Differential Expression patterns of the pathogenesis-related PR10 multigene family in *Pisum* and *Lathyrus*

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DIFFERENTIAL EXPRESSION PATTERNS OF THE PATHOGENESIS-RELATED PR10 MULTIGENE FAMILY IN *Pisum* and *Lathyrus*

AND

SANDHYA TEWARI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

GENERAL ABSTRACT

The major objective of this study was to analyze the expression patterns of the individual members of the pathogenesis-related PR10 multigene family in a set of recently diverged legume species. The pea PR10 probe hybridized to multiple bands in the genomic DNA from *Lathyrus* and wild *Pisum* ecotypes, indicating that this gene family is present in these taxa. Host/pathogen compatibility was evaluated between two forms of *F. solani* (f. sp. *phaseoli* and f. sp. *pisi*) and four *Pisum* (*P. sativum. P. humile, P. elatius* and *P. fulvum*) and two *Lathyrus* (*L. sativus* and *L. tingitanus*) taxa. The two *F. solani* forms germinated and proliferated to different extents on pod endocarp tissue from different *Pisum* and *Lathyrus* taxa. *P. sativum* was the only species that demonstrated complete resistance to *F. solani* f. sp. *phaseoli*. RNA gel blot analysis indicated a strong correlation between rapid accumulation of PR10 mRNA and inhibition of pathogen growth. This conclusion was also supported by the results from chemical treatments on *P. sativum* pod tissue. Unlike chitosan, which induces PR10 and also protects pea tissue from *F. solani* f. sp. *pisi*, application of salicylic acid (SA) to the pods resulted in an enhanced rate of pathogen growth. This enhancement of growth was concurrent with disappearance of PR10 transcript below detectable levels.

Subfamily-specific probes were used in RNA gel blot analysis to detect evidence for differential expression of two PR10 subfamilies. Messenger RNA hybridizing to the PR10.1 subfamily probe accounted for most of the PR10 expression.

Accumulation of specific PR10 transcripts was analyzed using a combination of cDNA synthesis followed by polymerase chain reaction (PCR) and chemiluminescent detection. Each of the four tested *Pisum* ecotypes accumulated different subsets of PR10 transcripts in response to a specific challenge. While ABR17 and ABR18 mRNAs accumulate in all the four ecotypes following fungal inoculation, PR10.3 mRNA was not detected in any of the ecotypes.

A time course of transcript accumulation in P. sativum in response to F. solani revealed that PR10.1 and ABR18 accumulate to high levels within 4-8 hours post inoculation. PR10.2 and ABR17 appear later and are induced only weakly. Salicylic acid, abscisic acid and chitosan, in general, were not as effective in inducing PR10 in *P. sativum* pods as fungal pathogens.

FOREWORD

This thesis is written in a paper format. The results are present in the form of two papers intended for publication. The first paper (chapter 3) has been submitted to *Physiological and Molecular Plant Pathology*. The second paper (chapter 4) is being submitted to *The Plant Cell* along with nucleotide sequences from PR10 homologs from wild peas. A general introduction and literature review precede the two papers. This is followed by a general discussion and a list of references cited throughout the thesis.

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Chapter 1

GENERAL INTRODUCTION

With the accumulation of analyzed sequence data, it is becoming increasing clear that while some eukaryotic genes are present in only one copy per haploid genome, many genes are present in multiple copies, either as tandem arrays at a single locus, or scattered at different loci. Several hypotheses have been put forward to explain the occurrence of closely-related multigene families (Smith, 1990). Multiple copies could facilitate higher levels of gene expression. Also, variants of a given protein might be made available in response to different endogenous and exogenous stimuli, allowing for more versatile genetic regulation. It is also possible that a gene copy encodes a non-functional protein and is only an artifact of gene amplification/deletion mechanisms and hence confers no selective advantage.

Differential expression of multigene family members may be a key component of phenotypic plasticity in plants (Smith 1990). In this view, differential expression of multigene family members may mediate specific phenotypic responses to environmental stimuli. One of the important exogenous stimuli that all plants must constantly respond to is the challenge by potential pathogens. Multiplicity of regulatory control over the expression of members of multigene families may be one of the means of enhancing flexibility of defense responses. For example, some members might be preferentially expressed in response to fungi and others to bacteria. Some members may be expressed early in the infection process, and others at later

times. Consistent with this idea, differential expression of specific members of defence-related genes is not uncommon (Ryder *et al.*, 1987; Corbin *et al.*, 1987; Shufflebottom *et al.*, 1993). However, such studies have focussed on a single host species or a cultivar. Speciation is accompanied by the random duplication and deletion of multigene family members (Maeda and Smithies, 1986). In the evolution of a multigene family the essential first step toward generating new genes with novel biological functions is the process that generates a duplicate gene or genes from a single ancestral gene. After a gene is duplicated, its two copies start to diverge by accumulating base-pair substitutions, deletions and insertions. Random mutations accumulate in the two copies as long as one copy fulfils the normal function of the organism for survival, the other copy can be silenced, or can fortuitously gain a new function or a regulatory pathway. Due to high degree of similarity between the genes, recombinational events between them can either increase or decrease the number of genes in a family. The processes of gene conversion and crossing over can contribute to the maintenance of homogeneity in the members of a multigene family within a single species. Thus, vastly different multigene families can be created as speciation proceeds. The question that arises then is whether the patterns of differential expression change drastically as well.

