Lloyd W. Sumner*

Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401 (M.A.F., B.E.D., M.N., R.A.D., L.W.S.); Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt 11562 (M.A.F.); Department of Biology, Colorado State University, Fort Collins, Colorado 80523 (B.E.D.); and Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Campus Pampulha, Belo Horizonte, Minas Gerais, 31270–901, Brazil (A.de.F.)

Metabolic profiling of elicited barrel medic (Medicago truncatula) cell cultures using high-performance liquid chromatography coupled to photodiode and mass spectrometry detection revealed the accumulation of the aurone hispidol (6-hydroxy-2-[4-(4-hydroxyphenyl)methylidene]-1-benzofuran-3-one) as a major response to yeast elicitor. Parallel, large-scale transcriptome profiling indicated that three peroxidases, MtPRX1, MtPRX2, and MtPRX3, were coordinately induced with the accumulation of hispidol. MtPRX1 and MtPRX2 exhibited aurone synthase activity based upon in vitro substrate specificity and product profiles of recombinant proteins expressed in Escherichia coli. Hispidol possessed significant antifungal activity relative to other M. truncatula phenylpropanoids tested but has not been reported in this species before and was not found in differentiated roots in which high levels of the peroxidase transcripts accumulated. We propose that hispidol is formed in cell cultures by hispidol. MtPRX1 and MtPRX2 exhibited aurone synthase activity based upon in vitro substrate specificity and product profiles of recombinant proteins expressed in Escherichia coli. Hispidol possessed significant antifungal activity relative to other M. truncatula phenylpropanoids tested but has not been reported in this species before and was not found in differentiated roots in which high levels of the peroxidase transcripts accumulated. We propose that hispidol is formed in cell cultures by metabolic spillover when the pool of its precursor, isoliquiritigenin, builds up as a result of an imbalance between the upstream and downstream segments of the phenylpropanoid pathway, reflecting the plasticity of plant secondary metabolism. The results illustrate that integration of metabolomics and transcriptomics in genetically reprogrammed plant cell cultures is a powerful approach for the discovery of novel bioactive secondary metabolites and the mechanisms underlying their generation.

Phenylpropanoid metabolism encompasses a complex network of branching biochemical pathways that collectively provide plants with thousands of compounds that have diverse functions in plants, most notably in defense, such as cell wall strengthening and repair (e.g. lignin and suberin), antimicrobial activity (e.g. furanocoumarin, pterocarpan, and isoflavonoid phytoalexins), and as signaling compounds such as luteolin (Peters et al., 1986) and apigenin (Peters and Long, 1988). In the core pathway, Phe is converted into p-coumaroyl-CoA, and the condensation of this molecule with three molecules of malonyl-CoA, via chalcone synthase activity, yields 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone), which is the primary C15 flavanone skeleton from which a large number of flavonoid subclasses diverge (Fig. 1). In legumes, the 6′-deoxy chalcone isoliquiritigenin is produced through the concerted activity of chalcone synthase and chalcone reductase (Ralston et al., 2005). This trihydroxychalcone can be further metabolized by chalcone isomerase to form liquiritigenin, a precursor for a range of 5-deoxy flavones, flavonols, and isoflavones (Fig. 1). Exceptions to this biosynthetic route are the aurones, a flavonoid subclass that can be directly synthesized from isoliquiritigenin (Strack, 1997). Aurones (Fig. 1) serve significant roles in flower pigmentation (Nakayama et al., 2001) and defense responses (Paré et al., 1991). In humans, aurones have recently drawn much attention for their therapeutic potential as anticancer, antidiabetic, antibacterial, antiparasitic, and antihormonal agents (Boumendjel, 2003). While flavone, flavonol, and isoflavone biosynthetic pathways have been extensively studied using different molecular and biochemical approaches, only a few studies on aurone biosynthesis have been reported.

Aureusidin synthase, a polyphenol oxidase (PPO) homolog, has been identified in snapdragon (Antirrhinum majus) as a key enzyme involved in aurone...
biosynthesis (Nakayama et al., 2000). The original mechanism for aureusidin synthase proposed the 3-hydroxylation and oxidative cyclization, involving 2',α-dehydrogenation, of naringenin chalcone to produce aureusidin (4,6,3',4'-tetrahydroxyaurone). It was subsequently shown that auresidin synthase could likewise form the corresponding aurone sulfuretin by similar 3-hydroxylation and oxidative cyclization of isoliquiritigenin (Fig. 1; Nakayama et al., 2001). An alternate mechanism proposed for aurone biosynthesis in soybean (Glycine max) seedlings involves a hydrogen peroxide (H₂O₂)-dependent peroxidase (PRX; Wong, 1967; Rathmell and Bendall, 1972) that catalyzes the oxidation of isoliquiritigenin to yield a 2-(α-hydroxybenzyl)coumaranone derivative, a hydrated form of aurone, which is then spontaneously dehydrated to form the aurone hispidol (Fig. 1). However, no plant PRX involved in aurone biosynthesis has been functionally characterized in legumes to date (Strack, 1997; Nakayama et al., 2001).

Plant PRXs (EC 1.11.17) are ubiquitous, heme-containing glycoproteins that catalyze the oxidation of diverse organic and inorganic substances at the expense of H₂O₂. Higher plants possess a number of PRX isoenzymes that are usually classified as anionic, neutral, or cationic based upon their pI (Barz et al., 1990). Anionic and neutral PRXs are usually cell wall bound, and cationic forms are typically confined to the vacuole (Kawalleck et al., 1995). Because of their location, anionic and neutral PRXs are believed to be
mainly involved in plant defense. PRXs can oxidize vacuolar phenolic pools and also play key roles in the polymerization steps involved in lignification and suberization of plant cell walls (Chittoor et al., 1997). Functional characterizations of PRXs have been based primarily upon genomic sequence mining and in vitro biochemical assays. However, the extremely wide substrate specificities of PRXs, the high number of PRX genes, and the diversity of PRX structures raise important questions about their true in vivo substrates and functions.

Large-scale metabolite profiling (metabolomics) is a powerful tool for analyzing metabolism and gene function (Sumner et al., 2003; Bino et al., 2004). Many early metabolomics studies were focused upon primary metabolites for plant genotyping (Taylor et al., 2003), detection of silent phenotypes in transgenic potato (Solanum tuberosum; Weckwerth et al., 2004), and the examination of stress responses (Broeckling et al., 2005). Parallel profiling of transcript and metabolites has also been applied to study the effect of cold acclimation (Kaplan et al., 2004), phosphorus stress (Hernandez et al., 2007), and arbuscular mycorrhizal interactions (Schaarschmidt et al., 2007) in plants. More recently, the profiling of natural products has also been incorporated into the metabolomics approach and integrated with transcriptome analysis to identify novel gene functions associated with flavonoid biosynthesis (Fridman and Pichersky, 2005). Such an integrated approach represents a powerful platform for the clarification of gene function in plant secondary metabolism (Fridman and Pichersky, 2005).

