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Proteome-Level Differences between Auxinic-Herbicide-Susceptible and -Resistant Wild Mustard (*Sinapis arvensis* L.)

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To identify proteins that may be involved in mediating auxinic herbicide resistance (i.e., resistance to dicamba, picloram, 2,4-D), we compared the proteomes of an auxinic-herbicide-susceptible (S) and -resistant (R) wild mustard (*Sinapis arvensis* L.) biotype at different developmental stages. Using two-dimensional electrophoresis and mass spectrometry, we identified 11 seedling and leaf proteins that showed reproducible differences in expression between the S and the R wild mustard biotype following application of dicamba. Our proteome-level studies revealed the increased expression of the enzyme peptidylprolyl cis−trans isomerase (PPIase), which has recently been implicated in auxin signal transduction. Juglone, an inhibitor of PPIase, interfered with the normal ability of R seeds to germinate in the presence of dicamba, whereas S seeds did not germinate in the presence of dicamba or dicamba plus juglone. When R and S plants (3−4 leaf stage) were treated with dicamba, S showed typical auxinic herbicide effects (e.g., epinasty) whereas R did not. However, the concomitant application of dicamba and juglone to greenhouse-grown R plants produced morphological changes that were consistent with known auxinic-herbicide-induced symptoms. This is the first report suggesting the potential involvement of differential expression of PPIase in mediating auxinic herbicide resistance.

KEYWORDS: Auxin; herbicide resistance; *Sinapis arvensis* L.; proteomics; peptidylprolyl isomerase

INTRODUCTION

While auxinic herbicides have been widely used for over 50 years, the exact mechanism of action that leads to the senescence and death of susceptible plants has yet to be clearly deciphered and remains an enigma for herbicide researchers. It has been suggested that because the cellular responses elicited by these herbicides are similar to those induced by indole-3-acetic acid (IAA), the phytotoxic consequence of auxinic herbicides is, in part, the result of a significant phytohormone imbalance within the cells of affected plants (*1*). Included among the known effects of exposure to auxinic herbicides are cell elongation, root initiation, hypertrophy, ethylene biosynthesis, severe downward twisting of the shoots (epinasty), early abscission of leaves, and faciation of the crown and leaf petioles (*2*). At the cellular level, research has demonstrated that following the detection of an auxinic herbicide by certain receptors, a series of signal cascades are stimulated, which results in a rapid uptake of calcium ions by the cells as well as an activation of the plasma membrane ATPase and nucleic acid and protein biosynthesis pathways (*2, 3*−*7*). The various changes regulated by auxinic herbicides ultimately contribute to the development of senescence and the eventual death of the affected plant. Auxinic herbicides are effective against many dicotyledonous plant species (*8*) and are administered in cereal crops for the selective eradication of undesirable weeds.

Although the use of auxinic herbicides has been extensive over the many years of its commercial use, there have not been many occurrences of resistant biotypes (*2, 9*). In 1990, a biotype of wild mustard (*Sinapis arvensis* L.) was found on farms in Manitoba, Canada, that was resistant to a commercial mixture of the auxinic herbicides dicamba (3,6-dichloro-2-methoxybenzoic acid), MCPA [(4-chloro-2-methylphenoxy) acetic acid], and mecoprop [(±)-2-(4-chloro-2-methylphenoxy) propanoic acid] that had previously been used to control wild mustard (*6, 10*). Research into the differences between susceptible (S) and resistant (R) wild mustard revealed that there were no differences between the two biotypes in terms of how several auxinic herbicides were absorbed, translocated, or metabolized (*11*). However, when compared to S plants there were numerous differences in morphological and physiological characteristics of the R wild mustard including shortness of stature, significantly more branching, and smaller root systems (*12*). Furthermore, the leaves were considerably smaller and darker green in color, the result of more chlorophyll (*12*). In addition, it was determined that the levels of calcium and the phytohormone cytokinin were elevated in the cells of R wild mustard plants

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compared with the S biotype (*11*). Since it is known that cytokinin in the presence of calcium can delay the onset of senescence (*11*), it was postulated that the significantly higher levels of calcium and/or cytokinin may be involved in regulating auxinic herbicide resistance (*12*). In many weeds, resistance to herbicides is mediated and inherited through a single, dominant nuclear allele. Consistent with this inheritance, Jasieniuk et al. (1995) also confirmed this mechanism for resistance of the wild mustard biotype to dicamba (*9*). However, the identity of the particular allele conferring resistance to auxinic herbicides in wild mustard has unfortunately eluded researchers.

The objective of the current study was to determine the proteome-level differences between the auxinic-herbicide-susceptible and -resistant biotypes of wild mustard at different stages of development upon exposure to the auxinic herbicide dicamba. It was hypothesized that the identification of proteins that differ between the two biotypes and how expression patterns were affected by dicamba could provide valuable information leading to the characterization or clarification of the mechanism involved in the auxin signal transduction pathway(s) as well as in the development of auxinic herbicide resistance. We utilized two-dimensional electrophoresis and mass spectrometry to identify the differentially expressed and dicamba-induced proteins. We found a protein that has recently been linked by Dharmasiri et al. (2003) to auxin signal transduction pathways yet has never before been implicated in regulating or causing auxinic herbicide resistance (*13*). In addition, we provide evidence that the activity of the protein may, in fact, be vital for the maintenance of an auxinic-herbicide-resistant phenotype.

MATERIALS AND METHOD

The proteome-level differences between the S and the R biotypes of wild mustard (*Sinapis arvensis* L.) were investigated using protein extracts from seedlings (shoots only) 1 week after germination in Petri dishes and leaf tissue from plants grown for 3 weeks in the greenhouse (3−4 leaf stage of development).