The study of this question requires a model system with several components. First, sequence data must be available for most or all members of a defense multigene family, in order to facilitate the detection of differential expression. Secondly, this gene family should be present in several very closely-related species, making it

possible to detect gain, loss, or mutation of family members. Finally, this set of species should exhibit differences in compatibility with a given pathogen.

Recognizing that demonstrating a causal link between the evolution of basic compatibility between plant and pathogen and the evolution of defense multigene families would require a long term effort, this project was undertaken to assemble the necessary pieces to begin such a study. The objective of this study was to evaluate compatibility between *F. solani* and a set of closely-related legume species and to analyze the expression patterns of individual members of the pathogenesis-related PR10 multigene family.

Pathogenesis-related (PR)-proteins, which accumulate in plants upon pathogen challenge, have been described from a wide variety of species. The pathogenesis-related PR10 proteins form one such group of proteins (Van Loon *et al.*, 1994). Although the function of the proteins encoded by this gene family is not known, close association is seen between the kinetics of accumulation of PR10 transcript and expression of resistance by pea pod tissue (Fristensky *et al*, 1985). Homologous transcripts also accumulate in other plant species under a variety of stress conditions (Matton and Brisson, 1989; Somssich *et al.*, 1988; Walter *et al.*, 1990, Warner *et al.*, 1993; Crowell *et al.*, 1992). Unfortunately, most expression studies tend to ignore the existence of individual members of the gene family. Although these studies indicate that PR10 is encoded by a family of 2-12 genes in all the species from which it has been identified so far, no detailed data are yet available on the expression and regulation of individual members of this family. Identifying family members whose

expression is closely correlated with resistance could facilitate manipulation of resistance using these genes; these genes could then be introduced into economically important crops to determine if their constitutive expression confers resistance to the plant. In view of this, the following objectives were set for the present study:

I. To determine if treatment of *P. sativum* pod tissue with salicylic acid or abscisic acid has any effect on the rate of *F. solani* f. sp. *phaseoli* or f. sp. *pisi* growth or PR10 mRNA accumulation.

II. To evaluate four *Pisum* and two *Lathyrus* ecotypes for resistance to two forms of *F. solani* (*F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli*) using a pod inoculation assay.

III. To determine if PR10 multigene family is present and expressed in the given *Pisum* and *Lathyrus* ecotypes.

IV. Using primers designed to detect each of the five cloned *P. sativum* PR10 genes in conjunction with polymerase chain reaction (RT-PCR), study the accumulation of PR10 mRNA

a) In wild *Pisum* ecotypes in response to fungal challenge.

b) In *P. sativum* in response to fungal as well as chemical treatments.

Chapter 2

LITERATURE REVIEW

Introduction

Plants are subjected to an ever-changing environment and need some ways to adapt/react to numerous external stimuli. One of the very important exogenous stimuli that all plants must constantly respond to is the challenge by potential pathogens. The vast majority of such encounters result in an incompatible interaction (host resistant, pathogen avirulent). This resistance is termed as basic or non-host resistance. A fraction result in a compatible (host susceptible) interaction where the pathogen is able to cause disease on a limited number of host species. This is termed as the host range of the pathogen. The outcome of encounters with specific pathogens is often determined by interaction of paired cognate genes in the host and the pathogen (Flor 1947). Susceptibility results if either member of this gene pair is absent or inactivated. Resistance in such interactions is often manifested in the form of the hypersensitive response (HR) which is characterized by rapid cell death around the site of infection which blocks further spread of the pathogen.

A number of physiological changes occur in plants upon challenge with potential pathogens. These changes include release of antimicrobial phytoalexins, synthesis of pathogenesis-related proteins, induction of the enzymes of the phenylpropanoid pathway, changes in the composition of cell walls and secretion of proteinase inhibitors and lytic enzymes (see Lamb *et al.*, 1989 for a review). These

collectively constitute the defense response.

I. The genus *Pisum*: interspecific relationships

Pea is one of the earliest cultivated crops and one of the most important seed legumes throughout the world (Marx 1977).

Pea belongs to the order Fabales, family Leguminosae (Stebbins, 1974). Other genera closely related to *Pisum* are *Cicer*, *Lathyrus*, *Lens* and *Vicia*. Classically, *Pisum* is considered to comprise the cultivated *P. sativum* L. and three wild taxa, *P. humile, P. elatius* and *P. fulvum* (Boissier, 1872). All the four forms are morphologically quite distinct. The major morphological character in which the cultivated forms differ from the wild forms is the lack of pod dehiscence. Cultivated forms have pods that do not split open on drying, while wild pods split suddenly along predetermined lines of weakness.

