University of Alberta

Proteome-level comparison of auxinic herbicide-susceptible and –resistant wild mustard (*Sinapis arvensis* L.)

by

William Yajima

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science

in

Plant Science

Department of Agricultural, Food and Nutritional Science

EDMONTON, ALBERTA

Fall 2004

Your file Votre référence
ISBN: 0-612-95885-X
Our file Notre référence
ISBN: 0-612-95885-X

Acknowledgments

First and foremost, I would like to express my sincere appreciation to my supervisor, Dr. N. Kav, for his invaluable guidance, encouragement, enthusiasm and financial support throughout my graduate studies program.

I wish to also thank Dr. W. Dixon and Dr. M. Deyholos for their helpful advice and suggestions during my studies.

Finally, I would like to convey my gratitude to those members of Dr. N. Kav's laboratory who have provided assistance throughout my studies, especially Mr. Sanjeeva Srivastava, Mrs. Shaheen Rahman, Dr. Phyllis Dale, Dr. Babu Subramanian, Miss. Nidhi Sharma, and Miss Kelley Dunfield.

Table of Contents

List of Tables

Page

List of Figures

Page

Note: Portions of the following have been included in a manuscript entitled "Proteome-level differences between auxinic herbicide-susceptible and –resistant wild mustard (*Sinapis arvensis* L.)" submitted to the Journal of Agricultural and Food Chemistry.

1.0 Introduction

1.1 Auxins

Research into auxins and their roles in plant growth and development was initiated in 1926 with Frits Went's description of a naturally occurring chemical found in plants that was involved in the bending of oat coleoptiles (Salisbury & Ross, 1992). The chemical that Went had discovered later became known as auxin or indole-3-acetic acid (IAA) (Sterling & Hall, 1997). While IAA is the most common naturally occurring auxin found in plants, there are other chemical compounds that elicit similar responses in plants and can be thought of as auxins and/or auxin precursors. Included among these compounds are phenylacetic acid (PAA), 4-chloro-indoleacetic acid (4-chloro-IAA), amino acid conjugates of IAA, and indolebutyric acid (IBA) (Sterling & Hall, 1997). IAA is endogenously produced from tryptophan by meristematic tissues and is then distributed to the rest of the plant, primarily to young and actively growing plant cells where the phytohormone is thought to have a greater effect than in mature tissues (Cobb & Kirkwood, 2000). It has been widely accepted that auxins have a significant role in regulating normal plant growth and development. Researchers have been able to demonstrate that this particular hormone is involved in activities such as the development of vascular tissues, fruit ripening, phototropic and gravitropic responses, maintenance of apical dominance, formation of lateral and adventitious roots, senescence and embryogenesis (Abel & Theologis, 1996; Gray & Estelle, 2000; Leyser, 2001; Walker &

Estelle, 1998; Worley et al., 2000). At the level of the cell, auxin is believed to modulate the rates of cell division, differentiation, and elongation, as well as cell and organ polarity and wound responsiveness (Walker & Estelle, 1998; Davies, 1987). Each of these cellular responses differs in terms of how rapid the response is following exposure to auxinic hormones. Typically, cell elongation and expansion occur with the shortest lag times, while the responses involving cell differentiation have the longest lag times following auxin hormone treatment (Macdonald, 1997). The precise activity of auxin is in part determined by cell type, growth and developmental stage, as well as environmental stimuli (Leyser, 2002). Another early auxin-regulated activity involves the expression of specific auxin-induced genes (Abel & Theologis, 1996). The rapid transcription of these genes does not require *de novo* protein synthesis as the necessary transcriptional machinery is already present in the cells and usually occurs within 5 – 60 minutes following auxin exposure (Key, 1989; Abel & Theologis, 1996). The expression of these genes produces proteins that help to complete the required cellular processes to generate the necessary responses. These auxin-induced genes can be categorized into distinct gene families and the best understood and characterized families include the GH3, small auxin up RNA (SAUR), and Aux/IAA families (Abel & Theologis, 1996). A significant amount of our knowledge about auxin activity has been the result of studies using mutants deficient in auxin perception and/or response as well as through the observation of changes in gene expression resulting from exogenous application of the hormone onto whole plants, individual plant cells and tissues (Sterling & Hall, 1997; Estelle & Somerville, 1987; Leyser, 2002). However, even with the wealth of information that has been uncovered concerning auxins and their actions, questions still

remain regarding certain aspects of auxin activity such as how the perception of the hormone is linked to the induction of known auxin-induced effects.

1.2 Auxin perception

A number of proteins with auxin binding activities have been identified from different plant tissues and species and they are typically localized in specific regions of the cell such as the endoplasmic reticulum, tonoplast vesicles, or plasma membrane (Dohrmann et al., 1978). While numerous auxin binding proteins have been identified, their precise involvement in auxin signalling pathways has not been established and continued research is required to critically examine whether every protein with affinity for auxin acts as a functional hormone receptor. It is also unclear whether auxin binding proteins are involved in a single auxin signalling pathway or whether the putative auxin receptors are part of multiple diverse signalling cascades. The endoplasmic reticulum-associated auxin-binding protein has been the subject of extensive research into the activities of the auxin binding proteins and, consequently, it is the best characterized and understood of all of the proteins with affinity for auxin. A dimeric (22 kDa subunits), soluble glycoprotein referred to as Auxin-Binding Protein 1 (ABP1) is a protein that contains the Lys-Asp-Glu-Leu (KDEL) amino acid sequence at its C-terminal region that allows it to be located primarily in the endoplasmic reticulum in a wide range of green plants (Macdonald, 1997). However, the ABP1 receptor also 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 (Macdonald, 1997). These plasma membrane-bound receptors are

thought to function in auxin uptake and efflux and to co-ordinate with an ATPase-mediated proton pump that is believed to be involved in auxin-regulated cell elongation (Rubery, 1981). Why the majority of the ABP1 protein is located in the endoplasmic reticulum, where the pH is not conducive to auxin binding, has not yet been determined. One possibility is that the endoplasmic reticulum lumen serves as a storage site for ABP1, which is exported to the cell membrane as needed. The pH in the vicinity of the cell membrane and cell wall is not optimal for the stability of the ABP1 protein (Macdonald, 1997). The potential for degradation of the cell membrane-associated ABP1 proteins may require continual replenishment via the export of ABP1 proteins from the endoplasmic reticulum, where the conditions do not adversely affect the stability of the receptor (Macdonald, 1997). It has been suggested that the rate of export of the ABP1 receptor to the plasma membrane is affected by factors such as environmental stimuli, plant developmental stage, and auxin concentration at the cell wall and membrane (Macdonald, 1997). It should be noted that the seemingly odd pattern of cellular distribution of this protein is not the only unusual property of this putative receptor. Researchers have reported that ABP1 does not exhibit many of the other characteristics common to proteins that function as receptors. In fact, ABP1 does not show any homology to any known family of receptors (Gray & Estelle, 2000). However, its role in auxin perception is not in doubt as it has been experimentally proven that interference of the ABP1 protein at the cell membrane results in a decrease in the rate of auxin-induced cell division and cell elongation (Chen et al., 2001). Furthermore, the consequence of complete elimination of ABP1 is death of the plant at the embryological developmental

stage, demonstrating the significance of ABP1 in normal plant growth and development (Chen et al., 2001).

ABP1 from maize has been crystallized and its structure characterized at 1.9 Å resolution (Woo et al., 2002). Each monomer of the dimeric maize ABP1 consists of 160 amino acids with amino acids 26-148 folding to create two anti-parallel β-sheets, which form a complex called a β-jellyroll barrel (Woo et al., 2002). Located within the β-barrel is a zinc-binding site composed of three histidine residues and one glutamic acid residue. It is this zinc metal ion that actually binds the auxin hormone within each receptor subunit. Studies utilizing the synthetic auxin 1-naphthalene acetic acid (1-NAA) have shown that a carboxylic acid moiety of the naphthalene interacts with the zinc metal ion (Woo et al., 2002). It has been reported that ABP1 binds auxins with high specificity and affinity with its K_D value for 1-NAA calculated to be 5×10^{-8}M (Napier, 1995). A binding pocket formed by a number of conserved hydrophobic amino acid residues (Trp151, Thr54, Pro55, Ile22, and Leu25) located in the vicinity of the zinc ion help to hold the auxin within the ABP1 protein and make the hormone inaccessible to solvent. Flanking the internal β-barrel are the N- and C-terminal extensions, which do not form any distinct structures except for a short β-strand in the N-terminal extension and a short α-helix within the C-terminal extension. The N- and C-terminal extensions are joined together by a disulfide bond, which is formed between Cys2 and Cys155. This disulfide bond is believed to restrict the movement of the N- and C-terminals, which helps to maintain the structure of ABP1 regardless of whether or not auxin is bound (Woo et al., 2002). There is evidence to support the idea that there is no substantial conformational change occurring as a result of auxin binding to ABP1. The crystal structures of ABP1

with and without bound auxin have been superimposed on each other and no significant conformational differences have been uncovered (Woo et al., 2002). Based on the structure of the ABP1 receptor, it is believed that the N-and C-terminal extensions form a readily accessible pathway that directs the auxin hormone towards the zinc metal ion (Woo et al., 2002).

The precise mechanism(s) by which the binding of auxin to ABP1 stimulates signalling cascades has not been conclusively deciphered. Although it is believed that the conformation of the ABP1 protein does not change significantly upon auxin binding, it has been hypothesized that minor alterations in the structure of the receptor act as a signal to the plasma membrane or to the adjacent proteins within the plasma membrane that auxin has been bound by the ABP1 (Woo et al., 2002). It has been postulated that the precise location of Trp151 within the auxin-binding site changes depending on whether auxin is present or not with the Trp151 residue shifting slightly away from the auxin-binding site when the receptor is free of auxin. This movement results in a concomitant minor shift in the C-terminal α-helix away from the auxin-binding site. Following the binding of auxin, both the Trp151 residue and the C-terminal α-helix move towards the auxin-binding pocket. This movement of the C-terminal α-helix, and the resulting movement of the N-terminal extension due to the disulfide linkage connecting the N-terminal and C-terminal extensions, is believed to act as a signal to nearby proteins or the plasma membrane itself that auxin has been bound by the receptor (Woo et al., 2002). The suggestion that the C-terminal portion of the ABP1 protein is involved in interacting with the plasma membrane or certain membrane-integrated proteins is strengthened by the discovery that monoclonal antibodies specific for the C-terminus of ABP1 cannot

bind to ABP1 at the cell membrane, which researchers felt indicated that the C-terminus is indeed buried within the plasma membrane (Macdonald, 1997). It has been proposed that the ABP1 receptor is associated with a specific plasma membrane-integrated protein called a "docking" protein, which anchors the receptor to the cell membrane so that the auxin-binding moiety of the receptor is situated facing the outside of the membrane (Macdonald, 1997). It is also believed that the docking protein belongs to the G-protein-coupled receptor (GPCR) family (Macdonald, 1997). Members of the GPCR family of receptors are typically made up of seven membrane-integrated domains, which are linked to their effector targets by G-proteins (Macdonald, 1997). These G-proteins are multi-subunit proteins, usually containing an α-subunit, which is capable of associating with guanine molecules as well as with the β- and γ-subunits of the G-proteins (Macdonald, 1997). The binding of auxin to ABP1 is thought to stimulate the binding of a GTP molecule to the α-subunit resulting in the dissociation of the α-subunit from the β- and γ-subunits and from the docking protein. This dissociation then leads to the activation and stimulation of the signalling cascades thought to be responsible for the auxin-mediated responses by the activated G-protein subunits (Macdonald, 1997). The re-association of the G-protein subunits to each other and to the docking protein occurs once the GTPase enzyme located within the α-subunit converts the bound GTP molecule to GDP (Macdonald, 1997). Zaina *et al.* (1990) have shown that in rice the binding of auxin by plasma membrane-associated ABPs leads to the activation of GTP-binding proteins, which suggests that these proteins are part of a signal transduction pathway that induces auxin-mediated cell elongation.

Although ABP1 is considered to function as a key auxin receptor, researchers have demonstrated that there are likely other auxin receptors that are involved in the induction of known auxin-regulated effects and that not all auxin-like molecules that have affinity for ABP1 stimulate auxin-mediated activities. For example, 2,6-dichlorobenzoic acid, which is known to possess auxin activity, does not appear to bind to ABP1, while 8-chloronaphthalene-1-acetic acid, which does not have any auxin activity, seems to have affinity for ABP1 (Sterling & Hall, 1997). Other cellular proteins that have been identified with affinities for auxin are thought to be involved in the polar transport of auxin, which in the plant is a phenomenon that is believed to allow the transport of auxin from the shoot apex, where it is synthesized, to the rest of the plant (Vogler & Kuhlemeier, 2003). This transport of auxin ensures that adequate concentrations of auxin are present where needed to allow auxin-regulated growth activities to take place in a temporally and spatially controlled manner throughout the plant lifecycle. A potential membrane-integrated, amino acid permease-like protein called AUX1 has been shown to be likely involved in the influx of protonated auxins into plant cells (DeLong et al., 2002). As well, membrane proteins belonging to the AGR/EIR1/PIN/WAV6 family (eg. PIN1) are believed to function as anion carriers, which facilitate the efflux of IAA^- out of plant cells (DeLong et al., 2002). Both AUX1 and PIN1 are proteins that are found distributed asymmetrically throughout the plant cell membranes. It is suggested that the particular pattern of distribution of these auxin influx and efflux carriers contributes to the polar transport of auxin by creating concentration gradients (Luschnig, 2002). Another type of transporter has also been implicated in the transport of auxin throughout the plant. In *Arabidopsis*, proteins that contain ATP-binding cassettes (ABC proteins)

have been shown to participate in the polar transport of auxin (Luschnig, 2002). ABC proteins are membrane proteins that are not exclusive to plants and, depending on the species in which they are found, are not only involved in the transport of auxin. As the name might suggest, the one common feature among all of the ABC proteins is the requirement of the hydrolysis of ATP during efficient protein activity. ABC transporters typically consist of two transmembrane domains (TMD), which are separated by a cytoplasm-associated nucleotide-binding fold (NBF) (Luschnig, 2002). A second NBF is usually present at the C-terminus of the ABC transporters and the complete ABC transporter is thought to contain two substrate-binding domains (Dey et al., 1997). One model to explain the activity of the ABC transporters suggests that one of the substrate-binding domains is present on the inner surface of the plasma membrane and the other substrate-binding domain, which also serves as the substrate-release domain, is situated on the outer surface of the plasma membrane. It is speculated that the hydrolysis of ATP is needed for the movement of the substrate between the two substrate-binding domains (van Veen et al., 2000) and the subsequent movement of the substrate from cell to cell or compartment to compartment.

Examination of the *Arabidopsis* genome has uncovered the existence of approximately 100 putative and confirmed members of the ABC protein family (Sanchez-Fernandez et al., 2001). One member of this family of proteins that is known to be involved in the polar transport of auxin has been designated AtMRP5. Studies that were instrumental in determining its role in the transport of auxin demonstrated that plants that were unable to produce wild-type AtMRP5 proteins exhibited altered phenotypes that were consistent with incorrect transport of auxin. These plants displayed

reduced elongation of the primary roots as well as premature formation of the lateral roots (Gaedeke et al., 2001). These phenotypes were found to be a consequence of abnormally increased concentrations of auxins in the affected plant cells resulting from the inactivity of the mutagenized AtMRP5 proteins (Gaedeke et al., 2001). Other *Arabidopsis* ABC proteins involved in the transport of auxin include the AtMDR1 protein and the closely related AtPGP1 protein (Noh et al., 2001). *Arabidopsis* plants that were mutagenized to produce defective AtMDR1 and AtPGP1 were similar in appearance to known auxin response mutant plants (Noh et al., 2001). These mutant plants, which exhibited decreased apical dominance, grew to have cotyledons that were significantly twisted (epinastic) and leaves that displayed abnormal wrinkling (Noh et al., 2001). Furthermore, double mutants (i.e. defective in both AtMDR1 and AtPGP1 proteins) were found to have more significant and noticeable defects when compared to single mutants (Noh et al., 2001). It has been experimentally determined that the rates of polar auxin transport in the mutagenized plants were significantly reduced and it is believed that the observed phenotypes of the mutant plants were the result of this abnormal transport of auxin (Noh et al., 2001). Studies on the AtMDR1 protein have proven that this membrane-associated protein is able to bind N-1-naphthylphthalamic acid (NPA), which is a known inhibitor of auxin efflux carriers (Luschnig, 2002). The fact that NPA has been shown to bind to specific plasma membrane proteins has provided some evidence that certain plasma membrane receptors, like AtMDR1, are almost certainly involved in the polar transport of auxin (Muday & Murphy, 2002). In fact, it has been demonstrated that a reduction in the number of NPA-binding sites within the plasma membrane is associated with a decreased level of auxin transport activity (Luschnig, 2002). In

addition, it has been suggested that the binding of NPA to AtMDR1 may influence or regulate the activities of other membrane-associated auxin efflux proteins such as PIN1 (Luschnig, 2002). It remains possible that the AtMDR1 protein functions as an NPA-binding site and is merely a subunit of a larger auxin efflux carrier. As such, the AtMDR1 protein could be viewed as the regulatory portion of the auxin efflux carrier complex. This regulatory subunit would then require some type of association with a protein like PIN1, which would function as the catalytic subunit of the auxin efflux complex.

1.3 Auxin-mediated responses

The perception of auxin by the various receptors induces signalling cascades, which in turn stimulate other cellular factors, which are required for the activation of the auxin-mediated responses. One of the earliest responses to auxin treatment involves the alteration of the plant cell membrane's electrochemical activity (Macdonald, 1997). Within minutes of auxin treatment, the auxin-induced proton-ATPase activity within the plasma membrane results in an increase in the rate of proton efflux from the cell (Macdonald, 1997). It has been suggested that the activation of the dissociated G-protein subunits of the aforementioned GPCR docking protein within the cell membrane causes the activation of a phospholipase A_2 (PLA_2) enzyme (Macdonald, 1997). The activation of the PLA_2 complex helps convert phospholipids to lysophospholipids and fatty acids. The lysophospholipid molecules then induce a membrane-integrated protein kinase (PK) to phosphorylate the proton-ATPase membrane protein, which stimulates the activity of the enzyme (Scherer & Andre, 1993). The products of the PLA_2 enzymatic activity are

also thought to possibly alter the elasticity of the cytoskeletal actin network (Grabski & Schindler, 1996). A consequence of the auxin-regulated proton efflux from the cell, which can occur as early as 7 to 8 minutes after auxin treatment, is a decrease in the pH of the cell wall region, which is believed to decrease the plant cell wall's rigidity and stimulate cellulases to cleave the hemicellulose chains within the cell wall (Fry, 1989). The decreased rigidity of the cell wall as well as the altered ion-channel activity resulting from auxin exposure also influences the transport of solutes into and out of the cell. This in turn can affect the turgor pressure within the plant cell (Macdonald, 1997) leading to the elongation of the cells, which is an observed auxin-mediated effect that occurs within 10 minutes of auxin exposure (Theologis, 1986). Auxins further affect the plant cell wall by modifying the secretion of cell wall components like lipids as well as altering the orientation of the cell wall polysaccharides (Macdonald, 1997).

