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Expression Analysis of Plant Defense Responses during the Establishment of Biotrophy and Role of Abiotic Stress in the Infection of Dyer's Woad (*Isatis tinctoria*) by *Puccinia thlaspeos*

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EXPRESSION ANALYSIS OF PLANT DEFENSE RESPONSES DURING THE

ESTABLISHMENT OF BIOTROPHY AND ROLE OF ABIOTIC STRESS

IN THE INFECTION OF DYER'S WOAD (*ISATIS*

TINCTORIA) BY *PUCCINIA THLASPEOS*

by

Elizabeth Thomas

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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2008

ABSTRACT

Expression Analysis of Plant Defense Responses during the Establishment of Biotrophy

and Role of Abiotic Stress in the Infection of Dyer's Woad (*Isatis tinctoria)*

by *Puccinia thlaspeos*

by

Elizabeth Thomas, Doctor of Philosophy

Utah State University, 2008

Major Professor: Dr. Bradley R. Kropp
Department: Biology

The kinetics and amplitude of the salicylic acid-responsive pathogenesis-related (*PR*) genes and the cytochrome P450 gene *ItCYP79B2* in the compatible interaction between *Puccinia thlaspeos* and dyer's woad (*Isatis tinctoria*) during the first 72 hours of inoculation were examined. Immediately following penetration of the host by the rust pathogen, there was a modest up-regulation of *PR* genes but a significant down-regulation of *ItCYP79B2* expression. During haustoria formation, a significant pathogen-mediated suppression of *PR* genes was observed with a corresponding up-regulation of *ItCYP79B2*. This potentially facilitates haustoria formation by *P. thlaspeos*. After haustoria formation, a more significant up-regulation of *PR* genes was observed that was followed by a second pathogen-induced suppression of both *PR* and *ItCYP79B2* genes.

This suppression of defense responses by the pathogen was sustained and was potentially responsible for successful infections in dyer's woad.

Exogenous application of salicylic acid was done to experimentally trigger the expression of defense-related genes in dyer's woad. In treatments not involving the pathogen, exogenous application of salicylic acid led to rapid activation of defense responses. In treatments involving both salicylic acid and the rust pathogen, a differential response was observed based on the timing of salicylic acid application. During the pre-haustorial and post-haustorial phases, the up-regulation of defense genes by salicylic acid application increased protection against rust. However, salicylic acid application during the haustorial formation could not override the pathogen-mediated suppression of defense responses. Suppression of pathogen-induced defense responses during and after haustoria formation is postulated to be vital in the establishment of biotrophy in this system.

In addition, the effects of varying levels of a number of different abiotic stresses (oxidative, salt, osmotic, dehydration, and cold stresses) on the host during the infection process were examined. While moderate-to-severe levels of salinity, osmotic, dehydration, and cold stress did not decrease infection, mild abiotic stress appears to help dyer's woad develop cross-tolerance to the rust pathogen, thereby affecting its efficacy as a biocontrol agent.

(163 pages)

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ABA   abscisic acid

Avr   avirulence

BTB-POZ   broad-complex, tramtrack, bric-a-brac/poxvirus, zinc finger

ChiA   acidic endochitinase

CHIB   chitinase B

COI1   coronatine insensitive 1

C_t threshold cycle

CYP   cytochrome P450 gene family

EDS   enhanced disease susceptibility

ET   ethylene

FAD   fatty acid desaturase

GST   glutathione-*S*-transferase

HEL   hevein-like protein

HR   hypersensitive response

I   inhibitor of avirulence

IAA   indole acetic acid

IAOx   indole-3-acetaldoxime

ICS1   isochorismate synthase 1

JA   jasmonic acid

JAR   jasmonic acid resistant

LOX	lipoxygenases
NPR1	nonexpresser of PR genes 1
PAD4	phytoalexin deficient 4
PCR	polymerase chain reaction
PDF1.2	plant defensin 1.2
PR	pathogenesis related
qPCR	quantitative real time polymerase chain reaction
R	resistance
ROS	reactive oxygen species
RT	reverse transcription
SA	salicylic acid
SAR	systemic acquired resistance
S-GT	S-glucosyltransferase
SID2	salicylic acid induction deficient 2
ST	sulfotransferase
THI2.1	thionin 2.1
TIR-NBS-LRR	Toll/Interleukin-1 receptor-nucleotide binding site- leucine-rich repeats
T_m	melting temperature

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Dyer's woad (*Isatis tinctoria* L.) is a biennial or short-lived perennial that belongs to the Brassicaceae. It was introduced into North America from Europe where it was initially raised as a source of blue dye during the colonial period (127). After arriving in the western United States, it has spread rapidly and is currently designated as a noxious weed in nine states (15, 26, 38, 39, 127, 141). Dyer's woad is endowed with good drought tolerance, high fecundity, and allelopathy, making it a formidable invasive species (141). Because it often invades remote and inaccessible terrain of low economic value, biological control may be the only viable option for arresting its spread.

During the 1970's, an indigenous species of rust fungus, *Puccinia thlaspeos* C. Schub. was found to cause rust disease on woad (84). *P. thlaspeos* is an autoecious, microcyclic rust that requires only one host (dyer's woad) to complete its life cycle (3). The first sign of infection is the yellow-orange spermatia produced in early spring followed by dark-brown teliosori as the season progresses. Diseased plants are stunted, chlorotic, malformed, and seed production is almost entirely eliminated. Because *P. thlaspeos* severely limits seed production and thus restricts the spread of dyer's woad, it has the potential of being an ideal biocontrol agent (69, 70, 71, 72, 73).

Members of the genus *Puccinia* cause some of the most devastating plant diseases in the world and are generally highly specialized biotrophs (97). Some of the characteristic features of biotrophy are: i) the development of a sophisticated infection structure, the appresorium, which helps in penetration of the host; ii) the development of haustoria that functions in the biotroph's nutrient absorption and metabolism; iii) the

presence of carbohydrate- and protein-enriched matrices that separate fungal and plant plasma membranes; iv) the durable suppression of host-defense responses; and v) the limited secretion of lytic enzymes, resulting in limited damage to host cell machinery (90).

In responding to a pathogen such as a rust, plants display a complex arsenal of defense responses that enable them to withstand the insult. Plants possess at least three genetically-unique inducible defense responses (53). Although distinct, these responses overlap partially, and should be considered as components of a large signaling network. They can be categorized into: i) resistance (R) genes associated with gene-for-gene responses; ii) salicylic acid (SA)-dependent responses; and iii) jasmonic acid (JA)- and ethylene (ET)-dependent responses. In addition, members of the Brassicaceae, including the model plant *Arabidopsis thaliana* and also *Brassica napus* (canola), possess a characteristic defense system. This brassicaceous defense response includes amino acid-derived secondary metabolites called glucosinolates, which are accompanied by α-thioglucoside glucohydrolase enzymes called myrosinases. In the following sections, each of these defense responses will be discussed in turn.

GENE-FOR-GENE RESISTANCE RESPONSE

Flor's postulation of the gene-for-gene theory of disease resistance is considered seminal in the field of plant pathology (44, 45). This theory proposed that successful disease resistance is triggered only if a resistance (R) gene product in the plant recognizes a specific avirulence (*Avr*) gene product from the pathogen. Pathogens that are recognized in this manner are called avirulent pathogens because they are unable to

initiate disease in the host plant and their interaction with the resistance (*R*) gene product is an incompatible one. However, non-recognition of the pathogen due to either the absence of the *Avr* gene in the pathogen or the absence of the corresponding *R* gene in the plants, leads to a compatible interaction. In a compatible interaction, the host is susceptible while the pathogen is virulent. In addition, a third type of interaction has been reported involving a dominant *Inhibitor of avirulence (I)* gene, which modifies the outcome of a specific *R-Avr* gene interaction from resistance to susceptibility (37, 63, 64, 77).

Several *R* genes and *Avr* genes have been identified to date. The majority of *R* genes identified thus far encode proteins that contain a predicted nucleotide binding site (NBS) that is followed by a series of leucine-rich repeats (LRR) at their C termini (9). The Avr protein is highly variable, indicating the extent to which plants are probed by a pathogen in its quest for a susceptible host (102). For instance, many of the *Avr* genes in bacterial pathogens encode type-III effectors which are essential for infection in hosts lacking the corresponding *R* genes (1). The first Avr protein identified from a rust was a small 127-amino acid mature peptide, AvrL567, from the flax rust *Melampsora lini* (29). The AvrL567 from flax rust is recognized by flax R proteins including L5, L6, and L7 NBS-LRR. Though the *R* gene and *Avr* gene have been cloned, the *I* gene has not yet been cloned (37).

Flor proposed two modes for pathogen recognition of avirulence products by plants, one direct and the other indirect (46). The plant R proteins can function by directly detecting the corresponding pathogen Avr protein in which the former functions as the receptor and the latter functions as the ligand. This direct interaction between

proteins leads to activation of resistance responses and has been called the "receptor-ligand model." This type of direct interaction was demonstrated in a study conducted by Dodds et al. (28), who examined the interaction between flax (*Linum usitatissimum*) and the flax rust fungus (*Melampsora lini*). In contrast to this, plant R proteins can function by perceiving alterations in plant components that are targeted by Avr proteins. This type of indirect interaction between the R protein and the Avr protein that results in the resistance response in the plant is often called the "guard hypothesis" (21, 87). For instance, in *Arabidopsis*, the R protein RPS2 in the plant detects the AvrRpt2-mediated elimination of *Arabidopsis* RIN4 (5, 85).

Recognition of the pathogen leads to the activation of host defense responses. There are two distinctive plant responses which signify the activation of gene-for-gene interaction. The first response involves the rapid production of reactive oxygen species (ROS) referred to as the oxidative burst that functions as a signal for the activation of other defense responses. The second of these responses is localized cell-death or the hypersensitive response (HR). It has been suggested that, in the absence of specific recognition, the plant defense response to a virulent pathogen referred to as basal resistance is activated to a certain degree and helps limit disease development (102). Furthermore, it has been proposed that many of the defense reactions that are in play during resistance responses are also seen during susceptible responses, albeit with altered timing and reduced magnitude. For instance, both virulent and avirulent pathogens are able to elicit an initial low-amplitude ROS accumulation. However, an avirulent pathogen elicits a sustained and higher magnitude accumulation of ROS which helps mount a successful defense (76). Development of HR is believed to limit the access of nutrients

and water to an obligate biotroph, thereby curtailing pathogen growth. HR also helps

activate the SA-dependent defense response.

SA-DEPENDENT SIGNALING

Pathogen attack leading to HR is believed to trigger the activation of SA-

dependent signaling. At the infection sites, increases in the levels of SA have been

reported during both compatible and incompatible interactions (112). Increases in SA

levels lead to the activation of various defense-related genes such as *PR-1*. The role

played by the phytohormone SA in local and systemic acquired resistance (SAR) has

been well-established (32, 86, 135). The requirement for SA in the expression of the *PR-*

1 gene and SAR was demonstrated by studying transgenic plants encoding *NahG* (22, 47,

78). The bacterial salicylate hydroxylase encoded by *NahG*, destroys SA by converting it

to catechol. In addition, two genes, *EDS1* (*enhanced disease susceptibility 1)* and *PAD4*

(*phytoalexin deficient 4*), are required for SA accumulation. These genes encode proteins

similar to triacyl-glycerol lipases (41, 61). EDS1 is found downstream of TIR-NBS-LRR

type *R* genes (43). It is necessary for development of HR, and is required early in plant

defense independently of PAD4. EDS1 and PAD4 have been found to interact with each

other leading to the accumulation of SA and subsequently to the intensification of the

defense response (43).

The majority of SA produced in *Arabidopsis* is produced from the shikimate

pathway and involves the chloroplast-localized isochorismate synthase 1 (ICS1) encoded

by *SID2* (136). However, since SA production is not completely abolished in the *SID2*

mutant, it has been proposed that SA production may involve the phenylpropanoid

pathway to a limited extent (91, 136). Movement of SA from the chloroplast to the cytoplasm is facilitated by a chloroplast-localized transmembrane protein encoded by *EDS5* (91, 100). EDS5, a member of the MATE family transporter is activated by pathogen attack leading to the production of SA (100).

Downstream from SA is *NPR1* (*nonexpresser of PR genes 1*). In the absence of the pathogen, low levels of *NPR1* are expressed throughout the plant. However, studies have found that pathogenic infection or treatment with SA led to a two- to three-fold increase in the levels of NPR1 (16, 113). At low levels of SA, NPR1 occurs in an oligomeric form in the cytoplasm. However as the levels of SA increase, reduction of disulfide linkages holding the oligomers together takes place leading to their disassociation into monomers. The NPR1 monomers move to the nucleus, where it interacts with TGA-type transcription factors (23, 24, 42, 62, 68, 98, 142). The NPR1 protein consists of an ankyrin-repeat domain, a BTB-POZ (*Broad-Complex, Tramtrack, Bric-a-brac/Poxvirus, Zinc finger*) domain, and two protein-protein interaction domains (16, 113). In addition, it also has a putative nuclear localization signal and phosphorylation sites (16, 113). The ankyrin-repeat domain, found in the middle of the NPR1 protein, is essential for binding TGA factors while the N-terminal region enhances binding (24, 142, 145).

The TGA factors bind to activator sequence-1 (*as-1*) or *as-1*-like promoter elements found in promoters of several plants (65). In *Arabidopsis*, NPR1 was found to interact with TGAs 2, 3, 5, 6, and 7 (24, 67, 142, 143, 145). The activation of *PR-1* expression by SA requires TGAs 2, 5, and 6 (143). On the other hand, during pathogen infection, the transcription factor WRKY70 is required for the activation of *PR-1*

expression. Although WRKY70 is SA-inducible and NPR-1 dependent, direct interaction between WRKY70 and NPR1 has not been reported (81). The sequence of events following pathogen recognition is depicted in Fig. 1.1.

Even though NPR1 has shown to be a positive regulator of *PR* genes, it can also play a role in the inhibition of transcriptional repressor. In the absence of SAR, SNI1 was found to prevent PR gene expression indicating that it is a negative regulator (82). SNI1 is not known to have a DNA-binding domain. This implies that repression of PR genes is brought about by interaction with factors other than binding to a promoter (31).

A few SA-dependent, but *NPR1*-independent, defense responses have been observed indicating the existence of a different branch of the SA-signaling pathway (19, 126). For instance, the transcription factor AtWhy required for SA induction of *PR-1* was induced by the pathogen *Peronospora parasitica* and by SA treatment. However, this induction was found to be *NPR1*-independent (25). From this it could be concluded that both the *NPR1*-dependent and *NPR1*-independent branches of the SA-dependent pathways are involved in SA-induced *PR-1* expression.

JA- AND ET-DEPENDENT SIGNALING

In addition to the classic SA-mediated SAR pathway, other signaling molecules such as jasmonic acid (JA) and ethylene (ET) have been implicated in plant defense responses (7). Jasmonic acid and its volatile methyl ester, methyl jasmonate (MeJA), are jointly referred to as jasmonates. Jasmonates are oxylipins that are derived from linolenic acid. They are released from the chloroplast membranes by lipase enzymes and later oxygenated to hydroperoxide-derivatives by lipoxygenases (LOXs) (132).
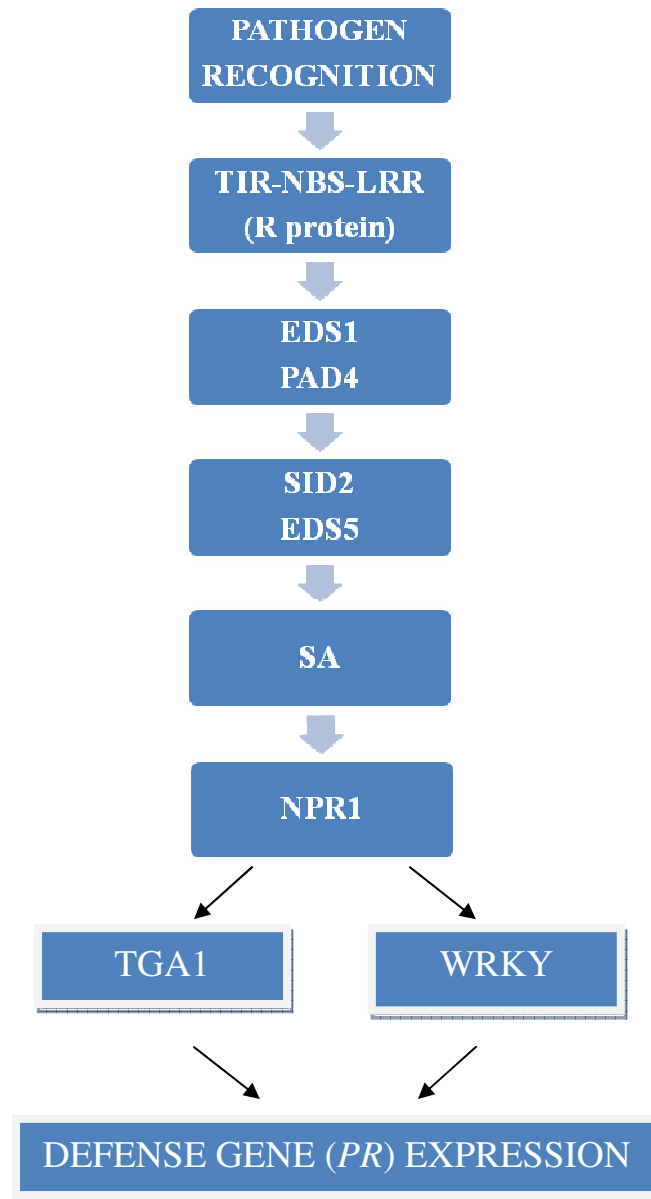
Fig. 1.1. A simplified model depicting the activation of plant defense responses following pathogen recognition. Abbreviations: TIR-NBS-LRR, Toll/Interleukin-1 receptor-nucleotide binding site- leucine-rich repeats; EDS, enhanced disease susceptibility; PAD, phytoalexin deficient; SID2, salicylic acid induction deficient 2; SA, salicylic acid; NPR1, nonexpresser of PR genes 1; PR, pathogenesis-related.

JAs influence several vital physiological processes, including plant defense against pathogens and insects, wound responses, and fertility (17). In addition, they also help plants to cope with environmental stresses, such as drought, low temperature, and salinity (17, 133). In response to pathogen attack, the level of JA synthesis increases leading to the expression of defense effector genes. These genes include *PLANT DEFENSIN 1.2* (*PDF1.2*) which encodes an antimicrobial defensin (106). Induced expression of *PDF1.2* also requires ethylene. Mutants of *A. thaliana* impaired in JA production or JA perception displayed enhanced susceptibility to necrotrophic pathogens such as *Alternaria brassicicola, Pythium sp.,* and the bacterial pathogen, *Erwinia carotovora.* An example of an *A. thaliana* mutant impaired in JA production is the triple mutant of *fatty acid desaturase* (*fad3/fad7/fad8*) while examples of *A. thaliana* mutants impaired in JA perception are the *jasmonic acid resistant 1* (*jar1*) and the *coronatine insensitive 1* (*coi1*) (103, 120, 123, 130). Other predominant JA-dependent genes which encode PR proteins include *thionin 2.1* (*THI2.1*), *hevein-like protein* (*HEL*), and *chitinase B* (*CHIB*). These genes along with *PDF1.2* are used to monitor JA-dependent defense responses (110).

Regulation of JA levels is brought about by cellulose synthases found in the plant cell wall. This was demonstrated by the use of cellulose synthase mutant *cev1* that exhibits constitutively high levels of JA and JA-dependent gene expression (36). The JA amino synthetase encoded by *JAR1* forms conjugates between JA and several amino acids including isoleucine, valine, and leucine. A JA-isoleucine conjugate may possibly be the active form of JA (117). In *Arabidopsis,* JA also requires the function of *COI1*; this encodes an F-box protein that is reported to act in proteolysis (140).

On the other hand, the role of ethylene (ET) is rather controversial. In certain interactions, it was responsible for inducing a response to *Botrytis cinerea* (124) and to *Erwinia carotovor*a (103). However, in other interactions, it was found to promote disease production by *Pseudomonas syringae* or *Xanthomonas campestris* pv. *campestris* (10). Both JA- and ET-signaling pathways are required for the induction of induced systemic resistance (ISR) that is activated by the root-colonizing bacterium *P. fluorescens* (107).

INTERACTIONS BETWEEN SA AND JA/ET SIGNALING

The interactions between SA and JA signaling appear to be extensive and complex with evidence for both positive and negative interactions between these pathways (53, 74, 114). However, the predominant interaction between these pathways appears to be that of antagonism. For instance, the antagonism between SA and JA signaling has been documented in tomato (27, 30). Studies have shown that the EDS4 and PAD4 mutants, that are impaired in SA accumulation, displayed enhanced responses to JA-mediated gene expression inducers (55). Studies also suggest that JA antagonizes SA signaling (101). Treatment of tobacco plants with elicitors from *E. carotovora* both activates JA signaling and inhibits SA signaling (129). Infection of transgenic plants carrying *NahG* (blocked in SA accumulation) with *P. syringae* led to enhanced JA-induced gene expression (116). Repression of JA by SA-induced expression is mediated by NPR1 through a cytosolic function. This is in contrast to the nuclear function that appears in SA-induced expression (116). Infection with *P. syringae*, which induces SA-mediated defense responses, made the plants more susceptible to *Alternaria brassicicola*.

This was due to the suppression of the JA signaling pathway brought about by NPR1 (115). The transcription factor WRKY70 is also believed to play a role in the antagonism observed between these two signaling pathways. Over-expression led to constitutive SA signaling, while antisense suppression led to the activation of COI1-dependent genes (81).

GLUCOSINOLATES AND PLANT DEFENSE

Apart from the SA and JA/ET signaling pathways present in most plants, members of the Brassicaceae also possess a unique defense system. This system is comprised of nitrogen- and sulfur-containing secondary metabolites, called glucosinolates. These secondary metabolites are produced by members of the order Capparales, including families such as the Brassicaceae, Gyrostemonaceae, and Capparaceae (111). As many as 120 glucosinolates have been identified so far, all of which share a common chemical structure comprising a β–D-glucopyranose residue linked to (Z)-N-hydroximinosulfate through a thioester bond (40). A variable R group is also present that is derived from one of eight amino acids.

There are three categories of glucosinolates: aliphatic, aromatic, and indolic. The aliphatic glucosinolates are derived from alanine, leucine, isoleucine, methionine, or valine while the aromatic glucosinolates are derived from phenylalanine or tyrosine. The indolic glucosinolates are derived from tryptophan. Some of the predominant indolic forms include glucobrassicin, neoglucobrassicin, and glucobrassicin-1-sulfonate (56).

The biosynthesis of glucosinolates can be divided into three separate stages (56). In the first stage, chain elongation by insertion of methylene groups is seen in certain

aliphatic and aromatic amino acids. In the second stage, the amino acid moiety undergoes a series of changes to produce the core structure of the glucosinolate. Finally, the R group in the nascent glucosinolates undergoes secondary transformations to produce the biologically active glucosinolate. Modifications in the R groups determine the direction of glucosinolate hydrolysis and the resulting biological activity of these hydrolysis products.

All glucosinolates share common intermediates during the biosynthesis of the core structure. These intermediates include aldoximes (from each of the respective aliphatic or aromatic amino acids, or tryptophan), *aci*-nitro or nitrile oxide compounds, *S*-alkyl thiohydroximates, thiohydroximic acids, and desulfoglucosinolates (Fig. 1.2). The genes involved in each of these steps have been identified with the exception of *S*-alkylation (56). The formation of the core structure of glucosinolates also involves three stages. In the first stage, the amino acids are converted into their respective aldoximes and cytochrome P450 from the CYP79 family is involved in this conversion (137). In the second stage, the aldoximes are converted to thiohydroximic acid via an *aci*-nitro compound and this is mediated by CYP83 (6, 57). In the third stage, the thiohydroximic acids are converted to glucosinolates and this is catalyzed by desulfoglucosinolate sulfotransferases (54, 108).

Indole glucosinolates are biosynthesized from tryptophan and involve the intermediate, indole-3-acetaldoxime (IAOx). Studies have found that CYP79B2 and CYP79B3 are involved in the conversion of tryptophan to IAOx (60, 92). Besides indole glucosinolates, IAOx (synthesized by CYP79 homologs) is involved in the synthesis of the distinctive sulfur-containing indole alkaloids found in the Brassicaceae (Fig. 1.3).
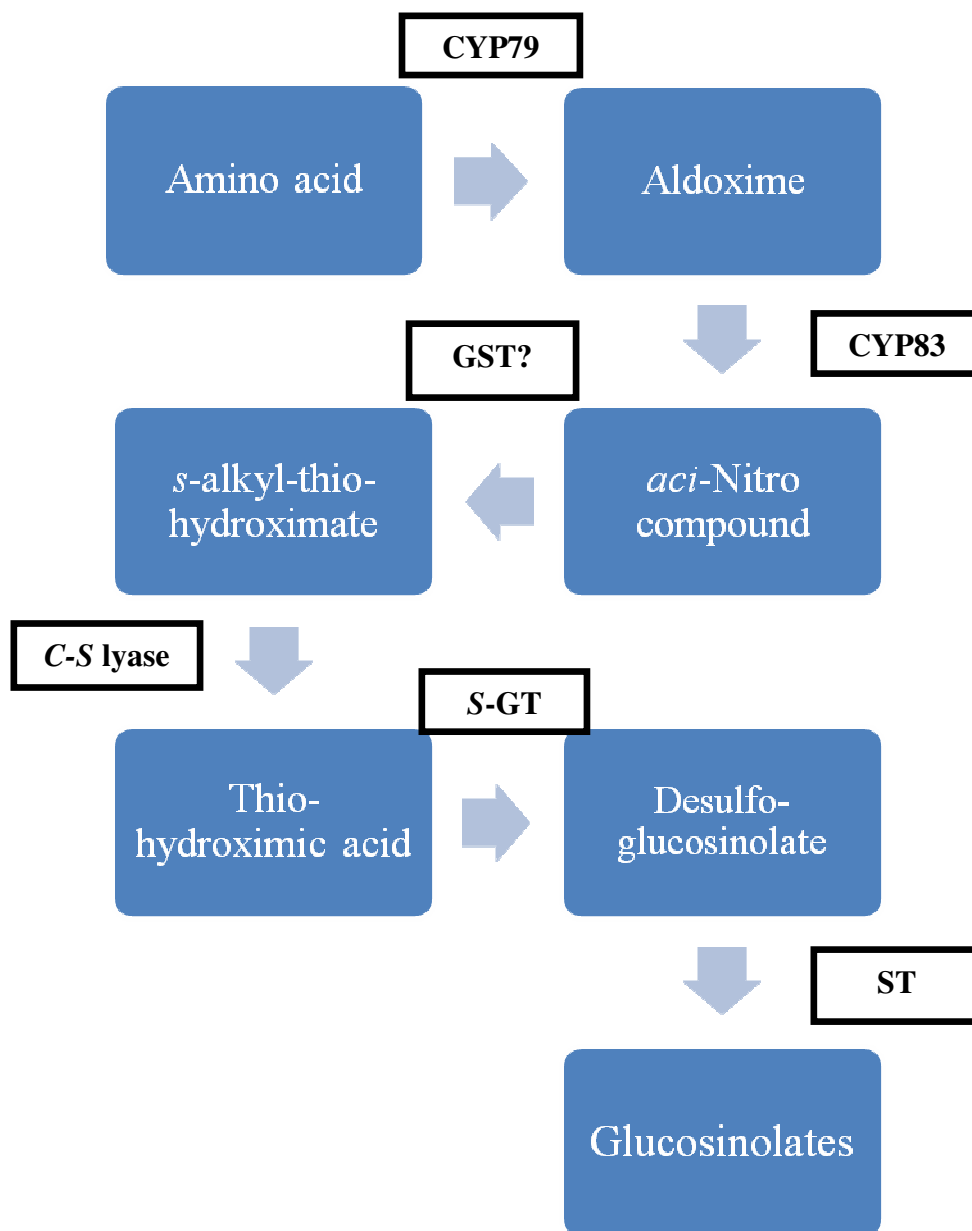
Fig 1.2. Biosynthesis of the core structure of glucosinolates. Abbreviations: GST, glutathione-*S*-transferase; *S*-GT, *S*-glucosyltransferase; ST, sulfotransferase.