The four *Pisum* taxa, in spite of being morphologically, cytologically and genetically distinct, form spontaneous hybrids. This has led taxonomists to question the merit of recognizing many species. Crosses between *P. sativum, P. humile* and *P. elatius* result in highly fertile hybrids that set abundant seed (Ben Ze'ev and Zohary, 1973). Crosses between genotypes of *P. fulvum* with genotypes of *P. sativum, P. humile* and *P. elatius* set seed only when *P. fulvum* is the male parent. The resulting hybrids are semi-sterile and set fewer seeds. Greater reproductive isolation of *P. fulvum* is also demonstrated by lack of spontaneous hybrids with other *Pisum* taxa. Only one *fulvum/humile* hybrid was found (Ben Ze'ev and Zohary, 1973), in sharp

contrast to the many hybrids found between *humile/elatius/sativum* at the edges of fields or in ecologically intermediate habitats. These results have led the authors to suggest inclusion of *P. sativum, P. humile* and *P. elatius* into a single aggregate, *P. sativum* and to consider *P. fulvum* to be a fully divergent species.

Evidence from other studies is consistent with this classification. Analysis of variation in chloroplast DNA from 30 *Pisum* genotypes indicated the presence of two lineages: one including all the genotypes of *P. fulvum* and the other including 12 of the 13 tested cultivated lines of *P. sativum* and a wild population of *P. humile* from northern Israel (Palmer *et al.*, 1985). Immunoelectrophoretic studies (Kloz, 1971) also reinforce the conclusion that *P. fulvum* is most diverged from other *Pisum* species.

II. The Pea-*Fusarium solani* interaction

Biology and disease symptoms

F. solani (Mart.) Appel & Wr. f. sp. *pisi* (F. R. Jones) Snyd. & Hans, an ascomycete (teleomorph: *Nectria haematococca*) is a soil-borne pathogen of peas and causes root-rot in the susceptible cultivars of pea (For a review see, Kraft 1981). This disease primarily affects the foot of the plant around the region where the cotyledons attach. Stomates on the epicotyl are thought to be the primary site of entry into the host plant (Bywater 1959). In the field, interactions with other root pathogens are important in determining the extent of damage (Burke and Kraft 1974). Genetic resistance to *F. solani* f. sp. *pisi* is dominant and is also influenced by cytoplasmic factors (Knavel, 1967).

F. solani f. sp. *pisi* produces sporodochia which are blue-green to buff in color (Jones, 1923). Macroconidia are septate, 4.4-5 µm by 27 to 40 µm, curved and hyaline. The optimum temperature for growth on agar is about 30° C.

F. solani f. sp. *phaseoli* (Burk.) Snyd & Hans. causes root-rot in beans. Pea is a non-host to this pathogen (Kraft *et al.*, 1981). Most isolates of this fungus produce appressed pseudopinnotal colonies which are blue or blue-green on carbohydrate media. Macroconidia are abundant while microconidia are rare.

The soil-borne nature of the pathogen makes it less amenable to studies of host-pathogen interactions at the molecular level for several reasons. Firstly, it is cumbersome to determine precisely when the pathogen actually attaches, enters and spreads through a host. Precise time course experiments are very important for dissection of molecular mechanisms involved in expression and regulation and of genes involved in resistance. Molecular analysis also require a system where there is minimal interference from external factors (such as changes in environment, interaction with other organisms etc.). Root inoculations also require a long time for the actual appearance of symptoms for scoring.

Pea inoculation bioassays

Several simplified assays have been used to study host-pathogen interaction between pea and *F. solani* f. sp. *pisi*. These include pea stem bioassy (Rogers *et al.* 1994), leaf assay (Stahl *et al.* 1994) and pod assay (Hadwiger *et al.*, 1970).

In the pea stem bioassay, stem sections are placed on water-saturated filter

paper in a sterile petri dish and a spore suspension is placed on the centre of each section. Significant variation in the frequency of lesion formation was observed in four different experiments when this assay was used to study the effect of cutinase gene disruption on the virulence of *F. solani* f. sp. *pisi* (Rogers *et al.*, 1994).

The leaf assay involves placing a conidial suspension on the upper surface of intact or needle pricked leaves of 13 day old plants. This assay has also been used to study the role of cutinase in the virulence of *F. solani* f. sp. *pisi* (Stahl *et al.*, 1994).

The pod assay involves spread of a measured quantity of pathogen or elicitor suspension on excised immature pods which have been split open along the suture lines (Hadwiger *et al.*, 1970). Pod tissue not only offers a wide and even surface for challenging host cells evenly and uniformly, but also facilitates precise time course experiments. Pod endocarp tissue is effectively sterile and the natural senescence process is relatively slower than that in other plant parts. The study of various biochemical (Hadwiger and Wagoner, 1983; Wagoner *et al.*, 1982), physiological (Hadwiger *et al.*, 1970; Hadwiger *et al.*, 1969; Hadwiger *et al.*, 1974; Hadwiger, 1975) and cytological (Hadwiger and Adams, 1978) changes in the host using this system have provided insights into many aspects of host-pathogen interactions such as importance of phytoalexins (Hadwiger, 1975), the use of heat-shock and protein synthesis inhibitors to demonstrate active nature of resistance (Teasdale *et al.*, 1974; Hadwiger and Wagoner, 1983).