Specific genes in *Arabidopsis* plants have been determined to be required for efficient auxin-mediated cellular responses. The loci of some of these genes have been designated AXR 1, 2, 3, 4, and 6 (Leyser, 2002). AXR 1, 2, and 3 have been reported to be involved in a specific pathway that regulates an auxin-mediated and ubiquitin-dependent protein degradation process (Leyser, 2002). The AXR2 and AXR3 gene products belong to the Aux/IAA protein family (Nagpal et al., 2000; Rouse et al., 1998). In *Arabidopsis*, 24 Aux/IAA proteins have been identified and these proteins, which are believed to be located within the nucleus (Leyser, 2001), are typically small (19 – 36 kDa) and hydrophilic (Abel & Theologis, 1996). A common feature among the proteins belonging to the Aux/IAA family is the presence of four highly conserved amino acid domains (Tiwari et al., 2001). Research has determined that within Domain 2 there exists

an amino acid sequence containing two highly conserved proline residues, which is required for an auxin-induced destabilization and subsequent degradation of Aux/IAA proteins (Dharmasiri et al, 2003; Tiwari et al., 2001). The Aux/IAA proteins act as transcriptional regulators through the formation of dimers with other Aux/IAA proteins to form homodimers as well as heterodimers (Leyser, 2002). These proteins are also able to form dimers with other transcriptional regulators, such as the auxin response factors (ARFs) through the binding of homologous Domains 3 and 4 (Ulmasov et al., 1997). The ARF transcriptional regulators, which also form homo- and heterodimers, are capable of binding to specific DNA segments called auxin response elements (ARE) located within the promoters of auxin-regulated genes and modifying the expression levels of those particular genes. Depending on the sequences of different ARFs, they may affect auxin-regulated gene expression by either suppressing or activating expression (Ulmasov et al., 1999). The aforementioned auxin-mediated, ubiquitin-dependent degradation of proteins involves the targeting of specific proteins by the incorporation of a polyubiquitin tag. The polyubiquitin tag is then identified by a 26S proteasome, which is responsible for the degradation of the tagged protein (Leyser, 2002). The initial step in the ubiquitin-dependent degradation process requires the activation of the ubiquitin molecule to prepare it for conjugation to the target protein. An enzyme called ubiquitin-activating enzyme (E1) binds the ubiquitin molecule by forming a high-energy thiol ester linkage between a cysteine residue on the enzyme and the ubiquitin molecule (Leyser, 2002). The ubiquitin molecule is then transesterified to a second enzyme (E2) called the ubiquitin conjugating enzyme (Gray & Estelle, 2000). E2, together with a ubiquitin protein ligase (SCF-type E3), conjugates the ubiquitin molecule to an ε-NH_2 group of a

lysine residue on the target protein (Gray & Estelle, 2000). The selection of the appropriate target protein is primarily the responsibility of the SCF-type E3 enzyme, which typically consists of four subunits. Two of the subunits, belonging to the Cullin (Cul) and RBX1 (Rbx) protein families, form a dimer that is capable of extending the ubiquitin chain conjugated to the target protein to form the observed polyubiquitin chains found on proteins targeted for degradation (Seol et al., 1999). The Cul subunit is modified by the addition of a ubiquitin-like protein called RUB1 (Leyser, 2002). The conjugation of RUB1 to the Cul subunit proceeds in a manner very similar to the conjugation of the ubiquitin molecule to the target protein and requires an AXR1 protein, which forms part of the RUB-activating enzyme (Walker & Estelle, 1998). It has been established that the AXR1 protein is predominantly found in cells undergoing division and elongation (Walker & Estelle, 1998). While the precise function of the RUB1 protein is not entirely clear, experimental evidence has demonstrated that a cycle of RUB1 conjugation and deconjugation enhances the efficiency of the ubiquitin-dependent protein degradation activity (Schwechheimer et al., 2001). The Cul-Rbx dimer is joined by a member of the SKP1 protein family (Skp1) and the multimeric SCF-type E3 enzyme is completed with the association of an F-box protein (eg. TIR1) to the Skp1 subunit. It has been reported that a leucine-rich region of the carboxyl terminus of the F-box protein interacts with the target protein during the ubiquitination of the target protein (Leyser, 2002). Consequently, it is the C-terminus of the F-box protein that varies between the different SCF-type E3 ligase enzymes enabling the ubiquitination of different target proteins. The target proteins of the TIR1 F-box proteins have so far been found to be some of the Aux/IAA proteins, including the previously mentioned AXR2 and AXR3

gene products (Leyser, 2002). Other suspected protein targets include members of the AGR/EIR1/PIN/WAV6 auxin efflux transporter family (Leyser, 2001). Clearly, by targeting auxin efflux carriers for degradation, the transport of auxin throughout the plant as well as the accumulation of auxin within specific tissues of the plant will be affected. It has been suggested that auxin regulates the ubiquitin-dependent protein degradation process by enhancing the affinity of the TIR1 F-box protein for the target proteins through the modification of the target protein, possibly within Domain 2 (Leyser, 2002). This hypothesis has been supported by the observation that in other F-box protein-type interactions the target protein is typically phosphorylated (Skowyra et al., 1997). Also, the PINOID (PID) serine/threonine kinase activity has been identified as likely being involved in auxin-regulated activities in plants since mutations to this gene have led to distorted auxin responses (Christensen et al., 2000). As a result, it was proposed that auxin may mediate some type of protein kinase cascade (eg. a MAP kinase pathway), which results in the phosphorylation of the target protein and a subsequent increase in the affinity of the TIR1 F-box protein for the target protein (Leyser, 2002; Mockaitis & Howell, 2000). However, it has been noted that the Domain 2 sequence of the Aux/IAA family of proteins, which is required for the auxin-inducible destabilization of proteins, does not contain any highly conserved phosphorylation sites and Dharmasiri *et al.* (2003) have determined that phosphorylation of this domain of Aux/IAA is not required for degradation. It should be noted that it remains possible that the auxin-regulated modification of the target protein may still involve phosphorylation, but the phosphorylation may be of a previously unidentified "adapter" protein that interacts with both the Aux/IAA target protein and the TIR1 F-box protein (Leyser, 2002). Speculation

by some researchers that the modification of the target protein may involve hydroxylation of the highly conserved proline residues of Domain 2 by a proline hydroxylase (Leyser, 2002) was disproved by Dharmasiri *et al.* (2003) who performed experiments using known prolyl hydroxylase inhibitors and demonstrated that the inhibitors did not affect Aux/IAA recognition by the SCF^{TIR} complex. Furthermore, it was reported that specific Aux/IAA proteins, with hydroxylated prolines within Domain 2, do not bind well to the SCF^{TIR} complex (Dharmasiri et al., 2003). Regardless of the precise modification that takes place, it is clear that auxin is somehow involved in the regulation of the degradation of target Aux/IAA proteins as indicated by demonstrations that the presence of auxin correlates with an increase in the number of Aux/IAA–TIR1 F-box protein complexes (Leyser, 2002) leading to an increase in the rate of degradation.

A model has been proposed to explain how the auxin-mediated ubiquitin-dependent protein degradation process is involved with the transcriptional regulation of auxin-induced genes by the Aux/IAA and ARF proteins (Leyser, 2002). According to this model, the ARF transcriptional regulators are permanently associated with the ARE sequences within the promoters of the auxin-regulated genes. At relatively low concentrations of auxin, the Aux/IAA transcriptional regulators are not subjected to auxin-induced destabilization and degradation by the ubiquitin-dependent protein degradation pathway. Therefore, the Aux/IAA proteins are able to form dimers not only with each other, but more importantly with the ARF transcriptional regulators, which results in the inhibition of the ARF activity. When the concentration of auxin increases, the Aux/IAA proteins are targeted to the TIR1 F-box proteins with greater efficiency due to the increased affinity of the TIR1 F-box proteins for the Aux/IAA proteins. This leads

to a significant increase in the degradation of the Aux/IAA proteins by the 26S proteasome. With a decreased concentration of available Aux/IAA proteins, a greater number of ARFs are able to form homodimers and the formation of these ARF homodimers at the AREs of auxin-regulated genes allows the ARFs to either activate or suppress the expression of the associated gene. One of the initial criticisms of the proposed model resulted from the discovery of ARE sequences within the promoters of many of the Aux/IAA genes. Therefore, it seemed somewhat counterproductive to destabilize and degrade existing Aux/IAA proteins if the subsequent activity of the ARF transcriptional regulators led to an increase in the abundance of transcripts encoding Aux/IAA proteins. It should be noted, however, that the Aux/IAA proteins that are degraded are not necessarily the same Aux/IAA proteins whose transcripts are amplified by the ARF transcriptional regulators (Leyser, 2002). Furthermore, it has been suggested that the transcription rate of an auxin-mediated Aux/IAA gene that increases immediately following auxin treatment may be repressed by the increased expression of another auxin-mediated Aux/IAA protein whose transcription rate increases following a lag period after auxin treatment (Leyser, 2002). In other words, the increased expression of some of the Aux/IAA proteins might serve to down-regulate the increased transcription of some of the other auxin-mediated genes. Further research has established that different Aux/IAA proteins show remarkable variability in terms of auxin-mediated temporal patterns of expression, auxin dose response kinetics, half-lives, dimerization affinities, and tissue specificities (Gray et al., 2001). Exactly how the altered expression of specific genes produces the observed auxin-regulated phenotypes is unknown in many instances and will require continued research in order to completely elucidate what is occurring.

1.4 Auxinic herbicides

By 1935, researchers in the United States had successfully synthesized the auxin phytohormone in the laboratory (Sterling & Hall, 1997). This accomplishment, along with the discovery that auxin-related compounds could selectively kill plant species, propelled scientists in North America as well as Europe to focus on the development of synthetic analogues that could mimic the effects of auxin for the purpose of being used as a weapon of biological warfare during World War II (Sterling & Hall, 1997). During the summer of 1944, American researchers made public their discovery that the herbicidal activity of the chemical 2,4-dichloro phenoxyacetic acid (2,4-D) was selective for specific plant species and that it was able to translocate to the roots of susceptible plants. By the end of the following year approximately 200 field experiments were performed throughout North America to evaluate the efficacy of 2,4-D as a herbicide and about 415,000 kg of the compound was synthesized (Peterson, 1967). The proven viability of 2,4-D as a herbicidal agent prompted an increase in its synthesis and by the mid-1960s the rate of production had approached 24,000,000 kg (Peterson, 1967). The successful use of 2,4-D led to the development of other auxinic herbicides, many of which are commonly used in present day agricultural practices (Sterling & Hall, 1997).

While a number of chemical compounds have been designated as auxinic herbicides, a common feature among them is their similarity to naturally occurring auxins with respect to structure and effects on plants. Auxinic herbicides are typically sprayed onto crops to eliminate weedy plant species, however, they can also be used for preemergent control. Auxinic herbicides are effective against dicotyledonous broadleaf

weeds and are generally used in grass crops like cereal grains (Sterling & Hall, 1997). These herbicides can be easily absorbed by the leaves, stems, and roots and they are transported throughout the treated plants in the phloem as well as the xylem (Sterling & Hall, 1997; OMAF, 2003). Similar to auxins, auxinic herbicides can migrate acropetally and basipetally within the phloem and tend to concentrate in actively growing tissues (Ashton & Crafts, 1981). At low concentrations both auxins and auxinic herbicides stimulate plant growth and development by way of cell elongation rather than cell division and it has been observed that in plant cell culture media, some auxinic herbicides, such as 2,4-D, dicamba, chloramben, and picloram, can substitute for IAA in the normal growth of plant cells (Fedtke, 1982). These herbicides can be classified as plant growth regulators and their herbicidal activities may be a consequence of uncontrolled growth of sensitive species resulting from the application of excessive amounts of the auxin-mimics. The effects of auxinic herbicides differ between tissue types as well as between similar tissue types at different stages of development (Sterling & Hall, 1997). The phenotypic symptoms displayed by susceptible plants following the application of phytotoxic levels of auxinic herbicides include cessation of terminal leaf growth, development of cupping and stunting of leaves, tissue proliferation along the stem, twisting (epinasty) of the leaves and shoots, thickening and stunting of the roots, development of adventitious roots, disintegration of the phloem and cortex, and rupturing of the stem tissue epidermis (Ashton & Crafts, 1981; Sterling & Hall, 1997; Foy, 1975). In addition to these symptoms, auxinic herbicides, like auxins, stimulate the production of ethylene prior to, or at the same time as, the development of auxin-induced symptoms, with increasing rates of ethylene production corresponding to increasing levels of auxinic

herbicides (Hall et al., 1985; Sterling & Hall, 1997). A comparison of auxinic herbicide-susceptible and –resistant species demonstrated a direct correlation between the onset of auxin-induced injury and elevated rates of ethylene biosynthesis in the susceptible species (Hall et al., 1985). Hall *et al.* (1985) have shown that inhibiting ethylene evolution following auxinic herbicide exposure can delay the onset of the phytotoxic symptoms as well as reduce the severity of the herbicidal effects. In addition to the induction of ethylene biosynthesis, other auxinic herbicide-induced effects that are similar to known auxin-induced effects include elevated levels of nucleic acid and protein synthesis as well as acidification of the cell wall matrix by way of an ATPase-regulated proton extrusion through the cell membrane (Sterling & Hall, 1997). Although the level of DNA synthesis increases, the increased levels of nucleic acid is observed mostly in the form of a rapid up-regulation of specific RNA, which combined with an increased production of functional ribosomal complexes, results in elevated expression of specific auxin-mediated genes (Chen et al., 1972; Caux et al., 1993). The abnormal expression of many of these auxin-regulated genes is thought to contribute to the development of aberrant cell growth and activity that can ultimately lead to the death of the affected plants. Following auxinic herbicide application, it is believed that plasma membrane ion channels are activated to allow a rapid influx of calcium ions into the cytoplasm leading to a concomitant efflux of protons, which is mediated by a plasma membrane ATPase that is activated as a result of the influx of calcium ions (Sterling & Hall, 1997). In addition to the acidification of the cell wall, the influx of calcium ions into the cell is believed to also somehow increase gene expression (Sterling & Hall, 1997). These herbicides have been divided into specific classes or categories depending on structural

characteristics such as the identity of the aromatic ring and/or the positioning of the carboxylate moiety relative to the aromatic ring. One classification scheme divides the herbicides into three groups, the pyridines, the phenoxyalkanoic acids, and the benzoics, while another scheme divides the herbicides into two groups based on the linkage between the carboxylate group and the aromatic ring. The phenoxyalkanoic acid herbicides and some of the pyridoxyalkanoic acid herbicides have an ether linkage spanning the aromatic ring and the carboxylate group and are grouped together while the herbicides in which the carboxylic acid moiety is bound directly to the aromatic ring, like the benzoic acids and some of the pyridine acids, are placed in a second group (Sterling & Hall, 1997). A relatively new herbicide family with auxinic herbicide activity has been developed called the quinolinecarboxylic acids, but unlike other auxinic herbicides, some grasses appear to be susceptible to this new family of herbicides (Sterling & Hall, 1997).

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is a chlorobenzoic acid herbicide and in Canada it has been registered for use since 1963 (Caux et al., 1993). It is a widely applied herbicide in the prairie provinces and Ontario and is one of ten herbicides accounting for over 80% of all herbicides used in Canada (Agriculture Canada and Environment Canada, 1988). While dicamba may be found in a variety of forms, like other acidic herbicides, it is commonly sold and used as a salt (sodium, dimethyl ammonium, mono- and diethanolamine, and dimethylamine), which provides improved solubility in water (Caux et al., 1993). It selectively targets broadleaf and woody plant species, brush, and vines and is often used as part of a mixture with herbicides like 2,4-D, glyphosate, metribuzin, MCPA, and paraquat for a more thorough control of unwanted weeds (Caux et al., 1993). It is typically applied for the post-emergence control of

young, small, and actively growing weeds in agricultural crops, such as rye, fescue, oats, barley, wheat, sorghum, corn, lowbush blueberries and canary grass (Alberta Agriculture, 1990; OMAF, 2003) as well as on non-crop areas, such as railways, utility rights-of-way, pastures, turf, forest and rangelands, and roadsides (OMAF, 2003). Dicamba-susceptible species include, among others, cleavers, cow cockle, field bindweed, Canada thistle, perennial sow-thistle, poverty weed, tartary and wild buckwheat, goldenrod, smartweeds, pasture sage, tansy ragwort, thyme-leaved spurge, corn spurry, diffuse knapweed, velvetleaf, and sheep sorrel (Caux et al., 1993; OMAF, 2003). This herbicide is readily absorbed by the roots, stems, and leaves of susceptible plants and it is quickly distributed to all plant tissues (Thompson et al., 1973). Unrelated plant species differ in the precise rate of uptake, translocation, and metabolism of dicamba and it is believed that these differences contribute significantly to the differential tolerance to this herbicide exhibited by some species. Furthermore, the ability to rapidly convert the herbicide to less toxic dicamba-conjugates or methoxy-dicamba metabolites is also credited with offering some protection to particular plant species from the herbicidal effects of dicamba (Caux et al., 1993).

The relatively high solubility of dicamba in water and the fact that it does not adsorb to soil particles to any appreciable degree are characteristics that allow it to be relatively mobile in soil (Frear, 1976). Its persistence in the soil environment is largely dependent on the type of soil as well as on environmental conditions like temperature, moisture content, pH, and microbial biota (Caux et al., 1993). Therefore, although it is considered to be a herbicide with a low level of persistence with a mean half-life in most agricultural soils of 25 days, the half-life can range from as short as 4 days to as long as

555 days (Altom & Stritzke, 1973). While hydrolysis, photodegradation, and volatilization do not significantly contribute to the degradation of dicamba in soils, microbial degradation by soil bacteria that are able to utilize dicamba as their sole source of carbon is believed to be the biggest factor contributing to the breakdown of this herbicide in soils (Caux et al., 1993). Soils with relatively high temperatures, moisture content, and percent organic matter likely provide ideal growth conditions for many of the bacteria responsible for the degradation of dicamba and it is expected that these types of environments are most conducive to dicamba dissipation. In Canada, under normal environmental conditions, it is expected that when it is applied at recommended dosages, any residual dicamba present in the soil is completely degraded within 6 months (Caux et al., 1993).

1.5 Auxinic herbicide resistance

The auxinic herbicides are effective against dicotyledonous broadleaf plants whereas monocots are better able to tolerate these herbicides (Hall et al., 1996). The morphological and metabolic differences between monocots and dicots are thought to be responsible for the observed susceptibility differences. For example, the phloem found in monocots is protected by a layer of sclerenchyma tissue and is arranged in scattered bundles, while the phloem of dicots is not covered in protective tissue and is therefore more vulnerable to degradation (Hall et al., 1996). In addition, the vascular bundles of monocots do not contain cambia or pericycles, which are targeted for destruction upon auxinic herbicide application in dicots (Hall et al., 1996). Furthermore, the distribution of auxinic herbicides within monocot species following uptake is inhibited due to the

intercalary meristem found in the shoots, which is not present in the shoots of susceptible dicot plants (Devine et al., 1993). Metabolism of the auxinic herbicides to non-toxic or unstable compounds is another method used by resistant species to tolerate otherwise phytotoxic levels of herbicide. The effects of these types of herbicides can be neutralized through chemical modification by oxidation, conjugation, and hydrolysis reactions (Hatzios & Penner, 1982). Tolerant monocots such as barley and wheat are known to irreversibly metabolize auxinic herbicides through hydrolysis reactions (Sterling & Hall, 1997). Amino acids, such as glutamic acid and aspartic acid, as well as carbohydrates like glucose and myo-inositol can be linked to the carboxylate moiety of the auxinic herbicide by a peptide or ester linkage, however, in some instances active auxinic herbicide can be regenerated from the conjugate compound by hydrolysis of the linkage (Sterling & Hall, 1997). Also, it has been reported that conjugation of the herbicide to amino acids does not necessarily eliminate the herbicidal activity of the compound (Davidonis et al., 1982). Auxinic herbicide resistance observed among different dicotyledonous species has also been the result of metabolism of the side chains of the herbicide (Luckwill & Lloyd-Jones, 1960). In addition to degradation of the side chain to create a non-toxic compound, incorporating a carbon chain to the carboxylate moiety of the auxinic herbicide has proven to be effective in the detoxification of 2,4-D (Sterling & Hall, 1997).