Barrel medic (Medicago truncatula) is a rapidly developing model for legume biology (Cook, 1999; Young et al., 2005) and an excellent species for studying the rich and unique secondary metabolism of legumes (Dixon and Sumner, 2003). We have recently utilized elicited M. truncatula liquid suspension cell cultures to study biotic stress responses using an integrated functional genomics approach that included transcriptomics (Suzuki et al., 2005), proteomics (Lei et al., 2005), and metabolomics (Broeckling et al., 2005; Farag et al., 2008). These studies involved independent applications of the phytohormone methyl jasmonate (MeJA) and a yeast cell wall preparation (yeast elicitor [YE], a fungal pathogen mimic) to M. truncatula root suspension cell cultures. We report here that the antimicrobial auran, hispidol, is a major YE-induced secondary metabolite in these cultures, and correlation analyses between metabolite and transcript profiles implicated specific PRXs in auran biosynthesis. The functionalities of these PRXs were subsequently confirmed by biochemical analysis of recombinant proteins. The integration of metabolomics and transcriptomics data has thus led to the discovery of both a novel bioactive secondary metabolite and a novel mechanism for its biosynthesis. These results are discussed in terms of biochemical and cellular responses to biotic stress.

RESULTS

Hispidol and Hispidol-4′-O-β-D-Glucoside Are Novel Phenylpropanoid Compounds Induced in the Response of M. truncatula to YE

A large-scale elicitation experiment was conducted using liquid suspension cell cultures and two elicitors (YE and MeJA) to generate an integrated global data set (transcriptome, proteome, and metabolome) and to facilitate gene discovery and novel insight into biotic and abiotic stress responses associated with natural product pathways in M. truncatula. Triplicate biological samples from control and elicited cell cultures were harvested at 21 different time points between 0 and 48 h postelicitation for each elicitor and each replicate from independent culture flasks (Broeckling et al., 2005). Global metabolite, protein, and transcript profiles were obtained for the sampled cells and specifically queried as related to the nature and extent of the effect on phenylpropanoid biosynthesis. The expression levels of approximately 16,000 tentative consensus sequences (TCs) were monitored using a custom M. truncatula cDNA oligonucleotide microarray. The accumulation patterns of approximately 1,000 proteins were monitored with two-dimensional gel electrophoresis (Lei et al., 2005). Approximately 180 secondary metabolites were profiled using HPLC coupled to photodiode and mass spectrometry detection (HPLC-PDA-MS; Farag et al., 2007, 2008), and approximately 500 primary metabolites were profiled by gas chromatography-mass spectrometry (GC-MS; Broeckling et al., 2005).

The effect on phenylpropanoid metabolite pools was most dramatic in cell cultures treated with YE. M. truncatula and alfalfa (Medicago sativa) cell cultures are known to respond to YE by transcriptional induction of isoflavonoid biosynthetic genes and the downstream accumulation of the isoflavonoid-derived pterocarpan phytoalexin medicarpin (Suzuki et al., 2005). Increased levels of several isoflavonoids were observed after exposure to YE in our cultures, with medicarpin notably induced 10-fold. However, two unknown compounds, P1 and P2, showed the largest fold inductions, up 45- and 15-fold, respectively, in response to YE, and their corresponding peaks were not found in the HPLC-PDA-MS chromatograms of control cells (Fig. 2A). Neither P1 nor P2 was induced in response to MeJA. In the negative ion electrospray ionization mode, the parent ion observed for P1 (retention time = 23.5 min) had mass-to-charge ratio (m/z) 415, with a major fragment ion at m/z 253. The m/z 162 difference between both ions suggests the loss of a hexose (Fig. 2B). P2 (retention time = 33.5 min) also had an ion peak at m/z 253, suggesting that P2 might be the aglycone moiety observed as part of the hexose conjugate in P1 (Fig. 2C). Enzymatic hydrolysis of P1 with β-glucosidase followed by HPLC-PDA-MS analysis yielded a single peak with the same retention time and spectral characteristics as P2. GC-MS analysis of
the sugar hydrolysate confirmed D-Glc as the hexose moiety in P1. The UV spectra for P1 (Fig. 2B, inset) and P2 (Fig. 2C, inset) were similar, with a \( \lambda_{\text{max}} \) at 390 nm, implying the presence of a highly conjugated chromophore typical of aurones. Aurones absorb near the visible region due to an extended conjugated double bond/pi system found in chalcones, flavones, or isoflavones (Mabry et al., 1970).

The basic aurone nucleus has a \( M_r \) of 222, and the 32-D mass difference between the aurone nucleus and P2 suggests the presence of two additional hydroxyl groups on either the A or B ring of P2. Several authentic aurone standards having a \( M_r \) of 254, the same as P2, were analyzed using the same HPLC-MS conditions, of which only hispidol (6,4'-dihydroxyaurone) matched P2 in retention time and spectral characteristics. Thus, P2 was identified as hispidol, a compound previously detected in soybean seedlings (Wong, 1967).

Higher intracellular levels of hispidol glucoside rather than hispidol were observed in response to YE (Fig. 2A). This might indicate a rapid in vivo conversion of hispidol to hispidol glucoside or a limiting availability of isoliquiritigenin. However, metabolite profiling of the extracellular medium from YE-treated cell cultures showed that hispidol accumulation was induced up to 50-fold (Supplemental Fig. S1), whereas, hispidol glucoside was below the detection limit of the HPLC-PDA-MS. The large accumulation of hispidol in the medium suggests that the lower intracellular levels of hispidol compared with hispidol glucoside are likely due to secretion of this compound into the medium.

**Antimicrobial Activity of Hispidol**

The induction of hispidol and hispidol glucoside in response to YE suggested that these compounds might be defense-related or antimicrobial compounds. Thus, antifungal activity assays were conducted with the fungal pathogen *Phoma medicaginis*, which causes spring black stem and leaf spot disease in alfalfa, to assess potential biological activities of hispidol and its glucoside. Other endogenous *Medicago* metabolites induced in response to YE were also tested and included formononetin, ononin, afromosin, iridisolide, and isoliquiritigenin. All phenolics were tested at a concentration of 100 \( \mu \text{M} \). Coumestrol, a known antimicrobial compound in alfalfa, was included in the assay as a positive control and showed the strongest inhibition of fungal growth, followed by hispidol, afromosin, ononin, and hispidol-4'-O-\( \beta \)-D-glucoside (Fig. 3). Thus, hispidol can be classified as a potential and relatively potent antifungal compound in *M. truncatula* cell cultures. The direct precursor of hispidol, isoliquiritigenin, showed very weak antifungal activity against *P. medicaginis*.

**Selection of Putative Hispidol Synthases**

Two primary mechanisms for aurone biosynthesis have been proposed based upon PPO (Nakayama
Potential involvement of PPOs in hispidol biosynthesis was investigated. BLASTn queries of the auruscinid synthase described previously (Nakayama et al., 2000) against the M. truncatula gene indices revealed three M. truncatula sequences with significant similarities (i.e. TC101697, P = 3.5 × 10^{-9}; BG644378, P = 2.7 × 10^{-12}; and CX521674, P = 9.9 × 10^{-10}). All three putative PPOs were present on the M. truncatula microarray, but the signals for all three were below the detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation. In addition, the literature reports that alfalfa leaves and stems detectable level in response to yeast elicitation.
Figure 4. Selection of candidate PRXs based upon microarray expression analysis. A and B, Microarray analysis results for putative PRXs based upon a 16K *M. truncatula* custom oligonucleotide chip. Color-coded and clustered histograms displaying Hispidol Biosynthesis in *Medicago truncatula* Cell Cultures.