As in other simplified assays, one disadvantage of pod inoculation is that it is far removed from the conditions occurring in fields. The host response has,

nevertheless, been observed to be similar, albeit modified in intensity (Hadwiger *et al.*, 1970). Using this assay, the pea pathogen, *F. solani* f. sp. *pisi*, is able to proliferate on pea tissue while the bean pathogen, *F. solani* f. sp. *phaseoli*, remains inhibited on pea endocarp for up to five days (Teasdale *et al.*, 1974). Symptoms indicate the relative virulence of the two fungi to peas. The epidermis of the pod shows pinhead-sized dark brown lesions and the endocarp appears darkened and slightly macerated by 42 hr after treatment with *F. solani* f. sp. *pisi* (Hadwiger *et al.*, 1970). At this time, the endocarps of the pods incubated with *F. solani* f. sp. *phaseoli* appear slightly discoloured only around the edge of the pod and in areas damaged during excision. Pea pod tissue also restricts the growth of other non-pathogens of peas such as *F. nivale, Ophiobolous graminis* and *F. roseum* f. sp. *cerealis* 'Culmorum' (Teasdale *et al.*, 1974).

Resistance is an active phenomenon

The inhibition of the bean pathogen on pea endocarp was shown to require protein synthesis. Protein synthesis inhibitors, applied to pods before inoculation with the incompatible pathogen, are able to block this resistance, enabling the pathogen to grow (Teasdale *et al.*, 1974). Similar results are observed when pods are heat shocked at 40 °C for 2 hours before inoculation (Hadwiger and Wagoner, 1983). Conversely, if the pods are inoculated with the incompatible pathogen prior to or with the compatible pathogen, the compatible pea pathogen remains inhibited on the pea tissue (Hadwiger and Beckman, 1980). Chitosan, a deacylated derivative of chitin, is a

component of cell walls of many fungi. This compound effectively mimics the non-pathogen in inducing many of the defense responses including phytoalexin production and elicitation of the hypersensitive response in pea pods (Hadwiger and Beckman, 1980). The application of chitosan (10 mg/ml) on pod tissue with or prior to inoculation with the compatible pathogen can protect it from the pathogen for at least 17 days after inoculation (Hadwiger and Beckman, 1980).

These results suggest that pea possesses the required mechanisms to resist *F. solani* f. sp. *pisi*. It is, however, prevented from doing so either because it fails to recognize the pathogen and mount a defense response, or the pathogen successfully overcomes this response. Regulation of these responses thus determines the outcome of the interaction.

Changes in the host in response to pathogens

Among the very first responses detectable within the first hour upon fungal challenge are structural changes in the host nucleus and membrane (Hadwiger and Adams, 1978). Challenge with *F. solani* spores leads to the reorganization of cytoplasmic network around the point of contact, distortions in the organization of host nucleus, condensation of chromatin and gradual disappearance of the nuclear membrane.

Pea pod tissue inoculated with *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli* accumulates the phytoalexin, pisatin. However, the host initially accumulates much higher levels of pisatin when inoculated with the incompatible pathogen (Christenson

and Hadwiger,1973). Eventually, the levels of pisatin exceed those in the host tissues inoculated with the compatible pathogen, presumably because the pathogenic form proliferates through a greater percentage of the tissue. Pisatin alone, however, does not determine the success or failure of a pathogen to invade pea tissue (Christenson and Hadwiger, 1973).

Some other host responses are also distinct in both interactions. Host cell viability is more rapidly reduced in pea pods inoculated with the compatible *F. solani* f. sp. *pisi* (Kendra and Hadwiger, 1987). A sharp increase was also observed in electrolyte leakage at 48 hours in the compatible interaction (Kendra and Hadwiger, 1987).

Resistance is associated with higher levels of specific proteins

Analysis of two dimensional electrophoretic patterns of *in vivo* and *in vitro* synthesized proteins from pea tissues inoculated with *F. solani* f. sp. *phaseoli* or chitosan had atleast twenty-five proteins that accumulate to higher levels than those found in water treated control (Wagoner *et al.*, 1982). These proteins were designated "resistance proteins" (Hadwiger and Wagoner, 1983). Some of these proteins are also induced initially by the compatible pathogen, but their accumulation diminishes between 24-96 hours, a period characterized by uninhibited pathogen growth.

A cDNA library constructed from *F. solani* f. sp. *phaseoli*-induced pea pod tissue RNA was used to isolate "disease resistance response" (drr) clones (Riggleman *et al.*, 1985). Eleven clones belonging to 7 distinct hybridization groups were chosen

for further study. Clones pI49, pI176 and pI206 showed a transcriptional increase within the first 6 h with either *F. solani* f. sp. *phaseoli*, *F. solani* f. sp. *pisi* or chitosan, although the induction by *F. solani* f. sp. *pisi* was not only lower in magnitude but also later than that with *F. solani* f. sp. *phaseoli*. (Fristensky *et al.*, 1985). In both compatible and incompatible reaction, pea actively represses the growth of the pathogen and non-pathogen during this period. Within 24 h, however, the true pathogen shows active growth. During this period, a second wave of induction of pI49, pI176 and pI206 is seen with chitosan and *F. solani* f. sp. *phaseoli* but not with *pisi*. Nucleotide sequence analysis of two of these clones, designated pI49 and pI176 revealed that these genes are 95% identical (Fristensky *et al.*, 1988). An additional member of this gene family, designated drrg49-c was isolated by screening a pea genomic library with the pI49a clone (Chiang and Hadwiger, 1990). This gene encodes a protein of 16.8 kD with a pI of 4.4. It shows 85% identity to pI49 and pI176 at amino acid level (93% when comparisons are based on functionally conserved amino acids). This gene was recently also cloned independently by Mylona *et al.*, (1994) while isolating root hair specific transcripts from pea.