Although the use of auxinic herbicides has been extensive over the many years of its commercial use, there have not been a plethora of examples of susceptible species developing resistance (Sterling & Hall, 1997; Jasieniuk et al., 1995). The relatively low levels of persistence in soils as well as the multiple modes of action of auxinic herbicides

are believed to be responsible for the infrequent occurrence of auxinic herbicide resistance. However, continual application of the same auxinic herbicides over a number of years have led to the development of some auxinic herbicide resistant plants in various parts of the world. In general, the susceptible biotypes are typically more fit than the resistant biotypes prior to auxinic herbicide application. Included among the naturally susceptible weed species that have auxinic herbicide resistant biotypes are *Sphenoclea zeylanica* Gaertn (Philippines), *Centaurea solstitialis* L. (United States), *Stellaria media* L. (United Kingdom), *Cirsium arvense* (L.) Scop. (Hungary), *Carduus nutans* L. and *Ranunculus acris* L. subsp. *acris* (New Zealand), and *Sinapis arvensis* L. (Canada) (Migo et al., 1986; Callihan et al., 1989; Lutman & Lovegrove, 1985; LeBaron, 1991; Bourdot et al., 1989; Heap & Morrison, 1992). 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, 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 (Peniuk et al., 1993; Deshpande & Hall, 1995). In many weeds, resistance to herbicides is mediated and inherited through a single, dominant nuclear allele, and this has also been demonstrated in the case of dicamba-resistant wild mustard (Jasieniuk et al, 1995). However, the identity of the particular allele conferring resistance to auxinic herbicides in wild mustard has unfortunately eluded scientists. 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 (Webb & Hall, 1995), which is consistent with the findings involving

auxinic herbicide resistance in *Stellaria media* L. (Coupland et al., 1991). There were, however, differences in the putative auxin receptor (ABP1) with respect to its affinity for auxin (Webb & Hall, 1995). It was discovered that the ABP1 receptors from the susceptible biotype had low or high affinity auxin-binding sites whereas the ABP1 from the resistant wild mustard had uniform binding sites whose levels of affinity for auxin were comparable to the low affinity auxin-binding sites of the S biotype ABP1 (Webb & Hall, 1995). The ABPs from the S and R wild mustard also differed in their sensitivities to inhibition of IAA binding by different auxinic herbicides. IAA binding to the ABP1 from S wild mustard was inhibited by picloram and dicamba more so than was the ABP1 from R wild mustard (Webb & Hall, 1995). Experiments exploring other differences between S and R wild mustard involving light scattering relaxation spectroscopy indicated the likelihood that picloram may differentially modulate calcium ion channels as well as differentially affect ATPase-dependent activities in the two wild mustard biotypes (Deshpande & Hall, 1995). When compared to S plants there were also numerous differences in morphological and physiological characteristics of the R wild mustard including shortness of stature, substantially more branching, and smaller root systems (Hall & Romano, 1995). However, there were no significant differences between the S and R wild mustard in terms of fresh or dry weights (Hall & Romano, 1995). In addition, the leaves were more numerous and were considerably smaller and darker green in colour in the R wild mustard biotype, as a result of a higher amount of chlorophyll (Hall & Romano, 1995). In addition, senescence occurred more slowly in leaf disks of R wild mustard, which also germinated better (at 30°C) and was more tolerant to low temperatures (-7 °C) at the 4-leaf stage of development compared to the S

biotype (Hall & Romano, 1995). Furthermore, levels of calcium and the phytohormone, cytokinin, were elevated in the cells of R wild mustard plants compared to the S biotype (Hall & Romano, 1995). Since it is known that cytokinin in the presence of calcium can delay the onset of senescence (Hall & Romano, 1995), it was postulated that the significantly higher levels of calcium and/or cytokinin may be involved in regulating auxinic herbicide resistance (Hall & Romano, 1995). In addition, there was a significant difference in the level of ethylene biosynthesis between the S and R wild mustard treated with auxinic herbicides (Hall et al., 1993). In fact, while the level of ethylene was elevated considerably in the S wild mustard, this phytohormone level in the R biotype did not increase significantly from the basal level of production upon herbicide treatment. Furthermore, fumigation of R wild mustard with ethylene produced phenotypic changes that were similar to those seen in S wild mustard following auxinic herbicide application (Hall et al., 1993). Continued research has demonstrated that the differences in ethylene evolution following auxinic herbicide exposure in the R wild mustard biotype is a consequence of a lack of an induction of the ACC synthase enzyme (Hall et al., 1993). While the importance of elevated levels of ethylene in many of the auxin-mediated effects has been demonstrated, the precise role of ethylene is not entirely clear. It is possible that ethylene functions as a secondary messenger that rapidly stimulates specific cellular pathways throughout the plant that contribute to the observed abnormal growth and activities, however, this hypothesis has yet to be conclusively proven.

1.6 Proteomics – 2D electrophoresis and mass spectrometry

Proteomics, which is a term used to describe the study of the complete protein composition within a cell type or organism in terms of function, cellular localization, regulation, and protein-protein interaction (Pandey & Mann, 2000), is a logical progression from the research that has been conducted on the genomes of various organisms. As the name might suggest, genomics involves the study of genes or DNA and while this field of study can provide significant insight into areas such as cellular function and disease research, there are limitations to what can be revealed about biological activity from the study of genes alone. Specifically, it is the proteins expressed from the genes that are the actual components that mediate or regulate most cellular activities and the presence or absence of a particular gene or its RNA transcript does not necessarily correlate with the levels of a particular protein within a cell. Also, examining the complete sequences of a genome does not provide any details about the specific types of post-translational modifications, such as glycosylation and phosphorylation, that may take place on expressed proteins (Pandey & Mann, 2000). Clearly, any post-translational modifications of an expressed protein can affect the efficient folding and final three dimensional structure of the protein, which in turn can have significant effects on its biological activity. Because genomics does not provide information about the post-translational modifications that take place, there cannot be any conclusive determinations made about the regulation or activity of a specific protein from the study of genomics alone. It is these limitations that necessitate the investigation of the properly folded and functioning proteins themselves in order to fully appreciate the activities that take place in the cells of all living creatures. Two-dimensional electrophoresis (2DE) and mass

spectrometry (MS) are included among the array of tools available to study the proteomes of organisms that range from single cell bacteria to multicellular plants, animals, and humans.

2DE allows the separation of proteins based on two unrelated parameters: the isoelectric point (pI) and relative molecular weight (M_r). 2DE was first described in 1975, and while a number of enhancements have been made in the decades since its development, its utility as a valuable and viable proteomics tool was clearly evident from its inception (O'Farrell, 1975). This experimental technique can separate or resolve more than 1000 proteins in a single experiment and depending on the specific staining protocols used, it can detect and quantify a protein corresponding to as little as 10^{-4} to 10^{-5} of 1% of the total protein sample used (O'Farrell, 1975). The initial step of 2DE involves the isoelectric focusing (IEF) of the proteins followed by a polyacrylamide gel electrophoresis step that separates the focused proteins according to relative size (O'Farrell, 1975). IEF separation or focusing of proteins utilizes specific characteristics common to all proteins regardless of species. The amphoteric nature of proteins means that they can have an overall charge that is positive, negative, or neutral, depending on the environmental pH (Berkelman & Stenstedt, 1998). The pH at which the net charge of the protein is zero (i.e. neutral) is referred to as the pI of the protein and proteins present in environments where the pH is greater than their pI will be negatively charged whereas proteins present in environments where the pH is less than their pI will be positively charged (Berkelman & Stenstedt, 1998). The carboxyl and amino moieties of the proteins as well as the side chains of the various amino acids that constitute a given protein can all carry specific charges and it is the sum of all of these positive and negative

charges that ultimately determines the overall net charge of a protein. The application of an electrical field to a solution of proteins present in a pH gradient results in the migration of the proteins through the gradient until they reach their pI values (Berkelman & Stenstedt, 1998). As positively charged proteins move towards the cathode, they continually become less positively charged until they become neutral at their pI and negatively charged proteins gradually become less negatively charged as they migrate in the opposite direction towards the anode until they attain a net zero charge at their pI. This migratory phenomenon forms the basis of the IEF technique.

Clearly, successful and efficient IEF requires a stable pH gradient and initial 2DE experiments utilized carrier ampholytes and polyacrylamide gel tubes to generate the necessary gradients (O'Farrell, 1975). Carrier ampholyte solutions consist of a large number (600 to 700) of amphoteric, small (M_r < 1 kDa), hydrophilic, and soluble compounds that do not interact with proteins and are able to function as buffers at pH values close to their pIs, which enables their use in the development of pH gradients (Berkelman & Stenstedt, 1998). The pI values of the buffering compounds in the carrier ampholyte solutions can cover a wide range (eg. pH 3 to 10), which allows for the generation of pH gradients that span an equally broad range, depending on the particular carrier ampholytes that are used. When a carrier ampholyte solution is exposed to an electrical field, the ampholyte molecules with lower pI and higher positive charge migrate towards the cathode while the ampholytes possessing higher pI and higher negative charge move towards the anode (Berkelman & Stenstedt, 1998). By utilizing ampholytes whose pI values span a desired pH range, a continuous pH gradient can be generated that is capable of resolving proteins from an applied sample whose pI values

fall within the specified pH range. Although carrier ampholyte solutions have been used successfully in IEF experiments, there are particular limitations with these reagents that necessitated the development of a more efficient and effective method of creating pH gradients. A particularly troublesome problem with carrier ampholytes is that because they are used within an aqueous solution they have a propensity to migrate over the course of time, which would adversely affect any IEF experiment since the pH gradient would not be stable during protein focusing. The use of immobilized pH gradients (IPG) was introduced to overcome the pH drift problem, thereby producing consistent and reproducible results (Bjellqvist et al., 1982; Gorg et al., 1988). Unlike IEF using carrier ampholyte solutions, the pH gradients in IPG IEF are incorporated directly into a polyacrylamide gel so that the various buffering moieties, which are either weakly acidic (a free carboxylic acid) or weakly basic (a tertiary amino group) molecules connected to a monomer of acrylamide, are immobile and therefore unable to drift (Berkelman & Stenstedt, 1998). The monomers of acrylamide with the covalently linked buffering molecules are polymerized with other monomers of acrylamide and bis-acrylamide crosslinkers with the aid of chemical catalysts to form a polyacrylamide gel with an embedded pH gradient (Berkelman & Stenstedt, 1998). The precise pH range of the gradient is determined by the concentrations of the different buffering compounds that are used. Once the polyacrylamide gels have been made, they can be applied to a plastic backing, dried, and then cut into strips of required length. An added benefit of the plastic backing is that it helps to minimize the possibility of stretching or breakage of the polyacrylamide gel during storage as well as during IEF. The strips can then be stored frozen until required, at which point the gels are rehydrated with appropriate rehydration

solutions that may contain the protein sample of interest although it is not absolutely necessary to apply the protein sample at the same time as strip rehydration. The solutions used for rehydration as well as for protein sample loading usually contain common elements to improve protein solubility and reduce protein aggregation, which are important factors to consider for effective IEF. In order to expose all of the ionizable groups within a protein and to ensure that each protein is found in only one configuration, the proteins are denatured with a relatively high concentration of urea (8 - 9.8 M), a neutral chaotropic agent (Berkelman & Stenstedt, 1998). Some sample solutions also contain thiourea, which serves the same purpose as urea and it also aids in the solubilization of highly hydrophobic proteins such as membrane proteins (Molloy et al., 1998; Rabilloud, 1998). In addition to a denaturant, protein sample solutions also typically contain reducing agents and detergents. Reducing agents help to break disulfide bonds that can form intramolecularly during protein folding as well as intermolecularly between unfolded proteins at exposed cysteine residues (Berkelman & Stenstedt, 1998). Maintaining proteins in a reduced state can prevent protein interaction or aggregation by disulfide bond formation so that each protein can migrate independently of all other proteins. Although tributylphosphine (TBP) has been shown to be a very effective reducing agent, because of its relatively unstable nature in solution, another reductant, dithiothreitol (DTT), is often used along with TBP to ensure that all proteins are in their reduced conformations during IEF (Berkelman & Stenstedt, 1998; Herbert et al., 1998). The detergents in the sample solutions help to maintain proteins in their soluble forms as well as aid in inhibiting hydrophobic interactions between proteins (Berkelman & Stenstedt, 1998). While sodium dodecyl sulfate (SDS) is one of the most effective

detergents at maintaining protein solubility, it is negatively charged and can interact with proteins, which would interfere with IEF since the overall charge of the proteins would be altered by its presence. Consequently, the concentration of SDS that is present in SDS-containing sample solutions is kept at a minimum and other, non-ionic or zwitterionic, detergents are used instead. The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and the non-ionic detergents like NP-40 and Triton X-100 can be utilized (O'Farrell, 1975; Perdew et al., 1983). Another component commonly added to protein sample solutions for IPG IEF is a solution of carrier ampholytes even though in IPG IEF the pH gradient is immobilized in the polyacrylamide gel. The addition of carrier ampholytes, while not required to generate the necessary pH gradient, has been found to improve IEF by creating a more uniform conductivity along the gradient as well as maintaining protein solubility by inhibiting charge-charge interactions between proteins, thereby enabling improved protein separations (Berkelman & Stenstedt, 1998).

Once the proteins have been separated or focused in the first dimension, they undergo a second dimension separation step called SDS-polyacrylamide gel electrophoresis (PAGE). Prior to SDS-PAGE, the focused proteins are equilibrated in appropriate buffers, which is required to ensure effective electrophoretic separation. The charged nature of SDS is utilized to give all of the denatured proteins an overall negative charge, thereby creating anionic molecules that have a constant charge per unit mass (Berkelman & Stenstedt, 1998). Because all of the proteins have a negative charge, the relative sizes of the proteins are determined by assessing the strength of the charge of each protein. Larger proteins will have a greater negative charge due to a larger number

of bound SDS molecules and this difference in degree of negative charge is used to separate the proteins in a polyacrylamide gel. These types of gels consist of polymerized acrylamide chains that are connected by a cross linker, such as N, N'-methylenebisacrylamide or piperazine diacrylamide (Sambrook et al., 1989). The ratio of acrylamide and cross linker used determines the relative sizes of the pores, which the proteins migrate through at different rates depending on their sizes (Sambrook et al., 1989). Polyacrylamide gels can therefore be viewed as sieving gels in which the proteins are separated based on differences in the rates of migration through the pores of the gel. Similar to the first dimension separation, urea and DTT are also used in the second dimension acrylamide gel separation to ensure complete denaturation and reduction of all disulfide bonds, respectively (Berkelman & Stenstedt, 1998). A subsequent saturation of the IPG strips in an equilibration buffer containing iodoacetamide results in the alkylation of the thiol groups of the reduced proteins as well as the excess DTT, which prevents reoxidation and protects the second dimension separation from the inhibitory effects of excess reducing agent (Berkelman & Stenstedt, 1998; Gorg et al., 1987). Furthermore, glycerol is present to increase the viscosity of the buffers, which, along with urea, is believed to reduce the effects of electroendosmosis (Gorg et al., 1985). Electroendosmosis is a term that describes a process that results in inefficient transfer of proteins from the IPG strip to the second dimension SDS-PAGE gel. Upon application of an electrical field during electrophoresis, the immobilized buffering groups of the IPG strips become negatively charged and in addition to the movement of hydration water, another consequence of this ionization is the migration of the strips towards the anode, which leads to the embedding of the strip into the second dimension gel (Gorg et al.,

1988). This "digging in" of the IPG strip into the PAGE gel is not conducive to efficient protein transfer and it has been determined that equilibration of the strips in a buffer containing approximately 30% glycerol as well as urea reduces the inhibitory electroendosmotic effects (Gorg et al., 1985). The use of an agarose overlay to cover the IPG strips immediately prior to electrophoresis can also reduce the tendency of the strips to migrate during electrophoresis. Once the protein-containing IPG strips have been adequately saturated with the equilibration buffers, the proteins separated by IEF are ready to be separated in the second dimension gel using tris-glycine buffers (Sambrook et al., 1989).

Upon completion of gel separation of proteins by 2DE, specific proteins can be selected for identification. While techniques such as Edman degradation have been used to ascertain the amino acid sequences of gel-separated proteins, automated analytical tools have been developed that can perform these identifications on a larger scale with perhaps greater sensitivity and speed than the traditional methods. One of the prominently used methodologies to identify proteins utilizes the mass spectrometer, which is capable of identifying proteins at subpicomole concentration levels (Shevchenko et al., 2000). While the name might suggest that the raw data generated by mass spectrometers are the masses of the analyte peptides, a more accurate description of the activity of mass spectrometers is that they measure the mass-to-charge ratio (m/z) of the peptides (Glish & Vachet, 2003). The actual molecular weights of the analyte proteins must be calculated from this raw data. In addition to analyte mass and relative amounts in a sample, mass spectrometric data can also provide information regarding protein structure, depending on how the mass spectrometer is constructed and used (Glish &

Vachet, 2003). Although there are key differences between the different types of mass spectrometers, all of them can be thought of as being comprised of three basic components, an ion source and a mass analyzer to ionize the analytes and determine the mass-to-charge ratios, respectively, in addition to a detector that measures and records the number of ions at each m/z value (Aebersold & Mann, 2003). The data generated by mass spectrometers are typically summarized and provided in a mass spectrum that plots the abundance of individual ions (on the y-axis) versus the mass-to-charge ratio (on the x-axis) (Glish & Vachet, 2003). The identification of gel-separated proteins requires an initial in-gel digestion of the analyte proteins, typically with sequence-specific proteases (Pandey & Mann, 2000). The most commonly used protease is the serine protease, trypsin, which cleaves proteins at the carboxy terminus of arginines and lysines, provided that the subsequent amino acid is not a proline (Covey, 1991). In addition to internal histidine residues and the amino terminus of the peptide, the basic arginine and lysine amino acids are probable sites of protonation during any subsequent electrospray ionization reaction (Covey, 1991). Other methods of in-gel digestion include the use of hydrochloric acid solutions or other enzymatic methods, such as digestion with the LysC serine protease (Shevchenko et al., 2000). The predictability of the cleavage sites of trypsin within a protein allows for a reasonably accurate estimation of the theoretical number of peptides a protein will produce following trypsin digestion as well as the sizes of these peptides. Therefore, these predicted peptide mass patterns can be compared with experimentally derived mass spectra and an identification can be made if a sufficient number of peptide masses (typically at least 4-6 peptides) match (Shevchenko et al., 2000; Pandey & Mann, 2000). This process of comparing and matching an

experimentally determined peptide mass spectrum with previously established peptide mass spectra of known proteins to identify a purified analyte protein is known as "peptide mass mapping" or "peptide mass fingerprinting" (Aebersold & Mann, 2003).