Response of selected peroxidases to YE or MeJA elicitors

<table>
<thead>
<tr>
<th>Response</th>
<th>YE</th>
<th>MeJA</th>
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<tbody>
<tr>
<td>TC110836 (Mt Prx1)</td>
<td>17.4</td>
<td>2.0</td>
</tr>
<tr>
<td>TC106484 (Mt Prx2)</td>
<td>1.4</td>
<td>2.4</td>
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<tr>
<td>TC106558 (Mt Prx3)</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>TC101009</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>TC107570</td>
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<td>TC102451</td>
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<td>TC106564</td>
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</tr>
<tr>
<td>TC102226</td>
<td>0.8</td>
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D-F, Bar graphs showing the relative ratio of actin in response to MeJA and YE for selected PRXs.

G, Graphs showing the relative abundance of Hispidol and Hispidol glucoside over time.
expression of these PRXs (Fig. 6). Relative expression level data were extracted from an expression atlas study where Affymetrix microarray data were collected for a series of M. truncatula tissues, including leaf, petiole, stem, vegetative bud, flower, pods, and unnodulated root, as well as nodulated root developmental time course and a seed developmental time course (Benedito et al., 2008). MtPRX1 was most highly expressed in flower, pods, and roots. MtPRX2 expression levels were approximately 200-fold higher in roots and nodules than in any other tissues. MtPRX3 expression levels were more evenly distributed throughout all tissues.

Biochemical Characterization of Recombinant PRX Enzymes

To determine the biochemical activities of each of the three candidate PRXs, their cDNAs were subcloned into a pET-28a expression vector and expressed in Escherichia coli BL21 (DE3) cells. Western-blot analysis of the total protein extracts from isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cells harboring MtPRX1, MtPRX2, and MtPRX3 clones showed the presence of a single protein band for each MtPRX between 34 and 39 kD and corresponding to the predicted size of the recombinant proteins (Fig. 7). No protein bands were detected in cultures harboring an empty pET-28a vector (Fig. 7). Recombinant PRX proteins in cell lysates were tested for enzymatic activity against guaiacol, a phenolic substrate commonly utilized in PRX enzymatic assays with equal amounts of total lysate protein used in each enzymatic reaction (see “Materials and Methods”). PRX enzymatic activity was detected with all recombinant proteins: MtPRX1, 111 ± 15 nmol mg−1 min−1; MtPRX2, 105 ± 21 nmol mg−1 min−1; and MtPRX3, 220 ± 44 nmol mg−1 min−1. It should be noted that variation in PRX enzymatic activity in cell lysates for MtPRX1, -2, and -3 could be attributed to minor differences in expression levels of the recombinant proteins, as purified recombinant proteins could not be isolated. No PRX activity was detected in the cell lysate containing the empty vector. To determine whether these PRXs could be involved in hispidol biosynthesis, isoliquiritigenin was tested as a substrate and the products were analyzed using HPLC-PDA-MS. In vitro assays showed the formation of hispidol from isoliquiritigenin using cell lysates expressing each of the three different PRXs (Fig. 8, B–D). No aurone products were observed in the in vitro assays of control cells expressing the empty vector (Fig. 8A). Although MtPRX2 and MtPRX3 protein sequences are 79% identical (Fig. 5A), their product profiles were quite different with isoliquiritigenin as a substrate. MtPRX2 yielded mainly hispidol (Fig. 8C), whereas MtPRX3 formed predominantly a quinol vinyl ether (QVE) and hispidol in lower amounts (Figs. 8D and 11). Furthermore, QVE was an observed product of horseradish peroxidase and not detected in elicited M. truncatula cell cultures.

Isoliquiritigenin-4-O-glucoside (Zhu et al., 2003) was also converted to hispidol glucoside by the recombinant PRXs (Fig. 11; Supplemental Fig. S2). A considerable proportion of the isoliquiritigenin glucoside was isomerized in all in vitro assays to liquiritigenin glucoside, as flavanones are the thermodynamically more stable isomers of chalcones (Boland and Wong, 1975). In the case of MtPRX3, a QVE glucoside was the major product (Fig. 11).

To further assess MtPRX1 to -3 as putative vacuolar PRXs, enzymatic assays were performed that monitored the formation of hispidol from isoliquiritigenin by MtPRX1 to -3 as a function of pH over the range of pH 3 to 9. Vacuolar enzymes tend to have lower optimum pH, consistent with the acidic environment of the vacuole, which is typically pH 5.5 (Taiz, 1992; Ros Barcelo et al., 2003), and is the case for snapdragon aureusidin synthase (Nakayama et al., 2000, 2001). Both MtPRX2 and -3 exhibited an activity optimum at pH 5, whereas MtPRX1 showed a slightly higher activity optimum at pH 6 (Supplemental Fig. S3). The pH profiles of MtPRX2 and -3 were bimodal, with a small peak at pH 7.

Substrate Specificities and Mechanisms of MtPRXs

The ability of the recombinant MtPRXs to synthesize aurones from a variety of chalcone substrates was evaluated to determine the specificity of these enzymatic reactions and the underlying mechanism(s) (Fig. 11). Chalcones tested either lacked one of the three (4, 2′, or 4′) hydroxyl groups present in isoliquiritigenin or contained an additional hydroxyl group on either the A or B ring. Chalcones lacking the 2′ or 4-hydroxyl functions (i.e. compounds 6, 7, and 20) generated no