Plant Material Preparation. R and S wild mustard seeds were surface sterilized for 1 min in 70% ethanol followed by two 5 min immersions in 0.3% sodium hypochlorite and a subsequent thorough rinse in sterile Milli-Q water. Seeds were placed in Petri dishes (10 seeds per Petri dish) on sterile filter paper, which was moistened with 3 mL of the control (sterile Milli-Q water) or treatment (10 μM dicamba; Micro Flo Co., Memphis, TN) solution, and germinated to obtain tissue material for protein extraction. To determine percent germination, another set of Petri dishes was prepared as previously described except 20 R seeds were placed in each dish on sterile filter paper that was moistened with 3 mL of the control (sterile Milli-Q water) or 3 mL of the treatment [10 μM dicamba, 0.2% ethanol, 10 μM juglone (Aldrich, Oakville, ON, Canada), 20 μM juglone, 10 μM dicamba and 10 μM juglone, or 10 μM dicamba and 20 μM juglone] solutions. The juglone in the treatment solutions was initially dissolved in 100% ethanol (10 mM) and then diluted appropriately to achieve the required juglone concentrations in the treatment solutions. All of the treatment solutions containing juglone also contained 0.2% ethanol. The Petri dishes were sealed with laboratory film and stored in the dark at room temperature (22 °C ± 2 °C) for 7 days after which photographs of representative seedlings were taken and the shoots from the control and dicamba-treated seedlings were collected, flash frozen in liquid nitrogen, and stored at −80 °C until the protein was extracted. For the germination studies, the percentage of seeds that germinated on each plate was calculated. If the seed coat was broken and the radicle was visible, the seed was considered to have germinated. The calculated germination percentages were statistically analyzed using the General Linear Model of SAS to determine if there were any significant differences among the percentage germination of the wild mustard seeds imbibed in the various treatment solutions. The means were categorized using a Student−Newman−Keuls test. Experiments were repeated at least twice.

For greenhouse experiments, R and S wild mustard seeds were sown in moistened Metro Mix 290 (Grace Horticultural Products, Ajax, ON, Canada) in 6 × 6 cm plastic containers and placed in the greenhouse (20 h photoperiod, 21 °C day/18 °C night cycle). The plants were watered as required for 3 weeks (until the plants were at the 3−4 leaf stage of development). The wild mustard plants were sprayed with water (control), dicamba (50 g ai ha^{-1}), 0.2% ethanol, 20 μM juglone, or dicamba (50 g ai ha^{-1}) and 20 μM juglone. Twenty-four hours after the plants were sprayed, photographs of representative plants were taken and the leaves from the control and dicamba-treated plants were removed, flash frozen in liquid nitrogen, and stored at −80 °C until all samples were ready for the protein extraction step. Experiments were repeated at least once.

Protein Extraction. Seedlings (5 pooled seedlings, shoots only, from the Petri dish experiments) or leaves (pooled leaf tissue from 6 plants grown and sprayed in the greenhouse) were ground in liquid nitrogen to a fine powder. Approximately 0.1 g of the ground tissue was resuspended in 1 mL of a solution consisting of 10% w/v trichloroacetic acid (TCA) and 0.07% dithiothreitol (DTT) in ice cold acetone in a 1.5 mL plastic tube. Following incubation at −20 °C for 1 h, the samples were centrifuged in a tabletop centrifuge (14 000 rpm, 10 min) and the supernatant was discarded. Each pellet was resuspended in 1 mL of 0.07% DTT in ice cold acetone and incubated at −20 °C for 1 h. The samples were centrifuged as previously described, and the pellets were resuspended in 1 mL of 0.07% DTT in ice cold acetone, incubated at −20 °C for 1 h, and centrifuged as previously described. The pellets were vacuum-dried for 30 min and then resuspended in 500 μL of Rehydration/Sample buffer (Bio-Rad, Mississauga, ON, Canada), which consisted of 8 M urea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte 3/10 (ampholytes), and 2 mM tributylphosphine (TBP). After vigorous vortexing of the samples for 5 min, the tubes were incubated at 4 °C overnight. The samples were centrifuged as previously described, and the protein concentrations of the collected supernatants (soluble protein extracts) were determined using a modified Bradford assay (*14*) using bovine serum albumin (Pierce Biotechnology Inc., Rockford, IL) as the standard.

Two-Dimensional Electrophoresis. The two-dimensional electrophoresis experiments were performed using the PROTEAN IEF cell (Bio-Rad) for the first dimension isoelectric focusing and the Mini-PROTEAN 3 system (Bio-Rad) to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension.

The first dimension separation, involving the isoelectric focusing of proteins present in the protein extracts, was performed using 7 cm (pH 3−10, nonlinear) immobilized pH gradient (IPG) strips (ReadyStrip IPG strips; Bio-Rad), which were passively rehydrated in 125 μL of the protein extract (in Rehydration/Sample buffer). This rehydration of IPG strips and concomitant loading of proteins took place overnight at room temperature in rehydration/equilibration trays. Depending on the source of the protein extract, a different amount of protein was loaded (seedlings, 65 μg/strip; leaves, 85 μg/strip) to generate ideal SDS-PAGE gels that contained numerous discrete protein spots. The isoelectric focusing of proteins was performed using Bio-Rad's preprogrammed four-step method. The initial conditioning step consisted of applying 250 V to the strips for 15 min followed by a voltage ramping step where the voltage was linearly increased from 250 to 4000 V over a 2-h period. These steps were followed by a final focusing step where 4000 V was applied for 20 000 Vhours after which the voltage was rapidly reduced from 4000 to 500 V. Upon completion of the isoelectric focusing step, the IPG strips were removed from the focusing tray and stored at −20 °C overnight. Prior to the embedding of the IPG strips on prepared SDS-PAGE gels for second dimension separation, the proteins underwent an equilibration step as described by the manufacturer. Briefly, the strips were initially saturated in 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT, with gentle agitation at room temperature two times for 15 min each. The strips were equilibrated again in 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 135 mM iodoacetamide, with gentle agitation at room temperature two times for 15 min each. Following the equilibration steps, the IPG strips were carefully embedded within a molten agarose solution (Bio-Rad) directly on top