Genes homologous to pI49 have been isolated independently from a wide variety of monocotyledonous and dicotyledonous species. These include the pathogenesis-related STH-2 in potato (Matton and Brisson 1989), PcPR1 and PcPR2 in parsley (Somssich *et al.*, 1988), PvPR1 and PvPR2 in bean (Walter *et al.*, 1990), AoPR1 in asparagus (Warner *et a*l., 1993), and PR 10 in alfalfa (Esnault *et a*l., 1993); stress-induced SAM22 and H4 in soybean (Crowell *et al.*, 1992); the major birch

pollen allergen *Betv*I (Breiteneder *et al.*, 1989) and abscisic acid (ABA)-responsive ABR17 and ABR18 in pea (Iturriaga *et al.*, 1994). These genes have recently been grouped under a common class and designated the pathogenesis related PR10 family (Van Loon *et al.*, 1994).

III. PR-Proteins

The pathogenesis-related (PR) proteins have been defined as plant proteins that are induced in pathological or related situations (Van Loon *et al.*, 1994). Pathological situations include both resistant and susceptible interactions with fungi, bacteria, viruses, nematodes, phytophagous insects and herbivores. Related situations include wounding or chemical treatments that mimic some aspect of pathogen infection (*e.g.* elicitors, salicylic acid) but do not include abiotic stress situations.

One of the conditions for a protein to be classified as a PR protein is that it should be newly expressed upon infection such that it is readily detected in an infected state but not in an uninfected state. Proteins like phenylalanine ammonia lyase (PAL), which are developmentally or environmentally regulated, are constitutively present in the cell, and are only increased upon infection, are not included. Specific isoforms of such proteins not normally present in the cells, which are induced in response to infection, however, are considered to be PR proteins.

The term pathogenesis-related proteins was first used for proteins induced in tobacco reacting hypersensitively to tobacco mosaic virus (Van Loon and Van Kammen, 1970). On the basis of sequence data and serological relationships they

were classified into five distinct classes. Since then many PR proteins, which do not fall into any of the existing five classes have been described from other plant species. The number of PR protein families have therefore been extended to include 6 new PR families. These groupings are based on similarities in amino acid sequences, serological relationship and/or enzymatic or biological activity (Table 1).

Families PR-1, PR-4 and PR-5 encode antifungal proteins of unknown function (Antoniw *et al.*, 1980; Van Loon, 1982). PR-3 encodes the hydrolytic enzyme β-1,3 glucanase, originally described by Antoniw *et al.*, (1980). Three of the PR families encode chitinases (PR-3, PR-8 and PR-11). Class I basic chitinases and class II acidic chitinases, despite their different physiological properties and sub-cellular localizations, possess similar enzyme activities and some other common characteristics. They have been grouped into a single family, PR-3 (Van Loon, 1982). Class III chitinases, possessing lysozyme activity, (one acidic and two basic isoforms) form the PR-8 family (Métraux *et al.*, 1988). Recently characterized class V chitinases fall into the PR-11 family (Melchers *et al.*, 1994). The family PR-6 is exemplified by the well characterized family of proteinase inhibitors first described by Green and Ryan (1972). PR-7 contains endoproteinases of the P_{69} type detected in tobacco (Vera and Conejero, 1988). Lignin-forming peroxidase is the "type member" of the PR-9 family (Lagrimini *et al.*, 1987). PR-10, which will be discussed in detail, contains proteins of unknown function which were originally identified in pea in response to fungal infection. Parsley "PR1" (Somssich *et al.*, 1986) has been designated the type member of this family.

Table 1. Recognized families of pathogenesis-related proteins*

Family	Type member	Properties	Reference
PR-1	Tobacco PR-1a	antifungal	Antoniw *et al.*, 1980
PR-2	Tobacco PR-2	β-1,3-glucanase	Antoniw *et al.*, 1980
PR-3	Tobacco P, Q	chitinase	Van Loon, 1982
PR-4	Tobacco "R"	antifungal	Van Loon, 1982
PR-5	Tobacco S	antifungal	Van Loon, 1982
PR-6	Tomato inhibitor I	proteinase inhibitor	Green & Ryan, 1972
PR-7	Tomato P_{6g}	endoproteinase	Vera & Conejero, 1988
PR-8	Cucumber chitinase	chitinase	Métraux *et al.*, 1988
PR-9	Tobacco lignin-forming peroxidase	peroxidase	Lagrimini *et al.*, 1987
PR-10	Parsley "PR1"	"ribonuclease-like"	Somssich *et al.*, 1986
PR-11	Tobacco class V chitinase	chitinase	Melchers *et al.*, 1994

* From Van Loon *et al.*, 1994.

IV. PR-10 Proteins

Substantial data in terms of the genomic organization, protein characteristics, induction patterns and regulation have become available in the decade since the gene encoding PR-10 protein was first isolated from pea tissue demonstrating non-host resistance to *F. solani* f. sp. *phaseoli* (Riggleman *et al.*, 1985; Fristensky *et al.*, 1988).