Figure 4. (Continued.)
the changes in putative PRX expression levels in response to YE (A) or MeJA (B) elicitor from 0 to 48 h postelicitation compared with control unelicited cells and significant at P > 0.05. Red indicates up-regulated and green indicates down-regulated. Suspected PRXs related to hispidol biosynthesis are underlined. C, Replicated Affymetrix microarray analyses at key time points (2 and 24 h postelicitation) confirm the significant fold changes in MtPRX1 and MtPRX2 following YE. Other PRXs with fold changes highlighted in gray were not significant. Correlation was seen between MtPRX transcripts and hispidol accumulation in M. truncatula cell cultures in response to YE or MeJA. D and F, qRT-PCR analysis of PRX transcripts in response to MeJA (D) or YE (F). E and G, Accumulation of hispidol and hispidol glucoside in M. truncatula cell suspensions in response to MeJA (E) or YE (G). The y axis values represent relative peak areas after normalization to the mean peak area for both compounds. Error bars represent ±S (n = 3). Means are separated using Duncan’s multiple range test at P < 0.05.
Figure 5. Sequence relatedness of *M. truncatula* PRXs to other functionally annotated PRXs. A, Amino acid alignments of *M. truncatula* PRXs generated using ClustalW. Solid line boxes represent conserved domains in class III PRXs; the dashed line box represents the C-terminal propeptide sequence targeting PRXs to the vacuole. Gaps are introduced to maximize the alignment. Conserved similarity shading is based on 100% (black), 60% (dark gray), and 30% (light gray). B, Unrooted tree dendrogram comparison of the amino acid sequences of *M. truncatula* PRXs with other functionally characterized plant PRXs. The genes are as follows: *N. tabacum* PRX (GenBank accession no. AB027752), *Ipomoea batatus* anionic PRX (AY206414), *Asparagus officinalis* basic PRX (AJ544516), *Quercus suber* cationic PRX (AY443340), *Arachis hypogaea* cationic PRX (M37636), *Gossypium hirsutum* bacteria-induced PRX (AF155124), *Linum usitatissimum* basic PRX (AF049881), *Scutellaria baicalensis* novel PRX (AB024439), *Pisum sativum* ascorbate PRX (X62077), *G. max* cytosolic ascorbate PRX (U56634), *G. max* ascorbate PRX (AF127804), *A. thaliana* ascorbate peroxidase cytosolic protein (X59600), *S. lycopersicum* ascorbate PRX cytosolic protein (Y16773), *A. thaliana* class III PRX (AF452388), *A. thaliana* class III PRX (AF452387), *Populus trichocarpa* xylem anionic PRX (X97351), *Populus nigra* anionic PRX (83225), *P. trichocarpa* xylem anionic PRX (X97350), *L. usitatissimum* anionic PRX (L07554), *G. max* seed coat PRX (L78163), *G. max* anionic seed coat PRX (U41657), *Medicago sativa* PRX (X90693), *M. sativa* PRX (X90694), *Lupinus albus* extensin PRX (AF403735), and *G. max* PRX (AF007211). The branch lengths are proportional to the degree of divergence, with a scale of 0.1 representing 10% change.
aurones, whereas the absence of a 4’ hydroxyl group (3) did not impede enzymatic activity and the respective aurone was produced as monitored by HPLC-PDA-MS. Interestingly, chalcone analogs having a Glc at position 4 (2) were also acceptable substrates for aurone production, whereas those with a methoxy group at this position (8) were not, highlighting the crucial role of ring substituents at this position on enzymatic activity. Interestingly, naringenin chalcone that serves as a substrate for aureusidin formation by PPO in snapdragon (Nakayama et al., 2000) generated no aurone when incubated with expressed MtPRX1 to -3 (Fig. 11). In cases where aurone formation was absent (i.e. substrates 6, 7, and 8), the corresponding flavanone dimers were produced with MtPRX2 and MtPRX3 but not with MtPRX1 (Fig. 11). The presence of extra hydroxyl groups at the 3 or 3 and 5’ positions in chalcones (4 and 5) did not prevent the formation of the corresponding aurones, although this occurred at lower amounts than with substrates 1, 2, and 3.

**DISCUSSION**

**Integrated Metabolite and Transcript Profiling Identify Hispidol and Putative PRXs**

PRX multigene families have been found in all species thus far; however, the assignment of individual PRXs to a specific in vivo function still remains difficult (Quiroga et al., 2000). The genetic approach used to assess the function of PRXs involves the production of transgenic plants either overexpressing or underexpressing a specific PRX gene (Kajita et al., 1994; McIntyre et al., 1996; Lagrimini et al., 1997), but this approach has often failed to provide definitive information, and the in vivo roles of many PRXs still remain elusive (Quiroga et al., 2000). Here, we describe an integrated functional genomics approach involving correlations between transcriptomics and metabolomics for the discovery of unique natural products and their biosynthetic mechanisms. This approach is similar to studies that coupled cDNA-amplified fragment length polymorphism or DNA microarray to metabolite profiling for gene discovery in alkaloid biosynthesis in *N. tabacum* (Goossens et al., 2003) and glucosinolate biosynthesis in Arabidopsis (*Arabidopsis thaliana*; Hirai et al., 2004, 2005). Global metabolic profiling of *M. truncatula* cell cultures first led to the identification of the novel phytoalexin hispidol, which was induced in response to YE but not to MeJA. Hispidol has been previously identified in soybean and is derived from isoliquiritigenin chalcone (Wong, 1967). However, the enzymology of this reaction was uncertain but likely to contain a PRX-mediated step (Strack, 1997). Through correlation analysis of hispidol accumulation and high-density oligonucleotide microarray analyses of YE- and MeJA-induced cell cultures, we were able to identify three PRXs that could be associated with hispidol biosynthesis. The potential roles of *MtPRX1*, *MtPRX2*, and *MtPRX3* in aurone biosynthesis were each tested by heterologous expression in *E. coli* and in vitro assays using isoliquiritigenin and isoliquiritigenin glucoside as substrates.

**Evidence Implicating *MtPRX1* and *MtPRX2* as Hispidol Synthases**

All cloned MtPRXs catalyzed the oxidation of isoliquiritigenin and its glucoside to the corresponding aurone as a single product or one of multiple products. However, only MtPRX1 and MtPRX2 are likely to be functional hispidol synthases. Although MtPRX3 catalyzes hispidol biosynthesis, it also catalyzes a greater quantitative production of a QVE that is believed to originate from the rearrangement of the 2-(α-peroxbenzyl)coumaranone (Wong, 1967). Less specific
PRXs such as commercial horseradish peroxidase were also tested and found to catalyze greater levels of QVE than hispidol from isoliquiritigenin. The fact that QVE was not observed in *M. truncatula* cell culture reduces the likelihood that MtPRX3 contributes to in vivo aurone biosynthesis. The specificity of MtPRX1 and MtPRX2 as hispidol synthases was supported by the coordinated accumulation of their transcripts in response to YE using the 16K *M. truncatula* oligonucleotide microarray data (Fig. 4) with hispidol and hispidol glucoside. This observation was confirmed in replicated Affymetrix microarray experiments.
analyses at 2 and 24 h postelicitation and by qRT-PCR. Other candidate *M. truncatula* PRXs were discounted based upon the nonstatistically significant accumulation of their transcripts. Examples include TC101009, TC107670, TC107346, TC106564, and TC102226.

In silico expression analyses were also performed for MtPRX1, MtPRX2, and MtPRX3 to evaluate the spatial expression of these PRXs (Fig. 6). MtPRX1 was most highly expressed in flower, pods, and roots. MtPRX2 expression levels were approximately 200-fold higher in roots and nodules than any other tissues. These data illustrate the high tissue specificity of MtPRX2, which would translate to the present cell cultures that were initiated from roots (Broeckling et al., 2005). Finally, MtPRX3 expression levels were more evenly distributed throughout all tissues, suggesting a more general PRX role and, therefore, a lower likelihood of a primary role in hispidol biosynthesis.

To better assess the substrate specificities of each of the PRXs by kinetic analysis, multiple approaches were exhaustively pursued to express and purify the recombinant MtPRX proteins. The proteins were heterologously expressed in *E. coli*, yeast, and insect cell systems. In all systems, only low levels of the PRXs were observed to accumulate, suggesting a potent cell toxicity of these enzymes in prokaryotic and eukaryotic systems. Enrichment attempts using immobilized metal affinity chromatography to purify the His-tagged proteins were also unsuccessful, and it is assumed that the His tag was nonaccessible for affinity binding and purification. Thus, quantitative kinetic data for these PRXs could not be obtained.