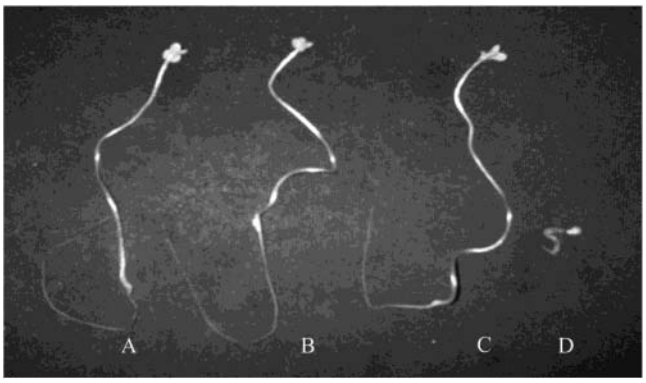

Figure 1. Effect of dicamba on representative auxinic-herbicide-susceptible and -resistant wild mustard seedlings germinated in Petri dishes containing R wild mustard in (**A**) water (control) or (**B**) 10 μM dicamba and S wild mustard in (**C**) water (control) or (**D**) 10 μM dicamba.

of a 1 mm 13% SDS-PAGE gel. The SDS-PAGE was performed at 150 V for 1.33 h. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 (Aldrich) followed by silver staining using the Silver Stain Plus kit according to the manufacturer's instructions (Bio-Rad).

Gel Analysis and Identification of Protein Spots. The gels were scanned using the GS-800 Calibrated Densitometer (Bio-Rad) to produce images that could be compared using the accompanying PDQuest 2D analysis software (Bio-Rad). Among other functions, the analysis software allowed for automated detection of protein spots followed by a process in which corresponding spots among selected gel images could be matched and marked. Visual analysis and manual correction of the automated spot detection and matching results were also required since the software, in some instances, misidentified and/or mismatched protein spots. Once all similar spots were detected and matched, the protein spots that were reproducibly unique or whose densities were reproducibly altered when compared with the control gel images were selected and carefully removed from the gels with sterile surgical blades for identification by mass spectrometry (MS).

The excised protein spots underwent a series of procedures within a MassPREP Station (Micromass, Manchester, U.K.) that ultimately produced protein samples that were destained, reduced, alkylated, and digested by sequencing-grade modified trypsin (Promega, Madison, WI). The liquid chromatography (LC) component of the LC/MS/MS analysis was performed using a capillary HPLC system (CapLC system; Waters Corp., Milford, MA), which utilized a PepMap C18 column (LC Packings, CA) for loading and desalting prior to reversed-phase separation of the peptides on a PicoFrit capillary column (New Objectives, MA). The separated peptides were applied to a Quadrupole Time-of-Flight 2 (Q-ToF 2) mass spectrometer (Micromass). The information generated by the Q-ToF 2 for the eluting peptides (tandem mass spectrum, MS/MS) was used to search appropriate NCBI protein databases to ascertain the expected identities of the corresponding protein spots. All of the mass spectrometry experiments were performed at the Institute for Biomolecular Design at the University of Alberta (Edmonton, AB, Canada.)

RESULTS AND DISCUSSION

Morphological Differences between S and R Wild Mustard. The effects of dicamba on the growth of representative S and R wild mustard seedlings are shown in **Figure 1**. While the herbicidal influence of dicamba on the susceptible seedlings is dramatic, the auxinic herbicide (10 μM) had little or no effect on the growth of the R seedlings. The morphological differences between the S and R plants (**Figure 2**) sprayed with dicamba are equally striking. S wild mustard plants became epinastic (within 24 h of application of dicamba), a characteristic symptom of the auxinic herbicides, while the R plants were unaffected. Therefore, the proteomes of the S and R tissues were compared to determine whether differences in their protein expression patterns could be linked with the response of the biotypes to dicamba.

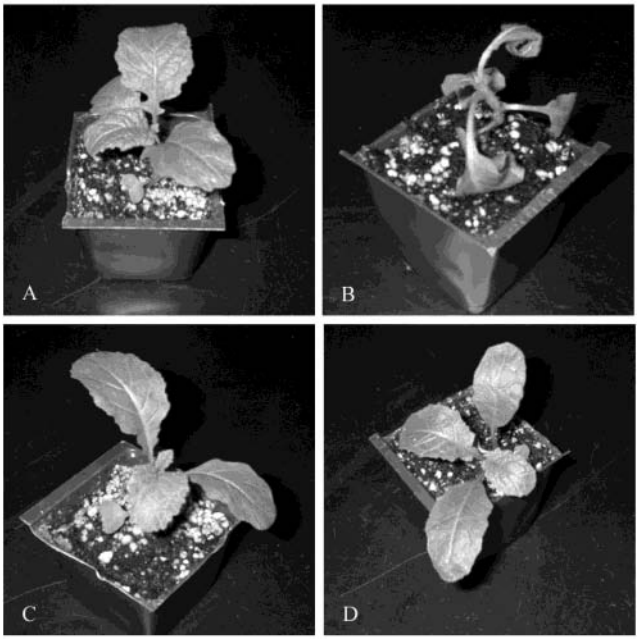

Figure 2. Effect of dicamba on auxinic-herbicide-susceptible and -resistant wild mustard plants 24 h after spraying: S wild mustard sprayed with (**A**) water (control) or (**B**) 50 g ai ha^{-1} dicamba and R wild mustard sprayed with (**C**) water (control) or (**D**) 50 g ai ha^{-1} dicamba.