PR-10 genes from different species have certain common properties. All genes share extensive sequence similarity, which is not restricted to specific regions but extends throughout the coding sequence. Occurrence of homologous genes in both monocotyledonous and dicotyledonous species which are activated by pathogens or their elicitor indicates that these genes are evolutionarily conserved and have probably evolved from a common ancestor. It also suggests that they play an important role in plant defense.

Protein characteristics

Comparative analysis of PR-10 proteins from different plant species reveals certain common characteristics. The size of the protein is about 17 kD (Table 2). Pea, parsley and potato PR-10 polypeptides show nearly identical hydropathic profiles (Matton and Brisson, 1989). All the proteins are slightly acidic. Parsley and bean PR-10 have five acidic residues surplus; Pea protein has four acidic residue surplus while birch has one; potato has one basic residue surplus but is still acidic since the weakly basic His cannot compensate for strongly acidic Asp and Glu (Walter *et al.*,

Table 2. The pathogenesis-related PR10 genes.

Source	Designation	Experimental system	Protein size (kD)	Copies[1]	Reference
Pea	pI49/PR10.1	pods/*F. solani*/elicitor	16.7	1	Fristensky *et al.*, 1988
	pII76/PR10.2	"	16.9	1	"
	drrg49-c/PR10.3	genomic library	16.8	1	Chiang & Hadwiger, 1990
	/RH2	roots/*Rhizobium leguminosarum*	14	-	Mylona *et al.*, 1994
	ABR17	embryos/Abscisic acid	17.2	1	Iturriaga *et al.*, 1994
	ABR18	"	18.1	8	"
Parsley	PcPR1	suspension cells/elicitor/*Pmg*[2]	16.5	3-6	Somssich *et al.*, 1988
	PcPR2	"	16.8	1	Van De Löcht *et al.*, 1990
Birch	Betv1	pollen	17.4	>12	Breitender *et al.*, 1989
	Betv1-Sc1	suspension cells/*Pseudomonas syringae*			Swoboda *et al.*, 1995
	Betv1-Sc2	"			"
	Betv1-Sc3	"			"
Potato	STH-2	tubers/elicitors/*Phytopthora infestans*	17	3	Matton & Brisson 1989
Bean	PvPR1	suspension cells/elicitor/*Colletotrichum lindemuthianum*	16.7	>12	Walter *et al.*, 1990
Asparagus	AoPR1	suspension cells/wounding			Warner *et al.*, 1992
Soybean	SAM22/H4	cytokinin-starved suspension cells	16.7	~10	Crowell *et al.*, 1992

[1]copies= represents the lower limit of actual copy number in the genome
[2]*Pmg*= *Phytopthora megasperma* f. sp. *glycineae*

1990). Twenty eight amino acids are strictly conserved in the PR-10 proteins from pea, parsley, potato, birch and bean. Glycines and charged amino acids are conserved to a much higher extent than other residues and charged amino acids are often exchanged in a conservative fashion. Among the conserved amino acids, six are glycines; four acidic glutamic acid residues, four aspartic acid residues and five basic lysine residues.

Unlike the tobacco PR-1 proteins, PR-10 proteins described so far do not contain a signal peptide which suggests that they may not be secreted and therefore function intracellularly.

Function

The function of PR10 is not yet known. The only clue about the function comes from the sequence similarity found between PR10 and a ribonuclease isolated from *Panax ginseng* calli (Moiseyev *et. al.*, 1994). This protein has an apparent molecular mass of 18 kD which is close to the mass of other PR-10 proteins. A ribonuclease function for PR10 homologs from different species suggests many interesting possibilities, as pointed out by Constabel and Brisson (1995). A ribonuclease activity (McClure *et al.*, 1989) has also been demonstrated for the S-glycoproteins of *Nicotiana tabacum* which are linked to self-incompatibility in *Nicotiana alata* (Cornish *et al.*, 1987). These results become even more interesting given that both plant-pathogen interactions and self-incompatibility involve specific recognition events. Developmental expression of PR10 homologs (Breiteneder *et al.*,

1989; Warner *et al.*, 1993; Constabel and Brisson, 1995; Walter *et al.*, 1995) and other PR-proteins (Atkinson *et al.*, 1993; Leung, 1992; Memelink *et al.*, 1990; Neale *et al.*, 1990; Ori *et al.*, 1990) in various reproductive tissues of many plant species has been reported. This reinforces the parallels between self-incompatibility and plant-pathogen interactions. Although a ribonuclease function appears attractive and plausible for PR10, a ribonuclease activity has not yet been demonstrated for any of the PR-10 proteins isolated so far (Constabel and Brisson, 1995). At this point, a possibility that a contaminant may have co-purified during purification procedures cannot be ruled out (Moiseyev *et al.*, 1995). It is therefore not confirmed if RNAse activity is an inherent property of the PR-like proteins from ginseng callus culture.

Role in defense

The induction of PR10 genes during pathogen-related stress situations suggests an important role for PR-10 in the defense response. However, such data are only correlations. A more direct strategy was adopted by Constabel *et al.*, (1993) who studied the effect of a constitutively expressed PR-10 gene in transgenic potato plants. To ascertain if this PR-10 protein has a direct inhibitory effect on pathogens, potato plants were transformed with a construct containing the coding region of PR-10 under the control of CaMV 35S promoter. Constitutive expression of the PR-10 gene did not have any significant effect on the resistance of potato to *Phytopthora infestans* or potato virus X.