Mechanism, Substrate Specificity, and Comparison of PRX- and PPO-Mediated Aurone Biosynthesis

The mechanism for the PRX-mediated conversion of isoliquiritigenin to hispidol proposed here is based upon that originally described by Wong (1967) and Wilson and Wong (1976). This mechanism involves the formation of a phenoxy radical (II) that undergoes further self-rearrangement, leading to the formation of a free radical (III; Fig. 9). The oxygenation of the α-carbon of III furnishes the peroxide IV, which, after intramolecular Michael-like addition, provides a dioxetane-type structure (V; Fig. 9). Intramolecular

**Figure 9.** A proposed biosynthetic pathway for isoliquiritigenin oxidation to hispidol and QVE as catalyzed by MtPRX1, MtPRX2, and MtPRX3 enzymes in *E. coli* cell lysates (modified from Wong, 1967). The pathway is initiated with the PRX-catalyzed generation of a phenoxy radical (II), and the subsequent steps most likely occur nonenzymatically. The additional steps include radical rearrangement (III) followed by oxygenation of the α-carbon (IV) and epoxide cyclization with the β-carbon (V). The epoxide radical is then utilized for the propagation of an additional substrate radical (i.e. conversion of I to II), which initiates a new cycle of the reaction, and it is converted to the neutral molecule. The neutral epoxide is then reduced and dehydrated to form VIII or the epoxide VII. These products then readily convert to hispidol or the QVE. The specific end products observed for each of the *M. truncatula* PRXs are indicated in the final steps.
opening of the dioxetane (V) results in a hydroperoxide 2-(α-peroxobenzyl)coumaranone (VI), which upon cleavage and dehydration yields hispidol (Fig. 9).

The substrate specificity of the MtPRXs differed from that of the snapdragon flower PPO. Interestingly, naringenin chalcone, which serves as a substrate for aureusidin formation by PPO in snapdragon (Nakayama et al., 2000), generated no aurone when incubated with expressed MtPRX1 to -3 (Fig. 11). However, naringenin chalcone does not accumulate in YE-elicited M. truncatula cell cultures (Farag et al., 2007). Thus, naringenin chalcone is less likely to be a primary in vivo substrate of the MtPRXs and/or is rapidly converted to the flavone via chalcone isomerase. In contrast, significantly elevated levels of isoliquiritigenin and its glucoside were observed and are more logical in vivo substrates for the MtPRXs.

Snapdragon aureusidin synthase also utilizes naringenin chalcone glucoside rather than naringenin chalcone as a substrate (Ono et al., 2006), whereas MtPRX utilized both isoliquiritigenin chalcone and its glucoside as substrates for the production of aurones.

Flavonoid glycosides colocalize in the vacuole with PRX and can serve as its substrate (Ros Barcelo et al., 2003). MtPRX2 and MtPRX3 contained a C-terminal propeptide that targets these PRXs for vacuolar import (Welinder et al., 2002), but this signal peptide was not present in MtPRX1 (Fig. 5A). However, the possibility

![Proposed Mechanism for the Production of Dimeric Flavonones, Aurones, and QVE from Chalcones Substituted at Position 4.](https://www.plantphysiol.org/)

**Figure 10.** Proposed mechanism for the production of dimeric flavonones, aurones, and QVE from chalcones substituted at position 4. Protection of the 4-hydroxyl group with a methyl group impedes the formation of a radical B ring species as described in Figure 9. Instead, a phenoxy radical species is initiated in the A ring at the 2'-hydroxyl, which would undergo further cyclization furnishing the pyrone radical (compound B), which can further dimerize (compound C). In contrast, the presence of a 4-hydroxyl sugar conjugate such as isoliquiritigenin-4-O-glucoside does not inhibit aurone formation. This may be possible through the homolytic cleavage of the sugar O-H bonds, which would further enable the formation of a radical species (compound E) followed by α-carbon oxidation and subsequent tautomerism, yielding hispidol-4'-O-β-D-glucoside (compound H) and QVE glucoside (compound I).
still exists that MtPRX1 is targeted to the vacuole due to its strong cationic nature (pI 8.9), as most cationic PRXs are vacuole localized due to the acidic nature of the vacuole (Ros Barcelo et al., 2003). In addition, the acidic pH optima for MtPRX1 to -3 are consistent with vacuolar localization. Vacuolar PRXs participate in the turnover and degradation of phenolic glycosides (Ros Barcelo et al., 2003). The oxidative breakdown of phenolics by PRXs begins with the enzymatic hydrolysis of glycosides to release the aglycones, which are then direct substrates for vacuolar PRXs. We hypothesize that *M. truncatula* PRXs utilize chalcone aglycones as substrates produced via cleavage of its glucoside in the vacuole under stress conditions to produce the antifungal agent “hispidol.” In onion (*Allium cepa*), a similar deglucosidation of quercetin glucosides to quercetin yields the antifungal agent 3,4-dihydroxybenzoic acid as catalyzed by the action of PRXs (Takahama and Hirota, 2000). The additional hydroxyl group of chalcones containing the functional motif for aurone conversion increased their reactivity for PPO but not for PRXs in *M. truncatula*. Both PPO and PRX mechanisms require the presence of 2′- and 4-hydroxy groups in the precursor chalcone.

Substitution of the 4-hydroxyl with a methoxy group (8) prevented conversion to aurone by any of the three MtPRXs, whereas Glc attachment at this position (2) did not. Furthermore, the 4-methoxy-2′,4′-dihydroxychalcone substrate only yielded a flavanone dimer (16) with MtPRX2 and MtPRX3 (Fig. 11); trace levels were detected with MtPRX1. Protection of the 4-hydroxyl group with a methoxy group inhibits the formation of a B ring radical species. Instead, a phenoxy radical species is initiated in the A ring at the 2′-hydroxyl, which undergoes further cyclization, furnishing the pyrone radical (Fig. 10, compound B) and dimerization to form compound C in Figure 10. Under our experimental conditions, the chalcones 6 and 7 (Fig. 11),

Figure 11. Substrates and related products formed during in vitro assays with recombinant *M. truncatula* PRXs. I, Aurone-producing chalcone substrates are listed with the amounts of aurones produced in each reaction expressed as a ratio relative to that obtained with an empty vector in the following order (MtPRX1, MtPRX2, MtPRX3). – indicates absence, + indicates ratio < 10, ++ indicates ratio = 10 to 20, and +++ indicates ratio > 20. The relative ratio of each product formed by MtPRX1, -2, and -3 does not solely reflect difference in enzymatic activities but also differences in expression levels. II, Chalcones determined to be inert as substrates for aurone or dimer formation. Names of the flavonoids are as follows: 1, isoliquiritigenin; 2, isoliquiriigenin–4-O-glucoside; 3, 2′,4′-dihydroxychalcone; 4, 2′,4′,3,4-tetrahydroxychalcone; 5, 2′,4′,5′,3,4-pentahydroxychalcone; 6, 2′-hydroxy-4′-methoxychalcone; 7, 2′,4′-dihydroxychalcone; 8, 2′,4′-dihydroxy-4-methoxychalcone; 9, hispidol; 10, hispidol–4′-O-β-D-glucoside; 11, 4′-hydroxyaurone; 12, 6,3′,4′-trimethoxy-2-hydroxychalcone; 13, biflavanone A; 15, biflavanone B; 16, biflavanone C; 17, 6-hydroxy-2-(4-hydroxy-phenoxymethylene)-benzofuran-3-one (QVE); 18, 6-hydroxy-2-(4-glucosyl-phenoxymethylene)-benzofuran-3-one (QVEG); 19, 7, 2′,4′,6′-trimethoxy-2-hydroxychalcone; 20, 4-methoxy-4′-hydroxychalcone.
which both contain 2’- and 4’-hydroxyl groups, also produced the corresponding dimers. This suggests that the absence of a hydroxyl group at C4 or its protection with a methyl group provides the mechanistic diversion toward dimer formation. In contrast, the presence of the sugar hydroxyl groups in isoliquiritigenin-4-O-glucoside may contribute to aurone formation. Homolytic cleavage of the O-H bonds of the sugar may also result in the formation of radical species (Fig. 10, compound E) that then follow a similar reaction mechanism, as proposed in Figure 9 and the previous literature (Wong, 1967), yielding hispidol-4’-O-β-d-glucoside (Fig. 10, compound H).