Table 1. Effect of Dicamba on the Expression of Selected Proteins and a Comparison of the Relative Protein Amounts between the Dicamba-Treated S and R Wild Mustard

spot no. from gels		effect of dicamba treatment[a]		final spot density ratios[b]
seedling	leaf	S wild mustard	R wild mustard	$S_{dicamba}/R_{dicamba}$
1		down-regulated	down-regulated	1:5.8
2		down-regulated	spot not detected	1:0
3		down-regulated	up-regulated	1:2.8
4		down-regulated	up-regulated	1:1.3
	1	down-regulated	up-regulated	1:4
	2	down-regulated	spot not detected	1:0
	3	spot not detected	down-regulated	0:0
	4	down-regulated	down-regulated	1:1.4
	5	down-regulated	spot not detected	0:0
	6	up-regulated	up-regulated	1:1.4
	7	up-regulated	up-regulated	1:2.2

[a] Down- or up-regulated refers to the change in protein quantities (i.e., spot densities) compared to the untreated control. [b] Spot densities listed were averaged from two gels as determined by the PDQuest software (Bio-Rad).

Proteome-Level Differences. Table 1 summarizes the changes in expression of the identified proteins resulting from exposure to dicamba and compares the ratio of the final spot densities for the S and R wild mustard samples as determined using the PDQuest software. These proteins include those identified from protein extracts prepared from seedlings germinated in the presence of dicamba and from leaves of plants sprayed with dicamba. Although other proteins were observed to be differentially expressed when various regions of the gels were scanned using the PDQuest software, they were not chosen for identification by MS/MS if the intensity of the protein spot was deemed to be too low for identification or if the observations were not reproducible. **Table 2** lists the amino acid sequence for each peptide, shown in **Table 1**, from the identified protein spots; the list includes proteins involved in metabolic pathways,

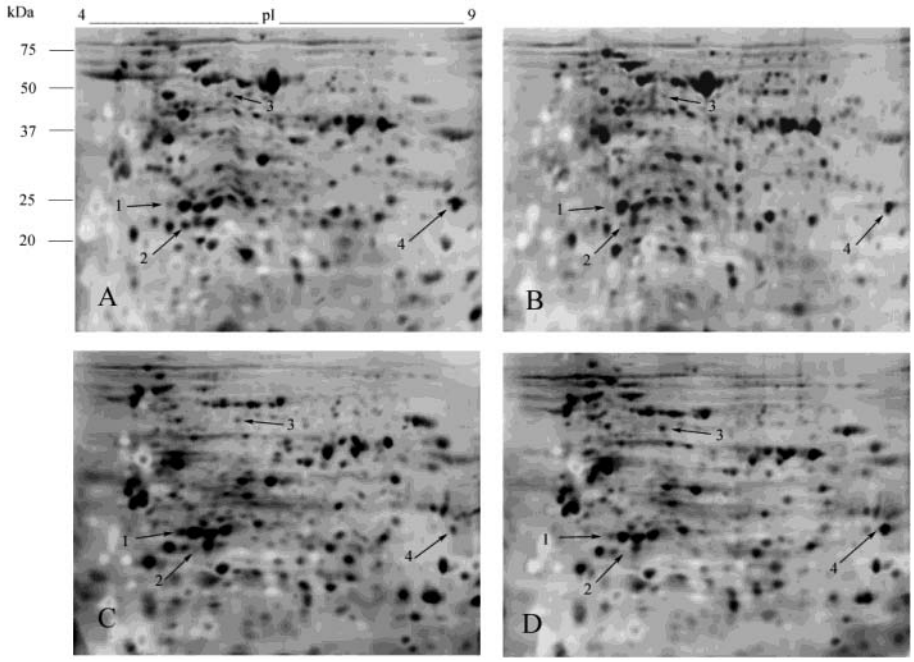

Figure 3. Coomassie blue and silver stained gel images of the R and S soluble seedling protein extracts separated by two-dimensional electrophoresis: S wild mustard germinated and grown in (**A**) water (control) or (**B**) 10 μM dicamba and R wild mustard germinated and grown in (**C**) water (control) or (**D**) 10 μM dicamba. Each arrow points to a protein that showed reproducible differences in expression between the S and R wild mustard treated with dicamba. Numbers correspond to the spot numbers for seedlings listed in **Tables 1** and **2**.

Table 2. Summary of Identities of the Proteins Shown in **Table 1**

spot no. from gels			MS/MS (ESI-Q-ToF)		
seedling	leaf	%[a]	sequence	identity	Mr/pI
1		8	DGSNYIALR TAGGLLLTETTK	chloroplast Cpn21 protein	26785/8.86
2		5	FLTQAVEEAYK	putative cytidine deaminase-like protein	20388/5.64
3		2	VACETCTK	methionine adenosyltransferase	43573/5.50
4		9	AIVNSDLGVTPNND GDVIR DLSSDLQK	expressed protein (*A. thaliana*)	30517/9.46
	1	7	TSWLDNK IVMGLFGEVVPK	peptidylprolyl isomerase	28532/8.83
	2	5	GPQSPSGYSCK	germin-like protein (AtGER3)	21993/6.26
	3	7	VLLTMEEK ISAADLSLAPK	dehydroascorbate reductase	27976/6.93
	4	3	SVGDLTSADLK YLIENGAK	phosphoglycerate kinase-related protein	50195/5.91
	5	3	NNLTNSMK ALAENEGEGIAK	dihydrolipoamide dehydrogenase 1	61004/8.13
	6	5	DSIMDSTVNCR GGAIDDSVITK	aminomethyl-transferase-related precursor protein	44759/8.55
	7	4	GGAIDDSVITK MPFVATK	aminomethyl-transferase-related precursor protein	44759/8.55