MS analyses are performed on gaseous ionized analytes within a vacuum and therefore the efficient ionization and volatilization of the analyte protein or peptide molecules is imperative to ensure an effective MS experiment. The most common ionization and volatilization techniques are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) (Fenn et al., 1989; Karas & Hillenkamp, 1988). In MALDI the analyte peptides are initially incorporated into a dry, solid matrix that is selected, in part, based on its ability to absorb the wavelength of a high energy laser (Glish & Vachet, 2003). These matrices can be made of an organic solution, for example, a mixture of 2,5-dihydroxybenzoic acid, acetonitrile, and water with the peptide samples that are added initially prepared in a solution of formic acid (Shevchenko et al., 2000). The laser that is used for ionization can be a nitrogen or UV laser that "fires" in a pulsed-laser mode, thereby generating ionized peptides, which are typically singly protonated, in discrete bundles rather than in a continuous stream as in ESI (Glish & Vachet, 2003). ESI also differs from MALDI in that the analyte peptides are maintained in a liquid phase prior to ionization and volatilization. ESI involves the spraying of a solution containing the analyte peptides through a capillary that has a very narrow internal diameter of less than 250 μm as well as an electrical potential difference at the tip (500V to 4500V) (Glish & Vachet, 2003). The applied voltage combined with the very narrow internal diameter of the capillary results in the formation of very small, charged liquid aerosol molecules containing charged analyte peptides (Glish & Vachet,

2003). The solvent then evaporates and the desolvated charged peptides are directed towards the mass analyzer portion of the mass spectrometer (Glish & Vachet, 2003). In some ESI ion sources, the capillary may be gently heated to accelerate the evaporation of the solvent from the ionized protein or peptide samples (Bottrill, 2000). In ESI, analyte peptides can be charged at more than one location and these peptides with different charge states are observed in the mass spectra as different peaks (Glish & Vachet, 2003). Typically, ions of trypsin-digested peptides occur as doubly charged molecules, although depending on the presence of any internal histidine residues, the degree of ionization might be higher (Covey, 1991). If the multiple peaks seen in a mass spectrum are of the same peptide, which differ only in the degree of protonation, they can all be used to more precisely calculate the mass of the analyte peptide or protein since each peak can serve as a separate mass-to-charge measurement (Glish & Vachet, 2003). The particular differences between MALDI and ESI provide specific advantages to each method of ionization. For example, the fact that the peptide samples are present in an aqueous solution for ESI has allowed the use of peptide separation methodologies such as reversed phase high performance liquid chromatography (RP-HPLC) prior to the ionization process. Because trypsin-digestion followed by an ionization technique can potentially generate peptides with different charged states, there exists the possibility that different peptides might possess the same or very similar mass-to-charge ratios (Covey, 1991). As a result, these different peptides may appear as part of a single peak in a mass spectrum and any attempts at determining the sequence of the peptides by mass spectrometry would be difficult because it would not be clear from the mass spectra data that the analyte consists of a mixed peptide solution. Therefore, any amino acid

sequences that are determined would be incorrectly based on the assumption that what is being sequenced is a single peptide species. A peptide separation step, such as RP-HPLC, prior to ESI-MS can ensure that only one peptide species is analyzed by the mass spectrometer at a time. Reversed phase protein or peptide separations are based on the differences in hydrophobicities between the solute samples applied in the mobile phase to the hydrophobic chromatographic column, which is referred to as the stationary phase (Corran, 1989). The hydrophobic ligands of reversed phase matrices typically consist of linear hydrocarbon chains with longer chains such as C18 commonly being used to separate small peptides, such as those that may be present in tryptic digested samples (Vydac, 1995). The binding of the proteins or peptides to the stationary phase and the subsequent gradual shift in the mobile phase conditions results in the elution of peptides from the column with the least hydrophobic molecules desorbing earlier than the more hydrophobic peptides. Initially, the mobile phase is a relatively polar solution to allow the binding of the peptide molecules to the stationary phase. In order to desorb the peptides from the column, the polarity of the mobile phase is reduced by the addition of organic modifiers such as acetonitrile as part of an increasing gradient (Corran, 1989). In addition to acetonitrile, which is the most commonly utilized organic modifier, other solvents such as methanol, ethanol, and isopropanol can also be used (Sussman, 1988; Ford & Smith, 1989). The reduced polarity results in the increased hydrophobicity of the mobile phase leading to desorption of the bound peptides from the hydrophobic resin.

Once the analyte proteins or peptides have been effectively ionized and volatilized, they are accelerated into a mass analyzer. There are four basic types of mass analyzers that are commonly used (ion trap, time-of-flight, quadrupole, and Fourier

transform ion cyclotron) that can be viewed as being either "trapping" analyzers or "beam" analyzers (Glish & Vachet, 2003; Aebersold & Mann, 2003). In trapping analyzers, such as the ion trap, the ionized analytes are held or "trapped" within the analysis chamber whereas in beam analyzers, like the time-of-flight (ToF) mass analyzers, the ionized samples flow through the analysis field from the ionization source towards the mass spectrometer's detector (Glish & Vachet, 2003). ToF mass analyzers differentiate between peptide ions by measuring the time required to travel a specified distance in the ToF chamber (Glish & Vachet, 2003). The analyte peptide samples are ionized simultaneously within the ion source and are then accelerated through a ToF chamber that is typically 0.5-2.0m in length until they strike the detector (Glish & Vachet, 2003). Ions with like charges travel through the ToF tube with similar kinetic energies and those ions with lower mass-to-charge ratios travel with greater velocity and therefore arrive at the detector of the mass spectrometer earlier than the ions with higher mass-to-charge ratios (Glish & Vachet, 2003). The m/z of the different ionized peptide samples are calculated from the time required for the ions to pass through the ToF tube to the detector.

The aforementioned different types of mass analyzers can be used individually, as in the MALDI-ToF, or together in a single mass spectrometer. One example of a mass spectrometer with mass analyzers used in tandem is the quadrupole time-of-flight mass analyzer (Q-ToF). A Q-ToF mass spectrometer is essentially a modified hybrid between a triple quadrupole mass analyzer and a ToF mass analyzer where the third quadrupole of the triple quadrupole has been replaced by a ToF chamber (Shevchenko et at., 1997). In addition, the Q-ToF mass spectrometer possesses a collision cell sandwiched between the

quadrupole mass filter and the ToF chamber (Loboda et al., 2000). Once the analyte peptides are ionized, they are passed through an initial radio frequency (r.f) ion guide quadrupole (Q0) that focuses the ions into a separating quadrupole mass filter (Q1) where peptides are resolved and ions with a specific m/z ("parent" or "precursor" ions) are selected and directed into the collision cell (q2) where they are fragmented (to produce "daughter" or "product" ions) prior to entering the ToF tube (Aebersold & Mann, 2003; Loboda et al., 2000; Chernushevich et al., 2001). The fragmentation of the precursor ions is typically performed using one of two methodologies. The more commonly used dissociation method is known as collision-induced dissociation (CID) and it involves the collision between the precursor ions and a gas, typically nitrogen or argon, that results in the conversion of some of the kinetic energy of the precursor ions to internal energy (McLuckey, 1992). The second possible dissociation method is called surface-induced dissociation (SID) and is similar to CID in that some of the kinetic energy of the precursor ions is converted to internal energy, but unlike CID, the conversion takes place as a result of the precursor ions colliding with a solid surface as opposed to a gas (Dongre et al., 1996). In both CID and SID a consequence of the conversion of a proportion of kinetic energy to internal energy is the dissociation or fragmentation of the precursor ions to form the product ions that are directed into the ToF chamber. The initial separation of the precursor peptide ions and isolation of ions of a particular m/z in the Q1 quadrupole can be thought of as comprising one stage of MS with the subsequent ToF analysis of the fragmented product ions consisting of a second MS stage that allows for the determination of amino acid sequence. As a result, the Q-ToF mass spectrometer can be described as enabling tandem mass spectrometry (MS/MS). Unlike peptide mass

fingerprinting, tandem mass spectrometry provides information about peptide amino acid sequence in addition to peptide mass, thereby allowing the identification of the analyte proteins or peptides based on more than one parameter, which can lead to more accurate or sensitive identifications.

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 whose expression patterns were affected by dicamba, could provide valuable information leading to the characterization or clarification of the mechanism(s) involved in the auxin signal transduction pathway(s) as well as in the development of auxinic herbicide resistance. We utilized 2DE and MS/MS to identify the differentially expressed and dicamba-induced proteins. Among others, 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. In addition, we provide preliminary evidence that the activity of the protein may, in fact, be vital for the maintenance of an auxinic herbicide-resistant phenotype.

2.0 Materials and Methods

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

seeds used in the experiments were kindly supplied by Dr. J. Christopher Hall (Department of Environmental Biology, University of Guelph, ON, Canada).

2.1 Plant material preparation

2.1.1 Seedlings germinated in Petri dishes

R and S wild mustard seeds were surface sterilized for 1 minute in 70% (v/v) ethanol followed by two 5 minute immersions in 0.3% (v/v) 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 the treatment (10 μM dicamba – Micro Flo Company, Memphis, TN, USA) solution and germinated to obtain tissue material for protein extraction. Our decision to use dicamba was based on dose-response experiments that demonstrated that R-wild mustard is 104 times more resistant to dicamba than S-wild mustard, while it is only 18 and 10 times more resistant to MCPA and 2,4-D, respectively, than S-wild mustard (Heap & Morrison, 1992). 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 and flash frozen in liquid nitrogen and stored at -80°C until the protein was extracted. Experiments were repeated at least once.

2.1.2 Greenhouse-grown pre-flowering plants

For greenhouse experiments, R and S wild mustard seeds were sown in moistened Metro Mix® 290 (Grace Horticultural products, Ajax, ON, Canada) in 6 x 6 cm plastic

containers. 1 seed per well was planted approximately 1 cm below the surface in each of the wells and after covering each seed with Metro Mix® 290, the surface was pressed gently to ensure the seeds were adequately buried. The plastic trays were placed in the greenhouse (20 hour photoperiod, 21°C day/18°C night cycle) and 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) or dicamba (50 g ai ha^{-1}). 24 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.

2.2 Protein extraction

2.2.1 Reagent 3 resuspension

Dry seeds (5 pooled seeds) or seeds at germination (5 pooled seeds from the Petri dish experiments) were ground in liquid nitrogen to a fine powder and resuspended in 300 μL Reagent 3 [5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 2 mM tributylphosphine (TBP), 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10 (ampholytes)], from the Ready Prep Sequential Extraction kit (Bio-Rad, Mississauga, ON, Canada.) After vigorous vortexing for 5 minutes, the samples were incubated at 4°C overnight. The solutions were centrifuged (14000 rpm, 10 minutes) after which, the protein concentrations of the collected supernatants were determined as described in a following section.

2.2.2 TCA-acetone precipitation of proteins

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), 0.07% (w/v) dithiothreitol (DTT) in ice cold acetone in a 1.5 mL plastic tube. Following incubation at -20°C for 1 hour, the samples were centrifuged (14000 rpm, 10 minutes) and the supernatant discarded. Each pellet was resuspended in 1 mL 0.07% (w/v) DTT in ice cold acetone and incubated at -20°C for 1 hour. The samples were centrifuged as previously described and the pellets resuspended in 1 mL 0.07% (w/v) DTT in ice cold acetone, incubated at -20°C for 1 hour, and centrifuged as previously described. The pellets were vacuum-dried for 30 minutes (until the pellets were completely dry). Germinated seedling samples were resuspended in 200 µL of Reagent 3 or Rehydration/Sample buffer (Bio-Rad) (8 M urea, 2% (w/v) CHAPS, 40 mM DTT, 0.2% (w/v) Bio-Lyte 3/10, 2 mM TBP). The leaf tissue samples were resuspended in 500 µL of Rehydration/Sample buffer (Bio-Rad). After vigorous vortexing of the samples for 5 minutes, the tubes were incubated at 4°C overnight. The samples were centrifuged as previously described and the protein concentrations of the collected supernatants were determined as described in the following section.

2.3 Protein concentration determination

The concentrations of the protein extract solutions were calculated using a modified Bradford assay using bovine serum albumin (BSA – Pierce Biotechnology Inc.,

Rockford, IL, USA) as the standard. The BSA protein standards (0 μg, 2 μg, 5 μg, 10 μg, and 15 μg) were prepared in disposable polypropylene cuvets containing 20 μL (final volume) Reagent 3 (Bio-Rad) or Rehydration/Sample buffer (Bio-Rad), depending on which solution the protein extracts were finally prepared in. The protein extracts were diluted 1:10 in cuvets containing 20 μL (final volume) of the same solution (Reagent 3 or Rehydration/Sample buffer.) 80 μL of 0.12 N hydrochloric acid (HCl) was added to each of the prepared protein standards and samples followed by the addition of 1 mL diluted (1:4 in water) Protein Assay dye reagent (Bio-Rad), which contains Coomassie blue. After thoroughly mixing the solutions and incubating them undisturbed for 5 minutes at room temperature, the absorbance values of the protein solutions at 595 nm were determined using the Cary 300 UV/vis spectrophotometer (Varian Inc., Palo Alto, CA, USA.) Coomassie blue organic dyes bind to the side chains of tyrosine amino acids and this binding affects the peak absorbance of the dye (Bradford, 1976). While Coomassie blue that is not bound to protein maximally absorbs light at a wavelength of 465nm, once the dye has bound to protein, it maximally absorbs at 595nm (Bradford, 1976). Furthermore, the greater the absorbance at 595nm, the greater the amount of protein present in the sample (Bradford, 1976). These characteristics as well as the relative stability of the dye-protein complex and the speed at which the binding occurs makes protein concentration determination using Coomassie blue dyes relatively simple and efficient (Bradford, 1976). Finally, the absorbance values of the BSA standards were used to construct a standard curve, which was then utilized to calculate the protein concentrations of the various protein extract solutions.

2.4 2-dimensional electrophoresis

The 2DE 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 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, non-linear) immobilized pH gradient (IPG) strips (ReadyStrip IPG strips; Bio-Rad), which were passively rehydrated in 125 μL of the protein extract (in Reagent 3 or 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 (dry seeds and seeds at germination – 200 μg/strip; seedlings – 65 μg/strip; leaves – 85 μg/strip) to generate ideal SDS-PAGE gels that contained numerous discrete protein spots. Once the proteins were loaded into the IPG strips, the strips were carefully transferred to the 7 cm focusing tray, which contained electrode wicks moistened with 6 μL nanopure water (Bio-Rad) covering the cathode and anode wires. After the IPG strips were oriented correctly within the channels of the focusing tray, mineral oil was added to submerge each strip to prevent evaporation of the samples or dehydration of the IPG strips during the focusing run. The interlocking lid was then securely placed on top of the strips and the tray was placed onto the Peltier platform of the IEF cell. Isoelectric focusing was performed using the manufacturer's pre-programmed 4-step method. The intial conditioning step, which applied 250 V to the strips for 15 minutes so that charged contaminants and/or salt ions could be removed, was followed by a voltage ramping step where the voltage was

linearly increased to 4000 V over a 2 hour period while ensuring that the applied current did not surpass a 50 μA/IPG strip limit. These steps were followed by a final focusing step where 4000 V was applied to the IPG strips for 20 000 Vhours to allow the proteins to migrate within the IPG strips to their pI values, at which point they became immobile. The final step, called the hold step, involved the rapid reduction of the 4000 V to a continuously applied 500 V and was an optional step whose purpose was to inhibit any diffusion of focused proteins and to prevent the over-focusing of proteins within the IPG strips. Upon completion of the isoelectric focusing step, the IPG strips were removed from the focusing tray and were placed, gel-side up, in the channels of a rehydration/equilibration tray, which was then stored at -20°C overnight. Prior to the embedding of the IPG strips onto prepared SDS-PAGE gels, the proteins underwent an equilibration step where the strips were initially saturated, with gentle agitation at room temperature two times for 15 minutes each, in a solution containing a detergent and a reducing agent (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, 130 mM DTT) and then saturated, with gentle agitation at room temperature two times for 15 minutes each, in a solution (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, 135 mM iodoacetamide) to ensure the complete alkylation of the proteins. Following the equilibration step, the IPG strips were carefully embedded within a molten agarose solution (0.5% (w/v) low melt agarose, 0.001% (w/v) bromophenol blue in a 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS solution) directly on top of 1 mm 13% SDS-PAGE gels [0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 13.3% (w/v) acrylamide/PDA, 0.1% (w/v) ammonium persulfate, 0.04% (v/v) tetramethylethylenediamine (TEMED)] and the SDS-PAGE was completed in an

electrode (running) buffer of 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS at 150V for 1.33 hours. Following electrophoresis, the gels were removed from the electrode assemblies and placed in separate, clean plastic containers filled with Coomassie Brilliant Blue R250 (Sigma-Aldrich, Oakville, ON, Canada) staining solution (0.05% Coomassie Brilliant Blue R250 in 50% (v/v) methanol and 10% (v/v) glacial acetic acid.) The gels were stained, with gentle agitation at room temperature, for 45 minutes after which, the gels were destained overnight by replacing the staining solution with a destaining solution (5% (v/v) methanol, 7% (v/v) acetic acid) in order to remove excess Coomassie Blue dye. Because the sensitivity of Coomassie Blue limits the detection of proteins to spots that are 100 ng or greater, a second, more sensitive staining solution was used to identify any protein spots in the gels that were present in amounts less than the 100 ng detection limit of Coomassie Blue. Therefore, following the destaining of the Coomassie Blue dye, the gels underwent silver staining using the Silver Stain Plus kit according to the manufacturer's instructions (Bio-Rad), which enabled the detection of protein spots present in amounts less than 10 ng. The Silver Stain Plus kit staining protocol allows staining and development to occur in a single step and is based on a previously developed procedure (Gottlieb & Chavko, 1987). Unlike some other silver staining methods, the staining procedure of the Silver Stain Plus kit does not necessitate the oxidation of proteins, which if required, would alter protein mass and adversely affect any downstream mass spectrometric analysis experiments. Instead, the gels were first flooded completely in a solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 10% (v/v) Fixative Enhancer Concentrate containing glycerol – Bio-Rad) for 20 minutes with gentle agitation and then rinsed twice for 10 minutes each time with gentle agitation in

Milli-Q water in order to remove excess acetic acid, which could interfere with the silver stain reaction. Once the gels were sufficiently rinsed, they were submerged in a solution of silver-amine-carrying colloidal tungstosilicic acid, from which the silver ions were transferred to the protein spots in the gels via an electrophilic or ion exchange mechanism. It is believed that the binding of the silver ions to the imidazole ring of histidine and to the carboxyl groups of aspartic acid and glutamic acid is especially strong (Rabilloud, 1990). The reduction of the silver ions to metallic silver [$Ag^+ + 1e^- \rightarrow Ag_{(metal)}$], which serves to visualize the protein spots in the gels, is facilitated by a reducing agent such as formaldehyde. The presence of substances such as sodium or potassium carbonate in the staining and development solution is necessary to ensure that the reducing activity of formaldehyde, which requires a relatively alkaline environment, can proceed optimally (Rabilloud, 1990). The silver stain reaction was stopped after approximately 10 minutes by removing the alkaline staining and development reagent and equilibrating the gels in a more acidic solution (5% (v/v) acetic acid).

2.5 Gel analysis and identification of protein spots

The Coomassie blue and silver stained 2DE gels from our experiments were scanned using a GS-800 Calibrated Densitometer (Bio-Rad) to produce images that could be compared using the 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 was 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.

The excised protein spots underwent a series of procedures within a MassPREP Station (Micromass, Manchester, UK) that ultimately produced protein samples that were destained, reduced (with 10 mM DTT), alkylated (with 55 mM iodoacetamide), and digested at 37°C for 5 hours by sequencing-grade modified trypsin (6 ng/μL in 50 mM ammonium bicarbonate - Promega, Madison, WI, USA.) LC/MS/MS analysis was completed on the prepared protein samples using a capillary HPLC system (CapLC system - Waters Corp., Milford, USA), which utilized a PepMap C18 column (LC Packings, CA, USA) for loading and desalting prior to reversed-phase separation of the peptides on a PicoFrit capillary (75 micron internal diameter x 10 cm, 15 micron tip) column (New Objectives, MA, USA) utilizing a linear gradient [water/acetonitrile (0.2% (v/v) formate).] The separated peptides were applied to a Quadrupole Time of Flight 2 (Q-ToF 2) mass spectrometer by electrospray ionization (Micromass, Manchester, UK.) The information generated by the Q-ToF 2 for the eluting peptides with a charge state of 2 or 3 (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 completed at the Institute for Biomolecular Design (IBD) at the University of Alberta (Edmonton, AB, Canada.)