Our data suggest that aurone biosynthesis is kinetically favored over the dimerization reaction, as dimers were only produced as alternative oxidation products by MtPRXs (Fig. 11) in situations where aurone formation was inhibited. Consistently, no dimers were detected for the in vivo MtPRXs substrate isoliquiritigenin and its respective glucoside. To the best of our knowledge, these structure-activity relationships provide the first mechanistic insights into aurone glucoside biosynthesis in legumes.

Hispidal biosynthesis in *Medicago truncatula* is unlikely to occur via a PPO mechanism. This conclusion is based upon the undetectable levels of all three putative PPOs present on the *M. truncatula* microarray and based upon our current mechanistic understanding. According to the reported PPO-catalyzed mechanism for aurone synthesis (Nakayama et al., 2001), the substrate must contain hydroxyl groups at the 2’ and 4’ positions, such that all products will then possess a 3,4-dihydroxy-substituted B ring (Fig. 1). Hispidol contains a 4-monoxyhydroxy B ring and lacks the 3-hydroxyl B ring group (Fig. 1); thus, the production of hispidol via a PPO-catalyzed mechanism is unlikely. This argument is further supported by the fact that the predicted substrate required for the PPO production of hispidol would contain an unsubstituted B ring (i.e. 2’,4’-dihydroxy chalcone or a 4-dehydroxy isoliquiritigenin), which has not been identified in legumes to date. Nakayama et al. (2000, 2001) also suggest that hispidol biosynthesis occurs via a PRX-mediated as opposed to a PPO-mediated mechanism. Furthermore, alfalfa leaves and stems have been reported to have relatively little if any PPO activity (Sullivan et al., 2004; Sullivan and Hatfield, 2006). The above evidence supports the role of PRXs and discounts the role of PPO-mediated hispidol biosynthesis in legumes.

Is Hispidol a True Phytoalexin in *Medicago*?

Production of hispidol and hispidol glucoside is likely associated with the oxidative burst and production of H₂O₂, which are key components of plant disease resistance (Lamb and Dixon, 1997). The presence of an oxidative environment is supported by increased H₂O₂ levels and PRX enzymatic activity detected in the *M. truncatula* culture medium in response to YE (Farag et al., 2008). Our data suggest that hispidol accumulation requires the buildup of isoliquiritigenin, which is normally and rapidly shunted into the (iso)flavonoid pathway by the activity of chalcone isomerase, in addition to induction of a specific set of PRXs. The accumulated isoliquiritigenin and/or isoliquiritigenin glucoside serve as scavengers for removal of H₂O₂ from the vacuole (Ros Barcelo et al., 2003) through the production of hispidol and hispidol glucoside by vacuole-targeted MtPRX1 and MtPRX2 activity. Many flavonoids and lignans have powerful antioxidant activity and can help remove H₂O₂ from acidic compartments such as the vacuole by acting as substrates for PRXs (Mehlhorn et al., 1996). Chalcones possess higher antioxidant activity than flavanones and isoflavones (Miranda et al., 2000). Thus, isoliquiritigenin and isoliquiritigenin glucoside are likely the best substrates for PRX-mediated H₂O₂ removal in *M. truncatula* cell cultures.

On the basis of hispidol’s relative antifungal activity and its inducible synthesis from distant precursors, hispidol meets the criteria for classification as a phytoalexin (VanEtten et al., 1994). The high antifungal activity of hispidol relative to its biosynthetic precursors shows that the compound be detected in interactions of *Medicago* with pathogens. To date, this has not been observed, but only a few interactions have been studied. Neither can hispidol be induced in roots or leaves of *M. truncatula* following exposure to YE (data not shown). Furthermore, the presence of these MtPRXs in tissues (e.g. roots) that do not appear to accumulate hispidol indicates that these enzymes have additional functions and that hispidol formation may not even be their true “function” but rather represents spillover of metabolite flux that cannot be rapidly incorporated into the main downstream branches of the phenylpropanoid pathway. However, the genetic reprogramming associated with the establishment and elicitation of cell cultures can provide substrate pools and catalysts for the generation of novel metabolites. Such flexibility of plant secondary metabolism provides a basis for the evolution of plant adaptation to changing environmental conditions, and the new knowledge gained from the observed plasticity of secondary metabolism can now be used for future metabolic engineering of this potential antifungal compound in planta.

**MATERIALS AND METHODS**

**Cell Cultures and Elicitation**

Cell cultures derived from barrel medic (*Medicago truncatula* ‘Jemalong’ line A17) roots were initiated, subcultured, elicited with YE and MeJA, and...
harvested at 21 time points as described previously (Broeckling et al., 2005). Tripleticate biological replicates were collected for both control and elicited samples at each time point, with each replicate collected from a separate culture flask. Thus, each elicitation time course contained 126 independent culture flasks and biological samples collected from each independent flask. Cell culture medium was also sampled, but at a lower temporal resolution, which included 0, 6, 12, 24, and 48 h postelicitation (Suzuki et al., 2005).

**Chemicals and Reagents**

Hispidol, coumestrol, aformosin, and isoliquiritigenin-4-O-glucoside were purchased from Apin Chemicals. Hispidol 4′-O-δ-glucoside and irisolindole were kindly provided by Dr. Tom Mahry (University of Texas, Austin). Guascil was purchased from Sigma-Aldrich. Other tested compounds were purchased from Indofine. Solvents used were of HPLC-grade purity. Naringenin chalcone was synthesized from naringenin according to the method reported by Shimokoriyama (1997).

**Extraction and HPLC-PDA-MS Analysis of Phenolic Compounds**

Phenolic compounds were extracted from lyophilized M. truncatula cells, 20–0.06 mg, with 1.8 mL of 80% methanol containing 2 μg of umbelliferone (as an internal standard) for 10 h at room temperature using an orbital shaker in the dark. Extract aliquots (1.4 mL) were centrifuged at 3,000g for 60 min, and the supernatant was evaporated under a nitrogen stream until dry. The residue was resuspended in 300 μL of 45% methanol, and samples were analyzed by HPLC-PDA-MS. For analysis of phenolic compounds in the culture medium, the medium was vacuum filtered from cell cultures and a 20-mL aliquot was extracted three times with 25 mL of ethyl acetate spiked with 2 μg of umbelliferone (internal standard). The extracts were pooled, evaporated to dryness using nitrogen, dissolved in 300 μL of methanol, and analyzed by HPLC-PDA-MS.