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                    │     TRYPTOPHAN      │
                    └─────────────────────┘
                              │
                              │      ╭──────────────╮
                              │      │   CYP79B2    │
                              ▼      │   CYP79B3    │
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                    │     INDOLE-3-       │
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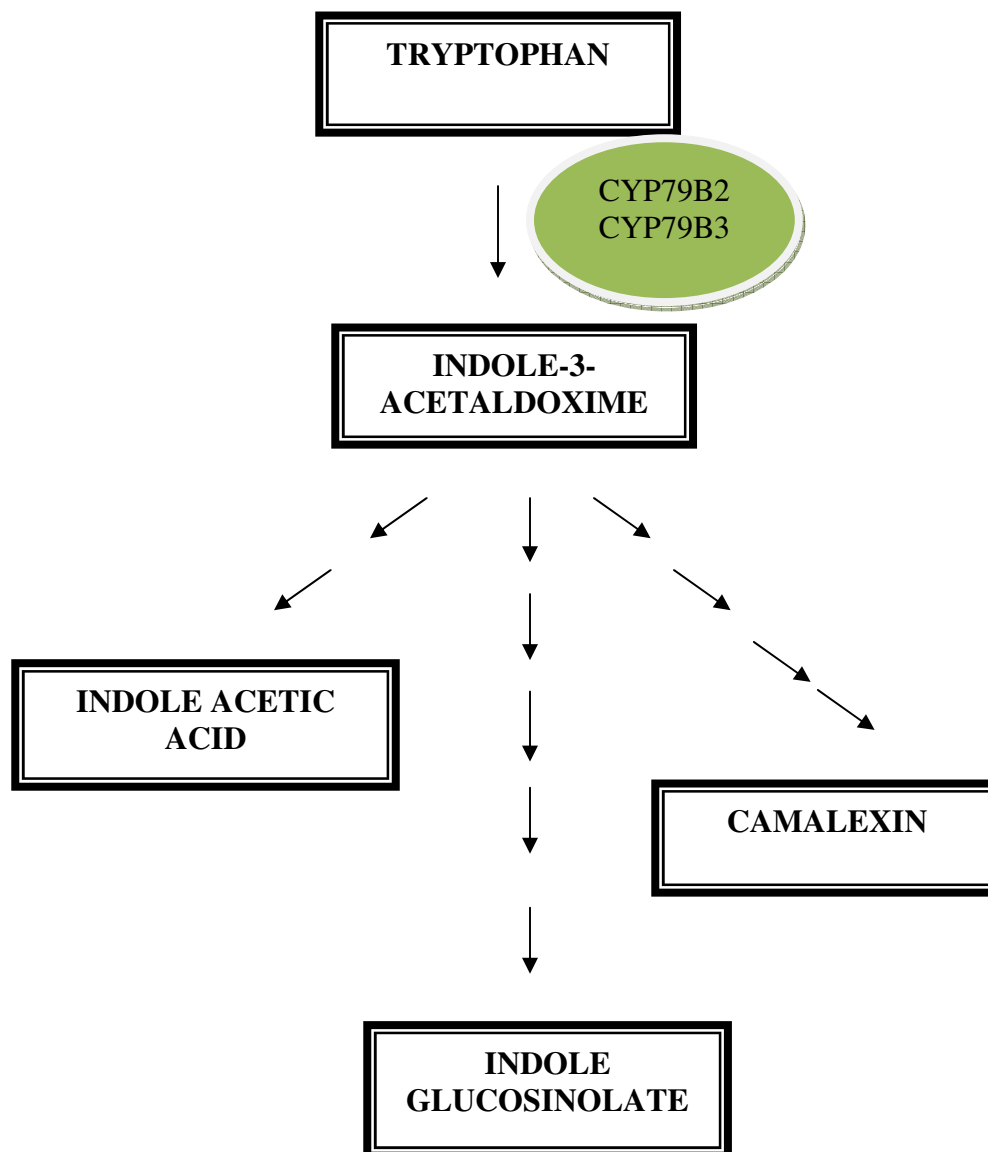
Fig. 1.3. Outline of biosynthetic pathways using IAOx as intermediates, in the synthesis of indole compounds in *Arabidopsis*

For instance, camalexin (49, 50, 51, 99), brassicin, and brassicin-derived phytoalexins (105) are synthesized from IAOx. The literature also suggests that, to a certain extent IAOx contributes to the production of the plant hormone indole-3-acetic acid in *Arabidopsis* (83, 144).

Induction of *CYP79* genes leads to the accumulation of indole glucosinolates and involves JA-dependent defense signaling (93). During defense, the hydrolysis products of glucosinolates are the determinants of the biological activities of these compounds. Hydrolysis of the thioglucoside linkage by myrosinases leads to the formation of glucose and an unstable aglycone (11, 109). The aglycone undergoes further rearrangement and, based on the structure of the side chain, forms different products such as isothiocyanates, nitriles, thiocyanates, and oxazolidinethiones (12, 18, 20). These derivatives are believed to be toxic at low levels to a wide range of organisms that includes plants, fungi, and insects (119, 128, 138, 139).

ABIOTIC STRESS AND PLANT DEFENSE RESPONSE

In the natural environment, plants are often subjected to simultaneous biotic and abiotic stresses. Phytohormones such as SA, JA, ET, and abscisic acid (ABA) regulate the protective responses of plants against biotic and abiotic stresses through cross-talk between the different signaling pathways. The phytohormone ABA plays a critical role in the launching of adaptive responses to various abiotic stresses such as drought, low temperature, and salinity. Further, abiotic stress leads to the accumulation of ABA which influences plant-pathogen interactions (88).

Levels of ABA have been found to vary in response to pathogens (13, 66, 134). Numerous studies have shown that changes in disease response could be produced by altering ABA levels in plants. In these studies, the levels of ABA were altered by the exogenous application of ABA, inhibiting ABA synthesis, or by the use of ABA-deficient mutants (4, 14, 33, 59, 80, 89, 94, 122, 131). It was found that enhanced ABA levels were associated with increased susceptibility while lower ABA levels were associated with increased resistance to several pathogens. Treatment of plants with ABA suppressed phytoalexin synthesis and inhibited the activity and the expression of phenylalanine lyase (59, 89, 131).

In tomato, an ABA-deficient mutant (*sitiens)* exhibited increased resistance to *Botrytis cinerea* that was accompanied by an increase in the activity of phenylalanine lyase (4). However, the exogenous application of ABA not only restored the susceptibility of *sitiens*, but it also increased the susceptibility of wild type plants to *B. cinerea*. Of particular interest is the observation that, in this work, the resistance of tomato against *B. cinerea* depended on SA and not on JA/ET signaling. Hence, exogenous application of the SA-functional analog benzothiadiazole, concomitantly led to increased levels of PR-1 and restored resistance to *B. cinerea* in wild-type plants. A different study found that the *sitiens* mutant was more resistant to *P. syringae* pv. *tomato* which was again SA-mediated (122). Similarly, in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato*, ABA was found to suppress SA and lignin accumulation (95). Taken together, these results suggest that the interaction between the SA and ABA pathways is antagonistic.

During plant development, ABA and ET signaling pathways were also found to interact antagonistically with each other (8, 49). Moreover, high ABA concentrations inhibited ET production (79). In the interaction between the ABA and JA signaling pathways, both synergistic and antagonistic effects have been reported (96, 118). In *Arabidopsis*, high ABA concentrations reduced the transcript accumulation of JA- and ET-dependent defense genes. At the same time, ABA-deficient mutants showed an increase in transcript accumulation of these genes (2). Exogenous application of methyl jasmonate or ET could not overcome the inhibitory effect of ABA. This implies that in plants, the ABA-mediated stress response is dominant over the defense response, and that abiotic stress signaling is able to override the biotic stress signaling when the plant is faced with simultaneous stresses.

JUSTIFICATION FOR THE PRESENT RESEARCH

The rust pathogen *Puccinia thlaspeos*, is an obligate biotroph that causes successful infections on dyer's woad. Numerous studies have examined the *R*-gene-mediated resistance response in plants (28, 29, 52). To a certain degree, nonhost resistances have also been examined (58, 125). In contrast, little attention has been paid to understanding the phenomenon of plant disease susceptibility (compatibility) in rust-host interactions. Two main reasons have been cited in the literature: i) obligate biotrophs such as the rusts and powdery mildews cannot be extensively cultured *in vitro*. Therefore their use in studying plant-biotroph interactions is challenging ii) although *Arabidopi*s has been used to examine resistance responses and nonhost responses, it has no available rust disease (104). In *Arabidopsis*, most of the gene-for-gene interaction studies have

been conducted using *Peronospora parasitica, Erysiphe* sp*,* and *Pseudomonas syringae* (53). Studies have suggested that the responses typical of a resistance response can also be expected to occur during a susceptibility response, albeit with slower kinetics and lower magnitude (75, 121). The infection of dyer's woad (also a member of Brassicaceae) by the rust *P. thlaspeos* provides an ideal platform to examine the dynamics of the susceptibility response.

The rust fungus, *Puccinia thlaspeos* C. Schub. has recently received much attention as a potential biological control agent for dyer's woad. Dyer's woad has also been shown to contain unusually high quantities of indolic glucosinolates (glucobrassicin, neoglucobrassicin, and glucobrassicin-1-sulfonate) (34, 35, 48). Although, the antifungal properties of glucosinolates have been observed in other plant-microbe interactions, it is not known how the rust is able to successfully infect this glucosinolate-producing plant. Though extensive studies have been conducted on the disease etiology, ecology, inoculation techniques, colonization, and dispersal of *P. thlaspeos* (69, 70, 71, 72, 73), very little is known about the effects of the environment on disease initiation. It is important to study the environmental modulation of disease in dyer's woad, since understanding the effect of abiotic stress on the infection process is essential for making predictions about the efficacy of a potential biocontrol agent.

OBJECTIVES

In the current study, the induction kinetics of plant defense responses during the rust infection of dyer's woad were determined along with the effect of abiotic stress on rust infection. The objectives of this research were to:

1) Understand the dynamics of teliospore and basidiospore germination in order to facilitate their use during the rust infection of dyer's woad.

2) Isolate the homologs of *PR-1*, *β-1,3-glucanase*, *ChiA*, *CYP79B2* (involved in synthesis of iodole glucosinolates), and *Actin* (to use as endogenous normalizer in gene quantification) from dyer's woad .

3) Correlate the induction kinetics of these selected defense-related genes with rust penetration, haustoria formation, and host colonization.

4) Induce SA-mediated defense responses by exogenous application of SA prior to and after rust inoculation in order to study the effect this has on long term rust establishment in dyer's woad.

5) Study the effect of different levels of abiotic stresses on rust infection of dyer's woad.

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CHAPTER 2

EXPRESSION ANALYSIS OF SALICYLIC ACID-MEDIATED PLANT DEFENSE

RESPONSES DURING THE ESTABLISHMENT OF BIOTROPHY BY THE

RUST PATHOGEN *PUCCINIA THLASPEOS*

ON DYER'S WOAD[1]

ABSTRACT

This study examined the kinetics and amplitude of the salicylic acid (SA)-responsive pathogenesis-related (*PR*) genes, *PR-1*, *β-1, 3-glucanase*, and *ChiA* in the compatible interaction between *Puccinia thlaspeos* and dyer's woad *Isatis tinctoria* during the first 72 hours of the infection process. Following initial penetration of the host by the rust pathogen, there was a modest up-regulation of *PR* genes. However, during haustoria formation a significant pathogen-mediated suppression of *PR* genes was seen, which potentially facilitates haustoria formation by obligate biotrophs such as rusts. After haustoria formation, there was another more significant up-regulation in each of these genes that was followed by a second pathogen-induced suppression of defense response. This final suppression of defense responses by the pathogen appears to be durable and allows successful infections in dyer's woad.

In order to reprogram the host toward exhibiting a resistance response to the rust pathogen, the defense-related genes were induced by the exogenous application of SA. In

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[1] Coauthored by Elizabeth Thomas, and Bradley R. Kropp, Expression Analysis of Salicylic Acid-Mediated Plant Defense Responses during the Establishment of Biotrophy by the Rust Pathogen *Puccinia thlaspeos* on Dyer's Woad.

uninoculated treatments, exogenous application of SA led to activation of defense responses by 8 hours after treatment. In treatments involving both SA and the rust pathogen, a differential response was observed based on the timing of SA application. The up-regulation of defense genes by the application of SA, during the pre-haustorial and post-haustorial phases, increased protection against rust. However, the application of SA during haustorial formation could not override the pathogen-mediated suppression of defense responses and, consequently, did not offer the host increased protection. In conclusion, suppression of pathogen-induced defense responses during and after haustoria formation is postulated to be vital in the establishment of biotrophy in this system.

INTRODUCTION

Puccinia thlaspeos is a potential biocontrol agent for a noxious weed, dyer's woad (*Isatis tinctoria* L.) which occurs in much of the western United States (4, 8, 13, 14, 32, 33, 34, 35). *P. thlaspeos* is an autoecious and microcyclic rust pathogen that cause disease on members of Brassicaceae (1). When it attacks dyer's woad, it becomes thoroughly colonized during the first growing season, and remains asymptomatic until the second year of its life cycle. When the plants bolt during the second growing season, the leaves become chlorotic and malformed. As the season progresses, spermatia develop on the leaves, followed by dark brown telia as the tissue matures. Bolted plants have twisted flower stalks and produce few seeds, making the rust pathogen a potentially effective biocontrol agent for dyer's woad.

Like all other rusts, *P. thlaspeos* is an obligate biotroph that benefits by keeping

its host alive until the completion of its life cycle. By nature, most obligate biotrophs

cause little damage to the host plant (6, 50), produce limited amounts of lytic enzymes (6,

47), and have a narrow host range (40). The genetic basis of resistance to biotrophs was

examined in the landmark study by H. H. Flor. The gene-for-gene theory of disease

resistance proposed by Flor was based on the interactions between the rust, *Melampsora*

lini, and flax (15, 16). This theory holds that successful disease resistance is triggered

only when a resistance (*R*) gene product in the plant recognizes a specific avirulence

(*Avr*) gene product from the pathogen. R proteins can function directly, by detecting the

corresponding avr proteins (9), or indirectly, by perceiving changes in plant components

that are targets of avr proteins (5, 7, 63). Pathogen recognition by the plant results in the

rapid activation of defense responses that include generation of reactive oxygen

intermediates and onset of cell death associated with the hypersensitive response (HR)

(22). The localized triggering of the HR leads to the induction of defense responses. A

significant response is the synthesis of pathogenesis-related (PR) proteins, which help in

containing the pathogen. The induction of defenses during the HR helps in establishment

of systemic acquired resistance (SAR) (54) that provides the plant with long-term

protection against a wide range of pathogens (11, 42, 58, 62). Salicylic acid is a key

signaling molecule in SAR that is implicated in the accumulation of several PR proteins

(11, 65).

PR proteins can be induced by signaling molecules such as salicylic acid,

ethylene, jasmonic acid, nitric oxide, and hydrogen peroxide. However, plants appear to

primarily use salicylic acid as a signaling molecule in response to obligate biotrophs (17,

60). PR proteins have been found to have antifungal properties *in vitro* through hydrolytic activity of cell walls (43, 56). Currently, there are 17 families of PR proteins that occur widely in plants, and represent a major aspect of plant defense responses (64, 65). They include PR-1 (unknown function), PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4 (chitinase), PR-5 (thaumatin-like), PR-6 (proteinase-inhibitor), PR-7 (endoproteinase), PR-8 (chitinase type III), PR-9 (peroxidase), PR-10 (ribonuclease-like), PR-11 (chitinase type I), PR-12 (defensin), PR-13 (thionin), PR-14 (lipid-transfer protein), PR-15 (oxalate oxidase), PR-16 (oxalate oxidase-like), and PR-17 (unknown function) (64, 65).

Studies have suggested that the changes in gene expression that occur during the *R*-gene mediated resistance response also occur during the susceptibility response. However, during the susceptibility response, the changes in gene expression have altered kinetics and reduced magnitude (38, 59). This could enable the pathogen to either avoid or suppress the induced host defenses that accompany HR. A significant amount of research has been devoted to understanding the *R*-gene mediated resistance upon pathogen recognition (9, 10, 15, 16, 17) and nonhost resistance has been studied to a lesser extent (27, 45, 61). However, in comparison, the susceptibility response to obligate biotrophs in general and the rusts in particular, has not been well defined (50). There are two primary reasons for this: i) *in vitro* cultivation of an obligate biotroph is very difficult; ii) although *Arabidopsis* has been exhaustively used as a model plant to study plant defense responses, it has no known rust pathogens. Thus the rust infection of dyer's woad (a relative of *Arabidopsis*) by *P. thlaspeos* provides an ideal system to explore the susceptibility response to a rust pathogen.

The aim of this study was to examine the response of dyer's woad to penetration and colonization by *P. thlaspeos*. The goals of the work were: i) to understand the dynamics of teliospore and basidiospore germination in order to facilitate rust infections, ii) to isolate homologs of *PR-1*, *β-1,3-glucanase*, and *ChiA* in dyer's woad, iii) to determine the induction kinetics of these three pathogen-inducible *PR* genes during rust penetration, haustoria formation, and colonization of dyer's woad, iv) to study the resistance response in dyer's woad by artificially reprogramming the susceptibility response to that of resistance, by the exogenous application of salicylic acid.

MATERIALS AND METHODS

Growth of Dyer's Woad

Silicles of dyer's woad collected from northern Utah in late summer were threshed manually to obtain seeds. The seeds were stored at 4°C until further use. The seeds were planted in 10 x 10 cm pots that contained steam-sterilized potting mixture, consisting of perlite, peat moss, and vermiculite, mixed in a ratio of 1:1.3:1, respectively. The plants were grown in a greenhouse using a regime of 16 h days at 25°C and 8 h nights at 18°C. Plants were watered until saturated on alternate days. In all treatments, fully expanded leaves from 4-week-old seedlings were used for experimentation.

Spore Calibration

To look at the host defense response to the pathogen, the rust fungus had to be characterized in terms of the number of viable basidiospores produced from each

teliosorus and the germination percentage. This was necessary to enable the consistent application of enough inoculum to produce 100 basidiospores per square millimeter of the host leaf surface with a minimum of 50% germination to all treatments.

Leaf pieces carrying teliosori were surface-sterilized for 2 minutes in 0.6% sodium hypochlorite and then rinsed thoroughly with sterile distilled water. Four leaf pieces, each carrying three teliosori, were then placed equidistantly, with the sori facing upwards in a Petri dish containing 1.5% water agar. A microscopic slide carrying four water agar disks 10 cm in diameter was taped to the inner side of the lid of the Petri dish. The dish containing the teliosori was then turned upside down over the lid with the leaf pieces directly above the water agar disks. This was done so that the basidiospores released during the germination of the teliosori could fall directly on the water agar disks. To germinate the teliospores, the plates containing the inoculum and the water agar disks were incubated in the dark, in a dew chamber at 15°C with parameters set for dew formation. Incubation was done for 8 h, 12 h, 16 h, 24 h, and 48 h, and after each time period, the water agar disks were stained and fixed with 1% lactofuschin. The number of basidiospores deposited on the disks, and the number that had germinated, were estimated by viewing 10 fields on each water agar disk under a microscope, using a 40 X objective lens. This was replicated three times and the average number of basidiospores and the germination count were estimated for each leaf piece carrying three teliosori.

Because the timely and uniform delivery of the basidiospores onto the host surface with minimal of lag time was critical, the effect of an additional incubation period of 6 h on teliospore germination was estimated. After surface-sterilizing the inoculum, it was soaked in sterile distilled water for three minutes and arranged, as before in the Petri

dish containing water agar. This time, it was incubated in the dew chamber at 15°C for 6 h. The dish containing the teliosori was then inverted over the lid carrying the microscope slide and the water agar disks, and was incubated for 8 h, 12 h, 16 h, 24 h, 36 h, and 48 h. At the end of each time period, the agar disks were stained and fixed with the lactofuschin. The total number of basidiospores and the germination percentage were estimated using the microscope.

Artificial Inoculation of Plants

Inoculum was prepared from diseased dyer's woad plants collected in northern Utah during spring. The leaves bearing teliosori were separated from the plants, air dried, and crushed into small fragments. To prevent or reduce extraneous microbial growth, the inoculum was surface-sterilized prior to inoculation with 0.6% sodium hypochlorite, soaked in sterile distilled water for 3 minutes, and rinsed thoroughly. To inoculate plants, the surface-sterilized inoculum was positioned over the surface of a 10-cm-diameter Petri dish containing 1.5% water agar. The leaf pieces were arranged with the teliosori facing upward, and the numbers of teliosori were kept constant for each treatment in order to produce 100 basidiospores per square millimeter of leaf surface. The teliosori were arranged on the agar to ensure that the apical two centimeters of the leaf would be saturated with basidiospores.

After placing the inoculum on the water agar, the plates were initially incubated for 6 h at 15°C ± 1 in a dew chamber to reduce the germination lag period before inoculating the plants. To inoculate plants, the Petri plates were inverted over selected leaves so that the basidiospores from germinating teliospores would land on the upper-

sides of the leaves. Care was taken to ensure that the leaf tissue was not damaged during

inoculation. Each inoculated leaf was tagged to ensure that the correct leaf was monitored

for changes in gene expression following rust infection. The plants and the water agar

were then transferred to the dew chamber for a period of 8 h and incubated in the dark.

Inoculations were always carried out in the night. Following inoculation, the plants were

transferred to the greenhouse, and incubated there until assayed.

Induction of Plant Defense Response with Salicylic Acid

As a positive control for the systemic activation of the pathogenesis-related genes,

dyer's woad seedlings were sprayed with 75mL of 1 mM of salicylic acid (SA) (Sigma,

St. Louis, MO) using a hand-held sprayer. The plants were then incubated for 8 h, 12 h,

16 h, 24 h, 36 h, 48 h, and 72 h under greenhouse conditions until assayed.

Primer Design

Since the sequences for defense-related genes were unknown for *I. tinctoria*,

primers were designed from the sequences of defense-related transcripts of *Brassica*

napus in Genbank (Table 2.1). Primers were designed using the program Primo Pro 3.4

from *B. napus* and *A. thaliana* sequences. After amplification, the double stranded PCR

product was purified using Wizard PCR Preps DNA purification system (Promega Corp.,

Madison, WI). From this purified product, both forward and reverse sequences were

obtained using the dye terminator method with an Applied Biosystems Inc model 3700

DNA sequencer (Foster City, CA). Sequence comparisons against databases were

performed using BLAST and BLASTX algorithms at the National Center for

Biotechnology Information. After ascertaining that the correct sequences were obtained

from *I. tinctoria*, new woad-specific primers were redesigned using Primo Pro 3.4 (Table

2.2). The woad-specific primers were used to amplify cDNA, following which both

forward and reverse sequences were obtained of the woad defense-related genes. After

validation, these primers were used for real time quantitative PCR.

Primer Validation

To avoid genomic DNA contamination, primers for quantitative real time PCR

(qPCR) were designed to span the introns of the relevant gene. However, several defense-

related genes in dyer's woad were found to be without introns. Care was taken to avoid

genomic DNA contamination at the time of RNA extraction and during reverse

transcription. Each PCR product was examined using melt curve analysis to detect any

contamination by genomic DNA.

Primer design strictly adhered to the following guidelines. Runs of a single

nucleotide, particularly those involving four or more guanines were avoided, and no more

than two guanines or cytosines were included in the last 5 nucleotides of the 3' end. To

avoid primer-dimer formation, the sequences of the forward and reverse primers were

both checked to ensure that there was no complementarity at the 3' end. Finally, the

primer pairs chosen were specific to the target gene and did not amplify pseudogenes or

other related genes. To determine the primer concentration that gave the lowest threshold

cycle and minimized nonspecific amplification, amplifications using different

concentrations of forward and reverse primers, ranging from 0.1 μM to 0.9 μM were

carried out.

TABLE 2.1. Primer sequences from *Brassica napus* used for identifying homologous sequences from dyer's woad

Gene	Primer	Primer Pairs	GenBank Accession No
PR-1	BnPR1AF1	CGACTTCTATTAATTTTGGCTG	U21849
	BnPR1AR1	AGGATCATAGTTGCAAGAAATG	
	BnPR1AF2	AATTTTGGCTGCCCTTGTGG	U21849
	BnPR1AR1	AGGATCATAGTTGCAAGAAATG	
β-1,3-glucanase	BnBGLF2	ACCACGGACACGTATCCTCC	AF229403
	BnBGLR2	CAACCGCTCTCTGACACCAC	
	BnBGLF2	ACCACGGACACGTATCCTCC	AF229403
	BnBGLR3	TGGCATTCTCCACACTCGTCC	
ChiA	BnCHAF1	GGGAAATCATCGGCACGTC	AF207560
	BnCHAR1	GTCTAGTTTTAAGGGCACTTC	
	BnCHAF1	GGGAAATCATCGGCACGTC	AF207560
	BnCHAR2	GCACTTCCCATTAGATCGTC	
Actin	BnACTF1	GAACTACGAGCTACCTGACGG	AF111812
	BnACTR1	ACTGTACTTCCTCTCAGGCGG	
	BnACTF2	TGTTGGTCGTCCTAGGCACAC	AF111812
	BnACTR3	TTCCAGGAGCTTCCATCCCC	

Detection of Rust in Infected Plants

Owing to the asymptomatic nature of the rust fungus, detection of the rust pathogen in plant tissues was done using the polymerase chain reaction (PCR). Universal primers that amplify a 620 base pair section at the 5' end of the large ribosomal subunit were used to insure that amplifiable DNA was present in the leaf extracts. To detect infections, rust-specific primers (Table 2.2) were used to selectively amplify a 560 base pair product from *Puccinia thlaspeos*.

TABLE 2.2. Woad-specific primer sequences

Gene	Primer pairs	Primer	Product length
Universal ITS	F63 R635	GCATATCAATAAGCGGAGGAAAAG GGGTCCGTGTTTCAAGACGG	620
Rust ITS	F63 Rust 1	GCATATCAATAAGCGGAGGAAAAG GCTTACTGCCTTCCTCAATC	560
PR-1	ItPR1AF2 ItPR1AR2	CTGCAGACTCGTACACTCC TACACCTCACTTTAGCACATC	210
	ItPR1AF3 ItPR1AR2	CACTCCGGTGGGCCTTAC TACACCTCACTTTAGCACATC	197
β-1,3-glucanase	ItBGLF3 ItBGLR2	CTCTGCTCTTGAATAACTACCC CCAACCGCTCTCTGACACC	214
	ItBGLF4 ItBGLR3	GAACAACATGAACGACATCCC TGGCATTCTCCACACTCGTC	216
ChiA	ItCHAF3 ItCHAR4	TAGGTGATGCTGTTCTTGATG TGGAAATGGACACTGAGGAG	147
Actin	ItACTF3 ItACTR4	GATGAAGCTCAGTCCAAGAG GAGGATAGCATGAGGAAGAG	364
	ItACTF3 ItACTR5	GATGAAGCTCAGTCCAAGAG ACCCTCGTAAATTGGCACAG	340

Extraction of DNA was done using the Extract-N-Amp Plant PCR kit (Sigma, St. Louis, MO). Leaf tissue was cut (0.5 cm diameter) using a standard paper punch and incubated in 100 μl of extraction solution for 10 minutes at 95°C. After vortexing, 100 μl of dilution solution was added to the extract in order to neutralize any inhibitory substances that might be present. The PCR was carried out using the Extract-N-Amp PCR reaction mix that contained Taq polymerase, buffers, salts, dNTPs and JumpStart Taq antibodies for hot start amplification. The final primer concentrations used were 0.4 μM of each of the forward and reverse primers. To set up the PCR, 10 μl of the Extract-

N-Amp PCR reaction mix, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse

primer, 4.4 µl of water, and 4 µl of leaf extract were combined, to create a 20 µl PCR

reaction mix. The amplification parameters used were an initial denaturation cycle at

94ºC for 3 minutes, 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC

for 1 minute, and extension at 72ºC for 2 minutes. Before termination, a final extension

step of 72ºC for 10 minutes was done.

Extraction of RNA

To study induction kinetics of defense-related genes in response to rust infection,

total RNA was extracted from rust infected plants at 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and

72 h after inoculation. Pathogen-free plants that had been mock-inoculated served as

negative controls with RNA being extracted at the same time intervals. RNA was also

extracted on the same schedule from SA-treated plants to serve as the positive control for

the stimulation of SA pathway. Each treatment consisted of three biological replicates.

Total RNA was carried extracted using RNeasy Plant Mini kit (Qiagen, Valencia,

CA). For each treatment, 100 mg of leaf tissue was flash frozen in liquid nitrogen and

ground to a fine powder. Following this, 450 µl of buffer RLT containing

mercaptoethanol was added to the sample, vortexed, and incubated at 56°C for 3 minutes

to disrupt the tissue. The lysate was then pipetted onto a QIAshredder spin column and

centrifuged at maximum speed for 2 minutes. This was followed by the addition of 225

µl of 100% ethanol and the application and centrifugation in an RNeasy column for 15

seconds. The flow-through was discarded and an on-column DNase digestion was carried

out to eliminate genomic DNA contamination. This was followed by the pipetting of 350

µl of buffer RW1 into the RNeasy mini column and centrifuged for 15 seconds.

Following this, a solution of 10 µl of RNase-free DNase I and 70 µl of buffer RDD was

applied directly for 15 minutes onto the silica gel membrane in the column. Next, 350 µl

of buffer RW1 were added to the column and centrifuged for 15 seconds. Subsequently,

the silica gel membrane was washed twice with 500 µl of buffer RPE, followed by

centrifugation each time, for 15 seconds. The RNeasy column was transferred to a new

collection tube and 50 µl of RNase-free water were applied to the column and centrifuged

for 1 minute at 14,000 rpm, in order to elute total RNA in the collection tube. The quality

of RNA was analyzed using the Bio Analyzer (Agilent 2100 Bioanalyzer, Agilent

Technologies, Waldbronn, Germany), and quantified spectrophotometrically, using a

NanoDrop ND100 (NanoDrop Technologies, Wilmington, DE).