2.6 Effect of PPIase inhibitors on dicamba-induced changes

To determine percent germination, a 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 solutions [10 μM dicamba, 0.2% (v/v) ethanol, 20 μM cyclosporin A (Sigma-Aldrich, Oakville, ON, Canada), 10 μM juglone (Sigma-Aldrich), 20 μM juglone, 10 μM dicamba and 20 μM cyclosporin A, 10 μM dicamba and 10 μM juglone, or 10 μM dicamba and 20 μM juglone]. The cyclosporin A and juglone in the treatment solutions were initially dissolved in 100% (v/v) ethanol (10 mM) and then diluted appropriately to achieve the required concentrations in the treatment solutions. All of the treatment solutions containing cyclosporin A or juglone also contained 0.2% (v/v) ethanol. The Petri dishes were sealed with laboratory film and stored in the dark at room temperature ($22^{\circ}C \pm 2^{\circ}C$) for 7 days after which, 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 (Statistical Analysis System, 1985) 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 wild mustard seeds were sown and grown as previously described (until the plants were at the 3-4 leaf stage of development.) The

wild mustard plants were sprayed with dicamba (50 g ai ha^{-1}), 0.2% (v/v) ethanol, 20 μM juglone, or dicamba (50 g ai ha^{-1}) and 20 μM juglone. 24 hours after the plants were sprayed, photographs of representative plants were taken. Experiments were repeated at least once.

3.0 Results and Discussion

3.1 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 hours 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.

3.2 Reagent 3 and Rehydration/Sample buffer

Our initial attempts at protein extraction of seedling samples utilized Reagent 3 as the final protein solubilization and IEF buffer. However, Figure 3A, which is a representative 2DE gel produced from protein extracts prepared in Reagent 3, clearly demonstrates that the quality of the gels was not optimal. There is excessive horizontal and vertical streaking and there are an insufficient number of clearly separated and

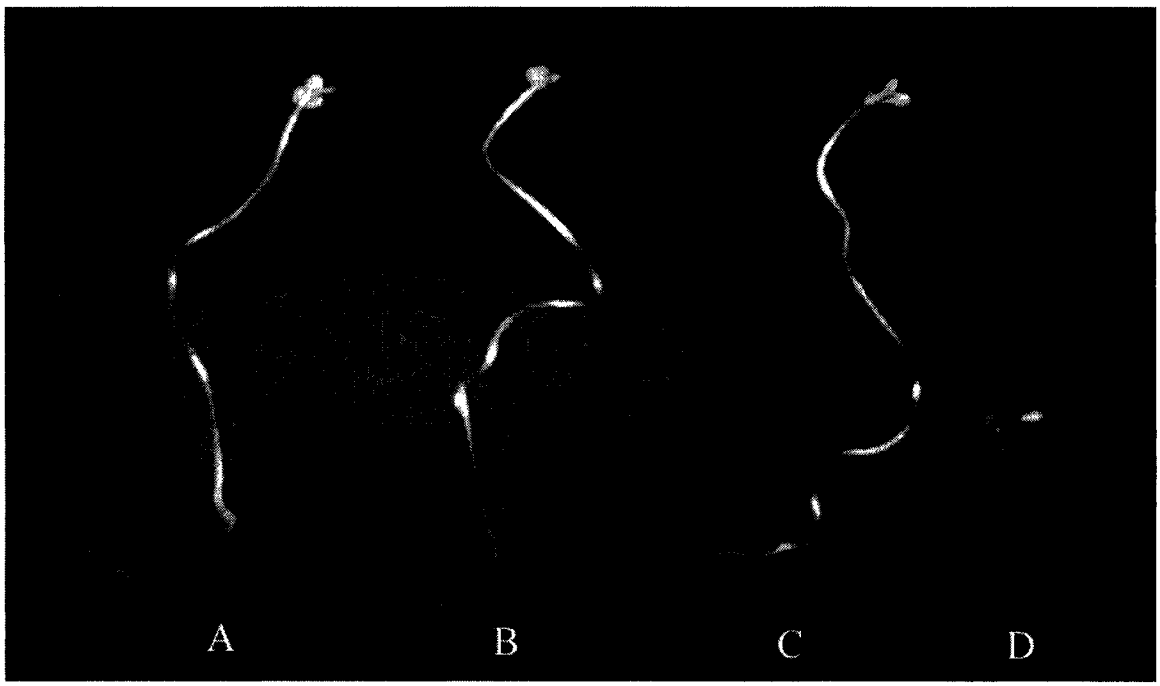

Figure 1. Effect of dicamba on representative auxinic herbicide–susceptible and -resistant wild mustard seedlings germinated in Petri dishes containing: R wild mustard in (A) water (control) or (B) 10 μM dicamba and S wild mustard in (C) water (control) or (D) 10 μM dicamba.

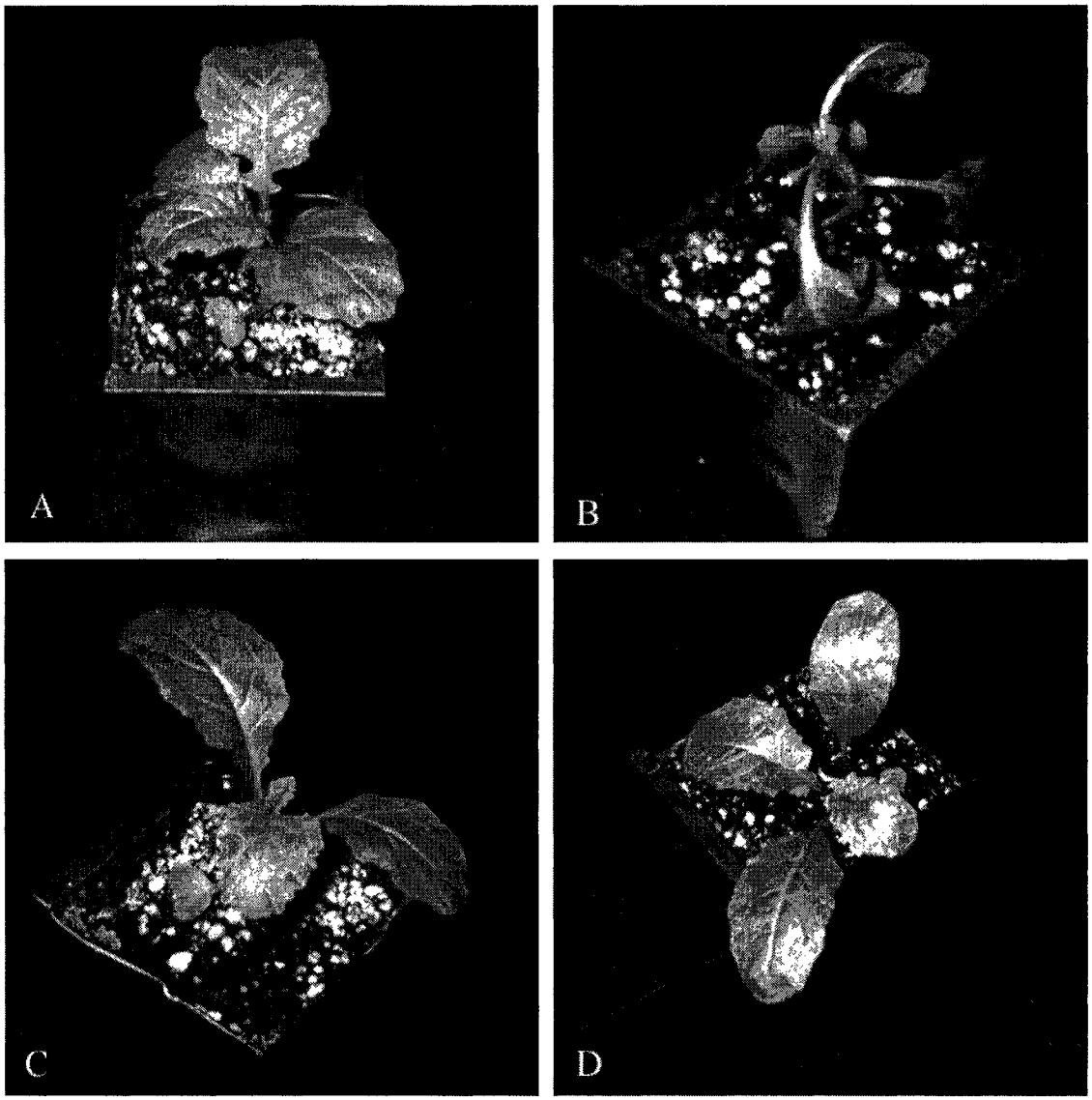

Figure 2. Effect of dicamba on auxinic herbicide –susceptible and –resistant wild mustard plants 24 hours after spraying: S wild mustard sprayed with (A) water (control) or (B) 50 g ai ha^{-1} dicamba and R wild mustard sprayed with (C) water (control) or (D) 50 g ai ha^{-1} dicamba.

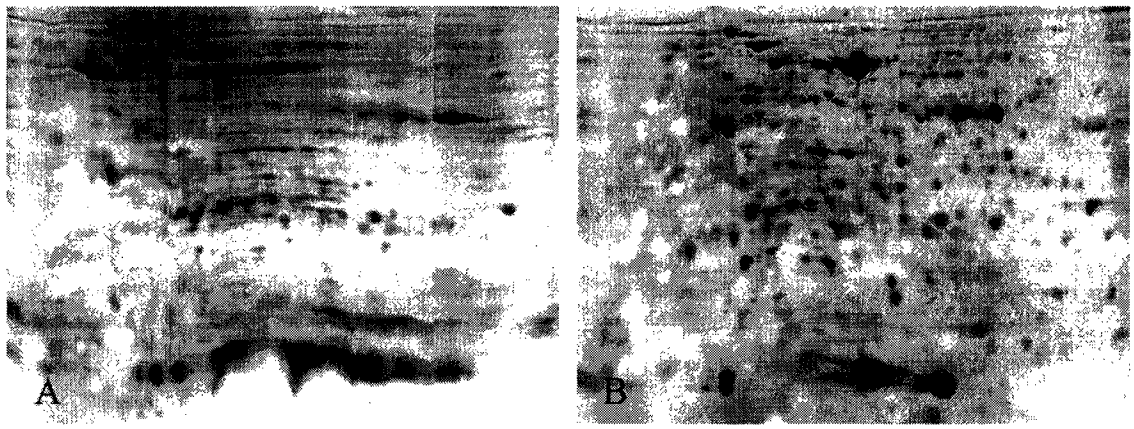

Figure 3. Coomassie blue and silver stained gel images of S seedling protein extracts separated by 2-dimensional electrophoresis using Reagent 3 (A) or Rehydration/Sample buffer (B) as the final protein extraction buffer.

discrete protein spots. Furthermore, the background staining or "noise" is not uniform throughout with the gel being significantly darker in certain regions. It was discovered by others in the laboratory that using Rehydration/Sample buffer in place of Reagent 3 could significantly improve 2DE gels in terms of number and quality of spots as well as reduced horizontal and vertical streaking. Figure 3B illustrates the quality of 2DE gels that could be produced simply by using Rehydration/Sample buffer as the final protein extraction buffer instead of Reagent 3. A comparison of the composition of the two buffers shows that they are very similar, however, a significant difference that may be at least partially responsible for the disparate quality of 2DE gels is the presence of 40 mM Tris base only in Reagent 3. Bases or buffers such as Tris are sometimes added to protein extraction buffers to maintain a basic environment, which can improve solubilization of some proteins (Berkelman & Stenstedt, 1998). However, it has been reported that the presence of Tris in protein extracts or IEF buffers can result in an increase in the electrical conductivity during the focusing of the proteins, which would then necessitate a longer focusing time in order to optimally separate proteins in the first dimension (Garfin & Heerdt, 2003). Therefore, it is possible that at least some of the problems associated with the gels of Reagent 3 extracts (eg. horizontal streaking) may simply be a consequence of insufficient focusing time. Increasing the duration of the focusing run was not attempted due to the suitable quality and reproducibility of the gels produced from Rehydration/Sample buffer extracts.

3.3 Proteome-level differences

3.3.1 Dry seed and germinated seed proteins

Our results show that there are not a significant number of proteins that are reproducibly differentially expressed between the S and R wild mustard dry seeds or seeds at germination. However, a comparison of Figure 4A and B indicates that there are two proteins that are present in the dry seeds of the R wild mustard that are not present in the S seeds. The proteins were identified as a heat shock protein (HSP) 22.0 (Figure 4; spot 1) and a cruciferin cru2/3 subunit of the cruciferin (12S globulin) seed storage protein (Figure 4; spot 2). Interestingly, it was discovered that germination of the R seeds in water or dicamba differentially affected the expression patterns of the two identified proteins. HSP 22.0 appears to be down-regulated when the seeds are germinated in water while the expression of the cruciferin subunit appears relatively unchanged, if not slightly up-regulated. The reverse seems to occur when the R seeds are germinated in 10 μM dicamba, with the cruciferin subunit protein being down-regulated and HSP 22.0 not significantly up or down-regulated.

The 12S globulin protein is the predominant seed storage protein in many dicotyledonous plant species, including *Brassica napus* (Canola) where the protein is referred to as a cruciferin (Sjodahl et al., 1991). The seed storage proteins accumulate during seed development and provide germinating seedlings with a source of carbon, nitrogen, and sulphur for proper growth and development (Sjodahl et al., 1991). Since the differentially-expressed protein was identified only as a precursor subunit of the cruciferin protein, our observation that the protein is found only in the R seed protein extract is not necessarily indicative of the presence or absence of the storage protein in

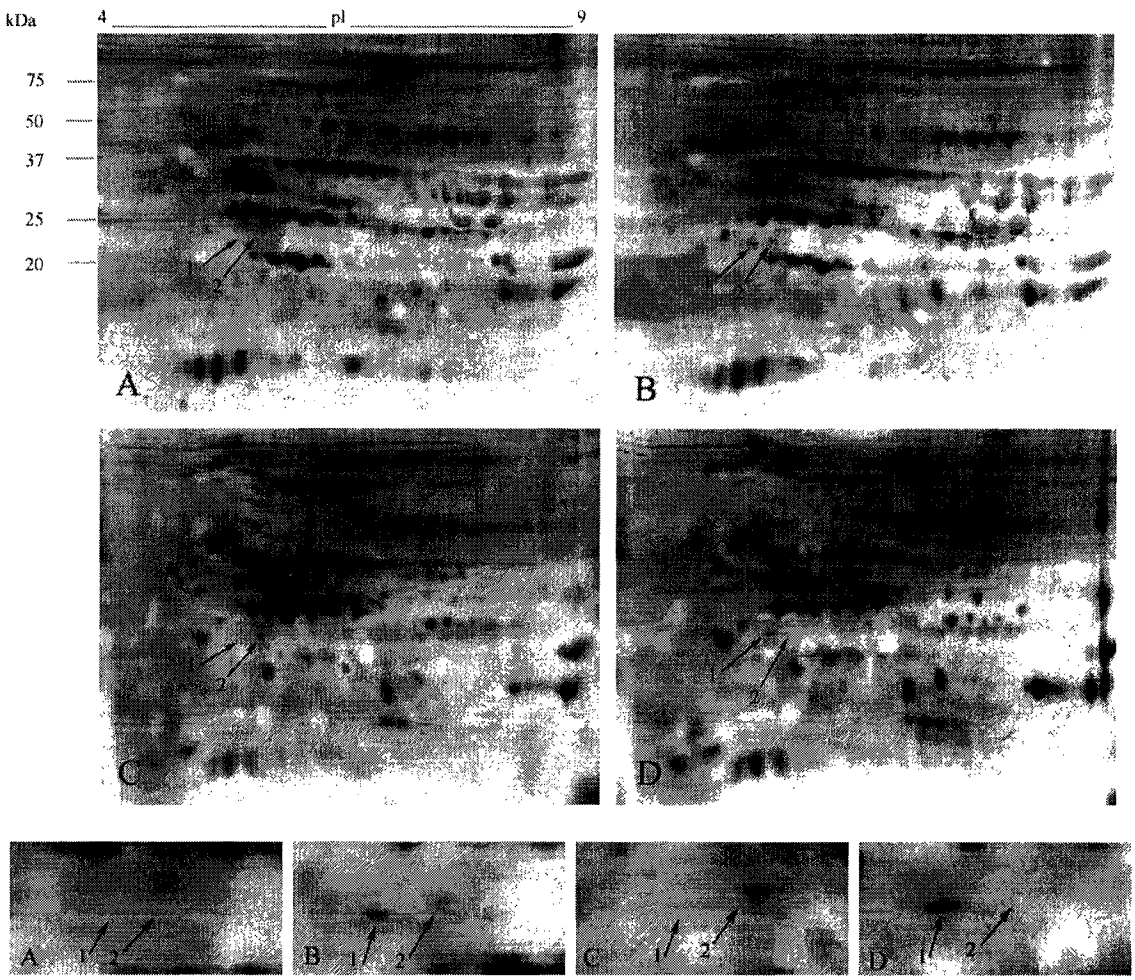

Figure 4. Coomassie blue and silver stained gel images of R and S dry seed and seed at germination protein extracts separated by 2-dimensional electrophoresis: S wild mustard dry seed (A) and R wild mustard dry seed (B) and R seed germinated in (C) water (control) or (D) 10 μM dicamba. Each arrow points to a protein that showed reproducible differences in expression between the S and R wild mustard. The sub-panels below the full gel images are enlarged images of the numbered spots for a clearer visual comparison of the spot intensities.

the two biotypes. It is possible that the cruciferin storage protein is present in both biotypes with an excess of the precursor subunit only in the R seeds. The fact that the cruciferin subunit is down-regulated or degraded upon herbicide application may simply be a consequence of herbicidal activity, and the differential expression of the cruciferin subunit in the S and R wild mustard may not have any role in the regulation of auxinic herbicide resistance.

Similar to other small heat shock proteins, the identified seed protein HSP 22.0 is likely involved in responses to heat or other environmental stresses such as dehydration or in dessication tolerance and dormancy (Wehmeyer et al., 1996). The known relationship between stress response and HSPs may offer some insight into the potential role of the differential expression of this protein in regulating auxinic herbicide resistance. The application of dicamba during germination of the seeds may impose a type of stress onto the developing seedlings that could be somewhat alleviated through the activity of HSP 22.0. Improved germination efficiency in dicamba or increased tolerance to the herbicide may result from the sustained presence or expression of HSP 22.0. The S seeds, which do not appear to possess any significant amounts of HSP 22.0, may not benefit from this protective activity. The observed down-regulation of HSP 22.0 in the R seeds germinated in water (control) is not an unexpected response since it has been established that there is normally a decline in the amounts of small HSPs during germination in other species, such as pea and sunflower (Wehmeyer et al., 1996). The potential connection, if any, between the observed differences in the protein content of the S and R wild mustard seeds and auxinic herbicide resistance is unclear and will likely require further examination.

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 # from gels		Effect of dicamba treatment		*Final spot density ratios
Seedling	Leaf	S wild mustard	R wild mustard	$S_{dicamba}$: $R_{dicamba}$
1		‡Down–regulated	Down-regulated	1:5.8 (1:6.2) (1:5.4)
2		Down–regulated	Spot not detected	1:0 (1:0) (1:0)
3		Down–regulated	Up–regulated	1:2.8 (1:3.1) (1:2.5)
4		Down–regulated	Up-regulated	1:1.3 (1:1.2) (1:1.4)
	1	Down–regulated	Up–regulated	1:4 (1:5) (1:3)
	2	Down-regulated	Spot not detected	1:0 (1:0) (1:0)
	3	Spot not detected	Down-regulated	0:0 (0:0) (0:0)
	4	Down-regulated	Down–regulated	1:1.4 (1:1.2) (1:1.6)
	5	Down–regulated	Spot not detected	0:0 (0:0) (0:0)
	6	Up-regulated	Up-regulated	1:1.4 (1:1.5) (1:1.3)
	7	Up-regulated	Up-regulated	1:2.2 (1:1.9) (1:2.5)

*Spot densities were averaged from 2 gels (values in parentheses) as determined by the PDQuest software (Bio-Rad).