Constitutive expression of a pea PR10 gene was found to enhance the

resistance of transgenic potato to *Verticillium* wilt, powdery mildew (Chang *et al.*, 1993) and early dying disease.

Induction, expression and regulation

All the PR10 genes isolated so far are either directly induced by pathogens or elicitors derived from pathogens. The pea PR10 genes are induced not only in pod tissue demonstrating non-host resistance to *F. solani* f. sp. *phaseoli*, but are also activated during race-specific resistance to *Pseudomonas syringae* pv. *pisi* (Daniels *et al.*, 1987). In parsley, two genes (designated PcPR1 and PcPR2) were identified from cultured parsley cells whose mRNA was elevated by treatment with fungal elicitor (Somssich *et al.*, 1988). Bean PR-10 genes (formerly PvPR1 & PvPR2) were isolated by differential screening of a library from elicitor-induced transcripts (Walter *et al.*, 1990). Transcripts accumulate as early as 30 minutes after elicitor treatment of bean cell suspension cultures. The potato PR-10a (originally called pSTH-2) was cloned by differential screening of a cDNA library prepared from elicitor treated potato tubers (Matton and Brisson, 1989). While no PR10a mRNA is detected in freshly sliced tubers, some signal appears around 6 hour in response to slice wounds. This signal is increased dramatically upon treatment with the elicitor arachidonic acid, reaching a peak at 24 hours that is sustained up to 72 hours. The PR10 homolog cloned from birch (Bet v 1) is a pollen allergen (Breiteneder *et al.*, 1989). When this gene is used as a probe in northern blots, signal is observed in response to pathogens (Swoboda *et al.*, 1994). RNAse protection assays have revealed that a subset of the genes

belonging to a Bet v 1 multigene family are induced in response to both compatible and incompatible bacterial and fungal pathogens in birch suspension cultures (Swoboda *et al.*, 1995). When birch leaves are infected with *Taphrina betulina* (a natural pathogen of birch) and *Fusarium solani* (a non-pathogen), all three genes are induced above the water levels found in control in the case of the pathogen but not with the non-pathogen.

The data obtained so far point to local induction of this gene around pathogen- or elicitor-treated sites. In contrast to that observed for the classical tobacco PR-proteins, systemic induction of these genes in response to pathogen-related challenges or chemical treatments has not been demonstrated so far. In situ hybridization of *Phytopthora megasperma* f. sp. *glycinea* infected parsley leaves showed heavy accumulation of PR10 transcripts around infection sites as early as 4 hours post inoculation (Schmelzer *et al.*, 1989). Accumulation of this transcript was shown to be, at least in part, to be due to transcriptional activation of PR10. *In vivo* footprinting identified a region of protein-DNA interaction at -130 relative to transcription start site that was elicitor dependent (Meier *et al.*, 1991). It was subsequently shown that an 11 bp DNA motif (CTAATTGTTTA) contained within this region binds specifically to proteins present in the nuclear extracts of parsley and *Arabidopsis thaliana* and is important for elicitor-mediated expression (Korfhage *et al.*, 1994). Complementary-DNA clones encoding these proteins having a high affinity to the above DNA motif were isolated. The proteins contain stretches of 61 amino acids that are characteristic of homeodomain proteins. Warner *et al.*, (1992), searching for wound-induced

transcripts in suspension cells of mechanically isolated asparagus seedlings, isolated a cDNA sharing similarity to other PR10 genes. Studies with the AoPR1 promoter fused to GUS reporter gene revealed strong activity localized to wound and pathogen invasion sites in transgenic tobacco (Warner *et al.*, 1993).

PR10 homologs also accumulate in response to several stress conditions. As mentioned earlier, the potato PR10 message is induced by wounding of potato tubers. Soybean PR-10 (SAM22) message appears in cytokinin-starved suspension cultures (Crowell and Amasino, 1991). SAM22 (starvation-associated message 22) mRNA accumulates in response to various chemical treatments usually indicative of and associated with stress (Crowell *et al.*, 1992). Transpiration-mediated uptake of salicylic acid, methyl viologen, chitosan, H_2O_2 and sodium phosphate buffer induces the accumulation of SAM22 mRNA in detached soybean leaves. Abscisic acid, however, does not have any effect on SAM22 abundance in this system. In general, PR10 proteins do not accumulate in response to stress factors not related to pathogenesis. For example heat shock has no effect on PR10 accumulation in potato and anaerobiosis inhibits the accumulation of PR10 mRNA induced by wounding or elicitor treatment (Constabel and Brisson, 1995). The transcripts homologous to the asparagus PR10 are only weakly induced following spraying asparagus seedlings with salicylic acid (Warner *et al.*, 1994).