[a] Sequence percentage coverage of the matched peptides.

antioxidant systems, storage, and those with unknown functions. Most of the identified protein spots have not been previously implicated in regulating auxinic herbicide resistance. Among the differentially expressed proteins listed in **Table 2**, one (seedling protein spot 3; methionine adenosyltransferase) is involved in ethylene biosynthesis whereas another (leaf protein spot 1; peptidylprolyl cis−trans isomerase) has recently been implicated in auxin signaling pathways (*13*). These two proteins are discussed below in the context of potentially contributing to auxinic herbicide resistance.

Methionine Adenosyltransferase (MAT). MAT is a protein identified in this study as being differentially expressed between the S and the R wild mustard seedlings that has a known association with an auxin signal transduction process. Our results (**Figure 3**, spot 3) show that this enzyme is present in the S and the R biotype seedlings 1 week after germination in water and is down-regulated in S biotype seedlings germinated in dicamba, while it is up-regulated in the R biotype seedlings germinated in the auxinic herbicide (**Tables 1** and **2**). MAT is an enzyme responsible for the conversion of methionine and ATP to *S*-adenosylmethionine (SAM), which is an important methyl donor as well as being part of the ethylene biosynthetic pathway found in many plants (*1*, *15*). 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase enzymes are responsible for

converting SAM to ACC, which is then either metabolized to 1-malonylaminocyclopropane-1-carboxylic acid (MACC) or converted to ethylene by ACC oxidase (*1*).

The synthesis of ethylene has been shown to increase in both biotypes, but this increase is significantly more in the S than the R biotype as early as 18−24 h following the application of auxinic herbicide (*10*). Furthermore, there was only a minor increase in ethylene biosynthesis in the R plant when compared to basal levels in the untreated control 96 h after treatment with the auxinic herbicide picloram (*1*). The difference between the induction of ethylene biosynthesis by auxinic herbicides in the S versus R biotypes may be key to auxinic herbicide resistance (*1*). Hall et al. (1985) treated rapeseed plants with aminoethoxyvinylglycine (AVG), a chemical known to interfere with the evolution of ethylene, and picloram and showed that inhibition of ethylene biosynthesis, albeit for a short time period, reduces the characteristic auxinic-herbicide-induced morphological changes observed in susceptible plants (*16*). In addition, Hall et al. (1993) showed that the quantity and the activity of the ACC synthase enzyme are significantly lower in the R biotype treated with an auxinic herbicide compared with the similarly treated S biotype (*1*).

A cursory review of our results may indicate that the observed changes in levels of MAT are counter to what would be expected, since ethylene biosynthesis would be expected to be higher in the S biotype seedlings treated with dicamba and therefore require greater quantities and/or activities of the MAT enzyme. However, one could argue that because of the rapid induction of ethylene production in susceptible plants treated with dicamba (i.e., within a few hours of treatment), the down-regulation of MAT in the extracts of S biotype seedlings may be a direct result of senescence due to this early induction of ethylene by dicamba. Conversely, due to little auxinic-herbicide-induced ethylene production in R and thus the absence of severe senescence, observed up-regulation of MAT in the dicamba-treated R biotype seedlings results in minor increases of ethylene biosynthesis. Nonetheless, we believe these small increases in ethylene above basal levels do not contribute significantly to phytotoxic symptoms in the R biotype, which leads us to conclude that the differential expression of MAT is not contributing to the resistance. Furthermore, Hall et al. (1985, 1993) showed that elevated ethylene levels are a result of susceptibility, not the cause of it (*1*, *16*)

Peptidylprolyl Cis−Trans Isomerase (PPIase). PPIases are present in all organisms and are responsible for the reversible isomerization of peptide bonds involving proline residues in proteins (*17* and references therein). PPIases can be grouped into three families based on structural differences and substrate specificities: cyclophilins, FK506-binding proteins (FKBP), and parvulins (*17*). The original PPIase that was identified was demonstrated to be identical to cyclophilin, a high-affinity receptor for the immunosuppressive drug cyclosporin A (*18*, *19*). The FKBP PPIases, which are unrelated in sequence to the cyclophilins, are also able to bind immunosuppressive compounds, such as rapamycin and FK506 (*17*). Parvulins comprise the third known family of PPIases; parvulins exhibit no sequence homology to either the cyclophilins or the FKBPs and are considerably smaller (*17*, *20*). However, it has been reported that members of the parvulin family share structural similarities with some of the FKBP PPIases (*17*).

Our results show that the expression pattern of a cyclophilin-type PPIase (**Table 2**; **Figure 4**, spot 1) varies between the S and the R wild mustard biotypes and it is differentially regulated by dicamba. PPIase is present in the leaf tissue of S controls (**Figure 4C**) and is down-regulated considerably within 24 h of dicamba application (**Figure 4D**). The opposite appears to be true for the R wild mustard biotype, where the expression of the PPIase is clearly up-regulated following dicamba exposure (**Figure 4G**,**H**). This result is particularly interesting since PPIases have recently been implicated in a mechanism that induces auxin-modulated activities (*13*) and have also been shown to associate with proteins that are thought to be involved in auxin transport (*21*) and ethylene response (*22*).