‡Down or up-regulated refers to the change in protein quantities (i.e. spot densities) compared to the untreated control.

Table 2. Summary of the identified seedling proteins shown in Table 1.

Spot # from gels	*%	MS/MS (ESI-Q-ToF) sequence	Identity	Mr/pI	Protein function
Seedling					
1	8%	DGSNYIALR TAGGLLLTETTK	Chloroplast Cpn21 protein	26785/8.86	Chloroplast co-chaperonin involved in refolding denatured ribulose-1,5-bisphosphate carboxylase
2	5%	FLTQAVEEAYK	Putative cytidine deaminase-like protein	20388/5.64	Catalyzes deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively
3	2%	VACETCTK	Methionine adenosyltransferase	43573/5.50	Conversion of methionine and ATP to *S*-adenosylmethionine
4	9%	AIVNSDLGVTPN NDGDVIR DLSSDLQK	Expressed protein (*Arabidopsis thaliana*)	30517/9.46	Unknown

*Sequence percentage coverage of the matched peptides

3.3.2 Seedling proteins

The comparison of the S and R wild mustard at the seedling stage of development (7 days post germination) also showed some reproducible proteome 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. 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, as well as listing known functions for each protein. Most of the identified protein spots have not been previously implicated in regulating auxinic herbicide resistance. Among the differentially expressed proteins listed in Tables 1 and 2, one (seedling protein spot 3; methionine adenosyltransferase) is known to be involved in ethylene biosynthesis. Our results (Figure 5, spot 3) show that this enzyme is present in the S and the R biotype seedlings one 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 (Hall et al., 1993; Gonzalez et al., 2003). ACC synthase enzymes are responsible for converting SAM to 1-aminocyclopropane-1-carboxylic acid (ACC), which is then either

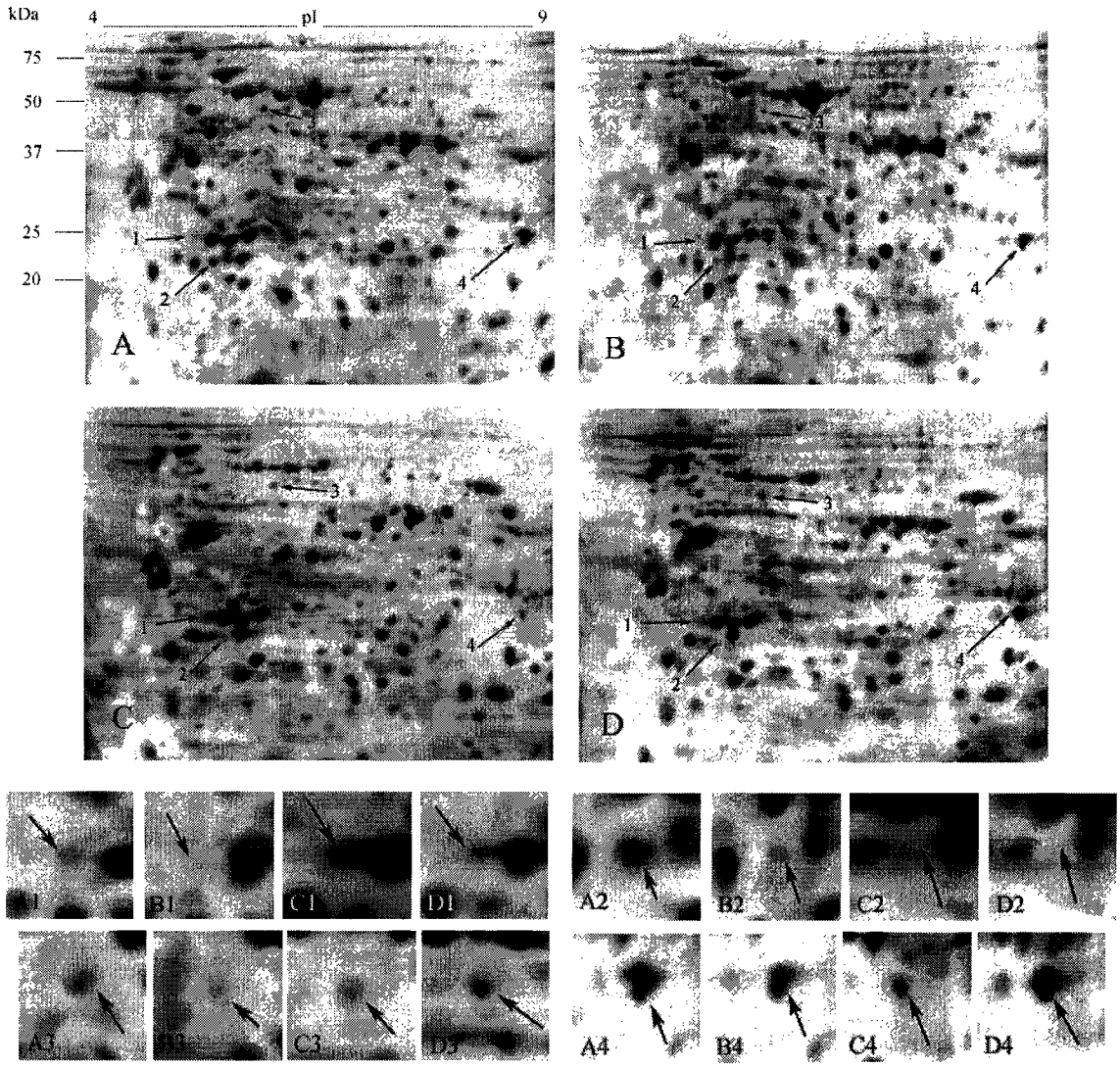

Figure 5. Coomassie blue and silver stained gel images of R and S seedling protein extracts separated by 2-dimensional electrophoresis: S wild mustard germinated and grown in (A) water (control) or (B) 10 μM dicamba and R wild mustard germinated and grown in (C) water (control) or (D) 10 μM dicamba. 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. The sub-panels below the full gel images are enlarged images of the numbered spots for a clearer visual comparison of the spot intensities.

metabolized to 1-malonylaminocyclopropane-1-carboxylic acid (MACC) or converted to ethylene by ethylene forming enzymes (EFE) (Hall et al., 1993). The reaction catalyzed by the ACC synthase enzyme is the rate-limiting step in the biosynthesis of ethylene (Abel and Theologis, 1996), which indicates the importance of this particular enzyme.

The synthesis of ethylene has been shown to increase in both biotypes but this increase is significantly more in S than the R biotype as early as 18 to 24 hours following the application of auxinic herbicide (Peniuk et al., 1993). Furthermore, there is only a minor increase in ethylene biosynthesis in the R plant when compared to basal levels in the untreated control 96 hours after treatment with the auxinic herbicide picloram (Hall et al., 1993). The difference between the induction of ethylene biosynthesis by auxinic herbicides in the S versus R may be key to auxinic herbicide resistance (Hall et al., 1993). Hall et al. (1985) treated rapeseed plants with aminoethoxyvinylglycine (AVG), a chemical known to interfere with the evolution of ethylene, as well as 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. In addition, Hall et al. (1993) showed that that the quantity and the activity of the ACC synthase enzyme is significantly lower in the R biotype treated with an auxinic herbicide compared with the similarly treated S biotype.

A cursory review of our results may indicate that the observed changes in levels of MAT is 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 lead 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. The auxinic herbicide-induced ethylene biosynthesis observed in the S wild mustard occurs as a result of *de novo* synthesis of ACC synthase, which does not take place in the R wild mustard.

3.3.3 Pre-flowering plant proteins

The proteome-level differences between S and R wild mustard at the 3-4 leaf stage of development is summarized in Tables 1 and 3. Similar to the identified seedling proteins, most of the identified leaf protein spots have not been previously implicated in regulating auxinic herbicide resistance. Among the differentially expressed proteins listed in Tables 1 and 3, one (leaf protein spot 1; peptidylprolyl cis-trans isomerase) has recently been implicated in auxin signalling pathways (Dharmasiri et al., 2003). Our results show that the expression pattern of a cyclophilin-type PPIase (Table 3; Figure 6, spot 1) varies between the S and the R wild mustard biotypes and is differentially regulated by dicamba. PPIase is present in the leaf tissue of S controls (Figure 6A) and is

Table 3. Summary of the identified leaf proteins shown in Table 1.

Spot # from gels	*%	MS/MS (ESI-Q-ToF) sequence	Identity	Mr/pI	Protein function
Leaf					
1	7%	TSWLDNK IVMGLFGEVVPK	Peptidylprolyl isomerase	28532/8.83	Catalyzes cis-trans isomerization of prolyl peptide bonds
2	5%	GPQSPSGYSCK	Germin-like protein (AtGER3)	21993/6.26	Generates hydrogen peroxide and is involved in abiotic and biotic stress response in plants
3	7%	VLLTMEEK ISAADLSLAPK	Dehydroascorbate reductase	27976/6.93	Reduces dehydroascorbate to ascorbate (antioxidant)
4	3%	SVGDLTSADLK YLIENGAK	Phosphoglycerate kinase-related protein	50195/5.91	Converts 3-phosphoglyceroyl phosphate to 3-phosphoglycerate producing ATP from ADP as part of glycolytic pathway
5	3%	NNLTNSMK ALAENEGEGIAK	Dihydrolipoamide dehydrogenase 1	61004/8.13	Catalyzes the reduction of the lipoamide group in the 2-oxoacid dehydrogenase and glycine decarboxylase multienzyme complexes
6	5%	DSIMDSTVNCR GGAIDDSVITK	Aminomethyl-transferase-related precursor protein	44759/8.55	Involved in catalyzing the degradation of glycine as part of the glycine cleavage multienzyme complex (mitochondria)
7	4%	GGAIDDSVITK MPFVATK	Aminomethyl-transferase-related precursor protein	44759/8.55	Involved in catalyzing the degradation of glycine as part of the glycine cleavage multienzyme complex (mitochondria)

*Sequence percentage coverage of the matched peptides

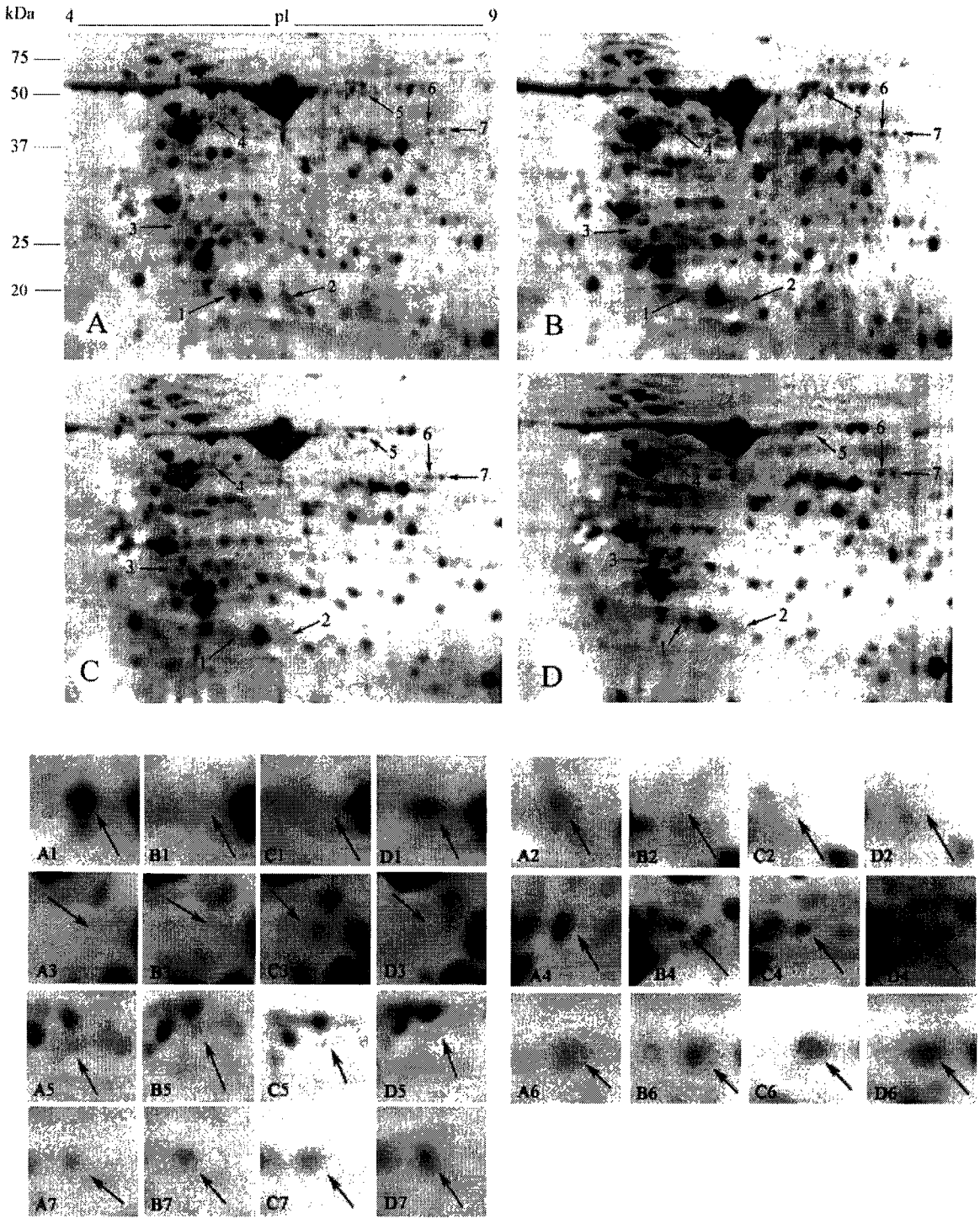

Figure 6. Coomassie blue and silver stained gel images of R and S leaf protein extracts separated by 2-dimensional electrophoresis: S wild mustard sprayed with (A) water (control) or (B) 50 g ai ha^{-1} dicamba and R wild mustard sprayed with (C) water (control) or (D) 50 g ai ha^{-1} dicamba. Each arrow 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 3. The sub-panels below the full gel images are enlarged images of the numbered spots for a clearer visual comparison of the spot intensities.

down-regulated considerably within 24 hours of dicamba application (Figure 6B). 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 (Figures 6C and D). This result is particularly interesting since PPIases have recently been implicated in a mechanism that induces auxin-modulated activities (Dharmasiri et al., 2003) and have also been shown to associate with proteins that are thought to be involved in auxin transport (Jackson & Soll, 1999) and ethylene response (Larsen & Cancel, 2003).

The planar peptide bonds that link individual amino acids together in proteins can occur in one of two conformations. The formation of peptide bonds in a *cis* or *trans* conformation affects the rate of correct protein folding, which can ultimately influence the functionality of the proteins. Due to the importance of correct folding, peptide bonds of most proteins are required to be in a well-defined and specific conformation. The determination of whether a peptide bond is in a *cis* or *trans* conformation is based on the positions of C_α atoms on adjacent amino acids and for almost all peptide bonds in correctly folded proteins the peptide bond is in the *trans* conformation. In fact, peptide bonds preceding a proline residue are the only bonds that have any significant degree of *cis* conformation. Prolyl peptide bonds that are in the *cis* conformation are believed to be conducive to the formation of tight turns in the vicinity of the proline residues, which can produce other structural changes to distant segments of the protein (Schmid, 2002). While approximately 7% of prolyl peptide bonds in properly folded proteins are found in a *cis* conformation, less than 0.5% of all other peptide bonds share this conformation (Scherer et al., 1998; Stewart et al., 1990; Macarthur & Thornton, 1991). *cis* peptide bonds are almost exclusively found in prolyl peptide bonds because in non-prolyl peptide

bonds the *cis* conformation is strained and energetically unfavourable relative to the *trans* conformation, whereas the energy difference is minimal for prolyl peptide bonds. However, in nascent or folding proteins, the tendency is for all peptide bonds to be in a *trans* conformation and therefore, in order to incorporate a *cis* peptide bond, specific enzymes are required for the conversion, which is a very slow reaction in the absence of enzymatic catalysis because of the need for rotation around a partial double bond (Schmid, 2002). Even with enzymatic catalysis, prolyl bond isomerizations are still relatively slow reactions that are believed to require 10 to 100 seconds at room temperature (Schmid, 2002). Peptidylprolyl *cis-trans* isomerases (PPIases), which are present in all organisms and in almost all cellular compartments, are the enzymes responsible for the catalysis of the reversible isomerization of peptide bonds involving proline residues (Schmid, 2002). PPIases are not restricted to ensuring correct folding of nascent proteins since they are believed to also regulate the activities of specific proteins through a conversion between active and inactive functional states by changing the conformation of the prolyl peptide bonds. It has been reported that PPIases can regulate the activities of growth factor receptors as well as ion channels in this manner (Schmid, 2002). Peptidylprolyl isomerization has also been implicated in calcium ion binding to plant and mammalian lectins, which has led to the suggestion that the activities of PPIases may somehow be involved in signalling pathways involving calcium ions (Schmid, 2002). All of the known PPIase enzymes can be divided into three families based on structural differences: the cyclophilins, FK506-binding proteins, and parvulins (Schmid, 2002).

Cyclophilins or cyclophilin-type domains were initially identified in 1989 due to their affinity for the immunosuppressive peptide cyclosporin A, which acts as a competitive inhibitor that binds to the PPIase in the vicinity of the peptidylprolyl isomerization active site (Galat, 1999; Schmid, 2002). Cyclophilins are known to be involved in protein folding activities as well as in mediating transport across plasma membranes (Gething & Sambrook, 1992). The inhibition of the seven different cyclophilin-type PPIases identified in yeast does not appear to affect the long-term viability of yeast, however, abnormal growth activities are among the deleterious effects attributed to non-functional cyclophilin-type PPIases (Dolinski et al., 1997). The protein domains possessing PPIase activity within the cyclophilin family typically include a β-sheet component consisting of approximately eight anti-parallel strands, which is associated with two α-helices (Schmid, 2002).

Jackson and Soll (1999) have identified a cyclophilin in *Arabidopsis* that has affinity for the A subunit of protein phosphatase 2A (PP2A), which may indicate that prolyl isomerization of a peptide bond within the A subunit is required for the efficient functioning of the phosphatase enzyme. The PP2A holoenzyme, which consists of a catalytic C subunit as well as regulatory A and B subunits, is responsible for the dephosphorylation of proteins, but it has also been implicated in auxin transport activities (Jackson & Soll, 1999; Smith & Walker, 1996). Rashotte et al. (2001) have reported that reduced phosphatase activity alters auxin transport and dependent physiological processes. Although it is not entirely clear what the precise effect cyclophilin has on the activity of PP2A, it is of interest to note that a mutation in the A subunit of PP2A results in an increase in the sensitivity of *Arabidopsis* seedlings to the auxin transport inhibitor

naphthylphthalamic acid (Garbers et al., 1996). It would seem plausible that the differential pattern of PPIase expression that we observed could affect the degree of peptidylprolyl isomerization within the A subunit of PP2A with a corresponding effect on the activity of the phosphatase enzyme as it pertains to auxin transport. This potential difference in auxin transport requires further examination and verification to determine if it is in fact involved in the development of auxinic herbicide resistance.