An Agilent 1100 Series II HPLC system (Agilent Technologies) equipped with a PDA detector was coupled to a Bruker Esquire ion-trap mass spectrometer equipped with an electrospray ionization source. UV spectra were obtained by scanning from 200 to 600 nm. A reverse-phase, C18, 5-μm, 4.6-× 250-mm column (J.T. Baker) was used for separations. Separations were achieved using a linear gradient of 5% to 90% (v/v) B in 70 min. The mobile phase consisted of eluent A (0.1% [v/v] acetic acid in water) and eluent B (100% acetonitrile), which was achieved using a linear gradient of 5% to 90% (v/v) B in 70 min. The mobile phase was maintained for approximately 3 years in subculture and pooled for use. Cy3 dye (Amersham Biosciences) was used for labeling reference RNA, and Cy5 dye (Amersham Biosciences) was used for experimental RNA. The oligonucleotide microarray data are publicly available in a custom database for “omics” data (DOME; http://medicago.vbi.vt.edu/data.html).

**Affymetrix M. truncatula Microarray Analysis**

The Affymetrix DNA microarray contained 61,200 probe sets. These were prepared from a common pool of reference cDNA, and a reference design of the microarray experiment was performed. Reference RNA was purified from separate M. truncatula ‘Jemalong’ (line A17) cell suspension cultures (maintained for approximately 3 years in subculture) and pooled for use. Cy3 dye (Amersham Biosciences) was used for labeling reference RNA, and Cy5 was used for experimental RNA. The oligonucleotide microarray data are publicly available in a custom database for “omics” data (DOME; http://medicago.vbi.vt.edu/data.html).

**Enzymatic Hydrolysis and Analysis of P1**

Collected P1 fractions were evaporated, resuspended in 200 μL of water, and subjected to β-glucosidase hydrolysis for 8 h at 37°C (Mahy et al., 1970). The aglycone was then partitioned from the aqueous phase into ethyl acetate (3 × 2 mL). The ethyl acetate fraction was evaporated to dryness under nitrogen, and the residue was resuspended in 100 μL of methanol and analyzed using HPLC-PDA-MS to characterize the aglycone. The aqueous phase following liquid-liquid extraction with ethyl acetate and containing the liberated sugar(s) was evaporated in a SpeedVac to complete dryness. The sugars were derivatized as described (Farag et al., 2007) and analyzed by GC-MS in parallel with authentic sugars (e.g. Glc, Gal, and malonic acid).

**DNA Microarray Analysis**

**RNA Isolation, cDNA Labeling, and Hybridization**

Total RNA was isolated from 0.5 g of M. truncatula suspension cells using 5 mL of TRI-Reagent (Molecular Research Center) following the manufacturer’s protocol. The Amino Allyl cDNA Labeling Kit (Ambion) was used to label 25 μg of total RNA following the manufacturer’s protocol. The product was resuspended in 70 μL of Microarray Hyridization Solution, version 2 (Amersham Biosciences), denatured at 95°C to 100°C for 2 min, pipetted onto the slides, and covered with a coverslip (Corning) before sealing in a hybridization chamber (Corning). The sealed chambers were wrapped in aluminum foil and incubated at 42°C for 16 h. The arrays were subsequently washed with 1× SSC, 0.1% (w/v) SDS, followed by a wash in 0.5× SSC, 0.01% SDS, and a third wash with 0.05× SSC, at room temperature for 5 min each. The slides were dried by centrifugation.

Custom M. truncatula Oligonucleotide Microarray Analysis

The 70-mer oligonucleotides representing 16,086 TCs from the Medicago Genome (Qiagen Operon Oligo Set version 1.0) were printed on Arrayit Brand SuperAmine Substrate glass slides (Telechem) using an OmnisGrid 100 (Gene Machine). The oligonucleotides were normalized to melting temperature, sequence, and concentration. The melting temperatures were within 78°C ± 5°C. Oligonucleotides corresponded to the sequences of TCs located within 1,000 bases from the 3′ ends, with less than 70% identity to all other genes and with less than 20 contiguous bases common to any other gene. The oligonucleotides also had no homopolymer tracts greater than eight bases and no hairpin stems longer than nine bases. The array dimensions were 39 mm long × 18 mm wide, with a spot size of approximately 100 to 110 μm in diameter and spot spacing of 185 × 185 μm. The array configuration used eight-by-four subarray grids. Each subarray grid was composed of 23 × 23 spots. Slides were stored in a vacuum-sealed box at room temperature and UV cross-linked at 200 mJ using a Stratalinker (Strategene) before use.

Each cDNA sample was hybridized against a reference cDNA sample that was prepared from a common pool of reference cDNA, and a reference design of the microarray experiment was performed. Reference RNA was purified from separate M. truncatula ‘Jemalong’ (line A17) cell suspension cultures (maintained for approximately 3 years in subculture) and pooled for use. Cy3 dye (Amersham Biosciences) was used for labeling reference RNA, and Cy5 was used for experimental RNA. The oligonucleotide microarray data are publicly available in a custom database for “omics” data (DOME; http://medicago.vbi.vt.edu/data.html).
Microarray Data Processing and Analysis

Arrays were imaged with a ScanArray 4000 scanner (Packard) at 10 μm resolution and variable photomultiplier tube voltage settings to obtain the maximal signal intensities. The fluorescence intensity for each label and each element on the array was captured using GenePix Pro 4.1 (Axon). Normalization of Cy3 and Cy5 signals was performed by adjusting the signal intensities of the two images using a Lowess (subgrid) method and GeneTraffic software (www.iobion.com). The local background was subtracted from the values of each spot on the array. The statistical ANOVA of normalized data was performed using GeneSpring software (www.silicongenetics.com).

qRT-PCR Analysis

Microarray results were confirmed by qRT-PCR using TC-specific primers for tissue collected at 0 min, 30 min, 2 h, 4 h, 12 h, and 24 h postelicitation. RNA was isolated from triplicate control and elicited cells. Ten micrograms of purified total RNA was treated with Turbo DNA-free DNase I (Ambion) according to the manufacturer’s protocol, purified by RNeasy MinElute columns (Qiagen), and checked for genomic DNA contamination by PCR using the M. truncatula-specific actin primers MtActin-for (5’-TCAATGTGGCTCCGATCCTGTATG-3’) and MtActin-rev (5’-ACTACACACCCGGTCAACAAACC-3’). RNA integrity was evaluated with an Agilent 2100 Bioanalyzer using RNA nanochips. RT reactions were performed using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. PCRs were performed on an optical 384-well plate with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems) using SYBR Green to monitor double-stranded DNA synthesis. PCRs (10 μL final volume) contained 5 μL of SYBR Green Master Mix reagent (Applied Biosystems), 1 μL of cDNA, and 200 ng of each gene-specific primer: MtPrx1for (5’-CTAGCCCCGGATGGTATCG-3’), MtPrx1rev (5’-CCGTTTTAGCATGTTGTCCT-3’), MtPrx2for (5’-GTGCTTACGGAAGAAAGGAGA-3’), MtPrx2rev (5’-AGCCGCTCAGAGCACAGATCA-3’), MtPrx3for (5’-GAAAACAGAGAGATTGAAAACA-3’) and MtPrx3rev (5’-CACTTGAGCTAACCATAACCCATCT-3’). PCRs were performed as described elsewhere (Chezcowski et al., 2005). Data were analyzed using SDS 2.2.1 software (Applied Biosystems). PCR efficiency was estimated using LinRegPCR software as described (Ramakers et al., 2003), and the transcript levels were determined by relative quantification (Pfaffl, 2001) using actin (TC107532) as a reference gene (primers as above).