In *Arabidopsis*, the cyclophilin-type PPIases are localized primarily in the cytoplasm; however, specific cyclophilins are also present in the chloroplast as well as the endoplasmic reticulum (ER) (*21*), where the majority of the auxin-binding protein 1 (ABP1) is located. However, the ABP1 receptor possesses a particular signal sequence at the N-terminal region, which enables a small proportion of the cellular ABP1 to migrate to the plasma membrane, where it is believed to function as a receptor for the auxin hormone (*23*). Webb and Hall (1995) have reported differences in auxin binding site affinities for the phytohormone between the auxinic-herbicide-susceptible and -resistant wild mustard biotypes (*11*), which may be a factor in the regulation of auxinic herbicide resistance. ABP1 from maize has been crystallized, and it has been reported that each monomer of the dimeric maize ABP1 contains two antiparallel β-sheets, which form a β-jellyroll barrel (*24*). Located within the β-barrel is a zinc-binding site where a zinc metal ion binds the auxin hormone. A binding pocket formed by a number of hydrophobic amino acid residues (Trp151, Thr54, Pro55, Ile22, and Leu25), including proline, located in the vicinity of the zinc ion helps to hold the auxin within the ABP1 receptor. In addition to proline being part of the hydrophobic binding pocket, there are other conserved proline residues present in ABP1 and at least two of these prolines form peptide bonds (Pro127 and Pro148) that are present in the cis conformation (*24*). Formation of these cis prolyl bonds may require action by a PPIase since the trans forms are more abundant in folding proteins and an enzyme is needed to catalyze the naturally slow trans to cis isomerization, which involves rotation around a partial double bond (*17*). Clearly, our observed differences in PPIase expression between the susceptible and resistant wild mustard biotypes could conceivably lead to differences in the degree of prolyl isomerization of the prolyl peptide bonds of ABP1, which could have dramatic effects on the activity of the auxin receptor and the induction of auxin-mediated symptoms and should be explored.

IAA-modulated activities also include the expression of auxin-regulated *Aux*/*IAA* genes as early as 5−60 min after the exposure to IAA (*25*). A common feature of the IAA-regulated Aux/IAA proteins is the presence of two highly conserved proline residues (*26*) that are part of a domain required for their recognition by an SCF^{TIR} complex, which is involved in the polyubiquitination of Aux/IAA proteins (*27*). The polyubiquitin labeling of Aux/IAA proteins targets them for degradation by a 26S proteasome. This ubiquitin-dependent protein degradation process is believed to be part of an auxin signaling pathway (*27*). Specifically, Aux/IAA proteins function as transcriptional regulators through the formation of dimers with other transcriptional regulators, such as the auxin response factors (ARFs) (*28*). The ARFs are capable of binding to specific DNA regions called auxin response elements (AREs) within the promoters of auxin-regulated genes where they act to modify the expression levels of these genes (*27*). It has been proposed that the ARFs are permanently associated with the ARE sequences and at relatively low concentrations of auxin, the Aux/IAA transcriptional regulators