Reverse Transcription

Synthesis of cDNA was done using the First Strand cDNA synthesis kit

(Fermentas Inc., Glen Burnie, MD). Briefly, 100 ng of RNA in 10 µl of RNase-free water

was added to 1 µl of oligo(dT) 18 primer and incubated at 70°C for 5 minutes. To this

mixture, 4 µl of 5X reaction buffer, 1 µl of ribonuclease inhibitor (20 U/µl), and 2 µl of

10 mM dNTP mix were added; the resulting mixture was incubated at 37°C for 5

minutes. In the final step, 2 µl of M-MuLV reverse transcriptase (20 U/µl) were added,

bringing the entire reaction mixture to a total volume of 20 µl; this was incubated at 37°C

for one hour. The reaction was stopped by heating at 70°C for 10 minutes. As a negative

control for reverse transcription and to check for the presence of genomic DNA

contamination in the RNA sample, 2 µl of water were added instead of the reverse

transcriptase. Each sample was amplified and checked by electrophoresis to ensure the absence of genomic DNA.

Quantitative Real Time PCR

To quantitate plant defense responses, quantitative real time PCR was carried out by using the Smart Cycler System (Cepheid, Sunnyvale, CA). Real time PCR was conducted using iTaq SYBR Green super mix with ROX (Bio-Rad Laboratories, Hercules, CA). In order to reduce pipetting errors and maximize the precision and accuracy of the assay, a reaction cocktail consisting of forward and reverse primers (0.5 μM for the gene *PR-1*, 0.4 μM for *β-1,3-glucanase*, 0.7 μM for *ChiA*, and 0.5 μM for *Actin*), water, and the 2X iTaq SYBR green mix was set up prior to carrying out the qPCR. Later, 23 μl of the cocktail were aliquoted into each smart cycler tube, to which 2 μl of the target cDNA were added as the final step, just before carrying out the assay. Negative controls without the cDNA template were run with every assay in order to assay for false positive signals. Each treatment at a specific time point consisted of three biological replicates. The target cDNA in each biological replicate that needed to be quantitated was assayed four times, amounting to four technical replications. Thus, each treatment at 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and 72 h consisted of 12 quantitative PCR amplifications (3 biological replicates x 4 technical replicates).

The amplification parameters consisted of one cycle at 95°C for 3 minutes, followed by 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. After amplification, melt curve analysis was performed by heating the reaction mixture from 60°C to 95°C at the rate of 0.2°C per second. Data analysis was performed

using the Smart Cycler software (version 2.0 d). For each sample, a threshold cycle (C_t)

was determined by using the exponential growth phase and the baseline signal. This in

turn, was derived from the plot of fluorescence versus cycle number. Every sample was

reviewed and considered positive only if the fluorescence exceeded the threshold value.

Melt curves which were unique for each gene of interest were transformed to the negative

first derivatives, and were used to identify the specific PCR product.

<center>Relative Quantitation of Gene Expression</center>

The induction kinetics of defense-related genes was studied by the relative

quantitation of gene expression, using the standard curve method (ABI Prism 7700

Sequence Detection System, User Bulletin 2. PE Applied Biosystems, Foster City, CA).

Before changes in gene expression could be computed, passive and active signals were

used to normalize the experimental results. The passive signal used in this study was the

ROX dye that was included in the PCR mix, which helped to normalize for non-PCR

related fluctuations in the generation of the fluorescence signal. The active signal was the

Actin gene, which helped in standardizing the amount of cDNA added in each reaction.

Actin was commonly chosen as the active signal because of its constant expression across

all test samples, and because its expression is usually not affected by the different

treatments in the study.

Quantification of gene expression in this study was done by using the relative

quantitation method. A standard curve was constructed by amplifying a known

concentration of the gene and determining its threshold cycle during quantitative PCR.

For each of the target gene and the endogenous control *Actin*, amplification was carried

out using the gene-specific primers with cDNA. The amplified product was run in a low-melt agarose gel and stained with ethidium bromide. After visualizing the DNA fragment using Ultra Violet (UV) light, it was excised and weighed. The product was purified by using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). For every volume of the agarose gel, 3 volumes of buffer QG were added and incubated at 50°C for 10 minutes. During this period, it was vortexed at intervals of 3 minutes to aid in dissolving the gel. This was followed by the addition of 1 volume of isopropanol to the sample, following which the entire sample was applied to a QIAquick spin column and centrifuged for one minute. The flow-through in the collection tube was discarded, and the column was washed with 0.75 ml of buffer PE and centrifuged for one minute. In order to elute the DNA from the column, 50 µl of water were added, incubated for one minute, and centrifuged for one minute. The quantity of DNA present in each sample was quantified spectrophotometrically using a NanoDrop ND100 (NanoDrop Technologies, Wilmington, Delaware). Prior to its use in constructing the standard curve, the standard curve gene product was sequenced. The concentrated DNA was then diluted 10^6 to 10^{12}-fold to ensure that it was at a concentration that was likely to be found in the biological sample.

In the construction of the standard curve, care was taken to ensure that the range of template concentrations of the unknown sample fell within the linear dynamic range of the known test samples. Typically each standard curve consisted of seven data points within a 2-fold dilution series, each of which, in turn, was amplified in triplicates. The standard curve was constructed by plotting the log of the starting quantity of the known sample against the average C_t values obtained during the amplification of that particular

dilution. After obtaining the standard curve, the equation of the linear regression line, along with Pearson's correlation (r) or the coefficient of determination (R^2) was obtained. The equation of the linear regression line that included the slope and the y-intercept of the standard curve line was then used to calculate the amount of target DNA, based on the threshold cycle generated during amplification.

Standard curves were constructed both for the target gene as well as the endogenous control, *Actin*. For each experimental sample, the amounts of the target and the *Actin* gene were estimated from the relevant standard curve. In the next step, the normalized values were obtained by dividing the amount of the target by the amount of the *Actin* gene. Once the normalized values were obtained, the relative changes in gene expression in rust treatments were computed by dividing the normalized amount in each of these treatments by the normalized amount in the healthy and untreated controls. From these values, the mean value and the standard error of the mean were estimated.

<div align="center">Effect of Salicylic Acid on Rust Infection Rates</div>

The induction of defense responses during key events in the pathogen establishment was examined by the exogenous application of 1 mM SA. SA was applied to 4-week-old dyer's woad plants before, and after rust inoculations and the effect this had on infection rates was studied. To study the effect of SA-induced defense response prior to rust inoculations, SA was applied at 12 h, 8 h, and 0 h prior to plant inoculations with the rust pathogen. Preliminary experiments indicated that SA inhibited basidiospore germination. In treatments where SA was applied either before, or at the time of rust

inoculation, the leaf that was going to be inoculated with the rust pathogen was covered with a plastic bag to ensure basidiospore germination was not affected.

The effect of SA-induced plant defense responses after rust inoculation, and the effect this had on infection rates were also examined. Plants were inoculated with the rust pathogen, following which, SA was applied to the plants at various time points including, 4 h, 8 h, 12 h, and 24 h after rust inoculations. The leaf that was inoculated with the rust pathogen was covered with a plastic bag in order to prevent the basidiospores from being washed off the leaf surface. In control treatments, plants were sprayed with water and inoculated with the rust pathogen. Each treatment had ten plants arranged in a completely randomized design. The plants were incubated for three weeks following which, DNA was extracted from the leaf away from the point of inoculation, and infection rates estimated.

Statistical Analysis

Changes in gene expression were computed using Microsoft Excel 2003 software (Microsoft Corporation, Redmond, WA). Fold changes in gene expression were reported as means ± SE. The results of chemical stimulation of plant defense responses before and after inoculation on infection rates were statistically analyzed using Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS, Chicago, IL). Using Welch's t-test with unequal variance (67), the differences between control groups and various treatment groups were examined. The significance level was set at $p < 0.05$.

RESULTS

Spore Calibration

When each leaf piece carrying three teliosori was incubated for 8 h, it released an average of 113.8 spores per leaf piece of which 18.3% germinated (Table 2.3). When the teliosori were incubated for 12 h, the number of basidiospores released went up to 445.7 of which 52.2% germinated. At an incubation period of 16 h, 793.1 spores were released of which 60.8%, germinated. When the incubation period of the teliosori increased to 24 h, the number of basidiospore was found to be an average of 1092.1 of which 76.6% germinated. Finally, when the teliosori was allowed to be on the water agar plates for 48 h, it led to the release of 1036.3 spores of which 94.3% had germinated.

In the next experiment, the teliosori were surface-sterilized, soaked in sterile distilled water for 3 minutes, and then placed on the water agar for 6 h. After incubation in the dew chamber, the teliosori were inverted to capture the basidiospores on the water agar disks. When individual leaf pieces carrying three teliosori were incubated for 8 h, it released an average of 1018.9 spores with 44.3% germination (Table 2.4). When the teliosori were incubated for 12 h, the number of basidiospores released went up to 1424.3, with 77.6% germination. At an incubation period of 16 h, 1763.2 spores were released, with 80.6% germination. When the incubation period of the teliosori increased to 24 h, the number of basidiospores was found to be an average of 1530.8, with 85.3% germination. At 36 h, 1645.7 were released, with 96.6% germination. Finally, when the teliosori was allowed to be on the water agar plates for 48 h, it led to the release of 1697.5 spores, with 97.9% germination. This experiment showed that surface-sterilized

inoculum soaked for an additional 3 minutes and incubated for an additional 6 h

displayed an increase in germination, when compared to surface-sterilized inoculum

placed directly on the water agar plate.

Isolation of Pathogen-Inducible PR Gene Homologs in Dyer's Woad

Since the homlogs of pathogen-inducible *PR* genes had not been identified in

dyer's woad, primers designed from *Brassica napus* (Table 2.1) were used for identifying

the sequences. The *PR-1* gene in dyer's woad had an identity of 91% with *A. thaliana* and

90% with *B. napus* at the nucleotide level (data not shown). The PR gene *β-1,3-*

glucanase, had an identity of 95% with *B. napus* and 81% with *A. thaliana* at the

nucleotide level. The *ChiA* gene from woad had an identity of 94% with *B. napus*, and

91% with *A. thaliana* at the nucleotide level.

Primer Validation

During primer validation, different concentrations of each primer, ranging from

0.1 μM to 0.9 μM, were used to obtain the lowest threshold cycle (C_t). For the gene *PR-1*,

use of 0.5 μM of forward and reverse primers gave the best results. For the gene *ChiA*,

0.7 μM gave the best results. Using 0.4 μM of forward and reverse primers gave the

lowest C_t number for *β-1,3-glucanase*. For the endogenous normalizer *Actin*, 0.5 μM of

forward and reverse primers gave the best results. Melting curve analysis was used to

determine the presence of non-specific amplification, and all PCR amplifications yielded

a single, specific product.

TABLE 2.3. Dynamics of *Puccinia thlaspeos* basidiospore germination without incubation

Basidiospores*	8 h	12 h	16 h	24 h	48 h
Total number	113.8	445.7	793.1	1092.1	1036.3
Number germinated	20.8	232.6	482.6	837.2	977.5
Germination percentage	18.3	52.2	60.8	76.6	94.3

* Basidiospores released from a leaf piece carrying three teliosori were captured on water agar disks. The average number of basidiospores was estimated by viewing 10 fields on each water agar disk under a microscope using a 40 X objective lens.

TABLE 2.4. Dynamics of *Puccinia thlaspeos* basidiospore germination with 6 h incubation in dew chamber on water agar plates

Basidiospores*	8 h	12 h	16 h	24 h	36 h	48 h
Total number	1018.9	1424.3	1763.2	1530.8	1645.7	1697.5
Number germinated	451.9	1105.7	1421.7	1306.1	1589.6	1662.7
Germination percentage	44.3	77.6	80.6	85.3	96.6	97.9

* Basidiospores released from a leaf piece carrying three teliosori were captured on water agar disks. The average number of basidiospores was estimated by viewing 10 fields on each water agar disk under a microscope using a 40 X objective lens.

TABLE 2.5. Primers and amplicon characteristics of defense-related genes and *Actin* in dyer's woad

Gene	Primer Pair	Oligonucleotide Sequence	Length (bp)	Amplicon T_m (°C)
PR-1	ItPR1F	CACTCCGGTGGGCCTTAC	197	82.6 ± 0.10
	ItPR1R	TACACCTCACTTTAGCACATC		
ChiA	ItCHIAF	TAGGTGATGCTGTTCTTGATG	147	79.9 ± 0.24
	ItCHIAR	TGGAAATGGACACTGAGGAG		
β-1,3-glucanase	ItBGLF	GAACAACATGAACGACATCCC	216	85.5 ± 0.10
	ItBGLR	TGGCATTCTCCACACTCGTC		
Actin	ItACTF	GATGAAGCTCAGTCCAAGAG	364	83.6 ± 0.13
	ItACTR	GAGGATAGCATGAGGAAGAG		

A
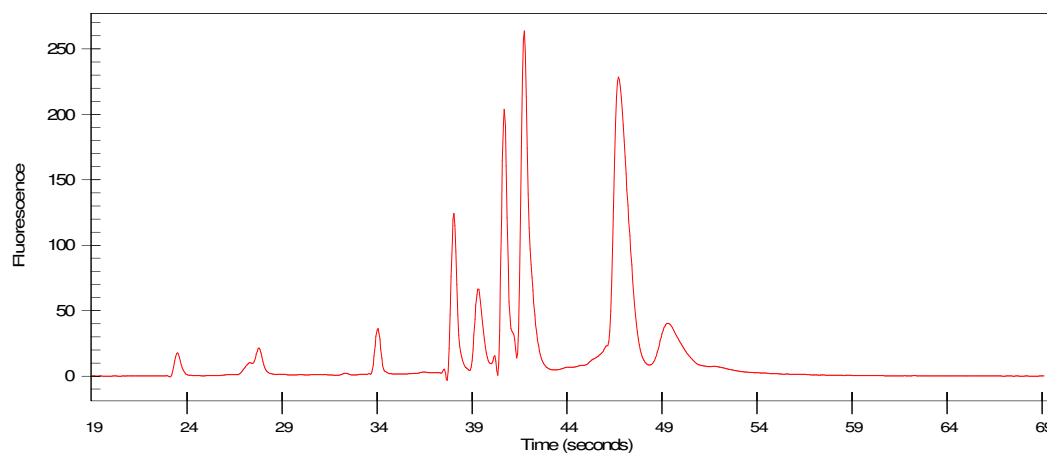
B
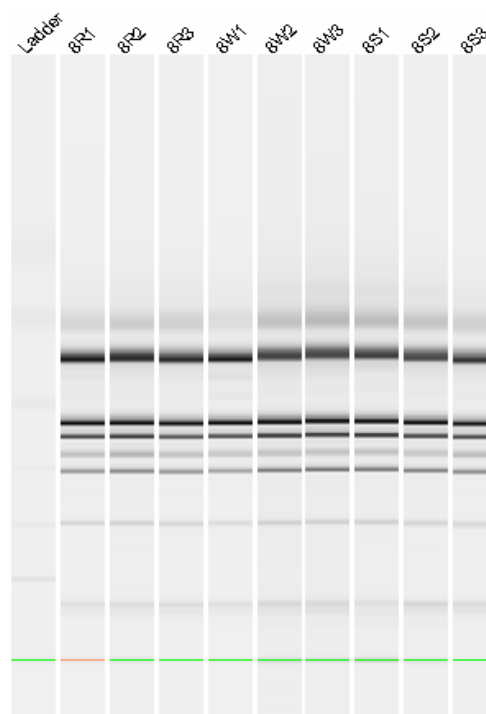
Fig. 2.1. Representative sample of total RNA extracted from treated dyer's woad. Total RNA was analyzed using Agilent 2100 BioAnalyser. Panel A is an electropherogram showing the distinctive peaks 18S and 25S at approximately 41 seconds and 46 seconds respectively for sample 8R1. Panel B shows the simulated gels generated by the Agilent BioAnalyser for the samples 8R1, 8R2, 8R3, 8W1, 8W2, 8W3, 8S1, 8S2, and 8S3 and includes the ladder in lane 1.

The melting temperatures for *PR-1, ChiA, β-1,3-glucanase,* and *Actin* were 82.6°C ± 0.1, 79.9°C ± 0.24, 85.5°C ± 0.1, and 83.6°C ± 0.13 respectively (Table 2.5). Furthermore, the no-template controls included in each assay indicated the absences of non-specific amplification and extraneous DNA in the PCR cocktail.

Evaluation of RNA Prior to Quantification

The quantity and quality of RNA extracted using RNeasy Plant Mini Kit was analyzed using the Agilent 2100 Bioanalyzer. A representative sample of the analysis of total RNA can be seen in Fig. 2.1. In the electropherogram (Fig. 2.1), the high-quality RNA is evidenced by the presence of flat baselines and a relatively flat valley between the strong fluorescent rRNA peaks. The undegraded total RNA sample shows distinct 16S, 18S and 25S rRNA subunit spikes at approximately 40, 41, and 46 seconds, respectively.

Relative Quantification Using a Standard Curve

The accuracy of gene quantification depends on the linearity and efficiency of the PCR amplification, both of which can be assessed by observing the standard curve. Standard curves were constructed using 6 to 7 data points within a 2-fold dilution series, each of which was amplified in triplicates. An example of standard curves for *PR-1*, *β-1,3-glucanase*, *ChiA*, and the normalizer *Actin* is depicted in Fig. 2.2. The standard curves were derived as a plot between the logarithm of the known quantity of cDNA and the threshold cycle it generates during the amplification. From the slope of the standard curve, percent efficiency was calculated. The efficiency of the PCR amplification in this set of standard curves was 106.3%, 97.22%, 99.63%, and 93.72% for *PR-1*,
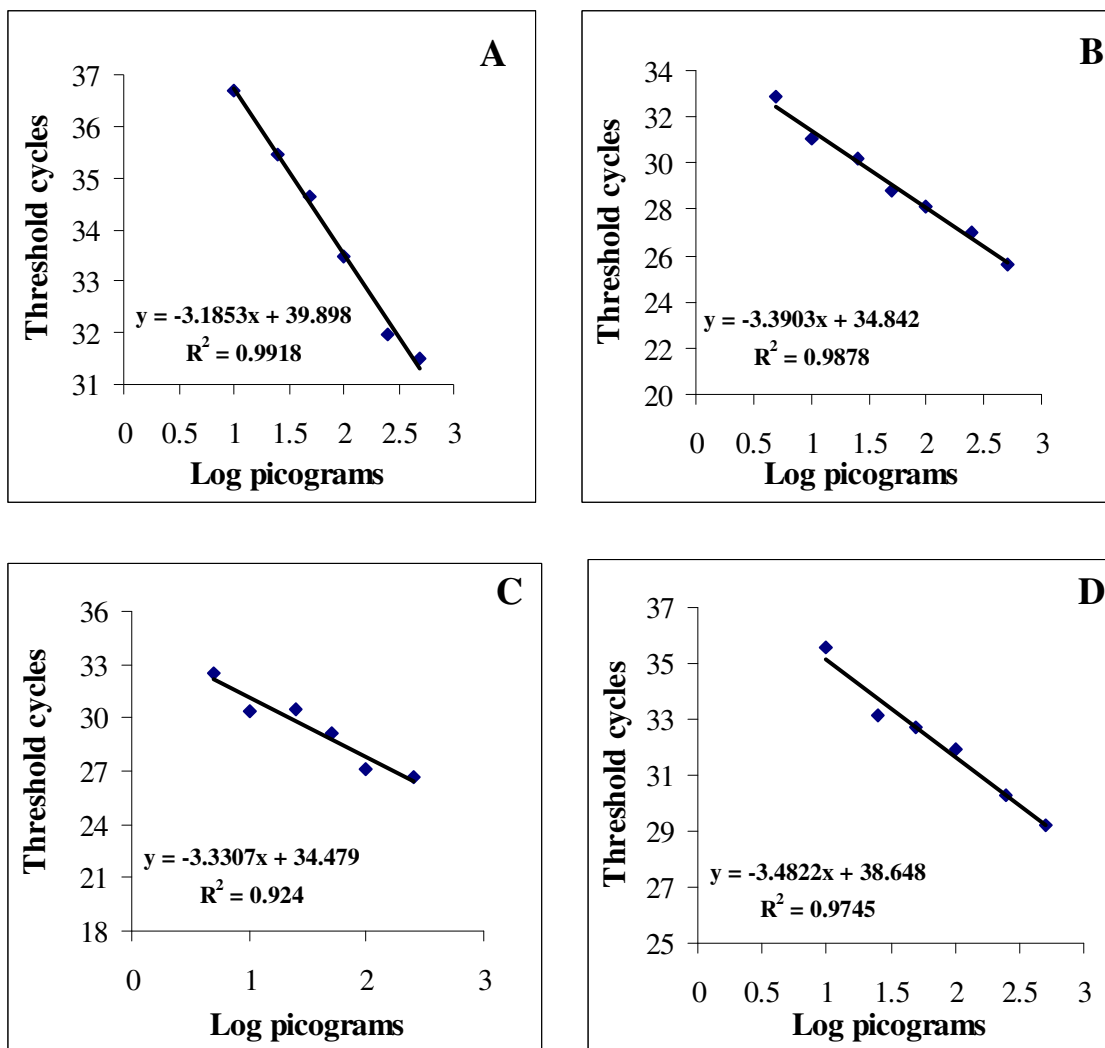
Fig. 2.2. Examples of standard curves for defense-related genes, including *PR-1* (A), *β-1,3-glucanase* (B), *ChiA* (C), and the endogenous normalizer, *Actin* gene (D). Known quantities of purified RT-PCR products were subjected to a two-fold dilution and used in the qPCR assay. Each data point is the mean of three technical replications generated in the same run. A linear relationship was obtained for each run by plotting the threshold cycle number (C_t) against the logarithm of the known quantity of the purified RT-PCR products. From the regression equation obtained, the slope and the intercept were used in determining the amount of target in the test sample.

β-1,3-glucanase, *ChiA*, and *Actin*, respectively. The linearity of the amplifications was assessed using correlation coefficients (*r*). The correlation coefficients were 0.9959, 0.9939, 0.9612, and 0.9872 for *PR-1*, *β-1,3-glucanase*, *ChiA*, and *Actin*, respectively.

Induction Kinetics of Defense-Associated Genes in Dyer's Woad

Induction kinetics of defense-related gene *PR-1* was followed over a period of 72 h after the rust infection (Fig. 2.3A), or after SA application (Fig. 2.3B). Untreated plants were used as the controls, and were the calibrators used to determine the fold-changes in *PR-1* expression over this period. When dyer's woad was infected with the rust pathogen, in the first 8 h, the expression of *PR-1* increased to 3.81 ± 1.32-fold (Fig. 2.3A). This level however, dropped down to 1.74 ± 1.07-fold in the next 4 h. The lowest level was reached at 16 h, when *PR-1* expression levels fell to 0.29 ± 0 .03-fold. On the other hand, at 24 h, *PR-1* expression levels started to increase and were 70.13 ± 42.64-fold; by the end of 36 h, they peaked at 81.69 ± 33.5-fold. The *PR-1* expression levels then began to decrease. At the end of 48 h, they decreased to 19.49 ± 2.78-fold, and at 72 h, to 1.08 ± 0.76-fold.

When plants were treated with SA, there was a 184.42 ± 4.53-fold increase in *PR-1* expression in the first 8 h (Fig. 2.3B). During the next 4 h, these levels fell to 5.65 ± 6.88-fold. At the end of 16 h, there was a 6.72 ± 8.23-fold expression, while at the end of 24 h, the expression levels were found to be 4.04 ± 2.48-fold. These levels continued to fall and were measured at 1.01 ± 0.93-fold, 0.08 ± 0.04-fold, and 0.23 ± 0.07-fold at the end of 36 h, 48 h, and 72 h, respectively.

Fig. 2.3. Induction kinetics of the defense-related gene *PR-1* seen as a result of treating dyer's woad either with the rust pathogen (**A**) or 1 mM salicylic acid (**B**). Transcript levels were determined using qPCR. Values were normalized with the *Actin* gene, and the fold-changes in gene expression were determined by dividing the normalized target by the normalized amount in the untreated control plants. Each data point was generated from 12 amplifications (3 biological replicates x 4 technical replicates) and is reported as mean (±S.E.).
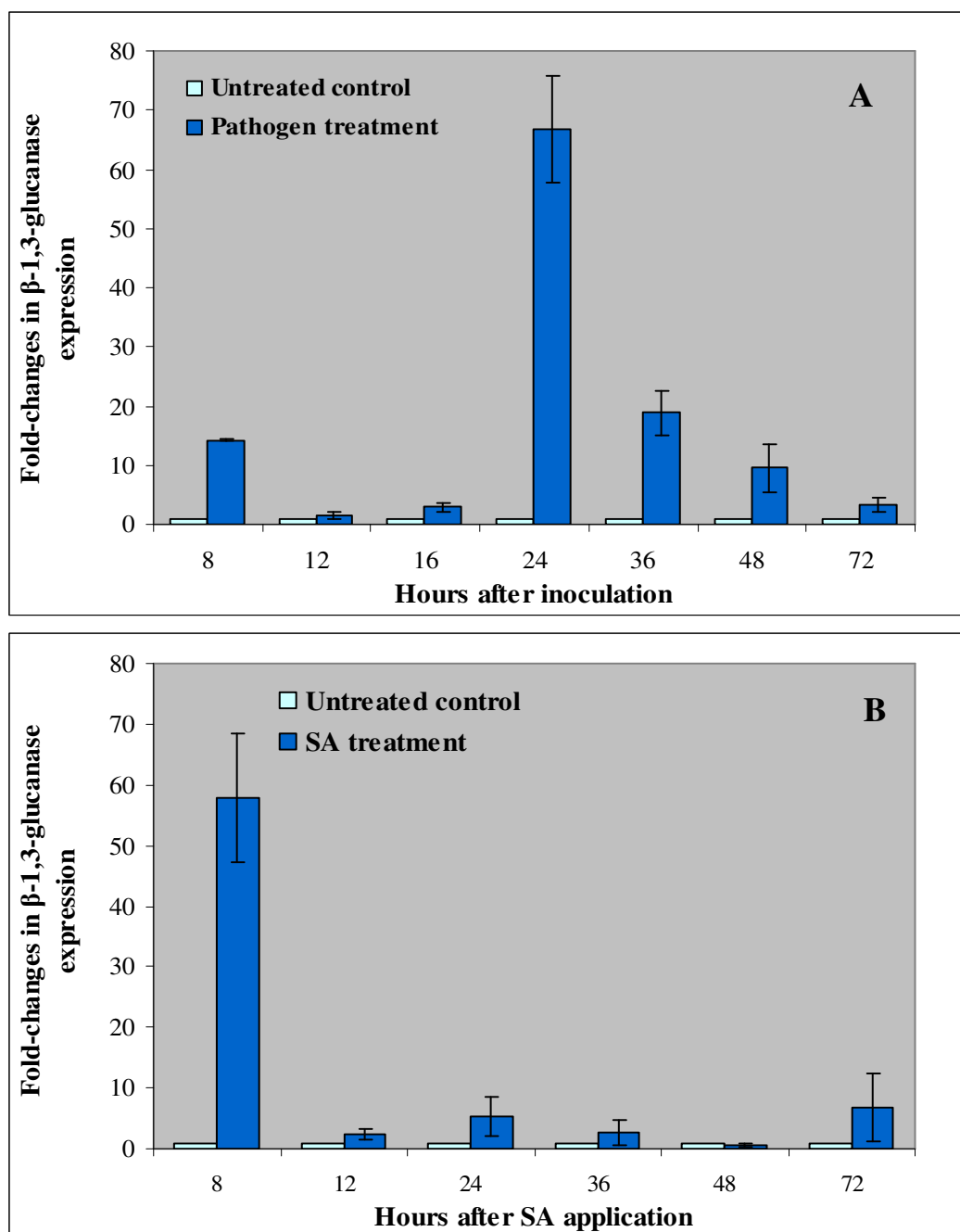
Fig. 2.4. Induction kinetics of the defense-related gene *β-1,3-glucanase* seen as a result of treating dyer's woad either with the rust pathogen (**A**) or 1 mM salicylic acid (**B**). Transcript levels were determined using qPCR. Values were normalized with the *Actin* gene, and the fold-changes in gene expression were determined by dividing the normalized target by the normalized amount in the untreated control plants. Each data point was generated from 12 amplifications (3 biological replicates x 4 technical replicates) and is reported as mean (±S.E.).

After infecting dyer's woad with the rust pathogen, levels of *β-1,3-glucanase* expression increased 14.22 ± 0.10-folds (Fig. 2.4A). However, these levels fell in the next 12 h and 16 h to 1.51 ± 0.47-folds and 2.88 ± 0.79-folds, respectively. At the end of 24 h, levels of *β-1,3-glucanase* expression peaked and were 66.77 ± 8.98-fold. At the end of 36 h, there was a 18.8 ± 3.88-fold expression of *β-1,3-glucanase*. At the end of 48 h and 72 h, these levels declined and were at 9.51 ± 4.09-fold and 3.31 ± 1.19-fold, respectively.