The observed differences in PPIase expression between the S and the R wild mustard could also be affecting another aspect of the auxin signalling pathway. Specifically, ethylene signalling and response could be affected due to the differences in the level of peptidylprolyl isomerization within the A subunit of PP2A resulting from the observed differential pattern of cyclophilin-type PPIase expression. Mutations to the A subunit of PP2A have been reported to affect ethylene signalling and response as demonstrated by the identification of an *rcn1-2* mutant *Arabidopsis* that exhibited enhanced ethylene responsiveness (Larsen & Cancel, 2003). The loss-of-function mutation to the A regulatory subunit was shown to inhibit the ability of the C catalytic subunit of PP2A to efficiently bind CTR1, which is a mitogen activated kinase kinase kinase (MAPKKK) that serves as a negative regulator of the ethylene response pathway (Larsen & Cancel, 2003). CTR1 is believed to repress ethylene responses by binding to ethylene receptors, and non-functional CTR1 proteins results in constitutive ethylene responses even in the absence of ethylene (Larsen & Cancel, 2003). It has also been reported that a consequence of the use of PP2A-inhibitors in wild-type *Arabidopsis* seedlings is the amplification of the ethylene response (Larsen & Cancel, 2003), which is an experimental observation that is consistent with what others have found in tobacco,

rice, and orchid flowers (Agrawal et al., 2002; Raz & Fluhr, 1993; Wang et al., 2001). It has been suggested that PP2A is involved in the activation of CTR1 and any inhibition of PP2A activity results in a deficiency of active CTR1, which consequently prevents the repression of the ethylene response (Larsen & Cancel, 2003). The aforementioned *rcn1-2* mutant *Arabidopsis* was observed to synthesize significantly more ethylene than the wild type (Larsen & Chang, 2001) and it has been proposed that the overproduction of ethylene is the result of inhibited PP2A activity leading to increased expression of an ethylene-mediated gene involved in the ethylene biosynthesis pathway (Larsen & Cancel, 2003).

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) (Jackson & Soll, 1999), where the majority of the ABP1 is located. 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, which may be a factor in the regulation of auxinic herbicide resistance. The previously described hydrophobic binding pocket of ABP1 from maize is known to contain a highly conserved proline residue, whose peptide bond conformation may be crucial for the maintenance of a functional 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 (Woo et al., 2002), which may require action by a PPIase for the incorporation of the correct conformation. 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.

Similar to the cyclophilin family of PPIases, the ubiquitous FK506-binding proteins (FKBP) occur as either small (approximately 12 kDa) monomers or as part of larger protein complexes and they also belong to a family of PPIases that includes a relatively large number of proteins (Schmid, 2002). Furthermore, these PPIases are also able to bind immunosuppressants, such as FK506 and rapamycin, which act as competitive inhibitors (Schmid, 2002). Although the FKBPs and cyclophilins share some common characteristics, they are structurally diverse with members of the FKBP family possessing a significant amount of irregular structure in addition to a short α-helical segment associated with a five stranded anti-parallel β-sheet (Van Duyne et al., 1993). Not unexpectedly, these structural differences contribute to differences in substrate specificities. Whereas the cyclophilins can catalyze the isomerizations of prolyl peptide bonds irrespective of the identities of the amino acids adjacent to the proline residue, the nature of the amino acids surrounding a proline significantly influence the efficiency of the isomerization reaction mediated by the FKBP PPIases with a clear preference for hydrophobic residues such as leucine and phenylalanine (Schmid, 2002).

The parvulins make up the third family of known PPIases and the enzymes of this group do not share any significant sequence homology to the proteins of the cyclophilin or FKBP PPIases. However, it has been reported that there are three-dimensional structural similarities between some of the parvulins and FKBPs (Schmid, 2002). It is

not entirely clear if these structural similarities correlate to similarities in the types of substrates that these PPIases interact with and modify. However, it has been determined that the parvulins and FKBPs are similar regarding the preference for hydrophobic amino acids adjacent to the proline residue (Schmid, 2002). The parvulins are generally considerably smaller than the enzymes of the other families of PPIases (Rahfeld et al., 1994). Ess1 (yeast) and Pin1 (human) are examples of two parvulin-type PPIases that have been relatively well characterized. Pin1 PPIases have an affinity for peptide bonds linking a phosphorylated threonine or serine to a proline residue, and it has been determined that unphosphorylated threonines or serines can decrease the efficiency of the *cis-trans* isomerization by as much as 1300-fold (Yaffe et al., 1997). The immunosuppressive peptide compounds that have been identified as being able to inhibit the activities of cyclophilin-type and FKBP-type PPIases do not appear to affect the parvulin-type PPIases (Hennig et al., 1998). However, it has been reported that juglone (5-hydroxy-1,4-naphthoquinone), which is a naturally occurring chemical produced by members of the Walnut family (Juglandaceae) of trees, negatively affects the activity of parvulin-type PPIases by covalently binding the side chains of two conserved cysteine residues (Hennig et al., 1998). Juglone is characterized as an allelochemical since it is involved in a natural phenomenon called allelopathy, which is a term used to describe the relationship or interaction between different living organisms and their naturally produced metabolites (Kocacaliskan & Terzi, 2001). Juglone is present in significant amounts in various parts of the walnut tree as hydrojuglone, which is the non-toxic form of the chemical (Kocacaliskan & Terzi, 2001). Upon release or exudation from the leaves, roots, and fruits into the environment, hydrojuglone is oxidized to form juglone,

which is phytotoxic to numerous species of woody and herbaceous plants by eliciting oxidative stress in addition to adversely affecting photosynthesis and respiration (Kocacaliskan & Terzi, 2001). The binding of juglone is specific for parvulin-type PPIases as it has been demonstrated that the compound does not affect the activities of the other types of PPIases (Hennig et al., 1998). Structural alterations resulting from the binding of juglone molecules are thought to inactivate parvulin-type PPIases through a modification of the active site or a destabilization of the overall protein structure potentially leading to increased proteolysis or degradation (Hennig et al., 1998).

Dharmasiri et al. (2003) have recently provided evidence that the activity of a parvulin-type PPIase may be essential for the efficient association of Aux/IAA proteins with the aforementioned SCF^{TIR} complex, 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. (2003) concluded that, of the different types of known PPIases, only the parvulin-type PPIases are involved in an auxin signalling 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 signalling along with the parvulin-type PPIase. It should also be noted that the structural differences between the three types of PPIases as well as the range of amino acids that can occupy the position adjacent to the prolines of target proteins may contribute to the determination of the type of PPIase that is involved in a particular prolyl peptide bond isomerization. Perhaps the structural nature

or amino acid content in the vicinity of proline residues of the specific Aux/IAA or other substrate proteins that are undergoing prolyl peptide bond isomerization and contributing to auxinic herbicide-resistance requires the activity of a cyclophilin-type PPIase. It is conceivable that in the untreated S wild mustard a PPIase (parvulin-, 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 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 (Hsu et al., 2001).

3.4 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 (2003), we initiated experiments to determine the effects of juglone on the activity of dicamba in the

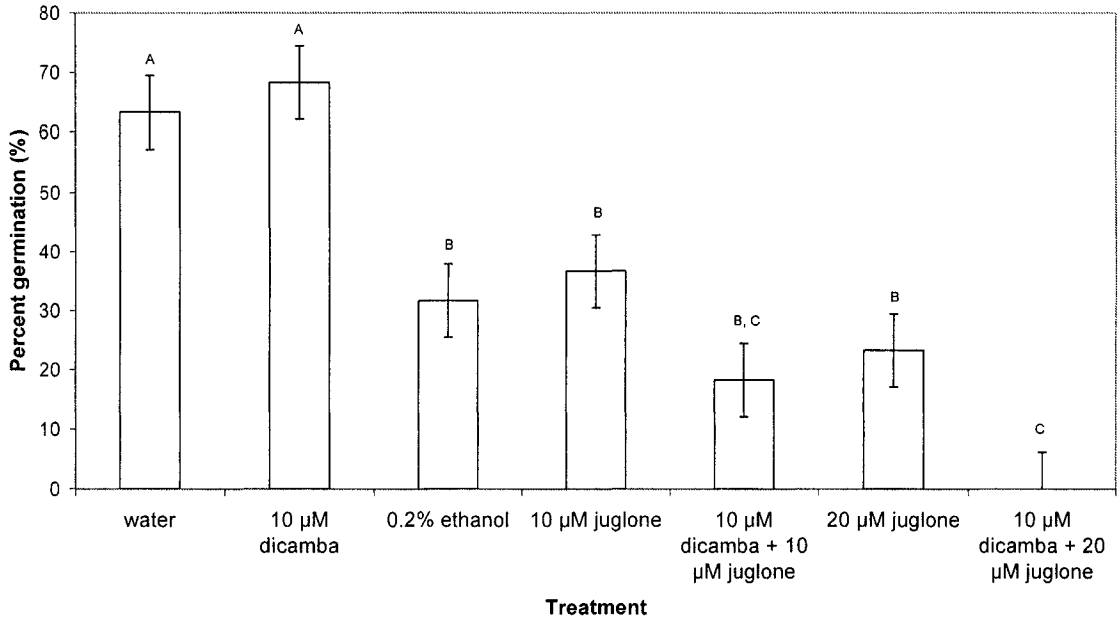

Figure 7. Effect of various treatments on the germination of R wild mustard seeds. Different letters above the standard error bars indicate which treatments are statistically different ($P < 0.05$) as determined by the Student-Newman-Keuls test.

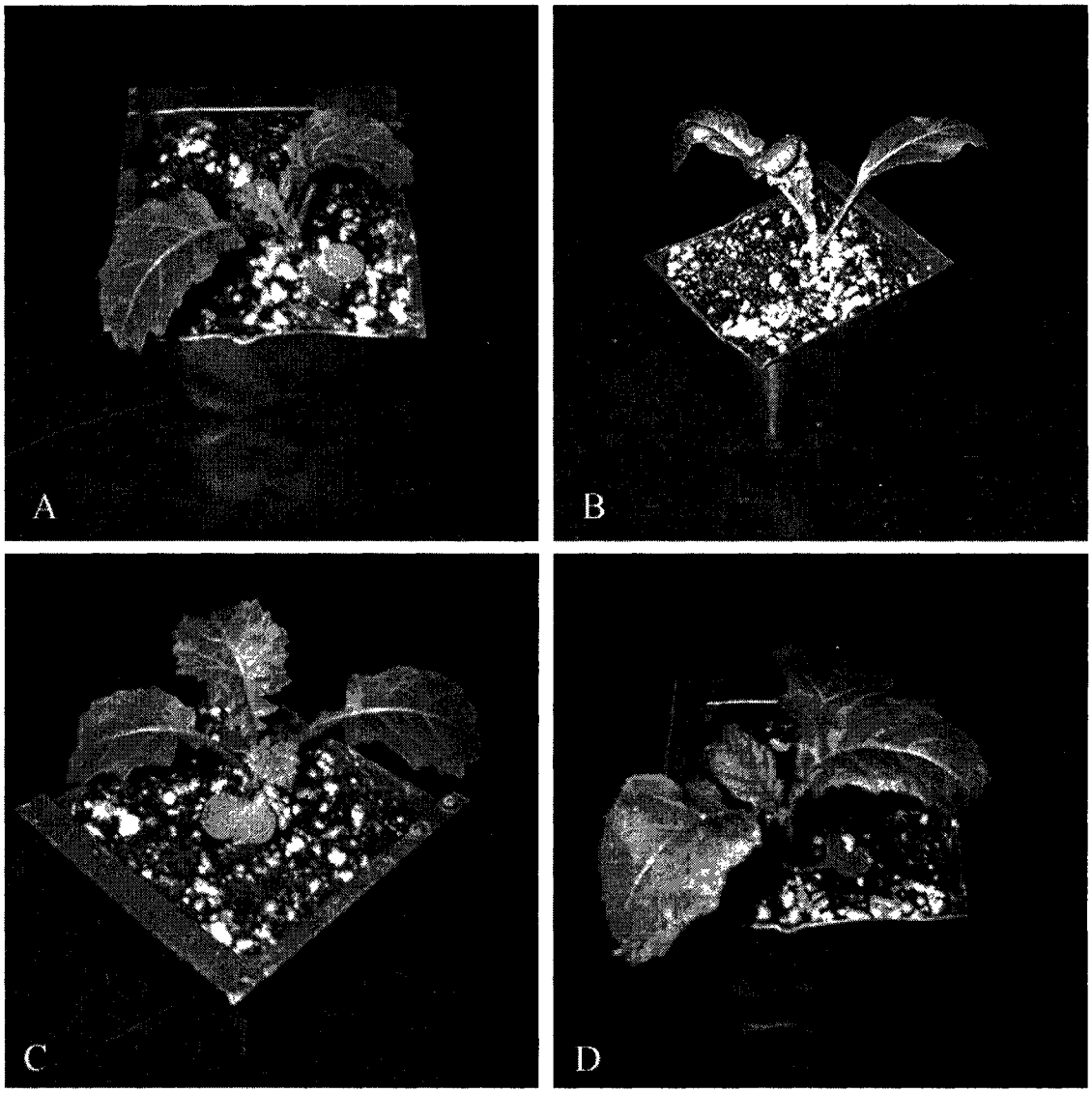

Figure 8. Effects of juglone on the morphology of 3-week old R wild mustard plants sprayed with (A) 50 g ai ha^{-1} dicamba; (B) 50 g ai ha^{-1} dicamba and 20 μM juglone; (C) 20 μM juglone; or (D) 0.2% ethanol.

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 7 and 8. 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 has an effect on germination rates). Furthermore, when dicamba plus juglone were applied to R wild mustard at the 3 to 4 leaf stage of development, typical auxinic herbicide symptoms (eg. epinasty; Figure 8) 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.

3.5 Phenotypic similarities between auxin resistant *Arabidopsis* and R wild mustard

Mutant *Arabidopsis* plants (*axr1*) have shown a reduced sensitivity to auxin likely resulting from an abnormality in the enzyme responsible for the activation of the RUB1 molecule (del Pozo et al., 1998) leading to the inefficient polyubiquitination of target proteins by the SCF^{TIR} complex. However, the defect is believed to only reduce the ubiquitin-dependent degradation process, not inhibit it completely (Leyser, 2001).

Morphologically, the *axr1* mutant *Arabidopsis* and R wild mustard biotypes share similar features. In both instances, the plants have smaller leaves and root systems, are shorter, and are more branched than their wild type versions (Hall & Romano, 1995; Estelle & Somerville, 1987). Also, genetic studies involving the *axr1* mutant *Arabidopsis* have led to the conclusion that the *axr1* mutation is responsible for regulating both the manifestation of the altered morphology as well as the resistance to auxin and auxinic herbicides (Estelle & Somerville, 1987). The fact that the *axr1* mutant *Arabidopsis* and the R wild mustard biotype are phenotypically alike might suggest that a similar mutation is responsible in both species for the observed morphology and auxin resistance. Our hypothesis that the altered expression pattern of PPIase is part of the auxinic herbicide resistance mechanism is consistent with the concept that there exists a similar mutation in the auxin resistant *Arabidopsis* and wild mustard. Although the *axr1* mutation interferes with the activity of the SCF^{TIR} complex itself and the differences in PPIase expression affect the Aux/IAA proteins, the consequence of both mutations is the reduced ability of the SCF^{TIR} complex to ubiquitinate target Aux/IAA proteins, which consequently leads to the inhibition of the induction of early auxin-mediated genes.

4.0 Conclusions

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 as well as their role is far

from being clear. Our experiments using the PPIase inhibitor juglone on both seedling 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 Rubisco in the region of the gel where parvulins would be expected to appear, we cannot see the effect on parvulin-type PPIase. Additional studies to establish the identities of the PPIases involved i.e., whether it is a cyclophilin- or a parvulin-type (or both), the identities of the target protein(s), including ABP1, modified by the PPIases as well as the regulation of expression of PPIases by auxins are warranted. Purification and characterization of the differentially-expressed PPIase through sequencing and crystallization experiments may further elucidate the structure and therefore, potentially the function of the protein as well. Clarifying the structure of the protein may also provide valuable clues to determine the precise nature or identification of the substrate proteins that are modified by the PPIase. Binding studies using wild mustard plant tissue extracts could also be performed to identify proteins that may have an affinity for the PPIase. Furthermore, the production of "knock-out" R wild mustard biotypes that do not express the identified PPIase or transgenic plant species that overexpress the PPIase with a subsequent treatment with auxinic herbicides can potentially further verify, or disprove, our suggestion that the differential expression of the identified PPIase is involved in the regulation of auxinic herbicide resistance. Finally, although we used dicamba as the representative auxinic herbicide, our experiments could also be repeated with other auxinic herbicides or IAA at various concentrations to ascertain if other

similar chemicals induce the same effects as dicamba or if there are other differences produced as well.

5.0 Literature Cited

Aebersold, R. and Mann, M. 2003. Mass spectrometry-based proteomics. Nature. 422: 198-207.

Abel, S. and Theologis, A. 1996. Early genes and auxin action. Plant Physiol. 111: 9-17.

Alberta Agriculture. 1990. Guide to crop protection in Alberta 1990. Part 1: Chemical herbicides, insecticides, fungicides, rodenticides for maximum economic yield. Agdex 606-1. Crop Protection Branch, Alberta Agriculture, Edmonton, Alberta.

Altom, J.D. and Stritzke, J.F. 1973. Degradation of dicamba, picloram, and four phenoxy herbicides in soils. Weed Sci. 21: 556-560.

Agrawal, G.K., Rakwal, R., Jwa, N.S. and Agrawal V.P. 2002. Effects of signalling molecules, protein phosphatase inhibitors and blast pathogen (*Magnaporthe grisea*) on the mRNA level of a rice (*Oryza sativa* L.) phospholipids hydroperoxide glutathione peroxidase (*OsPHGPX*) gene in seedling leaves. Gene. 283: 227-236.

Agriculture Canada and Environment Canada. 1988. Pesticide registrant survey 1988 report. Pesticides Directorate of Agriculture Canada and Commercial Chemicals Branch of Environment Canada, Ottawa. Confidential rept.

Ashton, F.M. and Crafts, A.S. 1981. Mode of Action of Herbicides. John Wiley and Sons, Inc.: Toronto, Canada.

Berkelman, T. and Stenstedt, T. 1998. 2-D electrophoresis: Principles and methods. Amersham Biosciences Handbook.

Bjellqvist, B., Ek, K., Righetti, P.G., Gianazza, E., Gorg, A., Westermeier, R. and Postel, W. 1982. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J. Biochem. Biophys. Methods. 6: 317-339.

Bottrill, A.R. 2000. High-energy collision-induced dissociation of macromolecules using tandem double-focusing/Time-of-Flight mass spectrometry. Ph.D. Thesis, University of Warwick.

Bourdot, G.W., Harrington, K.C. and Popay, A.I. 1989. The appearance of phenoxy-herbicide resistance in New Zealand pasture weeds. In *Proceedings of the British Crop Protection Conference – Weeds*. 1: 309-316.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Callihan, R.H., Prather, T.S. and Northam, F.E. 1989. Invasion by yellow starthistle. In *Proceedings of the 1989 Knapweed Symposium*. Montana State University, EB. 45: 73-78.

Caux, P.Y., Kent, R.A., Tache, M., Grande, C., Fan, G.T. and MacDonald, D.D. 1993. Environmental fate and effects of dicamba: a Canadian perspective. Rev. Env. Contam. Toxic. 133: 1-58.

Chen, L.G., Switzer, C.M. and Fletcher, R.A. 1972. Nucleic acid and protein changes induced by auxin-like herbicides. Weed Sci. 20: 53-55.

Chen, J.G., Ullah, H., Young, J.C., Sussman, M.R. and Jones, A.M. 2001. ABP1 is required for organized cell elongation and division in *Arabidopsis* embryogenesis. Genes Dev. 15: 902-911.

Chernushevich, I.V., Loboda, A.V. and Thomson, B.A. 2001. An introduction to quadrupole-time-of-flight mass spectrometry. J. Mass Spectrom. 36: 849-865.

Christensen, S.K., Dagenais, N., Chory, J. and Weigel, D. 2000. Regulation of auxin response by the protein kinase PINOID. Cell. 100: 469-478.

Cobb, A.H. and Kirkwood, R.C. (Eds) 2000. Herbicides and Their Mechanism of Action; CRC Press: Boca Raton, FL.