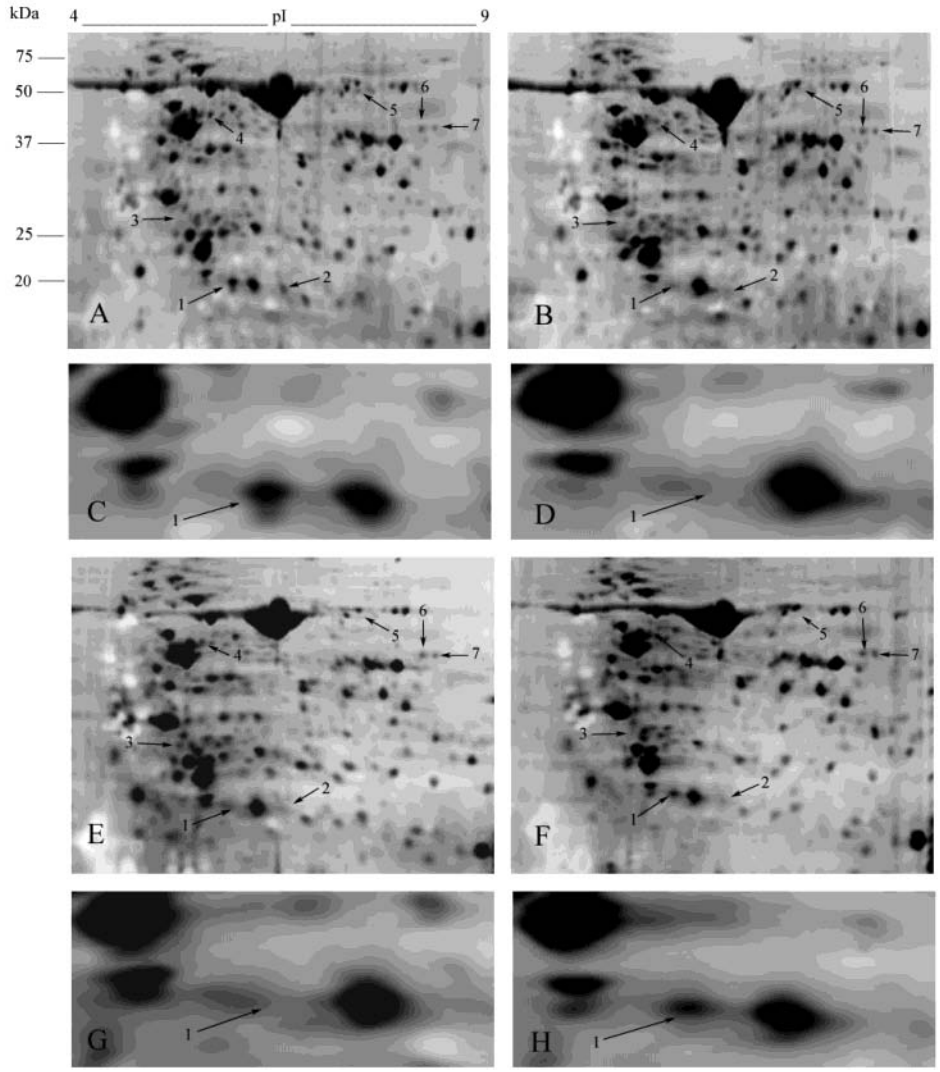

Figure 4. Coomassie blue and silver stained gel images of the R and S soluble leaf protein extracts separated by two-dimensional electrophoresis: S wild mustard sprayed with (**A**) water (control) or (**B**) 50 g ai ha^{-1} dicamba and R wild mustard sprayed with (**E**) water (control) or (**F**) 50 g ai ha^{-1} dicamba. Images **C**, **D**, **G**, and **H** are enlarged views of the gels shown directly above (A,B,E,F) to more clearly show the change in expression of spot 1 (peptidylprolyl isomerase). Each arrow points to a protein that showed reproducible differences in expression between the S and R wild mustard treated with dicamba. Numbers correspond to the spot numbers for leaves listed in **Tables 1** and **2**.

are not subjected to auxin-induced destabilization and degradation. As such, the Aux/IAA proteins are able to form dimers with the ARFs, which results in the inhibition of the ARF activity. When the concentration of auxin or auxin-mimics increases, as it may upon dicamba application, the Aux/IAA proteins are targeted to the SCF^{TIR} complex with greater efficiency, leading to a significant increase in their degradation of Aux/IAA proteins. With a decreased concentration of available Aux/IAA proteins, a greater number of ARFs are able to form homodimers, and it is the formation of these complexes at the AREs of auxin-regulated genes that allows for the modulation of the expression of the associated genes.

Dharmasiri et al. (2003) have recently provided evidence that the activity of a parvulin-type PPIase is essential for the efficient association of Aux/IAA proteins with the SCF^{TIR} complex (*13*). PPIases catalyze the reversible isomerization between the cis and trans conformations of peptide bonds involved with proline, implying that a particular conformation of Aux/IAA proteins influences the activity of the auxin-mediated, ubiquitin-dependent protein degradation process. Our results indicate that the expression pattern of a cyclophilin-type PPIase varies between the S and the R wild mustard biotypes and it is differentially regulated by dicamba. Although Dharmasiri et al. concluded that, of the different types of known PPIases, only the parvulin-type PPIases are involved in an auxin signaling pathway, the clear and dramatic changes in expression of a cyclophilin-type PPIase upon application of dicamba suggest the possibility that this type of PPIase could also be involved in mediating auxin signaling along with the parvulin-type PPIase.

It is conceivable that in the untreated S wild mustard a PPIase (parvulin-type, cyclophilin-type, or both) catalyzes the isomerization of the prolyl peptide bonds to produce the required Aux/IAA conformation that maximizes the identification and ubiquitination of these proteins for their subsequent degradation, thereby increasing ARF activity, largely causing repression of auxin-mediated transcription. PPIase is down-regulated in dicamba-treated S plants, resulting in little or no isomerization of Aux/IAA proteins, thereby allowing them to bind to ARFs, which largely results in activation of dicamba-mediated transcription. In R plants, this course of events is reversed in that there is little expression of PPIase in untreated R plants while PPIase is up-regulated in dicamba-treated R plants. In this case, ARF activity would decrease and increase in untreated and treated R plants, respectively. Therefore, in dicamba-treated R

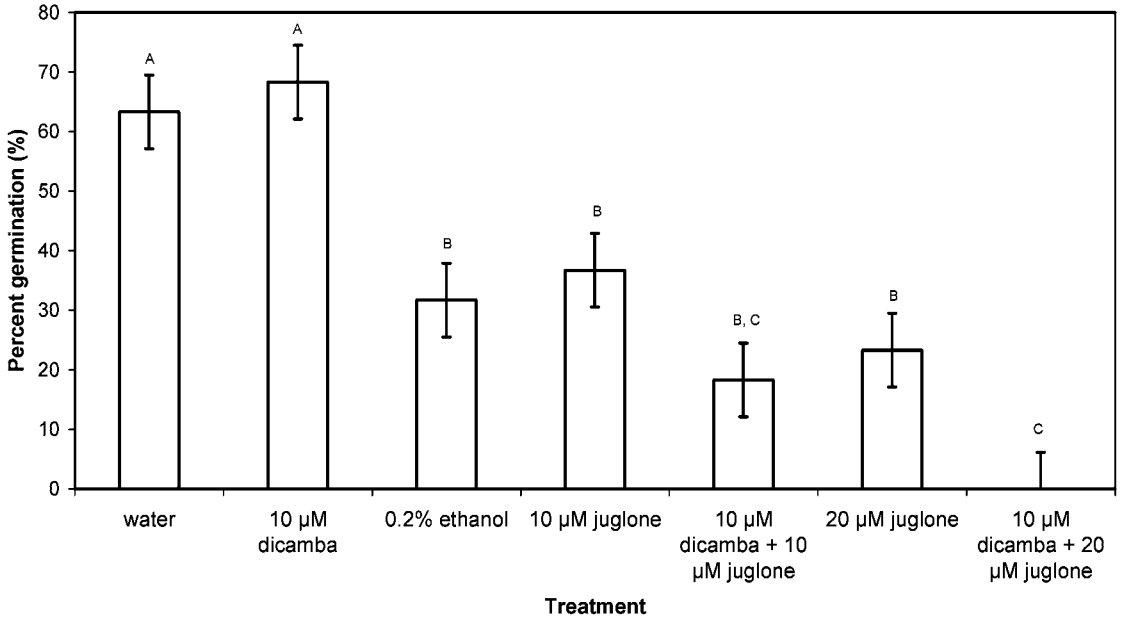

Figure 5. Effect of various treatments on the germination of R wild mustard seeds. Different letters above the standard error bars indicate which treatments are statistically different ($P < 0.05$) as determined by the Student–Newman–Keuls test.