On the other hand, application of SA led to peak *β-1,3-glucanase* expression levels at 57.8 ± 10.67-fold in the first 8 h (Fig. 2.4 B). At the end of 12 h, levels were 2.36 ± 0.93-folds. At the end of 24 h and 36 h, these levels were 5.32 ± 3.22-fold and 2.67 ± 2.11-folds, respectively. At the end of 72 h, levels of *β-1,3-glucanase* expression were found to be 6.78 ± 5.58-fold higher than the untreated control.

When dyer's woad was infected with the rust pathogen, in the first 8 h, the expression of *ChiA* increased 2.62 ± 0.3-folds (Fig. 2.5A). This level however, dropped down to 0.96 ± 0.37-fold in the next 4 h. Like *PR-1*, the lowest level was reached at 16 h, when *ChiA* expression levels fell to 0.76 ± 0.06-fold. On the other hand, at 24 h, *ChiA* expression levels started to increase and were 3.2 ± 1.74-folds and by the end of 36 h, they peaked at 7.09 ± 1.9-folds. At the end of 48 h, *ChiA* levels declined to 2.06 ± 1.23-fold, and at 72 h, to 0.55 ± 0.3-fold.

Exposing dyer's woad to SA led to a 5.89 ± 0.61-fold increase in the *ChiA* expression levels, compared to the calibrator (Fig. 2.5B). At the end of 12 h and 16 h of exposure, expression levels were 1.69 ± 0.79-fold and 1.17 ± 0.56-fold, respectively.
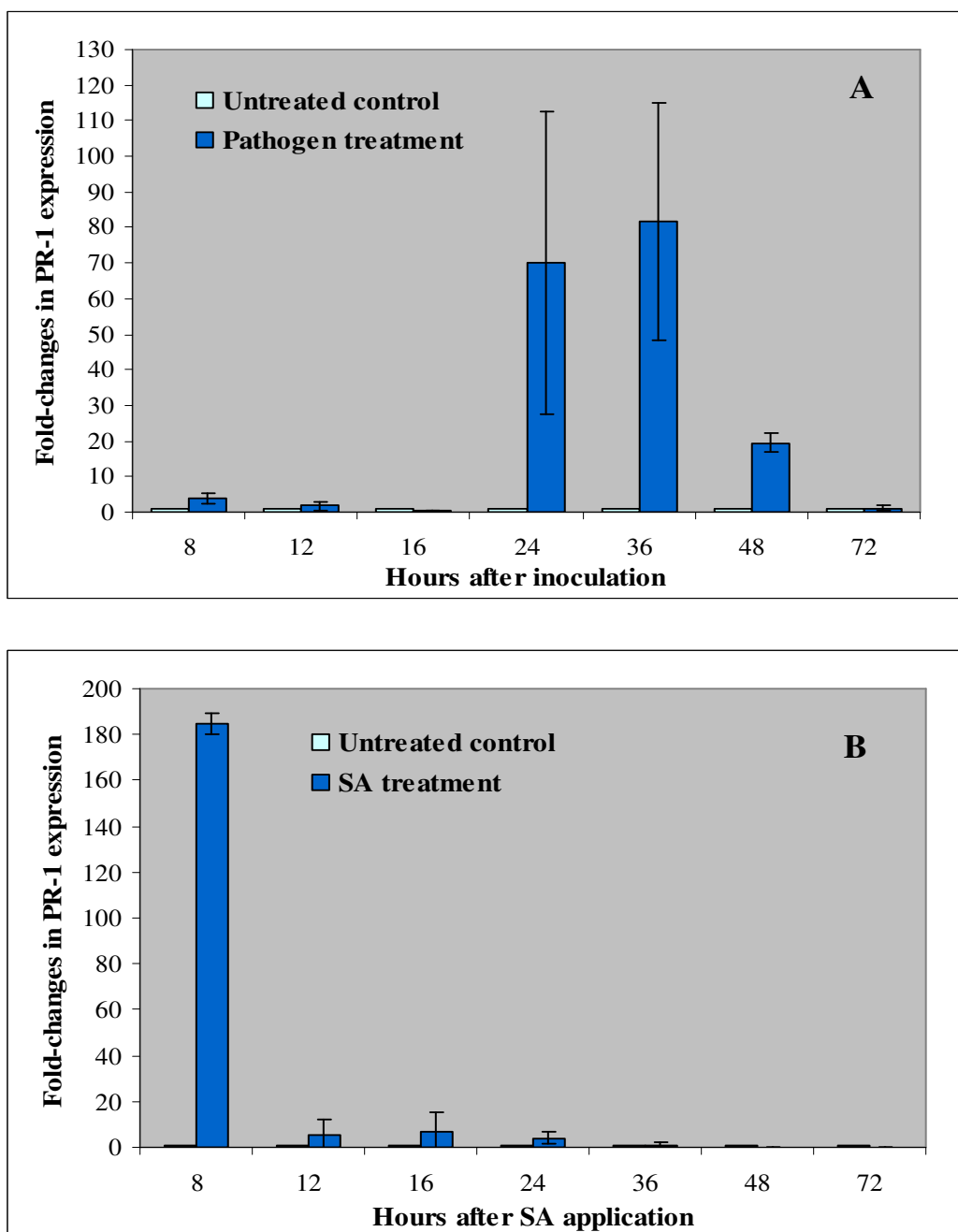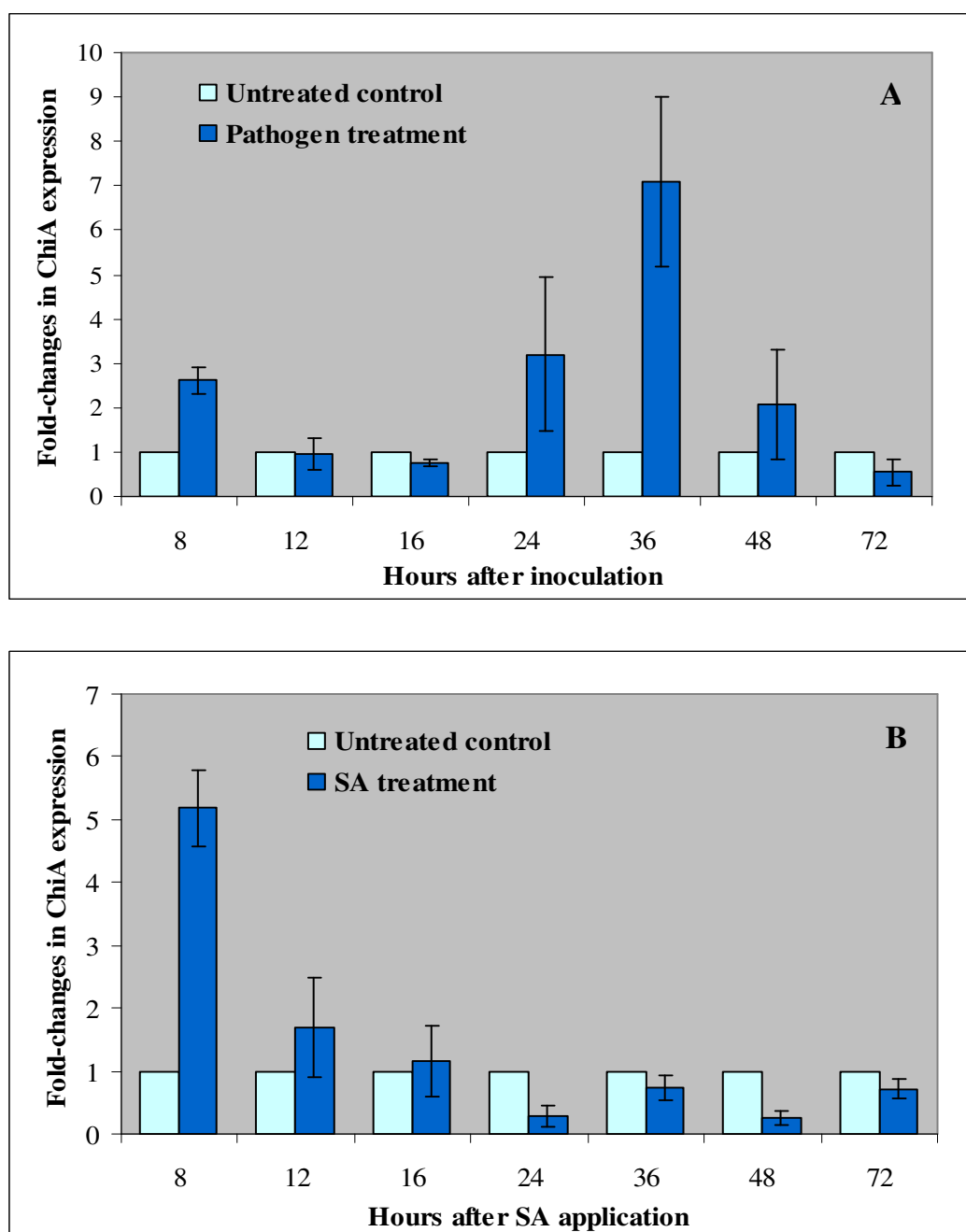
Fig. 2.5. Induction kinetics of the defense-related gene *ChiA* seen as a result of treating dyer's woad either with the rust pathogen (**A**) or 1 mM salicylic acid (**B**). Transcript levels were determined using qPCR. Values were normalized with the *Actin* gene, and the fold-changes in gene expression were determined by dividing the normalized target by the normalized amount in the untreated control plants. Each data point was generated from 12 amplifications (3 biological replicates x 4 technical replicates) and is reported as mean (±S.E.).

After a period of 24 h and 36 h of application, *ChiA* expression levels were measured at 0.28 ± 0.16-fold and 0.72 ± 0.2-fold, respectively. These trends of decline continued and at the end of 48 h and 72 h of exposure, *ChiA* expression levels were 0.26 ± 0.12-fold and 0.71 ± 0.16-fold, respectively.

Effects of Salicylic Acid on Rust Infection Rates

SA was applied to dyer's woad at various time points that coincided with the pre-penetration phase, penetration or the pre-haustorial phase, haustorial phase, and post-haustorial phase in the rust infection (Fig. 2.6A, 2.6B, 2.6C). Induction kinetics of *PR-1* was used to illustrate the events occurring in dyer's woad, as a consequence of SA application. SA was first applied 12 h prior to rust inoculation which included the pre-penetration phase. Following this application, the levels of defense-related genes reached a peak level 4 h prior to the actual event of rust inoculation (Fig. 2.7B). When the peak levels of defense-related gene expression were attained prior to inoculation, the infection rate was 90% (Fig. 2.7A) and there was no statistically significant difference between the treatment and the control groups in infection levels (using Welch's *t*-test, $p>0.05$).

In order to induce defense responses at the time of basidiospore germination and penetration into dyer's woad, SA was applied at either, 8 h before inoculation, or at the time of inoculation (Fig. 2.6A, 2.8). When SA was applied 8 h prior to rust inoculation, the timing at which peak levels of defense-related genes were observed coincided with the time of rust inoculation and penetration into the plant (Fig. 2.8B). The infection rates decreased to 70% (Fig. 2.8A); statistically significant differences were found between the treatment and the control group in infection levels (using Welch's *t*-test, $p<0.05$).

Fig. 2.6. Model depicting the cross section of dyer's woad leaf during penetration and pre-haustorial (A), haustorial (B), and post-haustorial (C) phases of *Puccinia thlaspeos* development in the host.
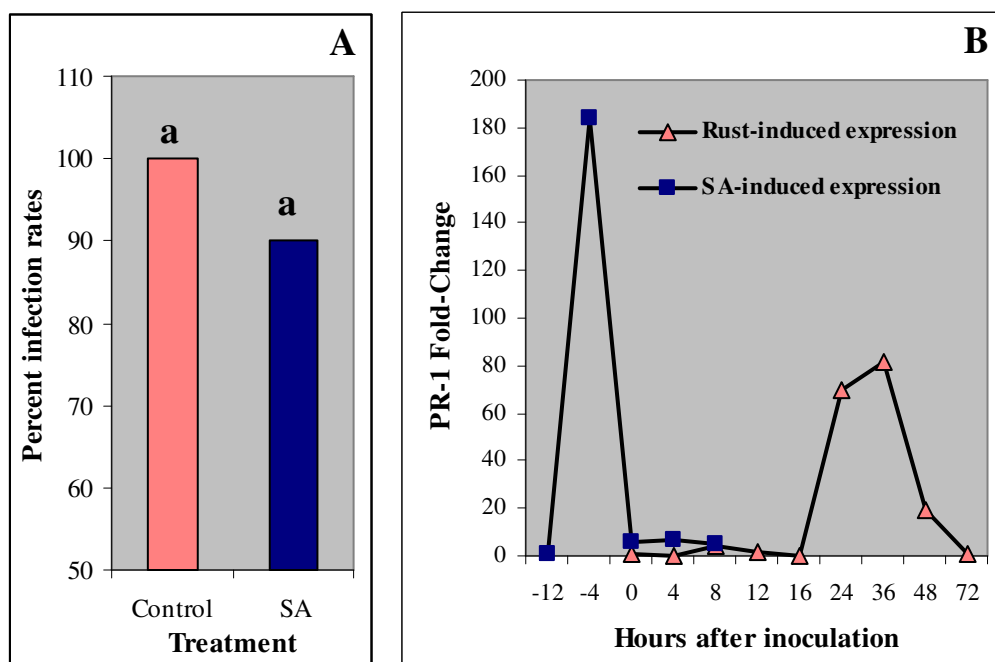
Fig. 2.7. Effect of SA-induced defense responses during pre-penetration phase of rust infection of dyer's woad. A 1 mM SA was applied to the plants 12 h before inoculating dyer's woad with the rust pathogen (**A**). Control treatments were sprayed with water in lieu of SA. Infection was detected by PCR using rust-specific primers three weeks after rust inoculation. Induction kinetics of *PR-1* as a result of SA application and rust inoculation obtained from previous experiments are represented here (**B**), indicating events occurring in the host during this time frame. Means were analyzed with Welch's *t* test ($p < 0.05$; *n*=10 plants). Means with the same letter (a, a) are not statistically different.

Fig. 2.8. Effect of SA-induced defense responses during penetration and pre-haustorial phase of rust infection. A 1 mM SA was applied to the plants, either 8 h prior to rust inoculation as seen in **A**, or at the same time as inoculation as seen in **C**. Control treatments were sprayed with water in lieu of SA. Infection was detected by PCR using rust-specific primers three weeks after rust inoculation. Induction kinetics of *PR-1* as a result of SA application and rust inoculation obtained from previous experiments are represented here (**B**, **D**), indicating events occurring in the host during this time frame. Means were analyzed with Welch's *t* test ($p < 0.05$; *n*=10 plants). Means with the different letters (a, b) indicate that they are statistically different.

When plants were treated with SA and rust inoculum at the same time, the peak values of defense response were attained within 8 h of inoculation and coincided with the pre-haustorial phase of rust infections (34) (Fig. 2.8D). In this situation infection rates were at 60% (Fig. 2.8C), and statistically significant differences were found between the treatment and the control group in infection levels (using Welch's t-test, $p<0.05$).

SA was also applied to dyer's woad after rust inoculation at time points that coincided with the intercellular hyphal growth and haustoria formation (34) (Fig. 2.6B, 2.9). Application of SA after 4 h of rust inoculation led to peak levels of defense response within 12 h of inoculation, at which time the fungus was expected to produce copious amounts of intercellular hyphae (34) (Fig. 2.9B). Activation of defense response at this time point led to infection rates of 89% (Fig. 2.9A) and no statistically significant differences were found between the control and the treatment group in infection levels (using Welch's t-test, $p>0.05$). Induction of defense responses by application of SA at 8 h after rust inoculation led to peak levels in defense-related gene expression within 16 h of inoculation (Fig. 2.9D). Infection rates in these plants were found to be at the same level as those of the control group (Fig. 2.9C).

SA was also applied to dyer's woad after rust inoculation at times when the rust pathogen had already produced the haustoria (34) (Fig. 2.6C, 2.10). SA was applied to dyer's woad 12 h after rust inoculation that led to peak levels within 20 h of inoculation (Fig. 2.10B). At this time point, infection rates were found to be at 55% (Fig. 2.10A). Statistically significant differences were found between the treatment and control group in infection levels (using Welch's t-test, $p<0.05$).
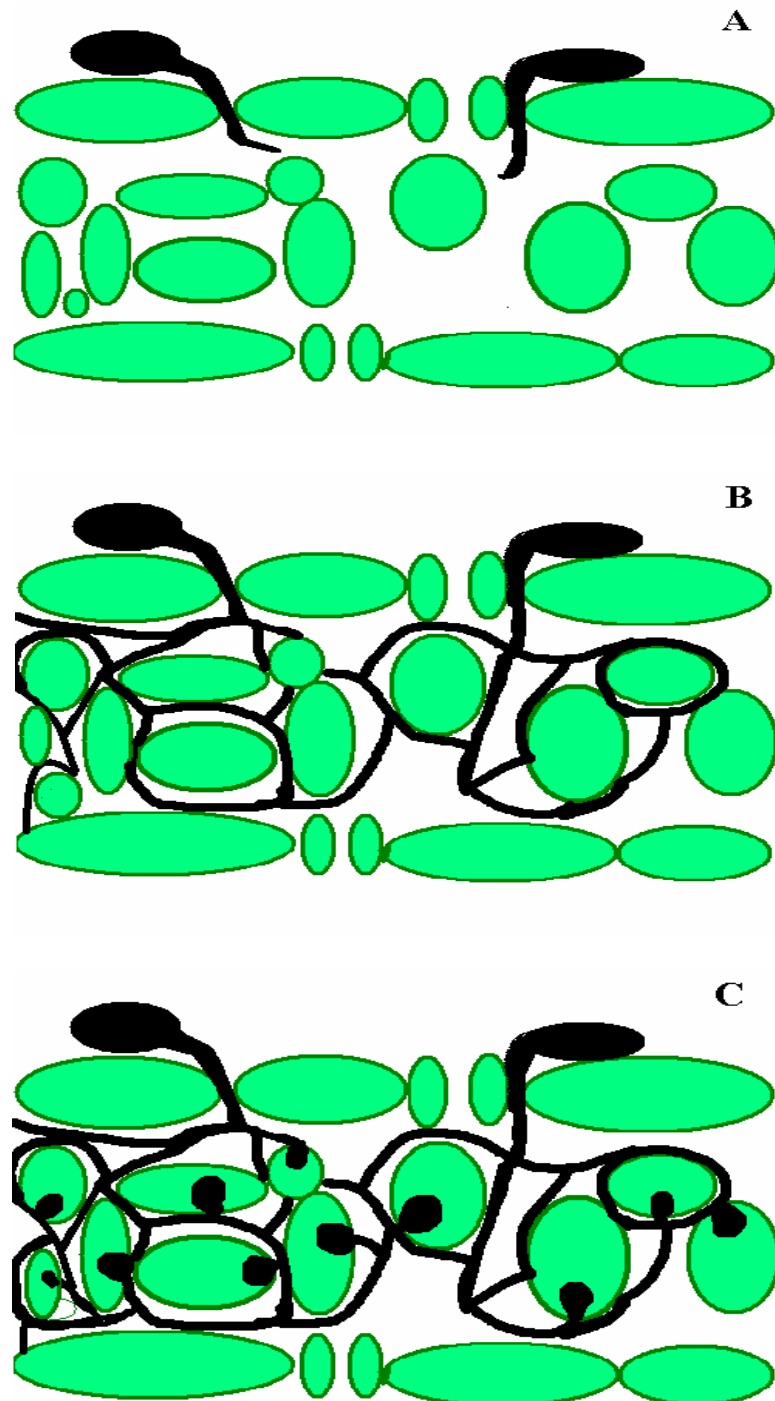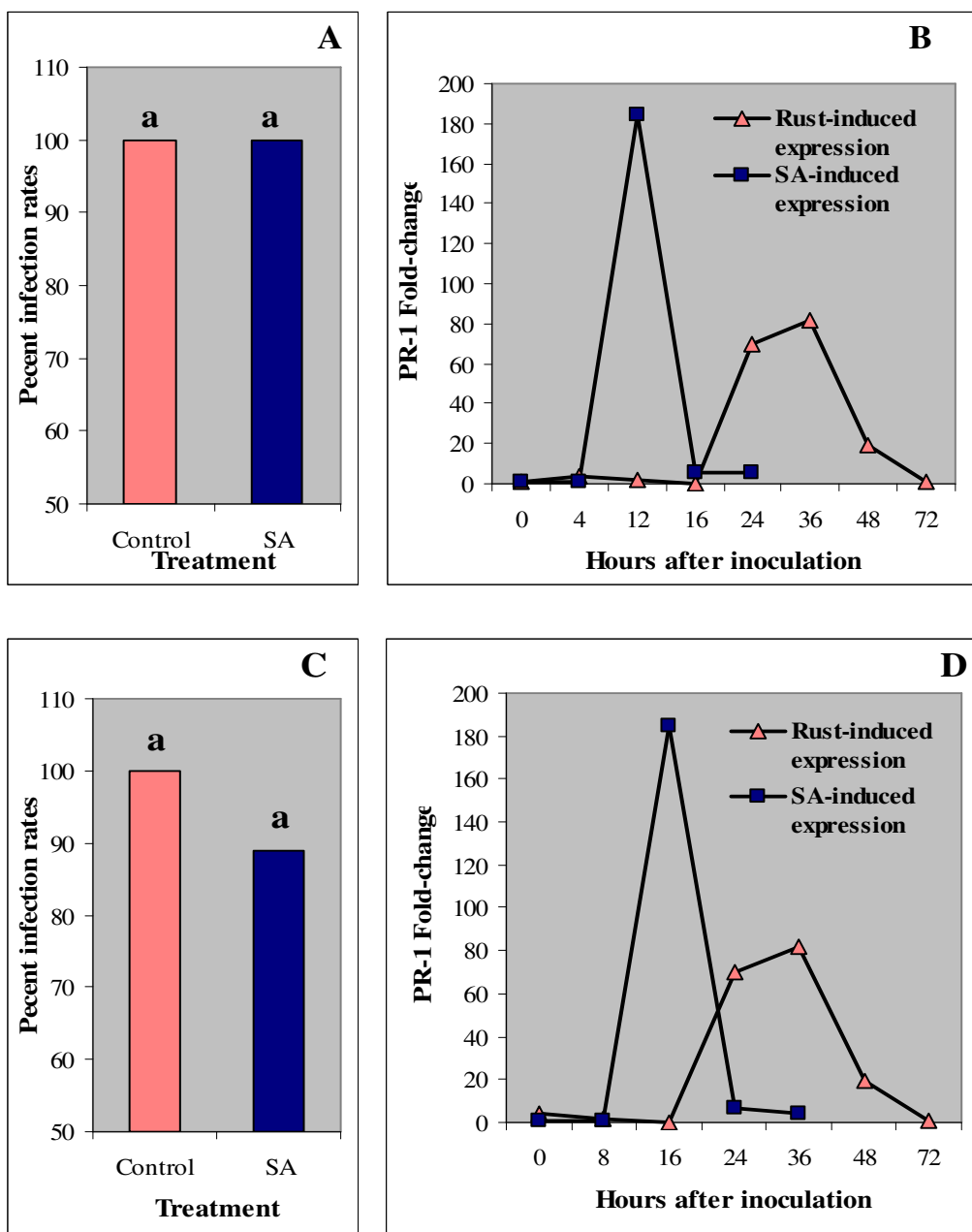
Fig. 2.9. Effect of SA-induced defense response on the haustorial phase of rust infection. A 1 mM SA was applied to the plants either 4 h after rust inoculation as seen in **A**, or at 8 h after rust inoculation as seen in **C**. Control treatments were sprayed with water in lieu of SA. Infection was detected by PCR using rust-specific primers three weeks after rust inoculation. Induction kinetics of *PR-1* as a result of SA application and rust inoculation obtained from previous experiments are represented here (**B**, **D**), indicating events occurring in the host during this time frame. Means were analyzed with Welch's *t* test ($p < 0.05$; n=10 plants). Means with the same letter (a, a) are not statistically different.
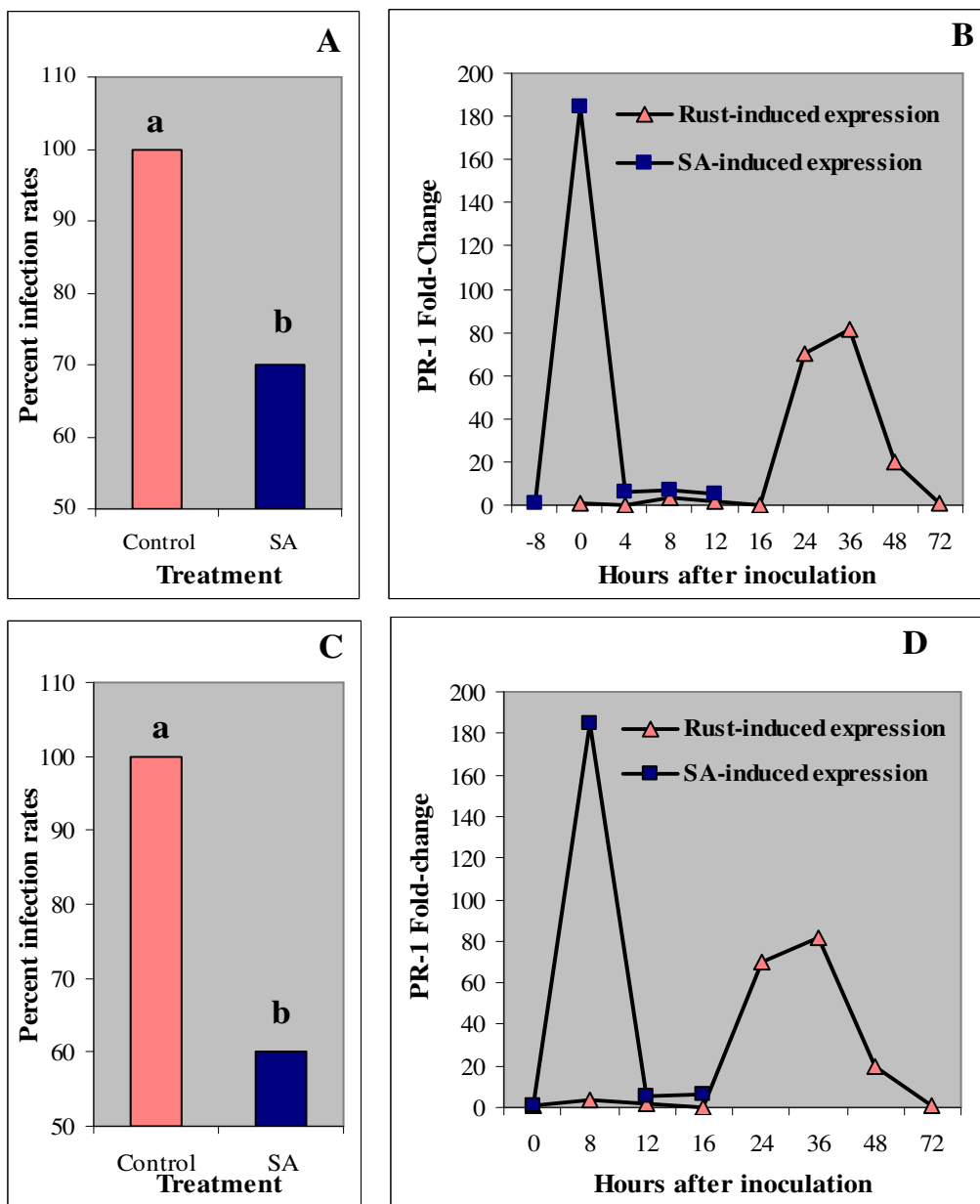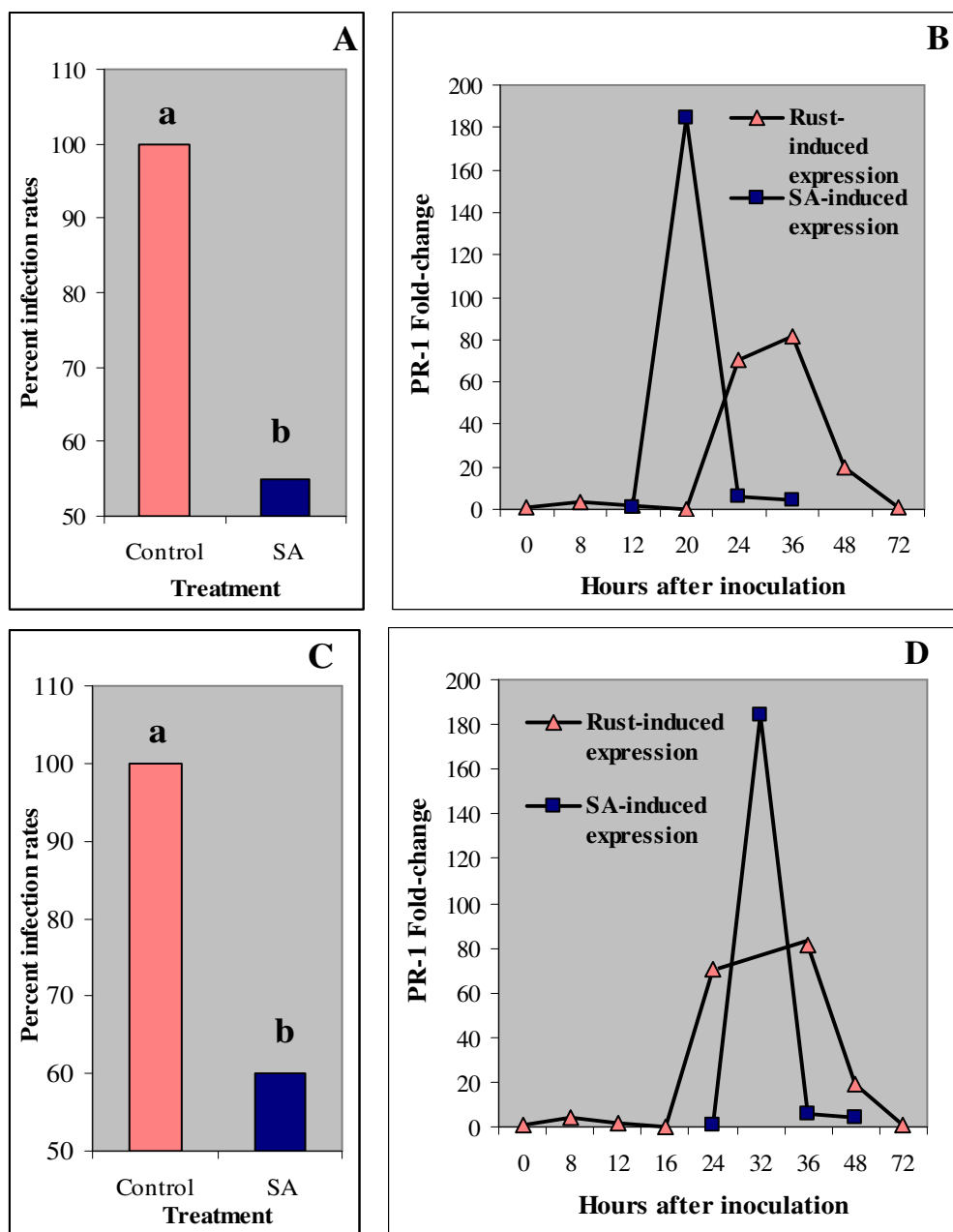
Fig. 2.10. Effect of SA-induced defense response on the post-haustorial phase of rust infections. A 1 mM SA was applied to the plants either 12 h after rust inoculation as seen in A, or at 24 h after rust inoculation as seen in C. Control treatments were sprayed with water in lieu of SA. Induction kinetics of *PR-1* as a result of SA application and rust inoculation obtained from previous experiments are represented here (B, D), indicating events occurring in the host during this time frame. Means were analyzed with Welch's *t* test ($p < 0.05$; *n*=9 plants in A; *n*=10 plants in B). Means with the different letters (a, b) indicate that they are statistically different.