Corran, P.H. 1989. Reversed-phase chromatography of proteins. In *HPLC of macromolecules, a practical approach*; Oliver, R.W.A., Ed.; IRL Press: Oxford, UK.

Coupland, D., Cooke, D.T. and James, C.S. 1991. Effects of 4-chloro-2-methylphenoxypropionate (an auxin analogue) on plasma membrane ATPase activity in herbicide-resistant and herbicide-susceptible biotypes of *Stellaria media* L. J. Exp. Bot. 42: 1065-1071.

Covey, T.R. 1991. Structural characterization of protein tryptic peptides via liquid chromatography/mass spectrometry and collision-induced dissociation of their doubly charged molecular ions. Anal Chem. 63: 1193-1200.

Davidonis, G.H., Hamilton, R.H. and Mumma, R.O. 1982. Metabolism of 2,4-D in resistant soybean callus. Plant Physiol. 70: 1982.

Davies, P.J. 1987. The plant hormones, their nature, occurrence and functions. In *Plant Hormones and Their Role in Plant Growth and Development*; Davies, P.J., Ed.; Martinus Nijhoff Publishers: Boston, USA.

DeLong, A., Mockaitis, K. and Christensen, S. 2002. Protein phosphorylation in the delivery of and response to auxin signals. Plant Mol. Biol. 49: 285-303.

del Pozo, J.C., Timpte, C., Tan, S., Callis, J. and Estelle, M. 1998. The ubiquitin-related protein RUB1 and auxin response in *Arabidopsis*. Science. 280: 1760-1763.

Deshpande, S. and Hall, J.C. 1995. Comparison of flash-induced light scattering transients and proton efflux from auxinic-herbicide resistant and susceptible wild mustard protoplasts: A possible role for calcium in mediating auxinic herbicide resistance. Biochim. Biophys. Acta. 1244: 69-78.

Devine, M.D., Duke, S.O. and Fedtke, C. 1993. Physiology of Herbicide Action. Prentice Hall Inc.: Englewood Cliffs, USA.

Dey, S., Ramachandra, M., Pastan, I., Gottesman, M.M. and Ambudkar, S.V. 1997. Evidence for two nonidentical drug interaction sites in the human P-glycoprotein. Proc. Natl. Acad. Sci. USA. 94: 10594-10599.

Dharmasiri, N., Dharmasiri, S., Jones, A.M. and Estelle, M. 2003. Auxin action in a cell-free system. Curr. Biol. 13: 1418-1422.

Dolinski, K., Muir, S., Cardenas, M. and Heitman, J. 1997. All cyclophilins and FK506-binding proteins are, individually and collectively, dispensible for viability in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA. 94: 13093-13098.

Dohrmann, U., Hertel, R. and Kowalik, H. 1978. Properties of auxin binding sites in different subcellular fractions from maize coleoptiles. Plant. 140: 97-106.

Dongre, A.R., Somogyi, A. and Wysocki, V.H. 1996. Surface-induced dissociation: an effective tool to probe structure, energetics and fragmentation mechanisms of protonated peptides. J. Mass Spectrom. 31: 339-350.

Estelle, M.A. and Somerville, C. 1987. Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol. Gen. Genet. 206: 200-206.

Fedtke, C. 1982. Biochemistry and Physiology of Herbicide Action. Springer-Verlag: New York, USA.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F. and Whitehouse, C.M. 1989. Electrospray ionization for the mass spectrometry of large biomolecules. Science. 246: 64-71.

Ford, J.C. and Smith, J.A. 1989. Synthetic peptide purification by application of linear solvent strength gradient theory. J. Chrom. 483: 131-143.

Foy, C.L. 1975. Picloram and related compounds. In *Herbicides: Chemistry, Degradation, and Mode of Action, Vol. 2, 2nd ed*; Kearney, P.C. and Kaufman, D.D., Eds.; Marcel Dekker Inc.: New York, USA.

Frear, D.S. 1976. The benzoic acid herbicides. In *Herbicides: Chemistry, Degradation, and Mode of Action, 2nd ed*; Kearney, P.C. and Kaufman, D.D., Eds.; Marcel Dekker Inc.: New York, USA.

Fry, S.C. 1989. Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. Physiol. Plant. 75: 532-536.

Gaedeke, N., Klein, N., Kolukisaoglu, U., Forestier, C. et al. 2001. The *Arabidopsis thaliana* ABC transporter AtMRP5 controls root development and stomata movement. EMBO J. 20: 1875-1887.

Galat, A. 1999. Variations of sequences and amino acid compositions of proteins that sustain their biological functions: An analysis of the cyclophilin family of proteins. Arch. Biochem. Biophys. 371: 149-162.

Garbers, C., DeLong, A., Deruere, J., Bernasconi, P. and Soll, D. 1996. A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in *Arabidopsis*. EMBO J. 15: 2115-2124.

Garfin, D. and Heerdt, L. (Eds) 2003. 2-D electrophoresis for proteomics: A methods and product manual.

Gething, M.J. and Sambrook, J. 1992. Protein folding in the cell. Nature. 355: 33-45.

Glish, G.L. and Vachet, R.W. 2003. The basics of mass spectrometry in the twenty-first century. Nat. Rev Drug Discov. 2: 140-150.

Gonzalez, B., Pajares, M.A., Hermoso, J.A., Guillerm, D., Guillerm, G. and Sanz-Aparicio, J. 2003. Crystal structures of methionine adenosyltransferase complexed with substrates and products reveal the methionine-ATP recognition and five insights into the catalytic mechanism. J. Mol. Biol. 331: 407-416.

Gorg, A., Postel, W. and Gunther, S. 1988. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis. 9: 531-546.

Gorg, A., Postel, W., Gunther, S. and Weser, J. 1985. Improved horizontal two-dimensional electrophoresis with hybrid isoelectric focusing in immobilized pH gradients in the first dimension and laying-on transfer to the second dimension. Electrophoresis. 6: 599-604.

Gorg, A., Postel, W., Weser, J., Gunther, S., Strahler, J.R., Hanash, S.M. and Somerlot, L. 1987. Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. Electrophoresis. 8: 122-124.

Gottlieb, M. and Chavko, M. 1987. Silver staining of native and denatured eucaryotic DNA in agarose gels. Anal. Biochem. 165: 33-37.

Grabski, S. and Schindler, M. 1996. Auxins and cytokinins as antipodal modulators of elasticity within the actin network of plant cells. Plant Physiol. 110: 965-970.

Gray, W.M. and Estelle, M. 2000. Function of the ubiquitin-proteasome pathway in auxin response. Trends Bio. Sci. 25: 133-138.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. 2001. Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. Nature. 414: 271-276.

Hall, J.C., Alam, S.M.M. and Murr, D.P. 1993. Ethylene biosynthesis following foliar application of picloram to biotypes of wild mustard (*Sinapis arvensis* L.) susceptible or resistant to auxinic herbicides. Pestic. Biochem. Physiol. 47: 36-43.

Hall, J.C., Bassi, P.K., Spencer, M.S. and Vanden Born, W.H. 1985. An evaluation of the role of ethylene in herbicidal injury induced by picloram or clopyralid in rapeseed and sunflower plants. Plant Physiol. 79: 18-23.

Hall, J.C. and Romano, M.L. 1995. Morphological and physiological differences between the auxinic herbicide-susceptible (S) and –resistant (R) wild mustard (*Sinapis arvensis* L.) biotypes. Pestic. Biochem. Physiol. 52: 149-155.

Hall, J.C., Webb, S.R. and Desphande, S. 1996. An overview of auxinic herbicide resistance: wild mustard (*Sinapis arvensis* L.) as a case study. In *Molecular Genetics and Evolution of Pesticide Resistance*; Brown, T.M, Ed.; American Chemical Society Symposium Series: Washington, USA.

Hatzios, K.K. and Penner, D. 1982. Metabolism of Herbicides in Higher Plants. Burgess Publishing Co.: Minneapolis, USA.

Heap, I. and Morrison, I.N. 1992. Resistance to auxin-type herbicides in wild mustard (*Sinapis arvensis* L.) populations in western Canada. Weed Sci. Soc. Amer. Abstr. 32: 164.

Hennig, L., Christner, C., Kipping, M., Schelbert, B., Rucknagel, K.P., Grabley, S., Kullertz, G. and Fischer, G. 1998. Selective inactivation of parvulin-like peptidyl-prolyl *cis/trans* isomerases by juglone. Biochem. 37: 5953-5960.

Herbert, B.R., Molloy, M.P., Gooley, A.A., Walsh, B.J., Bryson, W.G. and Williams, K.L. 1998. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis. 19: 845-851.

Hsu, T., McRackan, D., Vincent, T.S. and de Couet, G. 2001. *Drosophila* Pin1 prolyl isomerase Dodo is a MAP kinase signal responder during oogenesis. Nat. Cell Biol. 3: 538-543.

Jackson, K. and Soll, D. 1999. Mutations in a new *Arabidopsis* cyclophilin disrupt its interaction with protein phosphatase 2A. Mol. Gen. Genet. 262: 830-838.

Jasieniuk, M., Morrison, I.N. and Brule-Babel, A.L. 1995. Inheritance of dicamba resistance in wild mustard (*Brassica kaber*). Weed Sci. 43: 192-195.

Karas, M. and Hillenkamp, F. 1988. Laser desorption ionization of proteins with molecular mass exceeding 1000 daltons. Anal. Chem. 60: 2299-2301.

Key, J.L. 1989. Modulation of gene expression by auxin. BioEssays. 11: 52-58.

Kocacaliskan, I. and Terzi, I. 2001. Allelopathic effects of walnut leaf extracts and juglone on seed germination and seedling growth. J. Hort. Sci. Biotech. 76: 436-440.

Larsen, P.B. and Cancel, J.D. 2003. Enhanced ethylene responsiveness in the *Arabidopsis eer1* mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, *RCN1*. Plant J. 34: 709-718.

Larsen, P.B. and Chang, C. 2001. The *Arabidopsis eer1* mutant has enhanced ethylene responses in the hypocotyls and stem. Plant Physiol. 125: 1061-1073.

LeBaron, H.M. 1991. Distribution and seriousness of herbicide-resistant weed infestations worldwide. In *Herbicide Resistance in Weeds and Crops*; Caseley, J.C., Cussans, G.W. and Atkins, R.K.; Eds.; Butterworth-Heinemann Ltd.: Oxford, U.K.

Leyser, O. 2001. Auxin-signalling: the beginning, the middle and the end. Curr. Opin. Plant Biol. 4: 382-386.

Leyser, O. 2002. Molecular genetics of auxin signalling. Annu. Rev. Plant Biol. 53: 377-398.

Loboda, A.V., Krutchinsky, A.N., Bromirski, M., Ens, W. and Standing, K.G. 2000. A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance. Rapid Commun. Mass Spectrom. 14: 1047-1057.

Luckwill, L.C. and Lloyd-Jones, C.P. 1960. Metabolism of plant growth regulators. I. 2,4-Dichlorophenoxyacetic acid in leaves of red and black currant. Ann. Appl. Biol. 48: 613-675.

Luschnig, C. 2002. Auxin transport: ABC proteins join the club. Trends Plant Sci. 8: 329-332.

Lutman, P.J.W. and Lovegrove, A.W. 1985. Variations in the tolerance of *Galium aparine* (cleavers) and *Stellaria media* (chickweed) to mecoprop. In *Proceedings of the British Crop Protection Conference – Weeds*. 1: 411-418.

Macarthur, M.W. and Thornton, J.M. 1991. Influence of proline residues on protein conformation. J. Mol. Biol. 218: 397-412.

Macdonald, H. 1997. Auxin perception and signal transduction. Physiologia Plantarum. 100: 423-430.

McLuckey, S.A. 1992. Principles of collisional activation in analytical mass spectrometry. J. Am. Soc. Mass Spectrom. 3: 599-614.

Migo, T.R., Mercado, B.L. and DeDatta, S.K. 1986. Response of *Sphenoclea zeylanica* to 2,4-D and other recommended herbicides for weed control in lowland rice. Philippine J. Weed Sci. 13: 28-38.

Mockaitis, K. and Howell, S.H. 2000. Auxin induces mitogenic activated protein kinase (MAPK) activation in roots of *Arabidopsis* seedlings. Plant J. 24: 785-796.

Molloy, M.P., Herbert, B.R., Walsh, B.J., Tyler, M.I., Traini, M., Sanchez, J.C., Hochstrasser, D.F., Williams, K.L. and Gooley, A.A. 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis. 19: 837-844.

Muday, G.K. and Murphy, A.S. 2002. An emerging model of auxin transport regulation. Plant Cell. 14: 293-299.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C. et al. 2000. AXR2 encodes a member of the Aux/IAA protein family. Plant Physiol. 123: 563-573.

Napier, R.M. 1995. Towards and understanding of ABP1. J. Exp. Bot. 46: 1787-1795.

Noh, B., Murphy, A.S. and Spalding, E.P. 2001. Multidrug resistance-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell. 13: 2441-2454.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Ontario Ministry of Agriculture and Food (OMAF). 2003. Publication 75: Guide to Weed Control.

Pandey, A. and Mann, M. 2000. Proteomics to study genes and genomes. Nature. 405: 837-846.

Peniuk, M.G., Romano, M.L. and Hall, J.C. 1993. Physiological investigations into the resistance of a wild mustard (*Sinapis arvensis* L.) biotype to auxinic herbicides. Weed Res. 33: 431-440.

Perdew, G.H., Schaup, H.W. and Selivonchick, D.P. 1983. The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. Anal. Biochem. 135: 453-455.

Peterson, G.E. 1967. The discovery and development of 2,4-D. Agric. History. 41: 244-253.

Rabilloud, T. 1990. Mechanisms of protein silver staining in polyacrylamide gels: A 10-year synthesis. Electrophoresis. 11: 785-794.

Rabilloud, T. 1998. Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis. 19: 758-760.

Rahfeld, J.U., Schierhorn, A., Mann, K.H. and Fischer, G. 1994. A novel peptidyl-prolyl cis/trans isomerase from *Escherichia coli*. FEBS Lett. 343: 65-69.

Rashotte, A.M., DeLong, A. and Muday, G.K. 2001. Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. Plant Cell. 13: 1683-1697.

Raz, V. and Fluhr, R. 1993. Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell. 5: 523-530.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M. and Leyser, O. 1998. Changes in auxin response from mutations in an AUX/IAA gene. Science. 279: 1371-1373.

Rubery, P.H. 1981. Auxin receptors. Annu. Rev. Plant Physiol. 32: 569-596.

Salisbury, F.B. and Ross, C.W. 1992. Plant Physiology, Fourth ed.; Wadsworth Publishing Company: Belmont, USA.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual (2^{nd} ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA.

Sanchez-Fernandez, R., Davies, T.G.E, Coleman, J.O.D and Rea, P.A. 2001. The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J. Biol. Chem. 276: 30231-30244.

Scherer, G.F.E. and Andre, B. 1993. Stimulation of phospholipase A2 by auxin in microsomes from suspension-cultured soybean cells is receptor mediated and influenced by nucleotides. Planta. 191: 515-523.

Scherer, G., Kramer, M.L., Schutkowski, M.U.R. and Fischer, G. 1998. Barriers to rotation of secondary amide peptide bonds. J. Am. Chem. Soc. 120: 5568-5574.

Schmid, F.X. 2002. Prolyl isomerases. Adv. Prot. Chem. 59: 243-282.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S. et al. 2001. Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TIR1} in mediating auxin response. Science. 292: 1379-1382.

Seol, J.H., Feldman, R.M.R., Zachariae, W., Shevchenko, A., Correll, C.C. et al. 1999. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. 13: 1614-1626.

Shevchenko, A., Chernushevich, I., Ens, W., Standing, K.G., Thomson, B., Wilm, M. and Mann, M. 1997. Rapid '*de Novo*' peptide sequencing by a combination of nanoelectrospray, isotopic labelling and a quadrupole/time-of-flight mass spectrometer. Rapid Commun. Mass Spectrom. 11: 1015-1024.

Shevchenko, A., Loboda, A., Shevchenko, A., Ens, W. and Standing, K.G. 2000. MALDI Quadrupole Time-of-Flight mass spectrometry: A powerful tool for proteomic research. Anal. Chem. 72: 2132-2141.

Sjodahl, S., Rodin, J. and Rask L. 1991. Characterization of the 12S globulin complex of *Brassica napus*: Evolutionary relationship to other 11-12S storage globulins. Eur. J. Biochem. 196: 617-621.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell. 91: 209-219.

Smith, R.D. and Walker, J.C. 1996. Plant protein phosphatases. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 101-125.

Sterling, T.M. and Hall, J.C. 1997. Mechanism of action of natural auxins and the auxinic herbicides. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R.M., Burton, J.D. Kuhr, R.J., Eds.; IOS Press: Amsterdam.

Stewart, D.E., Sarkar, A. and Wampler, J.E. 1990. Occurrence and role of cis peptide bonds in protein structures. J. Mol. Biol. 214: 253-260.

Sussman, M.R. 1988. Purification of integral plasma membrane proteins by reverse-phase high performance liquid chromatography. Anal. Biochem. 169: 395-399.

Theologis, A. 1986. Rapid gene regulation by auxin. Annu. Rev. Plant Physiol. 37: 407-438.

Thompson, L., Slack, C.H., Augenstein, R.D. and Herron, J.W. 1973. Action and fate of 2,4-D and dicamba in trumpetcreeper. Weed Sci. 21: 429-432.

Tiwari, S.B., Wang, X.J., Hagen, G. and Guilfoyle, T.J. 2001. AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell. 13: 2809-2822.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. 1997. ARF1, a transcription factor that binds to auxin response elements. Science. 276: 1865-1868.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. 1999. Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA. 96: 5844-5849.

Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L. and Clardy, J. 1993. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. J. Mol. Biol. 229: 105-124.

van Veen, H.W., Margolles, A., Muller, M., Higgins, C.F. and Konings, W.N. 2000. The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism. EMBO J. 19: 2503-2514.

Vogler, H. and Kuhlemeier, C. 2003. Simple hormones but complex signalling. Curr. Opin. Plant Biol. 6: 51-56.

Vydac. 1995. The handbook of analysis and purification of peptides and proteins by reversed-phase HPLC, 2nd ed.

Walker, L. and Estelle, M. 1998. Molecular mechanisms of auxin action. Curr. Opin. Plant Biol. 1: 434-439.

Wang, N.N., Yang, S.F. and Chang, Y. 2001. Differential expression of 1-aminocyclopropane-1-carboxylate synthase genes during orchid flower senescence induced by the protein phosphatase inhibitor okadaic acid. Plant Physiol. 126: 253-260.

Webb, S.R. and Hall, J.C. 1995. Auxinic herbicide-resistant and –susceptible wild mustard (*Sinapis arvensis* L.) biotypes: effect of auxinic herbicides on seedling growth and auxin-binding activity. Pestic. Biochem. Physiol. 52: 137-148.

Wehmeyer, N., Hernandez, L.D., Finkelstein, R.R. and Vierling, E. 1996. Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. Plant Physiol. 112: 747-757.

Woo, E.J., Marshall, J., Bauly, J., Chen, J.G., Venis, M., Napier, R.M. and Pickersgill, R.W. 2002. Crystal structure of auxin-binding protein 1 in complex with auxin. EMBO J. 21: 2877-2885.

Worley, C.K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A., and Callis, J. 2000. Degradation of Aux/IAA proteins is essential for normal auxin signalling. Plant J. 21: 553-562.

Yaffe, M.B., Schutkowski, M., Shen, M.H., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J. et al. 1997. Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. Science. 278: 1957-1960.

Zaina, S., Reggiani, R. and Bertani, A. 1990. Preliminary evidence for involvement of GTP-binding protein(s) in auxin signal transduction in rice (*Oryza sativa* L.) coleoptile. J. Plant Physiol. 136: 653-658.