plants, we hypothesize that up-regulation of PPIase results in repression of transcription (i.e., little or no auxinic herbicide effects) similar to what happens with *Drosophila* PPIase Dodo, which has been implicated in degradation of the transcription factor CF2 (*29*). Since it is widely accepted that the expression of the early auxin-induced genes, which are involved in initiating the auxin-mediated activities, occurs rapidly after the application of auxin, we believe that the isomerization reaction, mediated by PPIase, occurs very quickly after the exposure to auxin or auxinic herbicide resulting in rapid down-regulation of PPIase (i.e., within 24 h).

Effect of Dicamba When Used in Conjunction with a PPIase Inhibitor. In an effort to further verify the involvement of a parvulin-type PPIase in the regulation of auxinic herbicide resistance as shown by Dharmasiri et al. (*13*), we initiated experiments to determine the effects of juglone, a known parvulin-type PPIase inhibitor (*30*), on the activity of dicamba in the R wild mustard biotype when dicamba and juglone are applied simultaneously. We hypothesized that if parvulin-type PPIases are involved in mediating auxinic herbicide resistance of R wild mustard, the presence of juglone may inhibit the ability of both seedlings and older plants to resist the effects of dicamba. The results of these experiments are summarized in **Figures 5** and **6**. As expected, the germination and subsequent growth of R wild mustard seeds in water (control) and dicamba were the same. Conversely, addition of 20 μm juglone with dicamba resulted in severe effects on R seedling growth similar to the effects of dicamba on S seedlings (germination in 0.2% ethanol served as a control to assess whether the ethanol present in the juglone-containing solutions had an effect on germination rates). Furthermore, when dicamba plus juglone was applied to R wild mustard at the 3−4 leaf stage of development, typical auxinic herbicide symptoms (e.g., epinasty; **Figure 6**) occurred. These results indicate that the inhibition of the PPIases by juglone interferes with the mechanism of auxinic herbicide resistance in growing plants as well. Similar experiments with cyclosporin could not be conducted because the seeds did not germinate in the presence of cyclosporin.

In conclusion, our research confirms the value and utility of a proteomics-based approach in the study of existing scientific questions in the fields of herbicide research and agriculture. Furthermore, we have demonstrated that PPIases may be involved in the mediation of resistance to auxinic herbicides in wild mustard. However, the specific type of PPIases that are involved in regulating auxinic herbicide symptoms and their role are far from being clear. Our experiments using the PPIase inhibitor juglone on both seedlings and older plants suggest that a parvulin-type PPIase may be involved in mediating resistance, whereas our proteome-level analysis identified a cyclophilin-type PPIase as being differentially regulated. It is possible that in leaves dicamba differentially regulates a parvulin-type PPIase as well; however, due to the overwhelming presence of the small subunit of ribulose bisphosphate carboxylase/oxygenase (Rubisco) in the region of the gel we cannot see the effect on this parvulin-type PPIase. Additional studies to establish the identi-

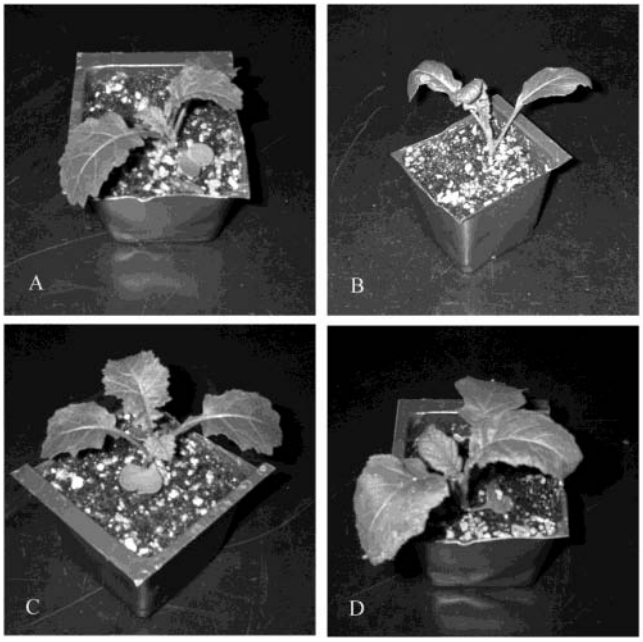

Figure 6. Effects of juglone on the morphology of 3-week-old R wild mustard plants sprayed with (**A**) 50 g ai ha^{-1} dicamba, (**B**) 50 g ai ha^{-1} dicamba and 20 μM juglone, (**C**) 20 μM juglone, or (**D**) 0.2% ethanol.

ties of the PPIases involved, that is, whether they are of cyclophilin- or parvulin-type (or both); the identities of the target protein(s), including ABP1, modified by the PPIases; and the regulation of expression of PPIases by auxins are warranted. These studies as well as those aimed at probing the calcium ion dynamics, which we have previously linked to the resistance of R wild mustard to dicamba (*3*−*7*), are in progress in our laboratories to determine whether PPIase and calcium effects are linked.

ABBREVIATIONS USED

DTT, dithiothreitol; TCA, trichloroacetic acid; TBP, tributylphosphine; IPG, immobilized pH gradient; SAS, Statistical Analysis System; MAT, methionine adenosyltransferase; PPIase, peptidylprolyl isomerase; Rubisco, ribulose bisphosphate carboxylase/oxygenase.

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