Finally, application of SA 24 h after rust inoculation induced peak defense responses within 32 h of inoculation (Fig.2.10D). Infection rates at this time point were 60% (Fig. 2.10C), and statistically significant differences of infection were found between the treatment and the control group (using Welch's *t*-test, $p<0.05$).

DISCUSSION

Spore Calibration

Rusts are obligate parasites and are challenging to work with because of their dependence on living hosts for nutrition and development. Studies on the uredial stage of the macrocyclic rusts have been extensive since this is one of the most economically destructive and easily studied stages of the rust cycle. On the other hand, studies on the teliospores, produced during the sexual stage of the rusts are limited (57). Teliospores are known to undergo a period of dormancy; consequently, the difficulty in achieving uniform germination has been a key deterrent in dissecting rust-host interactions. Obtaining uniform germination of teliospores and the release of basidiospores onto the host is vital for studying plant responses to basidiospore germination and biotrophic establishment. Studies have suggested that washing the teliospores, and stimulating teliospore germination in the dark, are some of the most effective treatments in overcoming dormancy (18). In the current study, spore washing alone caused the release of an average of 113.8 basidiospores, of which, 18.3% germinated in the first 8 h of release (Table 2.3). On the other hand, when spore washing was accompanied by a 6 h incubation on water agar, which helped to further soak the teliospores, a 10-fold increase

in the number of basidiospores released with an enhanced basidiospore germination of 44.3% was observed (Table 2.4). Therefore, soaking the teliospores under controlled conditions was helpful not only in achieving uniform germination of teliospores, but also in increasing the number of germinating basidiospores.

<center>Induction Kinetics of Pathogen-Inducible PR Genes</center>

Prehaustorial Phase

The events involved in basidiospore germination and colonization of dyer's woad by the rust pathogen have been described using scanning electron microscopy and PCR (34). As in other rusts, the basidiospores of *P. thlaspeos* require about 6 h for germination and penetration into the plant (34). The basidiospores and the germlings attach to the host epidermis with the help of an extracellular matrix. The germlings then enter the host by forming appresoria on an epidermal cell. However, germlings were not observed entering through open or closed stomata. The woad rust also causes both mechanical and enzymatic disruption of the epidermal wax layer (34).

The present work found that during germination and penetration into the plant, there was an up-regulation of pathogen-induced, SA-responsive *PR* genes. Both *PR-1* and *ChiA* showed modest increases of a 3.81-fold increase in *PR-1* and a 2.62-fold increase in *ChiA* expression levels (Fig. 2.3A, 2.5A). However, the up-regulation of *β-1,3-glucanase* by 14.22-fold was more pronounced (Fig. 2.4A). Despite the up-regulation of these antimicrobial compounds, rust infection was successful.

Fungal penetration failure is often seen in biotrophs that are unable to enter a nonhost (46), and previous studies have suggested that nonhost resistance to rust fungi is

generally expressed before the formation of haustorium (24). The current work both

complemented and contradicted existing studies of prehaustorial resistance. In one

published study of nonhost resistance of parsley to *Phytophthora sojae*, it was noted that,

while there was a modest increase in *PR-1* levels, the induction of *PR-2* (*β-1,3-glucanase*)

was not detectable (20). The current study mirrored the slight increase in *PR-1* expression

levels but the *β-1,3-glucanase* levels in dyer's woad were 14.22-fold.

Another study investigating the role of SA in the infection process of a nonhost by

using *sid2, NahG,* and *npr1* mutants in *Arabidopsis*, found that SA-inducible PR proteins

are likely to be involved in the nonhost resistance (45). In order to establish biotrophy in

a potential host, rust fungi have been observed to negate non-specific defense responses

in both resistant and susceptible cells early in the infection process (26). This suppression

of non-specific defense responses is brought about by a reduction of Hechtian strands in

the host, which in turn helps in lowering the cell wall-associated defense response (44).

It is not known whether there is an active suppression of SA-responsive genes by

the dyer's woad pathogen. However, exogenous application of SA appears to reprogram

the host in exhibiting increased resistance towards the rust pathogen, particularly during

basidiospore germination and host penetration. Exogenous application of salicylic acid on

healthy plants led to a 184.2-fold increase in *PR-1*, a 57.8-fold increase in *β-1,3-

glucanase*, and a 5.89-fold increase in *ChiA* (Fig. 2.3B, 2.4B, and 2.5B) during the first 8

h.

The timing of SA application appears to be critical, since the application of SA 12

h before inoculation did not affect infection rates (Fig. 2.7). However, application of SA

at the time of basidiospore germination and penetration into the plant, induced resistance

of the plant to the rust pathogen (Fig. 2.6A, 2.8A, 2.8C). These findings are similar to earlier studies by Rauscher et al. (53) who found that *Uromyces fabae* was inhibited immediately after penetration of the host. These authors attributed this inhibition to the antifungal activity of the PR-1 protein, brought about by the exogenous application of SA.

Haustorial Phase

   Puccinia thlaspeos produces abundant intercellular hyphae in tissues between 12 and 24 h after inoculation (34). Hautoria penetrate leaf mesophyll cells, particularly those adjacent to the veinlets, and range from simple coiled structures to more elaborate septate and branched forms (34). In the present work, the levels of *PR-1* were measured at 1.74-fold and 0.29-fold that of mock inoculated controls at 12 h and 16 h after inoculation, respectively (Fig. 2.3A). The levels of *β-1,3-glucanase* were measured at 1.51-fold and 2.88-fold (Fig. 2.4A), while the levels of *ChiA* were 0.96-fold and 0.76-fold (Fig. 2.5A) at 12 h and 16 h after inoculation, respectively. Thus, it is apparent that there is a suppression of PR genes that coincides with haustoria formation in dyer's woad.

   Suppression of defense responses during haustoria formation has been reported in other biotrophic interactions. For example, formation of intracellular haustoria by the rust *Uromyces* sp., has been described as a process that involves the suppression of defense responses (25, 28). In addition, the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* produces haustoria between 14 and 18 h after inoculation of barley and suppression of the basal defense responses necessary for the formation of haustoria were observed 16 h after inoculation (2, 3). In another study of powdery mildew infection of

barley, haustoria formation was proposed to be contingent upon the suppression of *PR* genes (50). Successful infections occurred when expression of *PR* genes such as the peroxidase-encoding gene, *Prx8* and the oxalate oxidase-encoding gene, *GRP94* were low (50). At the same time, failed infections corresponded with transcriptional up-regulation of these genes (19). Reduced levels of SA also promoted haustoria formation by *Uromyces* spp. in *Arabidopsis* mutants including *sid2* (SA induction deficient 2) and *NahG* plants (45).

For the current study, it was hypothesized that triggering defense gene expression during the haustorial phase by applying SA, would inhibit haustorium formation and lead to increased resistance. However, exogenous application of SA during the time of haustorium formation did not abolish the pathogen-induced suppression of defense response, as was reflected by the unchanged infection rates (Fig. 2.6B, 2.9A, 2.9C).

The formation of haustoria without promoting host defense responses is of vital importance to the establishment of biotrophy, as these specialized organs enable the pathogen's nutrient absorption (47, 48, 50, 51, 55). Haustoria are typically separated from the host cytoplasm by the extra haustorial membrane (host plasma membrane-derived) and the carbohydrate enriched-extra haustorial matrix (28, 50). As stated earlier, prior studies have suggested that nonhost resistance to rust fungi is generally expressed before the formation of haustoria (24). However, the disease resistance (*R*) triggered by the recognition of the pathogen avirulence (*Avr)* gene product that leads to HR occurs only after the formation of the haustoria (23). The fungal avirulence elicitors that are secreted by the haustoria reach the host intercellular spaces, where they are recognized by a host factor (9, 10, 12, 21). For example, a rust transfer protein RTP1 from *Uromyces* is

believed to play a vital role in the maintenance of biotrophy (31). Although, RTP1 is found initially in the extra haustorial matrix, it is later found in the host cell as the infection progresses (31). In a different study, Dodds et al. (10) reported that avirulence proteins from the rust fungus *Melampsora lini,* that are synthesized in the haustoria and secreted across the extracellular matrix, are recognized inside the plant cell.

Posthaustorial Phase

The present study shows that after haustoria formation, there was an induction of *PR* genes in infected dyer's woad. At this time, the levels of *PR-1* were found to be 70-fold and 81-fold, 24h and 36h after inoculation, respectively (Fig. 2.3A). While peak levels of *β-1,3-glucanase* occurred with a 66-fold induction at 24 h after inoculation (Fig. 2.4A), *ChiA* reached peak levels with a 7.09-fold induction at 36 h after inoculation, (Fig. 2.5A). Even though there was an induction of *PR* genes in the current study, Kropp et al. (34) did not find evidence of HR. This suggests that the interaction between the host and the pathogen is a compatible one, and leads to successful infections.

In the current study, after *PR* expression reached peak levels, there was a steady decline in expression as measured at 48 h and 72 h after inoculation. Similar declines in nonspecific defense responses in compatible barley- powdery mildew interactions have been noted (2). Also, wheat infected with powdery mildew showed an initial accumulation of *PR* genes *WIR1* and *WIR2* which later declined as the pathogen colonized the host (66).

Earlier studies suggest that compatible interactions could occur due to: i) non-recognition of pathogen-derived elicitors, or ii) ineffective signaling of PR genes, or iii)

suppression of host defense responses. One or more of these factors could help the pathogen to establish a biotrophic relationship (2, 55, 66). The suppression of host defense responses could be seen during the phenomenon of 'induced accessibility,' as seen in powdery mildew-barley interactions (36, 55). Induced accessibility refers to the state where a host that was successfully penetrated by a compatible pathogen becomes more susceptible to a different and otherwise avirulent pathogen, indicating that normal defense responses are suppressed. This pathogen-mediated suppression was found to target different types of resistance, including basal (41) and nonhost resistance (37). This implies that the ability of the pathogen to inhibit host defense responses could be a vital component in establishing biotrophic associations.

In the current study, although there was an accumulation of *PR* genes, infections were still successful. Exogenous application of SA at 12 h and 24 h after inoculation led to peak levels of PR genes at 20 h and 32 h after rust infections, and significantly lowered infection rates (Fig.2.10A, 2.10C). The antifungal activity of each of the PR genes used in the current study has been well documented (43, 53, 56). However, it is not known whether pathogen inhibition is dependent on the amount of *PR* expression. Exogenous SA application led to a 184-fold expression of *PR-1*, while the pathogen only induced an 81-fold expression of the same. It appears that pathogen-induced PR expression is insufficient to slow the fungal growth during the compatible interaction between dyer's woad and the rust pathogen.

Summary

Although the interaction between dyer's woad and *P. thlaspeos* is a compatible one, there was nonetheless an induction of SA-mediated defense responses. The pattern of induction seen during the prehaustorial phase was distinct from that seen at the posthaustorial phase. The resistance response seen during the prehaustorial phase was more muted compared to that of the posthaustorial event, and could be part of a basal response of the plant to the pathogen penetration. For instance, one study examining nonhost resistance to different species of rusts with the aid of *Arabidopsis* signaling mutants such as *sid2, NahG,* and *npr1* mutants in *Arabidopsis*, found that although SA-dependent responses were involved in suppressing haustorium formation, it was *NPR1-*independent (45). However, the *R*-gene mediated defense response seen after haustorium formation is typically *NPR1*-dependent (17, 52). Hence, it is possible that the induction events seen in dyer's woad before and after haustoria formation are brought about by two different mechanisms.

During rust infection of dyer's woad, the suppression of defense responses was seen during two phases, one at the haustorial phase and the other, after haustoria formation. Although the first pathogen-mediated suppression was short term and likely facilitated haustoria formation, the second suppression event was more significant because this suppression is a long term event brought about after haustoria development. Previous studies have implicated the haustoria not only in nutrient acquisition, but also in the suppression of defense responses during compatible interactions (50). The manner in

which *P. thlaspeos* brings about suppression of defense responses either at haustorial

phase or the posthaustorial phase is not yet known.

The suppression of defense responses could be brought about either by pathogen-

derived or plant-derived suppressor molecules. For example, the wheat stem rust *P.*

graminis f. sp. *tritici* appears to employ host-derived oligomers of galacturonic acid from

the host cell wall to suppress the activity of phenylalanine ammonia lyase and

peroxidases (49). Furthermore, in addition to the *R* gene and the *avr* gene, a third

dominant *Inhibitor of avirulence (I)* gene has been reported, which modifies the outcome

of a specific *R-Avr* gene interaction from resistance to that of susceptibility (29, 30, 39).

It is not known whether the susceptibility seen during the dyer's woad-rust interaction

involves the *I* gene.

Further investigation into the tools employed by the rust pathogen in suppression

of defense responses is important to more fully understand compatibility in host-pathogen

interactions and establishment of biotrophy. The interaction between dyer's woad and *P.*

thlaspeos provides an excellent platform to make such determinations.

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CHAPTER 3

ISOLATION AND EXPRESSION ANALYSIS OF *ITCYP79B2* DURING THE

RUST INFECTIONOF DYER'S WOAD (*ISATIS TINCTORIA*)

BY *PUCCINIA THLASPEOS* [2]

ABSTRACT

Glucosinolates and their hydrolysis products are part of a plant defense response to pathogens and insects. Dyer's woad (*Isatis tinctoria*) has been shown to have unusually high quantities of indole glucosinolates. CYP79B2 and CYP79B3 in *Arabidopsis thaliana* catalyze the conversion of tryptophan to indole-3-acetaldoxime during the biosynthesis of indole glucosinolates. In this study, a sequence encoding CYP79B2 was isolated from dyer's woad. *ItCYP79B2* from *I. tinctoria* shows 97% sequence identity to *CYP79B2* and 89% sequence identity to *CYP79B3* from *A. thaliana*. Because of the high sequence identity, it could be inferred that the dyer's woad sequence, like that of *A. thaliana*, is likely to be involved in the biosynthesis of indole glucosinolates. This study also examined the kinetics and amplitude of *ItCYP79B2* expression during the first 72 hours of the infection by the virulent pathogen *Puccinia thlaspeos.* During the first 8 hours which coincided with fungal penetration into the host, there was a significant down-regulation of *ItCYP79B2*. However, during the time that coincided with haustoria formation in dyer's woad there was an induction of *ItCYP79B2* which is in contrast to the suppression of basal defense-related genes seen in other

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[2] Coauthored by Elizabeth Thomas, and Bradley R. Kropp, Isolation and Expression Analysis of *ItCYP79B2* during the Rust Infection of Dyer's Woad (*Isatis tinctoria*) by *Puccinia thlaspeos*.

compatible interactions. After the formation of haustoria, the levels of *ItCYP9B2* were

suppressed during the period coinciding with successful colonization.

INTRODUCTION

Plant defense response to pathogens and herbivorous insects is complex and is

regulated by multiple signal transduction pathways. These pathways use salicylic acid,

jasmonic acid, and ethylene as signaling molecules. However, in the Brassicaceae there is

a unique plant defense response system that involves glucosinolates. Glucosinolates are

amino acid-derived secondary metabolites produced by members of the order Capparales

that include families such as the Brassicaceae, Gyrostemonaceae, and Capparaceae (36).

As many as 120 glucosinolates have been identified so far, all of which share a chemical

structure consisting of a variable R group, derived from one of eight amino acids. All are

linked to a glucose moiety bound to an O-sulfonate group through a thioester bond (11).

There are three categories of glucosinolates: aliphatic, aromatic, and indolic. Aliphatic

glucosinolates are derived from alanine, leucine, isoleucine, methionine, or valine, while

the aromatic glucosinolates are derived from phenylalanine or tyrosine. On the other

hand, indolic glucosinolates are derived from tryptophan. Some of the predominant

indolic forms include glucobrassicin, neoglucobrassicin, and glucobrassicin-1-sulfonate.

The intermediates in the indolic glucosinolate biosynthetic pathway from the amino acid

tryptophan include indole-3-aldoximes (IAOx), *aci*-nitro compounds, *S*-alkyl

thiohydroximates, thiohydroximic acid, and desufloglucosinolates (Fig. 3.1).
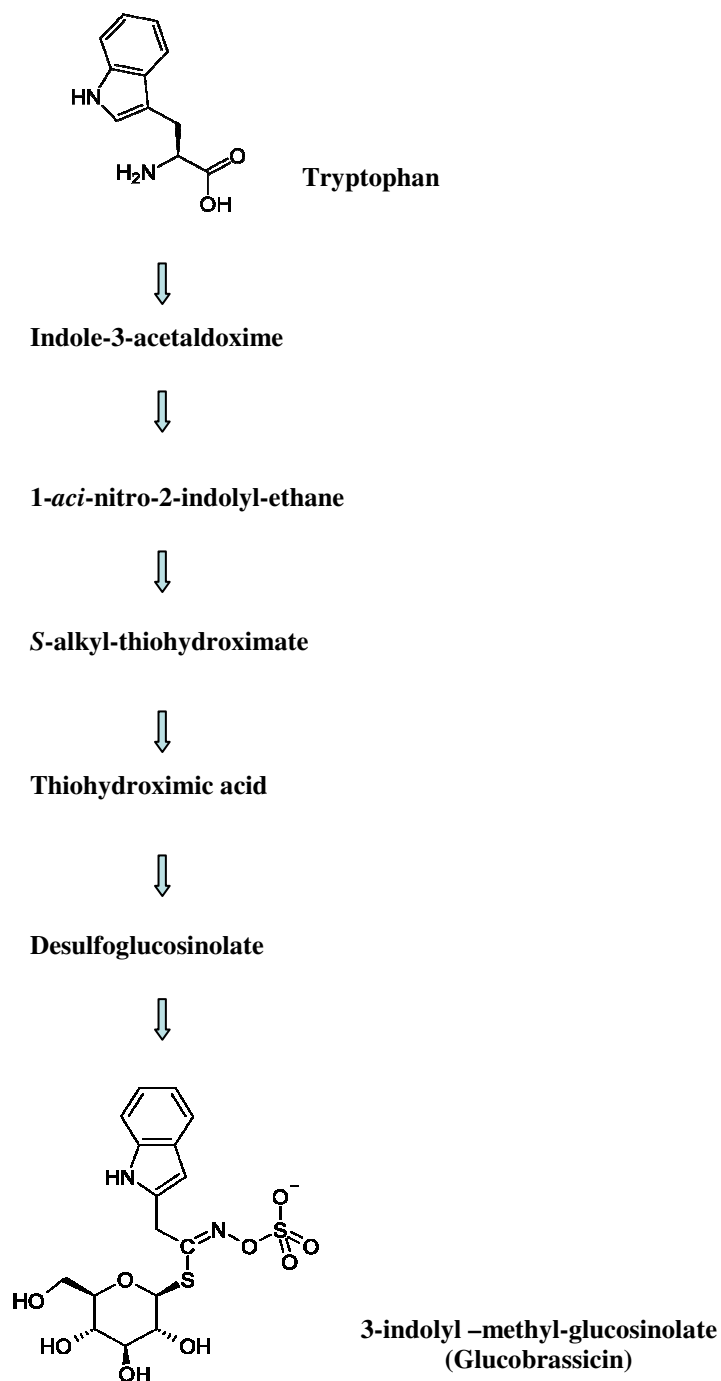
Fig. 3.1. Biosynthesis of the predominant indole glucosinolate (Glucobrassicin) from tryptophan (32, 34).

Glucosinolates are related to cyanongenic glucosides in that their first step in biosynthesis includes the conversion of amino acids to their corresponding aldoximes (22). The CYP79 family is related to the cytochrome P450 genes and is involved in the conversion of amino acids to aldoximes. Some of the predominant members of the CYP79 family that have been identified include CYP79B2 and CYP79B3 that metabolize tryptophan (20, 32), CYP79F1 and CYP79F2 that metabolize 1-6, or 5-6 homomethionine (6, 17), and CYP79A2 that metabolizes phenylalanine (46).

Glucosinolates are stored in idioblasts in an inactive form that exhibits very little biological activity (21, 41, 44). However, upon injury, these glucosinolates are hydrolyzed by the enzyme myrosinase (□-thioglucoside glucohydrolase) and are transformed into unstable glucosinolate aglycones. The aglycones spontaneously undergoes further rearrangement and, based on the structure of the side chain, forms different products such as isothiocyanates, nitriles, thiocyanates, and oxazolidinethiones (1, 7, 8, 12). These derivatives are believed to be toxic at low levels to a wide range of organisms that includes plants, fungi, and insects (38, 45, 47, 48).

Research has revealed that that the enzymatic breakdown of the glucosinolate, sinigrin, leads to the formation of allyl-isothiocyanate (AITC), that is highly inhibitory to the growth of the post harvest pathogen *Penicillium expansum* (30). Similarly, the enzymatic hydrolysis of glucosinolate, glucoraphenin producing isothiocyanates is inhibitory to pear postharvest pathogens *Botrytis cinerea, Monilinia laxa,* and *Mucor piriformis* (29). The breakdown products of thiofunctionalised glucosinolates, including glucoiberin and glucoerucin are effective in inhibiting *Phytophthora irregulare* oospore germination and *Rhizoctonia solani* soil colonization (28). By employing a mutant altered

in glucosinolate profile, Tierens et al. (43) demonstrated that 4-methylsulphinylbutyl

isothiocyanates, which are breakdown products of glucoraphanin, protect *Arabidopsis*

against *Fusarium oxysporum***.**

The levels of glucosinolates in plants prior to injury differ significantly from those

following injury (44). For instance, the levels of glucobrassicin in roots and hypocotyls of

Brassica rapa were found to be doubled 30 to 60 days after initial infection with

Plasmodiophora brassicae (3). Similarly, when inoculated with turnip mosaic virus,

progoitrin levels were higher in *B. napus* subsp. *napobrassica* than in uninouculated

controls (39). Likewise, large increases of indole glucosinolates were reported on *B.

napus* when infested with the cabbage stem beetle, *Psylliodes chrysocephala* (23).

Dyer's woad (*Isatis tinctoria* L.), a noxious weed in the Brassicaceae, contains

unusually high quantities of indolic glucosinolates (glucobrassicin, neoglucobrassicin,

and glucobrassicin-1-sulfonate) (9, 10, 14). Several studies have reported seasonal

variation in the indole glucosinolate content in dyer's woad (9, 10, 14). Research has

indicated that one mechanism of change in glucosinolate profiles in plants is the

alteration of expression levels of one or more *CYP79* genes (31).

A rust fungus, *Puccinia thlaspeos* C. Schub., has been found to be an effective

biological control agent of this noxious weed and although, the antifungal properties of

glucosinolates have been observed in other plant-microbe interactions, it is not known

how the rust is able to successfully infect this glucosinolate-producing plant. Even though

glucosinolates are believed to play a role in plant defense responses, it unknown whether

there is a specific induction of *CYP79* genes in dyer's woad that could lead to an

alteration of glucosinolate profiles during rust infection. The goals of this study were: 1)

to identify the homolog of *CYP79B2* in *I. tinctoria* and 2) to study induction kinetics of this gene in dyer's woad.

MATERIALS AND METHODS

Growth of Dyer's Woad

Silicles of dyer's woad collected from northern Utah were threshed manually to obtain seeds that were stored at 4°C until further use. The seeds were planted in pots that contained steam-sterilized potting mixture, consisting of perlite, peat moss, and vermiculite, mixed in a ratio of 1:1.3:1 respectively. The plants were grown in a greenhouse using a light and temperature regime of 16 h days at 25°C and 8 h nights at 18°C. Plants were grown in 10 x 10 cm pots and watered until saturated on alternate days. In all treatments, fully expanded leaves from 4-week-old seedlings were used for experimentation.

Artificial Inoculation of Plants

Inoculum was prepared from diseased dyer's woad plants collected in the field in northern Utah during spring. The leaves bearing teliosori were separated from the plants, air dried and crushed into small fragments. Each leaf piece was viewed under the microscope and the numbers of teliosori were estimated. To prevent or reduce extraneous microbial growth, the inoculum was surface-sterilized prior to inoculation with 0.6% sodium hypochlorite, soaked in sterile distilled water for 3 minutes, and rinsed thoroughly. During inoculation, the surface-sterilized inoculum was positioned over the

surface of a 10-cm-diameter Petri dish containing 1.5% water agar. The arrangement of

the leaf pieces was such that the teliosori were facing in the upward direction on the

surface of the agar. For each treatment, the numbers of teliosori were kept constant in

order to produce 100 basidiospores with a 50% germination rate per square millimeter of

the host leaf surface. The teliosori were also consistently arranged to ensure that the

apical two centimeters of the leaf would be saturated with basidiospores.

After the placement of the teliosori on the water agar, the plates were initially

incubated for 6 h in a dew chamber that was maintained at a temperature of 15°C ± 1 to

reduce the germination lag period before inoculating the plants. To conduct the

inoculation, the Petri plates were inverted over selected leaves to permit the direct

landing of the basidiospores from germinating teliospores onto the upper-side of the

leaves. Care was taken to ensure that the leaf tissue was not damaged during inoculation.

Furthermore, each inoculated leaf was tagged to ensure that the correct leaf was

monitored for changes in gene expression following rust infection. The plants and the

water agar were then transferred to the dew chamber for a period of 8 h. Inoculations

were carried out in the dark at night. The following morning, the plants were transferred

to the greenhouse, and kept there for the duration of 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and

72 h until assayed further.

Detection of Rust

Owing to the asymptomatic nature of the rust fungus, detection of the rust

pathogen was done using the polymerase chain reaction (PCR). Universal primers that

amplify a 620 base pair section at the 5' end of the large ribosomal subunit were used to

ensure that amplifiable DNA was present in the leaf extracts. To detect infections, rust-specific primers were used to selectively amplify a 560 base pair product from *Puccinia thlaspeos* (Table 3.1).