MtPRX Sequence Analyses

BLAST searches were performed using BLASTp version 2.2.2 (http://www.ncbi.nlm.nih.gov/BLAST). The amino acid sequences were aligned using ClustalW, and the similarities were calculated using Megalign (DNASTAR). Publicly available programs (http://www.expasy.ch/tools/pi_tool.html) and searchscripts (http://www.ncbi.nlm.nih.gov/blast/seq Crypt.html) were used to compute pl and Ms for the deduced amino acid sequences. The subcellular targeting of proteins was predicted using the programs PSORT (http://psort.ims.u-tokyo.ac.jp/form.html), TargetP (http://www.cbs.dtu.dk/services/TargetP/), and SignalP (http://www.cbs.dtu.dk/services/SignalP/)..

Functional Characterization of Recombinant PRXs Expressed in Escherichia coli

M. truncatula EST clones NF041088 (i.e. MtPRX1 corresponding to TIGR release 8.0 TC106836), NF092006 (i.e. MtPRX2 corresponding to TIGR release 8.0 TC106484), and NF089106 (i.e. MtPRX3 corresponding to TIGR release 8.0 TC106558) were sequenced from both ends using M13 primers and found to contain full-length sequences. cDNA sequences were PCR amplified from pBluescript II SK+ (Stratagene) using primers with EcoRI and XhoI (MtPRX1 and MtPRX3) or SacI and HindIII (MtPRX2) sites (underlined): MtPRX1F, 5’-AGGATATGACCCAGGATTGCCCCTCTAATTGTTATG-3’; MtPRX1R, 5’-TAACTACAGATGTGGATTGGTTTCTATCCAC-3’; MtPRX2F, 5’-CACTAGAATGCAAAATGAATCTCCCTTATG-3’; MtPRX2R, 5’-ACCCAAAGCTTGTAGTTAATACATCTAT-3’; MtPRX3F, 5’-TACGTAATACTGACATTTCCCTTATG-3’; and MtPRX3R, 5’-CACTACCGATGATCCATACCATACATACAT-3’. Purified PCR products were digested with appropriate restriction enzymes (Novagen) for 2 h and purified from an agarose gel using the QIAquick gel extraction kit (Qiagen). The excised PCR products were cloned into appropriately digested pET-28a (+) vector (Nova- gen). The resulting plasmids were sequenced to confirm identity.

Hispisol Biosynthesis in Medicago truncatula Cell Cultures

E. coli BL21 (DE3) cells harboring the pET-28a or MtPRX expression constructs were grown to an optical density of 600 nm of 0.8, and expression was initiated by addition of IPTG to a final concentration of 0.5 mM, with further incubation while shaking overnight at 20°C. Cultures were centrifuged, and pellets were resuspended in a lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, pH 8.0, 1% glycerol, 1% Tween 20, and 1 mM phenylmethyl-sulfonyl fluoride). Cells were lysed by sonication (Biologics) and centrifuged, and the resulting supernatant was removed and assayed for PRX activity against guaicol. For PRX activity assays, cell lysates (50 μg of protein equivalent) were added to 2 mL of 0.1% guaicol and 0.03% H2O2 in 50 mM potassium acetate, pH 6.0, and the increase in A520 over 3 min was measured using a Beckman Coulter DU800 spectrophotometer. For all PRX enzymatic reactions, equal amounts of protein were used as quantified using the Bradford (1976) assay and using a commercial dye reagent (Bio-Rad) with bovine serum albumin as the standard.

Product profiling of chalcone analogs incubated with recombinant PRXs was assayed in 500 μL of 10 mM potassium phosphate buffer, pH 6.7, containing 50 μg of protein equivalent of cell lysate, 8 μL of 5 mM substrate dissolved in dimethyl sulfoxide, and 1 μL of 100 mM H2O2. Reactions were carried out at 37°C for 2 h and stopped by adding 3 volumes of ethyl acetate. After vortexing, the ethyl acetate phase was removed and evaporated to dryness under nitrogen, and the residue was resuspended in methanol prior to HPLC-PDA-MS analysis using the same instrumental conditions mentioned previously. All enzyme assays were performed in duplicate.

Protein Analysis

E. coli cell lysates were separated on a 12% Tris-HCL gel (30 μg of protein loaded per lane) and electroblotted onto a nitrocellulose membrane (Transblot; Bio-Rad) according to the procedure of Sambrook and Russell (2001). Blots were incubated overnight at 4°C with the anti- His tag monoclonal antibody (Novagen) diluted 1:1,000 in 3% bovine serum albumin, followed by incubation with anti-mouse horseradish peroxidase (Amersham). The peroxidase color reaction was developed using a color reagent (Amer sham) according to the manufacturer’s instructions. Western-blot analyses were performed in duplicate with a representative blot presented.

Antifungal Agar Plate Bioassays

Antifungal assays were conducted essentially as described (Blosn et al., 1992). Stock plates of Phoma medicaginis grown on potato dextrose agar medium (Difco Laboratories) in sterile 100 × 15-mm petri dishes until the mycelia covered one-third of the plate. Compounds to be tested were dissolved in dimethyl sulfoxide (Sigma) at stock concentrations of 20 mM. The solvent (as a control) or compounds were then added to the melted agar medium at 5 μL mL-1 to give a final phenolic concentration of 0.1 μM. Five-milliliter samples of medium were aseptically pipetted into sterile 35–40-mm petri dishes and allowed to cool. Using a sterile 4-mm cork borer, agar discs with mycelia were cut from a stock fungal culture plate and placed on the center of treatment and control plates. Radial mycelial growth was measured from the edge of the disc to the outer edge of the mycelia, and the results of treatments were calculated as the relative percentages of their corresponding solvent controls.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: MtPRX1, EF456703; MtPRX2, EF456704; MtPRX3, EF456705.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Accumulation of hisisol in medium of YE-elicited and control M. truncatula cell cultures.

Supplemental Figure S2. In vitro enzymatic assays using recombinant MtPRX1, -2, and -3 with isoliquiritigenin-4-O-β-D-glucoside (ILG) revealing the reaction product hispidol-4-O-β-D-glucoside (HG).

Supplemental Figure S3. PRX activity of MtPRX1, -2, and -3 as a function of assay pH.
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LITERATURE CITED


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