DNA extraction was done using the Extract-N-Amp Plant PCR kit (Sigma, St. Louis, MO). Leaf tissue was cut using a standard paper punch (0.5 cm in diameter) and incubated in 100 µl of extraction solution for 10 minutes at 95°C. After vortexing the tube, 100 µl of dilution solution was added to the extract in order to neutralize any inhibitory substances that might be present. PCR was carried out using the Extract-N-Amp PCR reaction mix that contained Taq polymerase, buffers, salts, dNTPs, and JumpStart Taq antibodies for hot start amplification. The final primer concentrations used were 0.4 µM of each of the forward and reverse primers. To set up the PCR, 10 µl of the Extract-N-Amp PCR reaction mix, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse primer, 4.4 µl of water, and 4 µl of leaf extract were combined, to create a 20 µl PCR reaction mix. The amplification parameters used were an initial denaturation cycle at 94ºC for 3 minutes,, followed by 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes. Before termination, a final extension step was included, where the reaction was held at 72ºC for 10 minutes.

Primer Design and Validation

The sequence for the cytochrome P450 gene in dyer's woad was unknown. Consequently, primers were designed from the sequences of the *A. thaliana CYP79B2* gene (Genbank accession NM_120158) (Table 3.1). Similarly, primers for *Actin* were designed from *Actin* sequences from *B. napus* (Genbank accession AF111812). The

primers were designed using Primo Pro 3.4 and ordered from MWG Biotech, NC. After

amplification, the double-stranded PCR product was purified using the Wizard PCR

Preps DNA purification system (Promega Corp., Madison, WI). From this purified

product, both forward and reverse sequences were obtained using the dye terminator

method with an Applied Biosystems Inc. model 3700 DNA sequencer (Foster City, CA).

Sequence comparisons against databases were performed using BLASTN and BLASTX

algorithms at the National Center for Biotechnology Information. After ascertaining that

the correct sequence was obtained from *I. tinctoria*, new woad-specific primers were

redesigned from it using Primo Pro 3.4. Woad-specific primers were used to set up

amplification from cDNA following which, both forward and reverse sequences of the

woad defense-related genes were obtained. Different concentrations of both, forward and

reverse primers, ranging from 0.1 uM to 0.9 uM were used in order to determine the

primer concentration that gave the lowest threshold cycle while simultaneously

minimizing non-specific amplification.

Sequence Analysis

Sequences related to dyer's woad *ItCYP79B2* were downloaded from GenBank

and aligned using Clustal X (42). The alignment was finalized by hand by removing

uninformative portions of the sequence and then used to do a heuristic search to find the

most parsimonious trees using PAUP* (40). The search for optimal trees used 1,000

random addition sequence replicates, holding two trees at each step and the tree-

bisection-reconnection branch-swapping algorithm. The strength of the branches was

assessed by performing 100 bootstrap replicates using a heuristic search protocol.

Table 3.1. Primer sequences from *Brassica napus* and *Isatis tinctoria* used for PCR

Target	Source	Oligonucleotide sequence	Length (bp)
Universal	*I. tinctoria*	GCATATCAATAAGCGGAGGAAAAG GGGTCCGTGTTTCAAGACGG	620
Rust	*I. tinctoria*	GCATATCAATAAGCGGAGGAAAAG GCTTACTGCCTTCCTCAATC	560
CYP79B2	*A. thaliana*	AAGAGCCGTCCCGTTTTCC GGCTAGACTCCATCAGCTC	1328
ItCYP79B2	*I. tinctoria*	TACCGCCGATGAAATCAAAC GGATGTCGGATTCTTGGAC	183
Actin	*B. napus*	TGTTGGTCGTCCTAGGCACAC TTCCAGGAGCTTCCATCCCC	718
Actin	*I. tinctoria*	GATGAAGCTCAGTCCAAGAG GAGGATAGCATGAGGAAGAG	364

Extraction of RNA and Reverse Transcription

In order to study the induction kinetics of defense-related genes in woad in response to rust infection, total RNA was extracted from rust infected plants at 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and 72 h after inoculation. Plants that were completely free of the pathogen and had been mock-inoculated served as negative controls with RNA being extracted at the same time intervals. Each treatment consisted of three biological replicates.

Total RNA extraction was carried out using RNeasy Plant Mini kit (Qiagen, Valencia, CA). For each treatment, 100 mg of leaf tissue was taken and flash frozen in liquid nitrogen and ground to a fine powder. Following this, 450 µl of buffer RLT containing mercaptoethanol was added to the sample, vortexed, and incubated at 56°C for

3 minutes to disrupt the tissue. The lysate was then pipetted onto a QIAshredder spin column and centrifuged at maximum speed for 2 minutes. This was followed by the addition of 225 µl of 100% ethanol and the application and centrifugation in an RNeasy column for 15 seconds. The flow-through was discarded and an on-column DNase digestion was carried out to get rid of the genomic DNA contamination. This was followed by the pipetting of 350 µl of buffer RW1 into the RNeasy mini column and centrifuged for 15 seconds. Following this, a solution of 10 µl of RNase-free DNase I and 70 µl of buffer RDD was applied directly for 15 minutes onto the silica gel membrane in the column. Next, 350 µl of buffer RW1 were added into the column and centrifuged for 15 seconds. Subsequently, the silica gel membrane was washed twice with 500 µl of buffer RPE, followed by centrifugation each time, for 15 seconds. The RNeasy column was transferred to a new collection tube and 50 µl of RNase-free water were applied to the column and centrifuged for 1 minute at 14,000 rpm, in order to elute total RNA in the collection tube. The quality of RNA that was obtained was analyzed using the Bio Analyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany), and quantified spectrophotometrically, using a NanoDrop ND100 (NanoDrop Technologies, Wilmington, DE).

Synthesis of cDNA was carried out using the First Strand cDNA synthesis kit (Fermentas Inc., MD). Briefly, 100 ng of RNA in 10 µl of RNase-free water was added to 1 µl of oligo (dT) 18 primer and incubated at 72ºC for 5 minutes. To this mixture, 4 µl of 5X reaction buffer, 1 µl of ribonuclease inhibitor (20 U/µl), and 2 µl of 10mM dNTP mix were added; the resulting mixture was incubated at 37°C for 5 minutes. In the final step, 2 µl of M-MuLV reverse transcriptase (20 U/µl) were added, bringing the entire reaction

mixture to a total volume of 20 µl; this was incubated at 37°C for 1 h. The reaction was

stopped by heating at 70°C for 10 minutes. As a negative control for reverse transcription

and to check for the presence of genomic DNA contamination in the RNA sample, 2 µl of

water were added, instead of the reverse transcriptase. Under these circumstances, cDNA

could be synthesized from RNA only in the presence of the enzyme. Amplification of

DNA in the absence of the enzyme suggested the presence of genomic DNA in the RNA

sample. In this study, every sample was amplified and run in a 0.8% agarose gel to ensure

the absence of genomic DNA.

Quantitative Real Time PCR

In order to quantitate plant defense responses, quantitative real time PCR

(qPCR) was carried out by using the Smart Cycler System (Cepheid, Sunnyvale, CA).

Real time PCR was conducted using iTaq SYBR Green super mix with ROX (Bio-Rad

Laboratories, CA). In order to reduce pipetting errors and maximize the precision and

accuracy of the assay, a reaction cocktail consisting of forward and reverse primers (0.7

µM for the gene *ItCYP79B2* and 0.5 µM for *Actin*), water, and the 2X iTaq SYBR green

mix was set up prior to carrying out the qPCR. Later, 23 µl of the cocktail were aliquoted

into each smart cycler tube, to which 2 µl of the target cDNA were added as the final

step, just before carrying out the assay. Negative controls without the cDNA template

were run with every assay in order to assay for false positive signals. Each treatment at a

specific time point consisted of three biological replicates. The target cDNA in each

biological replicate that needed to be quantitated was assayed four times, amounting to

four technical replications. Thus, each treatment at 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and

72 h consisted of 12 quantitative PCR amplifications (3 biological replicates x 4 technical

replicates).

The amplification parameters consisted of one cycle at 95°C for 3 minutes,

followed by 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30

seconds. After amplification, melt curve analysis was performed by heating the reaction

mixture from 60°C to 95°C at the rate of 0.2°C per second. Data analysis was performed

using the Smart Cycler software (version 2.0 d). For each sample, a threshold cycle (C_t)

was determined by using the exponential growth phase and the baseline signal. This in

turn, was derived from the plot of fluorescence versus cycle number. Every sample was

reviewed and considered positive only if the fluorescence exceeded the threshold value.

Melt curves which were unique for each gene of interest were transformed to the negative

first derivatives, and were used to identify the specific PCR product.

Relative Quantitation of Gene Expression

The induction kinetics of defense-related genes was studied by the relative

quantitation of gene expression using the standard curve method (ABI Prism 7700

Sequence Detection System, User Bulletin 2. PE Applied Biosystems, Foster City, CA).

However, before changes in gene expression could be computed the values were

normalized to account for fluctuations during loading and amplification. Passive and

active signals were used to normalize the experimental results. The passive signal used in

this study was the ROX dye that was included in the PCR mix, which helped to

normalize for non-PCR related fluctuations in the generation of the fluorescence signal.

The active signal used in this study was the *Actin* gene, which helped in normalizing or

standardizing the amount of cDNA added in each reaction. *Actin* was chosen as the active
signal because of its constant expression across all test samples and because its
expression is usually not affected is by the different treatments in the study.

Quantification of gene expression in this study was done by using the relative
quantitation method, which employs the construction of a standard curve. The standard
curve was constructed by amplifying a known concentration of the gene and determining
its threshold cycle during quantitative PCR. For *ItCYP79B2* and the endogenous control
Actin, amplification was carried out using the gene-specific primers and cDNA. The
amplified product was run in a low-melt agarose gel and stained with ethidium bromide.
After visualizing the DNA fragment using Ultra Violet (UV) light, it was excised with a
clean and sharp scalpel; it was then weighed. The product was purified by using the
QIAquick Gel Extraction kit (Qiagen, Valencia, CA). For every volume of the agarose
gel, 3 volumes of buffer QG were added and incubated at 50°C for 10 minutes. During
this period, it was vortexed at intervals of 3 minutes to aid in dissolving the gel. This was
followed by the addition of 1 volume of isopropanol to the sample, following which the
entire sample was applied to a QIAquick spin column and centrifuged for one minute.
The flow-through in the collection tube was discarded, and the column was washed with
0.75 ml of buffer PE and centrifuged for one minute. In order to elute the DNA that was
present in the column, 50 µl of water were added, incubated for one minute, and
centrifuged for one minute. The quantity of DNA that was present in each sample was
quantified spectrophotometrically using a NanoDrop ND100 (NanoDrop Technologies,
Wilmington, DE). Prior to its use in the construction of the standard curve, the purified
product was sequenced, and the concentrated DNA was diluted 10^6 to 10^{12}-fold. This was

done to ensure that it was at a concentration that was likely to be found in the biological sample.

In the construction of the standard curve, care was taken to ensure that the range of template concentrations of the unknown sample fell within the linear dynamic range of the known test samples. Typically each standard curve consisted of seven data points within a 2-fold dilution series, each of which, in turn, was amplified in triplicates. The standard curve was constructed by plotting the log of the starting quantity of the known sample against the average C_t values obtained during the amplification of that particular dilution. After obtaining the standard curve, the equation of the linear regression line, along with Pearson's correlation (r) or the coefficient of determination (R^2) was obtained. The equation of the linear regression line that included the slope and the y-intercept of the standard curve line were then used to calculate the amount of target DNA, based on the threshold cycle generated during amplification.

Standard curves were constructed both for the target gene *ItCYP79B2* and the endogenous control, *Actin*. For each experimental sample, the amounts of the target and the *Actin* gene were estimated from the relevant standard curve. In the next step, the normalized values were obtained by dividing the amount of the target by the amount of the *Actin* gene. Once the normalized values were obtained, the relative changes in gene expression in rust treatments were computed by dividing the normalized amount in each of these treatments by the normalized amount in the healthy and untreated controls. From these values, the mean value and the standard error of the mean were estimated.

RESULTS

Isolation of *ItCYP79B2* from Dyer's Woad

The sequences isolated from dyer's woad were compared to other related

sequences from Genbank. A phylogenetic tree showing the relationships between the

different members of the CYP79 family, involved in the conversion of amino acids to

aldoximes, shows that the *I. tinctoria* sequence clusters with the CYP79B subfamily. All

members of this cluster use tryptophan as the precursor in the formation of indole-3-

acetaldoxime (IAOx) (Fig. 3.2).

The sequence of ItCYP79B2 was 100% homologous with that of CYP79B2 and

95% with that of CYP79B3 (Table 3.2). The sequence of ItCYP79B*2* was 86%

homologous with that of CYP79A1 from *Sorghum bicolor* and 78% homologous with

that of CYP79E1 from *Triglochin maritime*. CYP79A1 and CYP79E1 use tyrosine as a

precursor in the synthesis of the aldoxime. The sequence from dyer's woad was 87%

homologous with that of CYP79D1 and 81% homologous with those of CYP79A2 and

CYP79D4; CYP79D1 uses valine, while CYP79A2 and CYP79D4 use phenylalanine and

isoleucine respectively. Finally, the sequence from dyer's woad was 75% homologous

with that of CYP79F1. CYP79F1 uses methionine as precursors in the synthesis of its

aldoximes.

Amplification Parameters

During primer validation, different concentrations of the primer, ranging from 0.1

μM to 0.9 μM, were used to obtain the lowest C_t. The lowest C_t was obtained by using

A. thaliana (*CYP79F1*)   *A. thaliana* (*CYP79F2*)

L. corniculatus (*CYP79D4*)   100   *A. thaliana* (*CYP79C1*)

                                                                            75

                                                                    A. thaliana (*CYP79B3*)

M. esculenta (*CYP79D1*)   100   *I. tinctoria* (*CYP79B2*)
                                                                            S. alba (*CYP79B1*)
                                                                    92   *B. napus* (*CYP79B5*)
                                                            A. thaliana (*CYP79B2*)

                                                    75

T. maritima (*CYP79E2*)   100

T. maritima (*CYP79E1*)   *A. thaliana* (*CYP79A2*)

                                        100

B.ventricosa (*CYP79*)   *S. bicolor* (*CYP79A1*)

——— 10
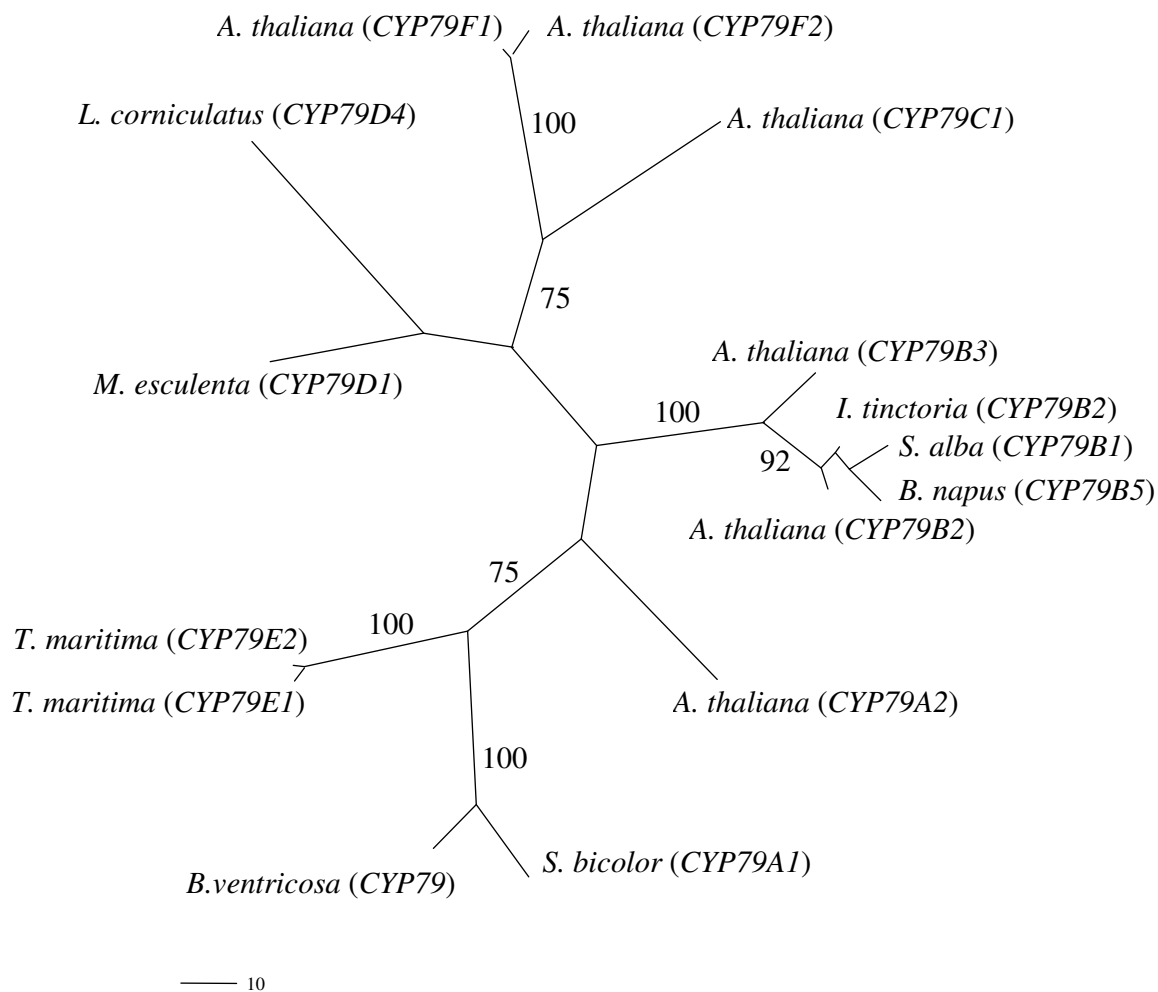
Fig. 3. 2. Unrooted phylogram of *CYP79* genes involved in the conversion of amino acids to aldoximes. Aldoximes are intermediates in both glucosinolate as well as cyanogenic glucosides pathways. Sequences were aligned using Clustal X following which maximum parsimony (MP) were performed using PAUP* 4.0 to find the most parsimonious tree(s), using 1000 replicates. The strength of the branches was assessed by performing 100 bootstrap replicates using a heuristic search protocol.

TABLE 3.2. Results of BlastX search of sequences homologous to *Isatis tinctoria* *ItCYP79B2*

Source	Accession number	Homologous protein	Positives (%)
Arabidopsis thaliana	NP_195705	CYP79B2	100
Sinapis alba	AAD03415	CYP79B1	100
Brassica napus	AAN76810	CYP79B5	98
Arabidopsis thaliana	NP_179820	CYP79B3	95
Manihot esculenta	AAV97889	CYP79D1	87
Sorghum bicolor	Q43135	CYP79A1	86
Bambusa ventricosa	ABD84027	CYP79	86
Triglochin maritime	AAF66543	CYP79E1	78
Triglochin maritime	AAF66544	CYP79E2	77
Lotus corniculatus	AAT11921	CYP79D4	81
Arabidopsis thaliana	NP_568153	CYP79A2	81
Arabidopsis thaliana	NP_563995	CYP79F2	76
Arabidopsis thaliana	NP_563996	CYP79F1	75
Arabidopsis thaliana	NP_178055	CYP79C1	71

0.7 µM forward and reverse primers for *ItCYP79B2* and 0.5 µM forward and reverse primers for *Actin*. Melting curve analysis was used to determine the presence of non-specific amplification. The melting temperature (T_m) is the temperature at which half of a duplex DNA strand becomes single stranded, and is dependent on GC content and length of the amplicon. In this study, the amplicon size and T_m of *ItCYP79B2* and those of *Actin* were 183 bp and 83 ± 0.08°C 364 bp, and 83.6 ± 0.13°C, respectively. All PCR amplifications led to a single and specific product. Furthermore, no-template controls that were included in every assay also indicated the absence of non-specific amplification and extraneous DNA in the PCR cocktail.

Relative Quantification Using a Standard Curve

The accuracy of gene quantification depends on the linearity and efficiency of the PCR amplification, both of which can be assessed by observing the standard curve. Standard curves were constructed using 6 to 7 data points within a 2-fold dilution series, each of which was amplified in triplicates. Examples of standard curves for *ItCYP79B2*, and the normalizer *Actin* are portrayed in Fig. 3.3. The standard curves were derived as a plot between the logarithm of the known quantity of cDNA and the threshold cycle it generates during the amplification.

Induction Kinetics of *ItCYP79B2* in Dyer's Woad

Infecting dyer's woad with rust pathogen led to an initial decline in the levels of *ItCYP79B2* and was measured at 0.38 ± 0.06-fold at the end of 8 h (Fig.3.4). In the next 4 h, these levels were found to be 0.73 ± 0.14-fold. By the end of 16 h, there had been a
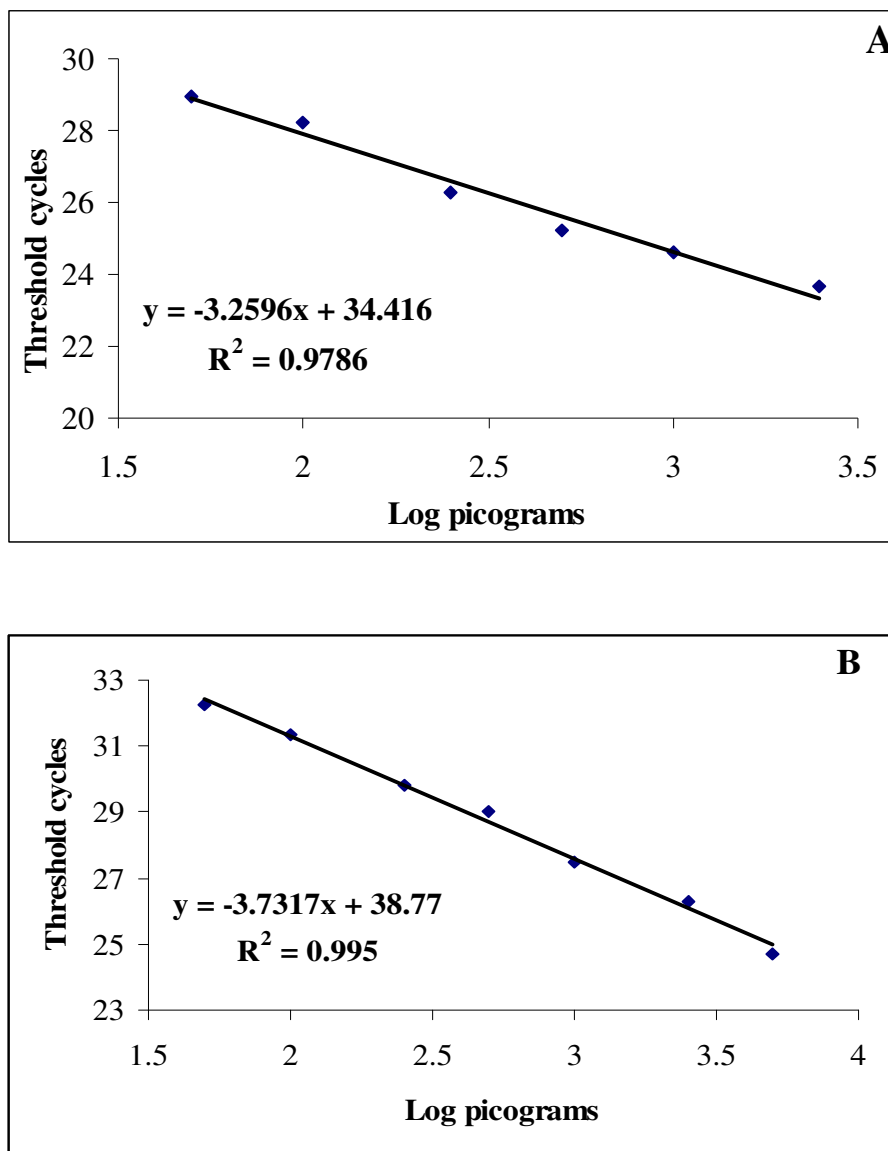
Fig. 3.3. Examples of standard curves for *ItCYP79B2* (**A**), and the endogenous normalizer, *Actin gene* (**B**). Known quantities of purified RT-PCR products were subjected to a two-fold dilution and used in the qPCR assay. Each data point is the mean of three technical replications generated in the same run. A linear relationship was obtained for each run by plotting the threshold cycle number (C_t) against the logarithm of the known quantity of the purified RT-PCR products. From the regression equation obtained, the slope and the intercept were used in determining the amount of target in the test sample.
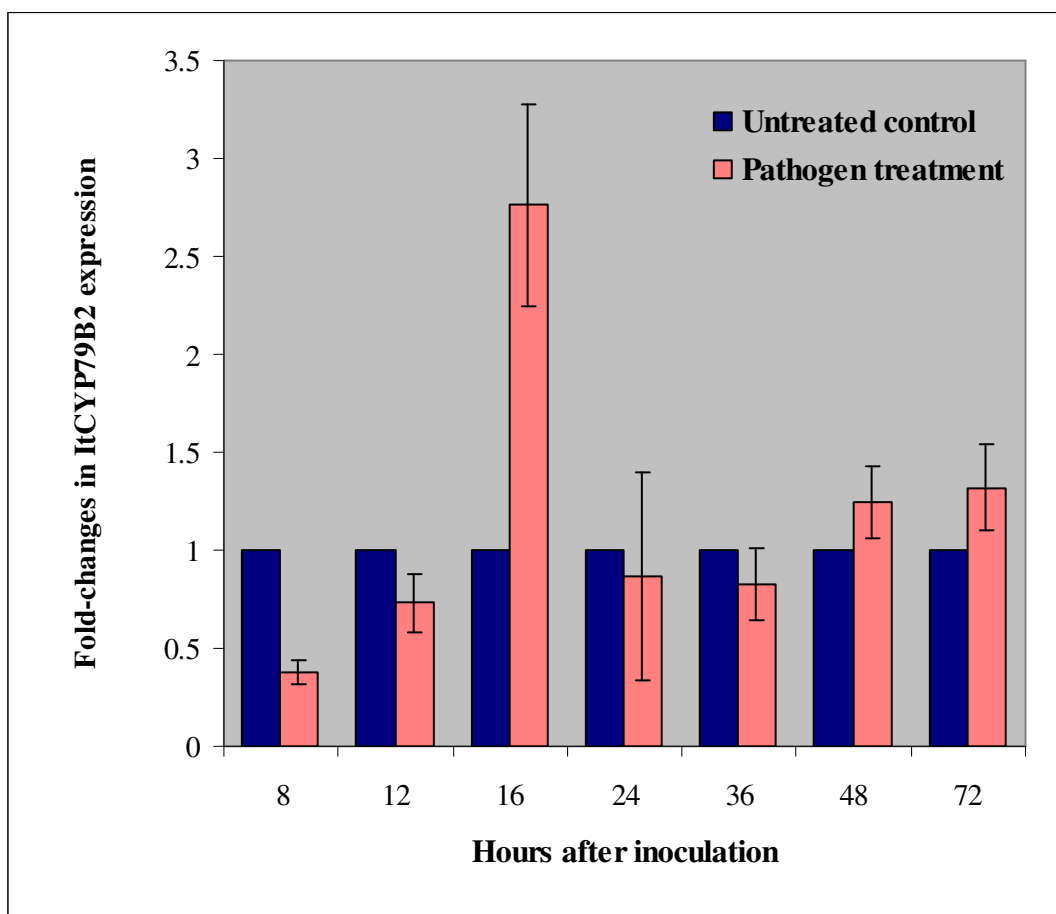
Fig. 3.4. Induction kinetics of defense-related gene *ItCYP79B2* following inoculation of dyer's woad with the rust pathogen. Transcript levels were determined using qPCR. The relative amount of the target was estimated from the slope and the intercept of the relative standard curve. Values were normalized with the *Actin* gene, and the fold changes in gene expression were determined by dividing the normalized target by the normalized amount in the untreated control plants. Each time point in the study had its own normalized calibrator. Each data point was generated from 12 amplifications (3 biological replicates x 4 technical replicates) and is reported as mean (±S.E.).

rapid increase in the levels to 2.76 ± 0.52-fold compared to control plants. However, these levels subsequently fell by 24 h and 36 h after inoculation when levels were quantified at 0.87 ± 0.53 and 0.82 ± 0.18-fold, respectively. At the end of 48 h and 72 h after inoculation, levels were found to be 1.24 ± 0.18 and 1.32 ± 0.22-fold, respectively.

DISCUSSION

In this study, a cDNA sequence encoding the enzyme *ItCYP79B2* was isolated from *Isatis tinctoria*. The sequence was found to cluster tightly with *CYP79B* genes of other members of the Brassicaceae, all of which use tryptophan as a precursor for indole-3-acetaldoxime (IAOx) synthesis (Fig. 3.2). At the same time, sequences for genes that use different amino acids in the synthesis of aldoximes (such as tyrosine, methionine and phenylalanine) were unrelated and clustered at different places on the phylogram. The *ItCYP79B2* sequence had 97 percent (at the nucleotide level) and 100 percent (at the amino acid level) identities to that of CYP79B*2* from *Arabidopsis thaliana*. Because of this, it can be inferred that the dyer's woad sequence is the homolog of *A. thaliana* CYP79B2, and is likely to use tryptophan as a precursor in the synthesis of indole glucosinolates.

Studies have shown that both CYP79B2, and its homolog CYP79B3, catalyze the conversion of tryptophan to IAOx in *Arabidopsis* (20, 32) and that mutations in either of these genes lead to modest reductions in the synthesis of IAOx (51). However, in *CYP79B2/CYP79B3* double-knockout mutants, indole glucosinolate synthesis was eliminated (51). Although other plasma membrane-bound peroxidases that could oxidize tryptophan to IAOx have been found (26, 27), IAOx formed by the catalytic action of

CYP79B2/CYP79B3 appears to be the main source of indole glucosinolate synthesis in *A. thaliana*. Both of these enzymes are essential in the synthesis of indole glucosinolates, but it appears that they play different roles in its synthesis. *CYP79B3* is induced in response to methyl jasmonate (33), while *CYP79B2* is induced by both eukaryotic and prokaryotic pathogens leading to the production of the *Arabidopsis* phytoalexin, camalexin (15, 16). Studies also suggest that *CYP79B2* is induced by aphid feeding particularly near the probing site (25).

The initial events in the colonization of dyer's woad by *P. thlaspeos* have been well characterized. Kropp et al. (24) determined that woad rust required around 6 h after inoculation to penetrate into dyer's woad, and that the rust produced abundant intercellular hyphae within 12 to 24 h of inoculation. Based on this knowledge, the inoculum used in the current study was incubated before treating the plants so that basidiospores would be released within 2 h after inoculation. During the first 8 h following treatment, the expression of *ItCYP79B2* in rust inoculated dyer's woad plants was found to be down-regulated to 0.38 ± 0.06-fold compared to the controls (Fig. 3.4). Thus, the down-regulation of this gene appears to be linked to the penetration of the fungus into the plant.

Similar results were obtained in a gene expression profile study conducted by Zou et al. (52). In their study, they examined the susceptible and resistance response of soybean to *Pseudomonas syringae* pv. *glycinea* that either lacked or carried the avirulence gene *avrB*. It was found that within 8 h of inoculation, there was a down-regulation of 94 chloroplast-associated genes specific to the resistance response. In cDNA microarray studies conducted on plants subjected to biotic and abiotic stress,

down-regulation of genes associated with photosynthesis including chlorophyll a/b binding protein and components of photosystem I and II has been observed (13). Studies conducted on *CYP79B2* have found that it is located in the chloroplast (20, 34). Hence, it is possible that when woad plants are facing biotic stress in the first 8 h of inoculation, the down-regulation of *ItCYP79B2* is part of a general down-regulation of chloroplast associated genes.

However, the current study found that following the initial down-regulation of *ItCYP79B2*, there was a steady increase in its expression levels that peaked at 16 h after inoculation. This coincides with the formation of haustoria by *P. thlaspeos* (28). In similar work, Hull et al. (20) found that induction of *CYP79B2* was observed in *Arabidopsis* within 12.5 h of inoculation with the virulent *P. syringae* pv. *maculicola*. These authors also suggested that the induction of *CYP79B2* in *A. thaliana* is similar to the induction of anthranilate synthase genes *ASA1* and *ASB1* in *Arabidopsis* and they implied that the expression of tryptophan biosynthetic genes and tryptophan-metabolizing genes like *CYP79B2* may be coordinated. Additional studies have also suggested that genes involved in tryptophan biosynthesis and its secondary metabolites, including camalexin, are induced by pathogens, salicylic acid, and oxidative stress (20, 35, 49, 50). A study conducted by Brader et al. (2) found that elicitors from plant pathogen *Erwinia carotovora* triggered the coordinated induction of the tryptophan biosynthesis pathway and the tryptophan metabolizing pathway; this was also accompanied by the accumulation of indole glucosinolates.

On the other hand, induction of pathogen-induced defense gene such as *ItCYP79B2* at haustorium formation is contrary to what has been reported in other

interactions (4, 5). In one rust fungus, a *Uromyces* spp, formation of intracellular haustorium involves the suppression of defense responses (18, 19). Suppression of basal defense responses necessary for the formation of haustoria was also observed 16 h after inoculation of barley with powdery mildew during compatible interactions (4, 5).

Although the current study has not examined the effect of rust infection either on the expression of tryptophan biosynthetic genes, or the levels of indole glucosinolates in dyer's woad, we can infer their likely involvement based on the induction kinetics of *ItCYP79B2*. Though dyer's woad has been shown to have high levels of indole glucosinolates, this does not appear to inhibit successful asymptomatic colonization of the host by *P. thlaspeos*. No evidence of necrosis was seen in woad during colonization (24). Because biotrophic pathogens avoid causing cell damage, indole glucosinolates may not interact with myrosinase and thus avoid the toxic breakdown products of glucosinolates. Furthermore, *Leptosphaeria maculans* that infects *B. napus* produces an enzyme that could convert glucosinolate breakdown products into less toxic components (37). At present, it is not known whether such a mechanism might be employed by *P. thlaspeos* in the asymptomatic infection of dyer's woad but it appears that the fungus is able to circumvent these defense compounds and establish a biotrophic interaction with its host.

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CHAPTER 4

ROLE OF ABIOTIC STRESS IN THE RUST INFECTION OF DYER'S WOAD

(*ISATIS TINCTORIA*) BY *PUCCINIA THLASPEOS*, AND

IMPLICATIONS FOR BIOCONTROL[3]

ABSTRACT

Dyer's woad (*Isatis tinctoria*) is a biennial noxious weed prevalent in the Western United States. It is parasitized by an autoecious microcyclic rust pathogen, *Puccinia thlaspeos*, and is used as a biocontrol agent of this weed. Though extensive studies have been conducted on the environmental factors that favor the infection process in dyer's woad, nothing is known about the environmental modulation of the host that could affect the infection process. This study examined the effect of varying levels of different abiotic stresses (oxidative, salt, osmotic, dehydration, and cold stresses) on the host during the infection process. Plants facing moderate-to-severe levels of oxidative stress had increased protection against the rust pathogen. Moderate-to-severe levels of salinity, osmotic, dehydration, and cold stress did not affect the infection process. However, with the exception of oxidative stress, plants facing mild and sub-lethal forms of each of the stresses had significantly lowered infection rates, compared to the control treatments. Mild abiotic stress appears to help the plant in developing cross-tolerance to the rust pathogen, thereby affecting its efficacy as a biocontrol agent. Though cross tolerance to multiple stresses is a desirable trait in plants of economic importance, in a noxious weed

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[3] Coauthored by Elizabeth Thomas, and Bradley R. Kropp, Role of Abiotic Stress in the Rust Infection of Dyer's Woad (*Isatis tinctoria)* by *Puccinia thlaspeos*, and Implications for Biocontrol.

such as dyer's woad, it is a cause for concern because of its potential to affect the achievement of desired biocontrol.

INTRODUCTION

Dyer's woad (*Isatis tinctoria* L.) is an invasive biennial or short-lived perennial plant that belongs to the Brassicaceae. Though dyer's woad was introduced into North America during the colonial period to exploit its commercial value as a source of blue dye (36), it is believed to have entered the western part of the continent in the early 1900's as a contaminant in alfalfa (5). Since then, it has spread rapidly and has been reported in most parts of the Western United States, leading to its designation as a noxious weed in nine states (5, 9, 11, 12, 36, 40).

In agricultural areas, dyer's woad can be controlled with herbicides or other traditional control methods. However, many large infestations of dyer's woad occur in remote, inaccessible or even environmentally sensitive areas where traditional methods cannot be used. Under these circumstances, biological control is an attractive alternative that can be used in the management of this noxious weed.

The rust fungus *Puccinia thlaspeos* C. Schub. has recently received much attention as a potential biological control agent for dyer's woad (25). This rust fungus infects rosettes of dyer's woad in the first year of its life cycle, and when the plants bolt during the second year, diseased plants are severely stunted, chlorotic, and malformed, with very little seed production (22, 23, 24). Though extensive studies have been conducted on the ecology, disease etiology, inoculation techniques, colonization,

establishment, and dispersal of *P. thlaspeos* (13, 21, 22, 23, 24, 25), very little is known about the effects of the environment on disease initiation.

Plants are subjected to numerous biotic and abiotic stresses in the completion of their life cycles. Abiotic stresses include drought, salinity, extreme temperatures, oxidative stress, and flooding while biotic stress is the result of challenges from pathogens as well as from herbivores. An abundance of research exists on the effects of individual stresses on plant growth and productivity (8, 14). However, in nature, the plants are often exposed to the simultaneous occurrence of several stress factors. Plant survival depends on their response to these multiple stresses.

Studies have shown that plants respond to different unfavorable conditions like drought, salt stress, and low temperatures in similar ways; hence plants which are resistant to one type of stress can develop cross-tolerance to others. For example, studies conducted by Yalpani et al. (39) showed that exposing tobacco to ultraviolet radiation increased tolerance to tobacco mosaic virus due to the accumulation of pathogenesis-related proteins. Similarly, exposing *Arabidopsis* plants to sub-lethal doses of ozone-induced plant defense responses brought about tolerance to *Pseudomonas syringae* (32); inoculation of *Arabidopsis* with the plant growth-promoting rhizobacterium, *Paenibacillus polymyxa*, made the plants more tolerant to drought stress and to challenges from *Erwinia carotovora* (35). This phenomenon of cross-tolerance leads us to believe that the signaling pathway leading to the appropriate response of plants to abiotic and biotic stress are interconnected at several levels and overlap greatly (14, 31)

Although much research (13, 21, 22, 23, 24, 25) has focused on the establishment and dispersal of *P. thlaspeos* under field conditions and on the environmental conditions

favoring infection of dyer's woad, nothing is known about how environmental conditions alter the host response, and subsequent effects on infection. Furthermore, under natural conditions, it is likely that almost any time an infection occurs, the plant could be facing mild, to moderate, to severe abiotic stress. The goal of this study was to examine the effect of different levels of abiotic stresses (namely, oxidative, salt, osmotic, dehydration, and cold stresses) on the infection process.

MATERIALS AND METHODS

Growth of Dyer's Woad

Silicles of dyer's woad were threshed manually to obtain seeds. Seeds were stored at 4°C until further use. The seeds were planted in pots that contained steam-sterilized potting mixture, consisting of perlite, peat moss, and vermiculite, mixed in a ratio of 1:1.3:1, respectively. The plants were grown in a greenhouse using a light and temperature regime of 16 h days at 25°C and 8 h nights at 18°C. Plants were grown in 10 x 10 cm pots and watered until saturated on alternate days. In all treatments, fully expanded leaves from 4-week-old seedlings were used for experimentation. In all treatments, plants were arranged in a completely randomized design.

Artificial Inoculation of Plants

Inoculum was prepared from diseased dyer's woad plants collected in the field in northern Utah during spring. The leaves bearing teliosori were separated from the plants, air dried and crushed into small fragments. Each leaf piece was viewed under the

microscope and the numbers of teliosori were estimated. To prevent or reduce extraneous microbial growth, the inoculum was surface-sterilized prior to inoculation with 0.6% sodium hypochlorite, soaked in sterile distilled water for 3 minutes, and rinsed thoroughly. During inoculation, the surface-sterilized inoculum was positioned over the surface of a 10-cm-diameter Petri dish containing 1.5% water agar. The arrangement of the leaf pieces was such that the teliosori were facing in the upward direction on the surface of the agar. The teliosori were also consistently arranged to ensure that the apical two centimeters of the leaf would be saturated with basidiospores.

After the placement of the teliosori on the water agar, the plates were initially incubated for 6 h in a dew chamber that was maintained at a temperature of 15°C ± 1 to reduce the germination lag period before inoculating the plants. To conduct the inoculation, the Petri plates were inverted over selected leaves to permit the direct landing of the basidiospores from germinating teliospores onto the upper-side of the leaves. Care was taken to ensure that the leaf tissue was not damaged during inoculation. The plants and the water agar were then transferred to the dew chamber for a period of 12 h. Inoculations were carried out in the dark at night. After incubation, the plants were transferred to the greenhouse, and grown there for the duration of 3 weeks, until assayed further.

Detection of Rust

Rust infections were identified after inoculation using polymerase chain reaction (PCR) to detect fungal DNA. Previous studies (22) have indicated that, after inoculation, the rust fungus moves at the rate of 0.25 cm/week in the dyer's woad tissue. Since the

plants in the present study were incubated for three weeks after inoculation before being sampled, the fungus would be expected to move at least 0.75 cm away from the point of inoculation, during that time (22). To determine whether infections had occurred, sampling was conducted at 0.75 cm away from the point of inoculation. Infections were considered to be successful only if the fungus could be found in this zone alone; care was taken to avoid collecting tissue from the point of inoculation in order to avoid false positives. As an added precaution, the sampled leaf tissue was rinsed thoroughly for 90 seconds under running cold water before DNA was extracted in order to get rid of any potential remaining inoculum on the plant surface.

Extraction of DNA was done by using the Extract-N-Amp Plant PCR kit (Sigma, St Louis, MO). Leaf tissue (one-half centimeter in diameter) was cut using a standard paper punch and the leaf disks were incubated in 100 μl of extraction solution, for 10 minutes at 95°C. After vortexing the tube, 100 μl of dilution solution was added to the extract in order to neutralize any inhibitory substances that might be present. PCR was carried out using the Extract-N-Amp PCR reaction mix that contained Taq polymerase, buffers, salts, dNTPs and JumpStart Taq antibody for hot start amplification. Two universal primers, F63 (5'-GCATATCAATAAGCGGAGGAAAAG- 3') and R635 (5'-GGGTCCGTGTTTCAAGACGG-3') were used to amplify a 620 base pair portion of the large ribosomal subunit (21). The universal primers were used as a check to insure the presence of amplifiable DNA in the leaf samples. A second rust-selective primer set, F63 and Rust1 (5-GCTTACTGCCTTCCTCAATC-3'), that amplified a 560 base pair length of DNA was used to detect the presence of rust infections in the leaf samples.

The final primer concentrations used were 0.4 μM each of the forward and reverse primers. To set up the PCR, 10 μl of the Extract-N-Amp PCR reaction mix was added to 0.8 μl of 10 μM forward primer, 0.8 μl of 10 μM reverse primer, 4.4 μl of water, and 4 μl of leaf extract leading to a 20 μl PCR reaction mix. The amplification parameters used were an initial denaturation step at 94ºC for 3 minutes for 1 cycle, followed by 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes. Before termination, a final extension step was included where the reaction was held at 72ºC for 10 minutes.

Abiotic Stress Treatments

To study the effects of abiotic stress on rust infection rates in dyer's woad, plants were subjected in turn to: oxidative stress, cold stress, dehydration stress, osmotic stress, and salinity stress. Each stress category was tested at four different levels to determine whether there was a dose-dependent effect on infection rates. Control plants were not subjected to any abiotic stress, but were exposed to only the rust pathogen. Each level of stress and control treatments included ten plants that were arranged in a completely randomized design. Following stress treatments, plants were watered on a regular basis to ensure that the treatments in each category as well as in control treatments did not undergo any further stress that might affect the outcome of the study.

Oxidative stress

To study the effect of oxidative stress on infection, dyer's woad was exposed to different levels of paraquat (methyl viologen; Sigma, St. Louis, MO) (29, 37). Dyer's

woad was sprayed with either 10 µM, 20 µM, 50 µM, or 100 µM paraquat in 0.1%

Tween 20 solution using a hand held sprayer with 100 ml sufficient to ensure complete

coverage of the plant. Since preliminary trials had indicated that plants exposed to 200

µM paraquat were severely scorched and lost leaves, plants in this study were exposed to

no more than 100 µM of paraquat. Control plants were treated with 0.1% Tween 20

solution. After plants were sprayed with different levels of paraquat, they were incubated

for 2 h in the greenhouse before inoculation with the rust pathogen. After inoculation, the

plants were then transferred back to the greenhouse and incubated for 3 weeks before

assessing infection rates.

Salinity stress

The effect of salinity stress on rust infections of dyer's woad was examined by

watering dyer's woad plants with salt solutions. Plants were watered twice with sodium

chloride solution of different concentrations (50 mM, 100 mM, 200 mM, or 300 mM) to

create varying stress levels. Each pot was watered with 100 ml of the saline solution 24 h

prior to inoculation, and then again with 100 ml of saline solution 2 h prior to inoculation.

This was done to ensure that the plants were in a state of salinity stress from the time of

basidiospore germination up to the time of intercellular mycelial growth in the host.

Saline solutions above 300 mM were not used since higher concentrations caused plants

to dry within a week. After treatment, the plants were allowed to recover by watering 24

h after inoculation and infection rates were determined 3 weeks after inoculation.

Osmotic stress

The effect of osmotic stress on infection rates was studied by watering the plants with 25 mM, 50 mM, 100 mM, or 200 mM mannitol. Concentrations above 200 mM caused the plants to dry within a week. All plants within each treatment were watered twice with 100 ml of the different mannitol solutions. The first treatment was done 24 h prior to inoculation so that the plants would be stressed at the time of inoculation, and the second application was done 2 h prior to inoculation to ensure continued stress at the time of basidiospore germination and intercellular fungal growth in the host. All plants were watered 24 h after inoculation and infection rates were determined after three weeks growth.

Dehydration stress

The effect of dehydration stress on infection rates was examined by withholding water for a period of 3, 4, 6, or 7 days. Control plants were watered on a daily basis. Plants that were not watered for more than 7 days did not recover. In all treatments involving dehydration stress, inoculation was carried out 24 h before the end of the stress period to ensure that the plants continued to face stress during the infection period. After the end of the stress period, all plants were watered regularly to ensure the recovery of the plant. At the end of 3 weeks, the inoculated leaves were recovered and PCR was carried out to measure infection rates.

Cold stress

To examine the effect cold stress on infection rates, dyer's woad plants were subjected to 4ºC for 6 h, 12 h, 24 h, or 48 h. After the plants were subjected to cold stress, they were immediately transferred to the dew chamber and inoculated without allowing for recovery. Control plants were left in the greenhouse until they were used for inoculations. All plants were transferred to the greenhouse and incubated for 3 weeks at the end of which infection rates were ascertained by PCR.

Statistical Analysis

Statistical analysis of the affect of abiotic stress on infection rates was done using SPSS Exact tests, version 13.0 (SPSS, Chicago, IL). The results were analyzed using the Jonckheere-Terpstra test designed to detect the statistical significance of the sequentially ordered data (20, 33). Pair wise comparisons between control groups and the various treatment groups were analyzed using SPSS version 15.0 (SPSS, Chicago, IL). A Welch's *t* test with unequal variance, between control group and various treatment groups with a $P < 0.05$, was considered statistically significant (38).

RESULTS

To determine the effects of varying levels of abiotic stress on rust infections in dyer's woad, trends in infection were analyzed using the Jonckheere-Terpstra test. This test results revealed the presence of statistically significant trends for oxidative and osmotic stresses, but not for salt, dehydration, and cold stresses (Table 4.1). To further

explore the effect of specific stresses on rust infection, within-group comparisons were also conducted, where each treatment group was compared to the control group, for all 5 stresses, using Welch's t-test of unequal variances. Trends in stress effects (Table 4.1) and within-group comparisons are described below (Fig.4.1-4.5).

Effect of Oxidative Stress on Rust Infection of Dyer's Woad

The Jonckheere-Terpstra test indicated the presence of a statistically significant trend ($p<0.001$) in the effect of increasing doses of paraquat on infection rates (Table 4.1). To further determine within-group differences, Welch's t-tests were used. This test showed that there was a statistically significant difference in infection rates between the control group and the group of plants sprayed with 100 μM of paraquat ($p<0.05$) with infection rates of 55 percent (Fig. 4.1). No statistically significant differences were found in infection rates for plants exposed to other levels (10 μM, 20 μM, and 50 μM) of paraquat treatment. The infection level for plants sprayed with 10 μM and 20 μM of paraquat was 100 percent and the rate for plants sprayed with 50 μM of paraquat was 80 percent (Fig. 4.1).

Effect of Salinity Stress on Rust Infection of Dyer's Woad

For plants exposed to increasing levels of salt stress, a statistically significant trend was not found when the Jonckheere-Terpstra test was conducted ($p>0.05$) (Table 4.1). However, to further determine within-group differences Welch's t-tests were used (Fig. 4.2). Statistically significant differences in infection rates were found between the control group and the group of plants watered with 50 mM of sodium chloride ($p<0.05$).

Table 4.1. Effect of different levels of abiotic stress on the rust infection of dyer's woad

Abiotic Stress	Level	J-T statistic (significance *)
Oxidative	Control	[a]-3.144 ***
	10 μM	
	20 μM	
	50 μM	
	100 μM	
Salinity	Control	[b]0.625
	50 mM	
	100 mM	
	200 mM	
	300 mM	
Osmotic stress	Control	[c]-2.139 **
	25 mM	
	50 mM	
	100 mM	
	200 mM	
Dehydration	Control	[d]-0.146
	3 days	
	4 days	
	6 days	
	7 days	
Cold stress	Control	[e]0.511
	6 hours	
	12 hours	
	24 hours	
	48 hours	

* Levels of significance one tailed: *$p<0.05$, **$p<0.01$, ***$p<0.001$
a: J-T statistic is based on Monte Carlo assumptions using 10,000 sampled tables with starting seed 2,000,000.
b: J-T statistic is based on Monte Carlo assumptions using 10,000 sampled tables with starting seed 926,214,481.
c: J-T statistic is based on Monte Carlo assumptions using 10,000 sampled tables with starting seed 299,883,525.
d: J-T statistic is based on Monte Carlo assumptions using 10,000 sampled tables with starting seed 1,314,643,744.
e: J-T statistic is based on Monte Carlo assumptions using 10,000 sampled tables with starting seed 624,387,341.
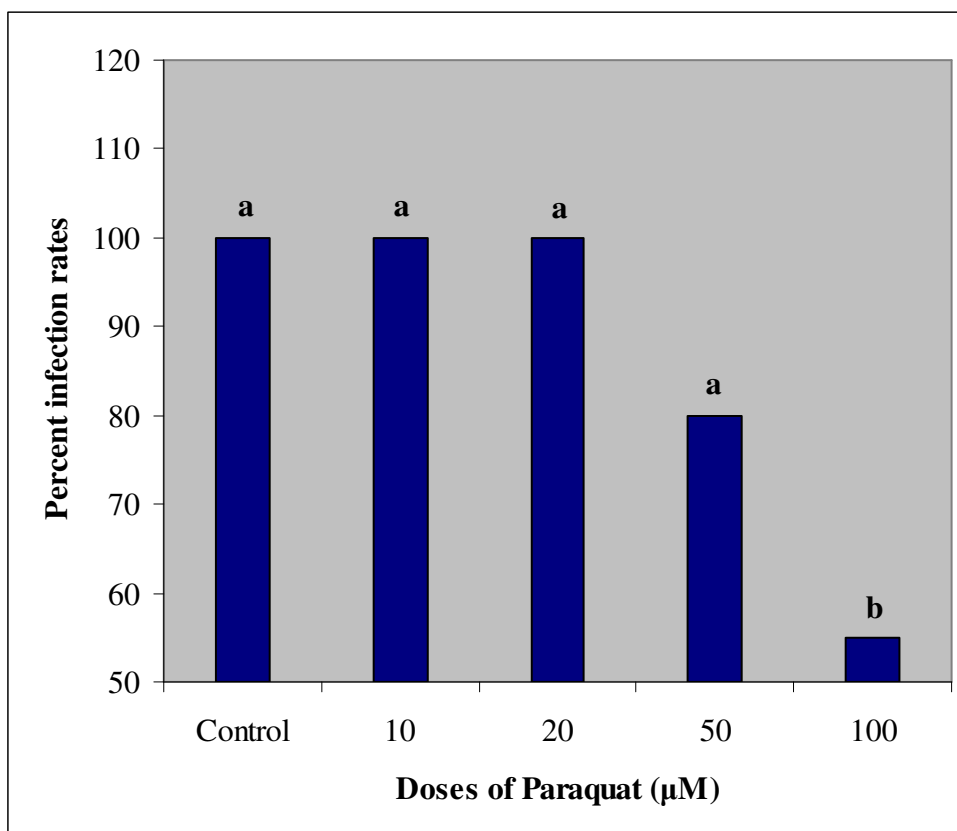
Fig. 4.1. Effect of oxidative stress on rust infection of dyer's woad. To induce oxidative stress on dyer's woad, paraquat (in 0.1% Tween 20) was applied at concentrations of 10 µM, 20 µM, 50 µM, and 100 µM using a hand held sprayer 2 h before inoculation. Control plants were sprayed with water (in 0.1 % Tween 20). Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means were analyzed with Welch's t test ($p < 0.05$; n=10 plants in control, 10 µM, 20 µM, and 50 µM treatments; n=9 plants in paraquat 100 µM). Means with different letters (a, b) are statistically different.

The infection rate in this treatment was found to be 60 percent (Fig.4.2). No statistically significant differences were found in infection rates for plants exposed to other levels (100 mM, 200 mM, and 300 mM) of sodium chloride treatment. The infection rate for plants watered with 100 mM was 75 percent while the rate for plants watered with 200 mM and 300 mM were 90 and 100 percent respectively (Fig.4.2).

Effect of Salinity Stress on Rust Infection of Dyer's Woad

For plants exposed to increasing levels of salt stress, a statistically significant trend was not found when the Jonckheere-Terpstra test was conducted ($p>0.05$) (Table 4.1). However, to further determine within-group differences Welch's t-tests were used (Fig. 4.2). Statistically significant differences in infection rates were found between the control group and the group of plants watered with 50 mM of sodium chloride ($p<0.05$). The infection rate in this treatment was found to be 60 percent. No statistically significant differences were found in infection rates for plants exposed to other levels (100 mM, 200 mM, and 300 mM) of sodium chloride treatment. The infection rate for plants watered with 100 mM was 75 percent while the rate for plants watered with 200 mM and 300 mM were 90 and 100 percent respectively (Fig.4.2).

Effect of Osmotic Stress on Rust Infection of Dyer's Woad

The Jonckheere-Terpstra test indicated the presence of a statistically significant trend ($p<0.001$) in the effect of increasing doses of osmotic stress on infection rates (Table 4.1). To further examine within-group differences, Welch's t-test was used.
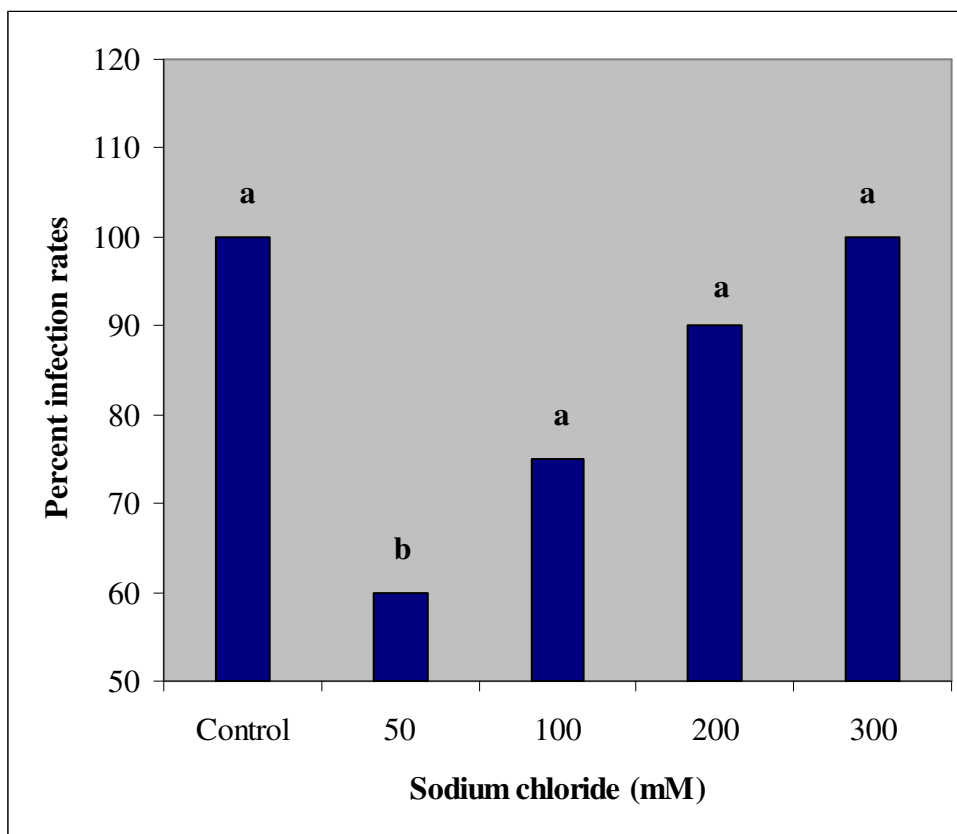
Fig. 4.2. Effect of salinity stress on rust infection of dyer's woad. To induce salinity stress on dyer's woad, each plant grown in a 10 x 10 cm was watered with 100 ml of sodium chloride solution at concentrations of 50 mM, 100 mM, 200 mM, and 300 mM. Control plants were watered regularly and not subjected to stress of any kind. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means were analyzed with Welch's *t* test ($p < 0.05$; n=10 plants in control, NaCl 50 mM, and 200 mM treatments; n=8 in NaCl 100 mM and 300 mM treatments). Means with different letters (a, b) are statistically different.
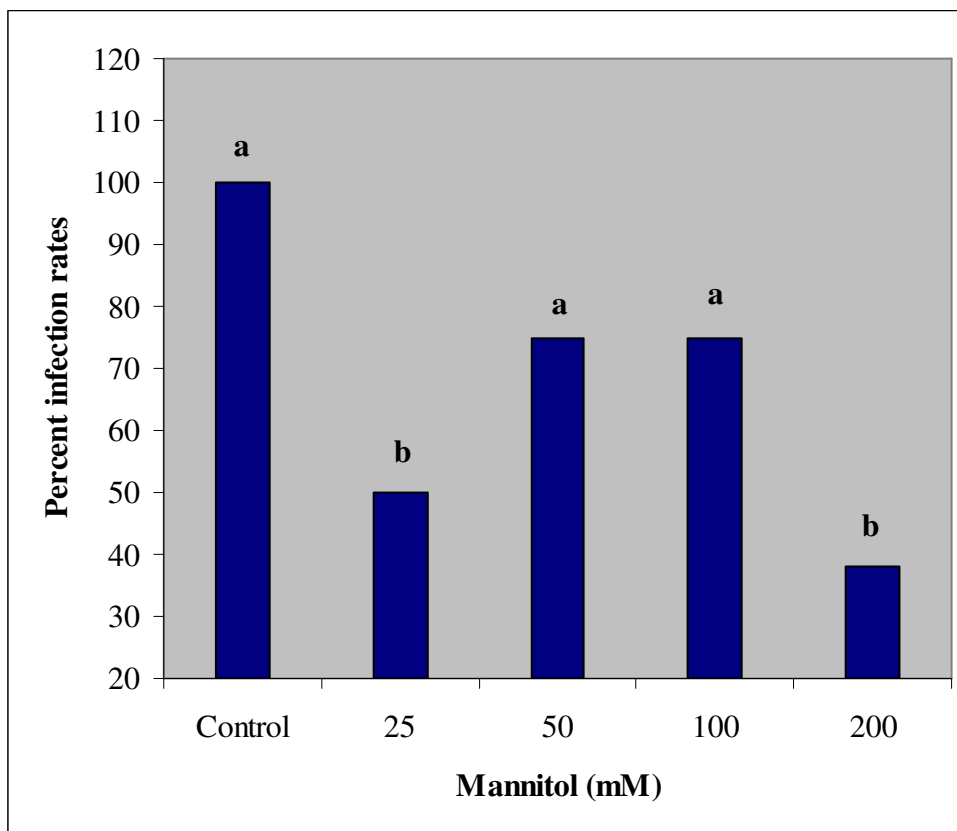
Fig. 4.3. Effect of osmotic stress on rust infection of dyer's woad. To induce osmotic stress on dyer's woad, each plant grown in a 10 x 10 cm was watered with 100 ml of mannitol solution at concentrations of 25 mM, 50 mM, 100 mM, and 200 mM. Control plants were watered regularly and not subjected to stress of any kind. Plants were exposed to mannitol twice, once 24 h prior to inoculation, and once 2 h prior to inoculation. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means were analyzed with Welch's t test ($p < 0.05$; $n=8$ plants). Means with different letters (a, b) are statistically different.

Statistically significant differences in infection rates were found between the control

group and the group of plants watered with 25 mM mannitol ($p<0.05$) and also between

the control group and the group of plants watered with 200 mM mannitol ($p<0.05$). No

statistically significant differences were found in infection rates for plants exposed to

other levels (50 mM and 100 mM) of mannitol treatment. The infection level for plants

watered with 50 mM and 100 mM of mannitol was 75 percent (Fig. 4.3).

Effect of Dehydration Stress on Rust Infection of Dyer's Woad

A statistically significant trend in infection rates for plants exposed to dehydration

stress was not found when the Jonckheere-Terpstra test was conducted ($p>0.05$) (Table

4.1). The within-group differences between different treatments were examined using

Welch's *t*-tests (Fig. 4.4). Statistically significant differences in infection rates were

found between the control group and the group of plants which were withheld water for 3

days ($p<0.05$). The infection rate in this treatment was found to be 70 percent. However,

no statistically significant differences were found in infection rates for plants which were

withheld water for 4 days, 6 days, or 7 days and the infection rates were 89 percent (Fig.

4.4).

Effect of Cold Stress on Rust Infection of Dyer's Woad

The Jonckheere-Terpstra test revealed that the trend in infection rates for plants

exposed to cold stress was not statistically significant ($p>0.05$) (Table 4.1). However,

within-group differences using Welch's *t*-test revealed that there was a statistically

significant difference in infection rates between the control group and the group of plants
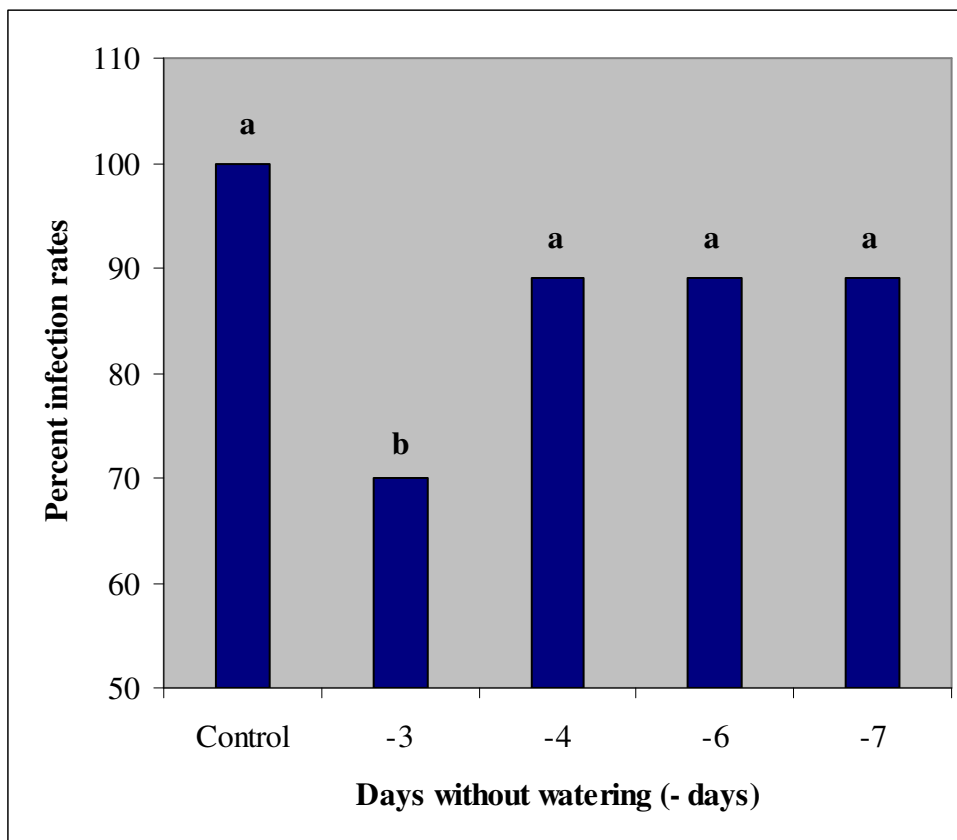
Fig. 4.4. Effect of dehydration stress on rust infection of dyer's woad. To induce dehydration stress on dyer's woad, each plant grown in a 10 x 10 cm was withheld water either for 3 days, 4 days, 6 days, or 7 days. Control plants were watered regularly and not subjected to stress of any kind. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means were analyzed with Welch's t test ($p < 0.05$; n=10 plants for control and -3 days; n=8 for -4 days, -6 days, and -7 days treatment). Means with different letters (a, b) are statistically different.
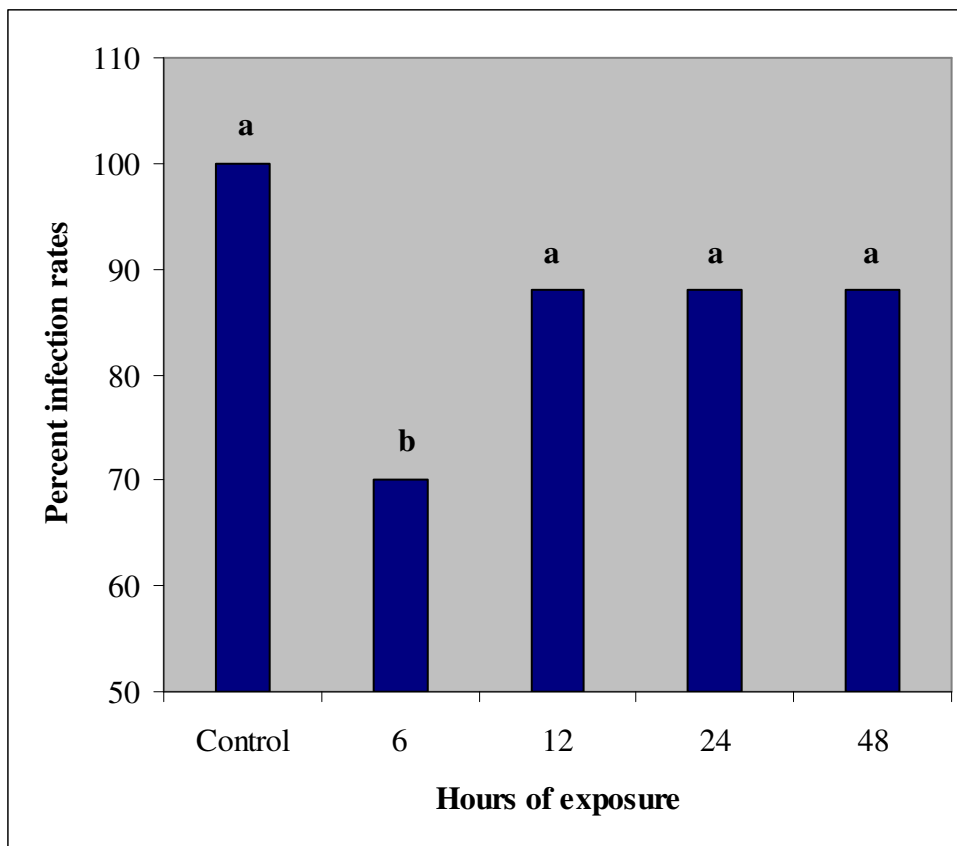
Fig. 4.5. Effect of cold stress on rust infection of dyer's woad. To induce cold stress on dyer's woad, each plant was subjected to 4° C for 6 h, 12 h, 24 h, or 48 h. Control plants were maintained in the greenhouse not subjected to stress of any kind. Immediately after exposure to the cold stress, plants were inoculated with the rust pathogen. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means were analyzed with Welch's *t* test ($p < 0.05$; n=10 plants for control and 6 h exposure; n=8 for 12 h, 24 h, and 48 h of exposure). Means with different letters (a, b) are statistically different.

exposed for 6 h ($p<0.05$) with an infection rate of 70 percent (Fig. 4.5). No statistically significant differences were found in infection rates for plants exposed for longer periods of time (12 h, 24 h, and 48 h). The infection level for plants exposed for 12 h, 24 h, and 48 h was 88 percent (Fig. 4.5).

DISCUSSION

In the natural environment, plants are subjected to various biotic and abiotic stresses. The perception of stress and the relay of such information within the plant are brought about by various signal transduction pathways. Increasing evidence suggests that, when subjected to multiple stresses at the same time, there is a considerable amount of crosstalk between stress signaling pathways (14, 31). Some of the key players involved in the crosstalk between biotic and abiotic stress signaling include phytohormones and reactive oxygen species (ROS). The rust fungus, *P. thlaspeos* is a virulent pathogen because it is not recognized by its host which is consequently unable to mount a successful defense response against the pathogen. Recognition of a pathogen leads to the activation of the plant's plasma membrane NADPH oxidases, cell wall bound peroxidases, and amine oxidases, resulting in the production of ROS (18, 19). The production of ROS is vital in key processes such as programmed cell death and hypersensitive responses that curtail the growth of the pathogen (27).

Application of paraquat is known to create oxidative stress leading to the accumulation of ROS in the apoplast (29, 37). In the current study, application of low doses of paraquat did not affect infection rates (Fig. 4.1). However, as the doses of paraquat increased, infection rates decreased accordingly. This is presumably because the

accumulation of ROS led to the activation of the plant defense responses which in turn lowered infection rates. The rust fungus is a virulent pathogen of woad in the absence of stress. However, under oxidative stress, the rust pathogen is unable to infect the plant thus reducing the efficacy of its role as a biocontrol agent.

In the current study, exposing dyer's woad to different doses of abiotic stress produced a differential response. This study found that when woad was exposed to moderate to severe levels of abiotic stress, including dehydration stress, salinity stress, osmotic stress, and low temperature stress, infection rates were no different from those of the control (Figure 4.2, 4.3, 4.4, 4.5). This could be explained in the context of the role played by phytohormones in the biotic-abiotic stress interaction. Studies have shown that plants facing salinity stress (10), dehydration stress (41), or low temperature stress (7) have elevated levels of abscisic acid (ABA). The role played by this phytohormone in stress response is recognized in other work also (6, 14, 16). On the other hand, some of the phytohormones involved in defense responses to a biotic challenge include, salicylic acid, jasmonic acid, and ethylene (17). Several studies have shown that ABA and defense resistance pathways act antagonistically to each other (1, 28). A study conducted by Audenaeart et al. (2) using ABA-deficient tomato mutant *sitiens*, found increased levels of SA-mediated resistance to the pathogen *Botrytis cinerea* which was abolished upon the exogenous application of ABA. Another study which used the same mutant found that it was more resistant to *Pseudomonas syringae* pv *tomato* than wild-type plants; this mechanism was also SA-mediated (34). These studies suggest that elevated levels of ABA inhibit SA-mediated responses in certain plants. Several studies have also established the antagonistic interactions between abscisic acid and jasmonate/ethylene

signaling (1, 3, 15) where ABA-deficient mutants displayed greater resistance to the pathogens, however this resistance was abolished on ABA application. These studies also concluded that the ABA-mediated stress response was dominant over the defense response when the plants faced abiotic and biotic stresses at the same time. Based on these reports, it is not unreasonable to suppose that the level of ABA could be elevated when dyer's woad faces moderate to severe abiotic stress. This in turn might suppress SA-mediated defense responses which would make the plant even more vulnerable to the biotic-abiotic stress combination. Hence the basidiospore, upon landing on an already stressed plant would face little challenge because of the suppressed defense response.

An unexpected finding in the current study was that woad exposed to mild or sub-lethal doses of salinity, osmotic, dehydration, and cold stresses, had lowered infection rates (Figure 4.2, 4.3, 4.4, 4.5). Mild challenge appears to tilt the balance in favor of the host as reflected in lowered infection rates. This has been reported in few other instances. Mittra et al. (30) found that wheat exposed to mild doses of the heavy metal toxin cadmium developed resistance to subsequent infections by *Fusarium oxysporum* (30). Their research suggests that this was due to the accumulation of cadmium stress-associated protein (CSAP) found in plants exposed to low levels of cadmium, which helped the host to fight plant infections. Similarly, in one study, winter wheat subjected to cold acclimation developed resistance to pink snow mould, *Microdochium nivale* (26). The data from this study show that cold acclimation leads to the accumulation of thaumatin-like proteins (i.e., pathogenesis-related proteins) in the apoplast which contributes to resistance to *M. nivale*. Studies have also shown that the exposure of tobacco plants to sub-lethal levels of ultra violet and ozone stimulated the accumulation

of salicylic acid and pathogenesis-related proteins which conferred resistance to the tobacco mosaic virus (39).

From these studies, it appears that exposure of plants to mild forms of abiotic stresses helped them develop resistance to a biotic challenge. This phenomenon, referred to as cross-tolerance, is of considerable importance in agriculture because it helps the plant breeder to develop and release varieties which show tolerance to multiple stresses (4). In the current study, exposure of woad to mild and sub-lethal levels of stress appeared to acclimate it and develop cross-tolerance to rust as reflected by lowered infection rates. The reasons for the lowered infection rates are not known although one can develop hypothetical explanations for this based on the available literature.

Cross-tolerance is an undesirable event in the biological control of a noxious weed. Much of the work in the selection and isolation of biocontrol agents, such as fungi, bacteria, or insects, is done under carefully controlled conditions that do not reflect field conditions. The modulation of the target by environmental stress leads to acclimation and development of cross-tolerance in the target; this could reduce the success of biocontrol. This factor must be taken into consideration during the development and release of potential biocontrol agents.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Obligate biotrophs are some of the most challenging plant pathogens to study, primarily because they cannot be extensively cultured in the absence of the host (11). In addition, the rust studied in this dissertation, *Puccinia thlaspeos*, produces only the telial stage and a major difficulty in working with teliospores is their uneven germination which leads to erratic basidiospore formation. Achieving uniform germination of teliospores is vital for accurately analyzing plant responses to the rust pathogen. In this study, by soaking the teliospores for varying lengths of time, uniform release of basidiospores was achieved (Chapter 2). This enabled the examination of the host response to the penetration and colonization by the pathogen using standardized inoculum (Chapters 2 and 3).

Prior to the current study, none of the plant defense response genes in dyer's woad had been sequenced. Primers were initially designed from the sequences of defense-related transcripts of *B. napus* and *A. thaliana* and submitted to Genbank (Table 2.1). Following this, gene sequences in dyer's woad homologous to plant defense response genes were obtained. The gene sequences from dyer's woad were 89% to 97% homologous with those of *A. thaliana* (Chapters 2 and 3).

In an earlier study, Kropp et al. (5) had traced the movements of the rust pathogen as it penetrated and colonized dyer's woad. In the current study, the host's defense-responses correlating to key events in the establishment of the rust pathogen in its host were examined. During the penetration of dyer's woad, and prior to haustoria formation,

there was an up-regulation of SA-responsive *PR* genes (Chapter 2). However, during haustoria formation, between 12 h and 16 h following inoculation there was a suppression of *PR* genes. This phenomenon has also been reported in the powdery mildew-barley interactions (1, 2) where pathogen-mediated suppression of defense response genes appears to be vital to the successful formation of haustoria. The production of haustoria appears to be vital not only to fungal acquisition of nutrients, but also to the durable suppression of host defense responses in biotrophic interactions. The *R* gene-mediated response is triggered by the recognition of the *Avr* gene product and occurs only after the formation of haustoria (3). In this research, a significant up-regulation of SA-responsive *PR* genes following haustoria formation was found potentially as a consequence of the delivery of pathogen virulence factors into dyer's woad.

At the same time, there was a second-wave of pathogen-mediated suppression of host defense responses that led to successful infections following 72 h of inoculations. It is not known how *P. thlaspeos* brings about this suppression but the wheat stem rust, *P. graminis* f. sp. *tritici*, has been found to use host-derived oligomers of galacturonic acid from the host cell wall to suppress the activity of phenylalanine ammonia lyase and peroxidases (9). The oomycetous pathogen *Phytophthora sojae* produces glucanase inhibitor proteins that inhibits the host's β-1,3-glucanases; this prevents the degradation of β-1,3- and β-1,6-glucans found in its cell walls (12). It is possible that *P. thlaspeos* might use similar means in suppression of defense responses. In addition, it is known that with respect to gene-for-gene interactions, some rusts possess an *I* gene in addition to the *R* and *Avr* genes that modifies the result of specific *R-Avr* gene interactions from resistance to susceptibility (4). However, further studies will be required to learn whether

P. thlaspeos possesses this *I* gene, and whether it enables this rust to be a virulent pathogen of dyer's woad.

The induction kinetics of *ItCYP79B2* was markedly different from those of SA-responsive genes (Chapter 3). During the fungal penetration of the host, there was a significant down-regulation of this gene. However, of particular interest was the fact that at the time of haustoria formation there was an induction of *ItCYP79B2* followed by a sustained suppression of this gene after haustoria formation. Prior work suggests that this defense gene is JA-responsive (8) and when the results obtained from the SA-responsive *PR* induction (Chapter 2) are compared with JA-responsive *CYP79B2* induction (Chapter 3), it appears that the up-regulation of *ItCYP79B2* directly corresponds to suppression of the SA-responsive genes (Fig. 5.1). Interestingly this was evident during haustoria formation. On the other hand, up-regulation of the SA-responsive gene as seen during the pre- and post-haustorial phases was accompanied by a marked suppression of the JA-responsive gene. This result could be indicative of a potential antagonism between these two pathways.

This possibility is supported by work that has analyzed glucosinolate production in *Arabidopsis* using defense signaling mutants, such as *NahG* (low levels of SA accumulation), *mpk4* and *cpr1* (overproduces SA), *npr1* (non-expresser of PR), and *coi1* (blocked JA signaling) (7, 8). These studies found that an over-production of SA led to a decline in glucosinolate accumulation. At the same time, mutants that were affected in SA signaling such as *npr1*, had increased glucosinolate accumulation. Further studies are needed to determine whether these pathways are antagonistic and if so, what significance this has to rust disease of dyer's woad.
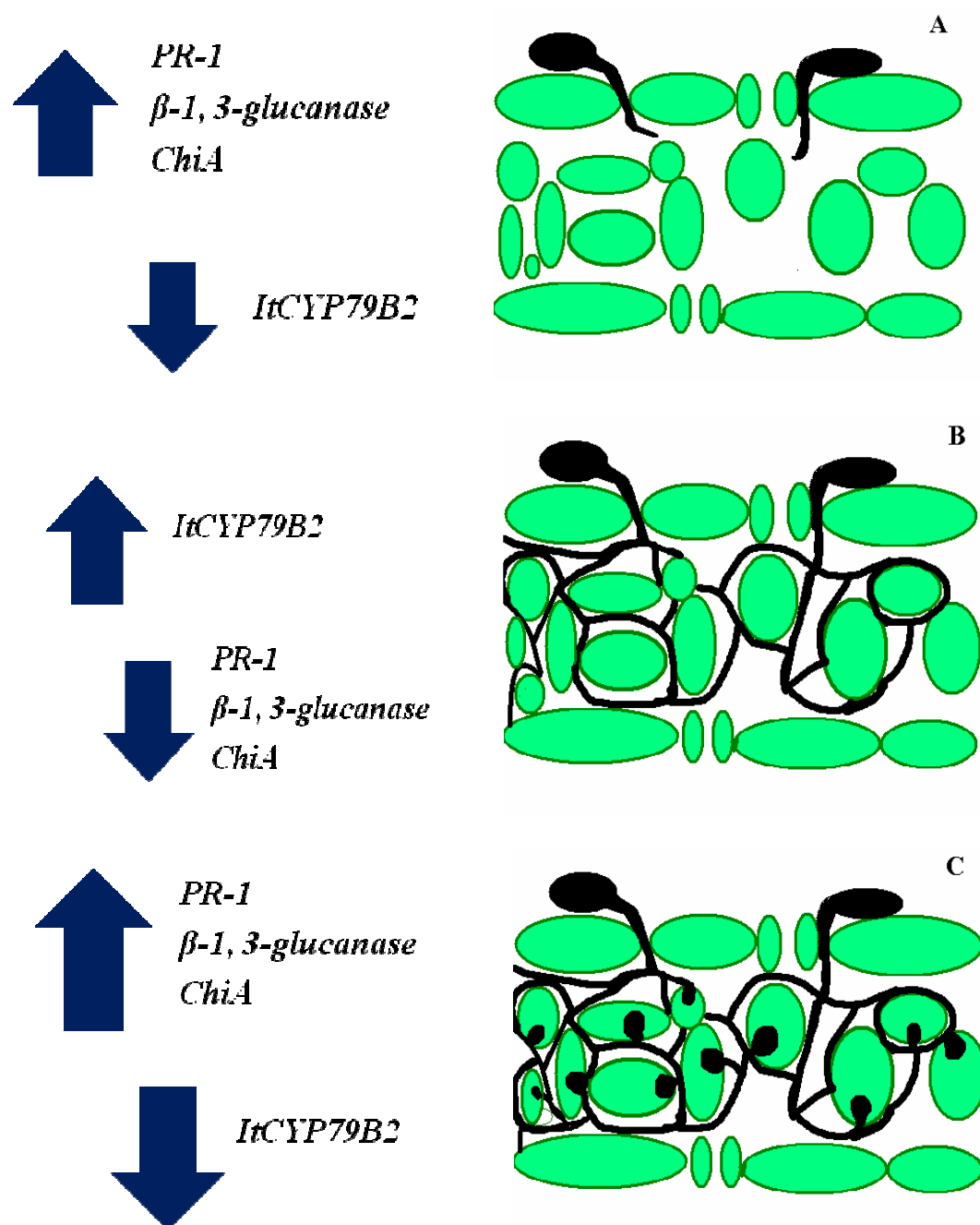
Fig. 5.1. Model depicting sequence of events during the penetration (A), haustorial formation (B), and post-haustorial (C) phases of rust infection of dyer's woad.

The effect of abiotic stress on rust infection was also examined in this dissertation (Chapter 4). It was learned that moderate-to-severe abiotic stresses, such as dehydration, osmotic, salinity, and cold stresses, did not affect disease development. Prior studies indicate that abiotic stress leads to accumulation of ABA which, in turn, influences plant-pathogen interactions (6). The literature also suggests that the interaction between the SA and ABA pathways is antagonistic and that the ABA-mediated stress response is dominant over the defense response (10, 13). However, mild abiotic stress appears to provide protection against the pathogen. The reason behind this is not known and warrants future investigation because of its relevance to biological control.

In conclusion, biotrophy is one of the most sophisticated plant-microbe interactions and the dialog between an obligate biotroph and its potential host is extremely complex. This dissertation offers an insight into the series of events that occurs during successful colonization of dyer's woad by *P. thlaspeos* the host. Although some evidence of host resistance to infection was seen, the host resistance was apparently not sufficient to deter the pathogen. The mechanisms that govern both short term and long term suppression of defense responses are poorly known and need more study before we will fully understand the complex interplay of events that occurs during the plant-microbe encounter.

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CURRICULUM VITAE

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EDUCATION

Ph.D., Biology, Utah State University, Logan.
>   Dissertation: Expression Analysis of Plant Defense Responses during the Establishment of Biotrophy and Role of Abiotic Stress in the Infection of Dyer's Woad (*Isatis tinctoria*) by *Puccinia thlaspeos*

MS, Biology, Utah State University, Logan.
>   Thesis: Phytotoxicity of *Pseudomonas chlororaphis* Strain O6: Role of Phenazines and Implications for Biocontrol.

PROFESSIONAL HIGHLIGHTS

Doctoral Research
Identification of dyer's woad homologs of defense-related genes
- Primer design in Arabidopsis and Canola
- Carried out DNA extractions and PCR of dyer's woad
- Analyzed sequences from purified PCR product
- Comparative analysis of dyer's woad sequences with that of other closely related members using bioinformatics tools

Expression analysis of defense response during rust infection of dyer's woad
- Assessed the suitability of a variety of commercially available RNA extraction kits during extraction of RNA from dyer's woad
- Examined kinetics and magnitude of various defense-related genes using real time quantitative PCR

Examined the effect of different abiotic stresses on rust infections
- Propagated dyer's woad under controlled environmental conditions
- Created a range of abiotic stresses on dyer's woad
- Examined the interaction between abiotic and biotic stress on dyer's woad

Characterization of rust pathogen *Puccinia thlaspeos*
- Collected inoculum under field conditions and optimized it for future use
- Developed a method for consistent inoculum delivery onto host
- Studied the dynamics of teliospore and basidiospore germination

Master's Research
Examined the antimetabolite activities of Phenazine
- Characterized the nutritional requirements for Phenazine production by the bacteria *Pseudomonas chlororaphis*
- Conducted fungal inhibition assays of *Fusarium culmorum* by phenazine-producing *P. chlororaphis*
- Examined the effect of phenazine on germinating wheat
- Carried out Southern analysis of mutants that had lost phenazine-producing ability due to random transposon insertion mutagenesis
- Restored phenazine production by tri-parental mating to mutants impaired in phenazine production using genomic library

WORK/LEADERSHIP HISTORY

General Graduate Assistant   August 1995 - Present
- Involved in the planning, organizing, coordinating, and directing the media preparation services for the Department of Biology
- Experience with, and knowledge of sterilization processes
- Knowledge of aseptic procedures and techniques
- Maintained a library of protocols used in the laboratory
- Initiated a database of cultures
- Management and purchases of resources
- Monitored and maintained the quality of products delivered to microbiological teaching laboratories
- Supervised student laboratory assistants
- Mentored undergraduate student research

ABSTRACTS AND PRESENTATIONS

Thomas, E, and Kropp, B. R. 1996. Potential phytotoxicity of Biocontrol agent O6 on wheat. Presented at the 1996 Annual Meeting of the Phytopathological Society in Indianapolis, IN.

Thomas, E. and Kropp, B. R. 2007. Induction kinetics of defense-related genes in rust infected dyer's woad. Presented at the 2007 North American Cereal Rust Workshop in St Paul, MN.

HONORS

- Nominated for the National Dean's list in the 17th edition 1993-1994
- Received a Graduate Student Senate Travel award for North American Cereal Rust Workshop in 2007
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- Graduate student representative in the search committee in hiring an Extension Plant Pathologist at Utah State University for the State of Utah in 2004.
- Judge in the Poster competition held for students for Microbial Physiology BIOL 5300 during 2002 and 2003