The expression of PR10 genes during different developmental stages has also been reported. The soybean SAM22 message accumulates predominantly in the roots of young seedlings, and in senescing cotyledons (Crowell *et al.*, 1992). No

accumulation is seen in the hypocotyls and leaves of young seedlings. The message appears in the leaves of older (about 1 month old) plants. The authors speculate that this expression pattern is consistent with a role of these genes in stress since some wounding of the main root occurs as young lateral roots penetrate through the body of the main root. Stress responses may also be triggered during programmed cell death characteristic of senescing tissues. Recently, detailed analysis of bean PR10c promoter-GUS fusion in transgenic tobacco revealed transcript accumulation in roots, senescent leaves, mature pollen and styles. Strong induction this gene was also observed in response to deprivation of light (Walter *et al.*, 1995). Elevated transcript levels were also observed in diurnal cycles during the night. In asparagus, PR10 message is detected in mature pollen grains, which is consistent with the existence of functional homology between members of the PR10 family since PR10 homologs isolated from birch encode pollen allergen (Warner *et al.*, 1993). Further studies revealed spatial and temporal expression pattern remarkably similar to genes coding for the enzymes in the phenlypropanoid pathway (Warner *et al.*, 1994). PR10 message was found in stems undergoing secondary thickening, in anthocyanin containing regions of developing petals which correlated with onset of anthocyanin synthesis; in nectaries and stigma surface; in immature seed testas prior to visible pigment accumulation. One of the pea PR10 homologs is also developmentally expressed (Mylona *et al.*, 1994). Transcripts for this gene (designated RH-2) were detected specifically in the root-epidermis and root hair and also in the parts of pea embryo determined to form the root. Inoculation of pea roots with *Rhizobium leguminosarum*

bv. *viciae* did not have any appreciable effect on expression of this gene, as detected using reverse transcription polymerase chain reaction (RT-PCR). Analysis of PR10a expression using PR10a promoter-GUS fusion revealed that in healthy potato plants this gene is not expressed in any tissue, except the stigma, during the normal development of the plant (Constabel and Brisson, 1995).

Potato PR-10a promoter-deletion analysis has identified a region of 50 bp, located between positions -155 and -105, necessary for the elicitor responsiveness of the GUS reporter gene in transgenic potato plants (Després *et al.*, 1995). A part of this sequence is specifically recognized by two nuclear factors, PBF-1 and PBF-2. Further, PR-10a activation requires phosphorylation of PBF-1. This result is further supported by inhibition of PR-10 protein activation by protein kinase inhibitors.

Based on their results, the authors have proposed a working model for PR10a gene activation. According to this model, the elicitor is first perceived by the cell, possibly through an interaction with a receptor, which activates a staurosporine-sensitive protein kinase. This kinase, either directly or through a cascade of protein kinases, stimulates the DNA binding activity of transcription factor PBF-1 by phosphorylating it. This, in turn, activates PR-10a transcription. Down regulation of the pathway could occur by dephosphorylation of PBF-1 by the action of an okadaic acid-sensitive protein phosphatase.

V. Differential Expression of defense multigene families

Eukaryotic organisms possess large numbers of multigene families coding for

RNAs or proteins. Some examples of proteins encoded by multigene families are: structural proteins involved in chromosomal organization and segregation, the nuclear and extracelluar matrix, the cytoskeleton and those involved in cell division, communication and motility (Dover, 1986). Individual members within a multigene families may be subject to differential expression wherein specific members of a gene family may be induced in response to different signals. Examples of well studied plant multigene families shown to be differentially expressed include: chlorophyll a/b binding proteins (Cab), Rubisco small subunit (*rbcS*) and other photosynthesis-associated genes (Simpson *et al.*, 1986); α-amylase (Lazarus *et al.*, 1985); alcohol dehydrogenase (Rogers, 1985). In addition to these, many defense-related genes induced in plants in response to pathogens are present as multigene families, including phenylalanine ammonia-lyase (Cramer *et al.* 1989), chalcone synthase (Koes *et al.*, 1989), chalcone isomerase (Van Tunen *et. al.* 1988) hydroxyproline-rich glycoproteins (Corbin *et al.*, 1987), 4-coumarate CoA ligase (Douglas *et al.*,1987) β-1,3 glucanase (Ward *et al.*, 1991), PR1 (Rigden and Coutts, 1988), peroxidase (Harrison *et al.* 1995) and many others. It has been proposed that differential expression of multigene families may be the molecular basis of the characteristic property of phenotypic plasticity in plants (Smith 1990). Rather than attempting a comprehensive review of differential expression within multigene families, differential regulation of the chalcone synthase multigene family is discussed here in relation to the functions of this enzyme in adaptative and protective responses to diverse environmental stresses.

CHS genes have been isolated from a number of plant species (Grab *et al.*

1985; Reiff *et al.*, 1983; Reimold *et al.*, 1983; Sommer and Saedler, 1986; Weinand *et al.*, 1986; Ryder *et al.*, 1987; Koes *et al.*, 1989; Junghans *et al.* 1993). In bean, CHS is encoded by a family of 6-8 genes (Ryder *et al.*, 1987). *In vitro* translation and S1 nuclease protection analysis of suspension cells and hypocotyls reveal a complex pattern of differential expression of specific CHS transcripts . In elicitor-treated cells, specific transcripts are differentially induced with respect to both the extent and kinetics of accumulation. In hypocotyls, wounding and infection activate several CHS transcripts with marked differences in the pattern of accumulation of specific transcripts, indicating operation of more than one signal for defense gene activation.

In *Petunia hybrida*, CHS is encoded by a family of 8-10 genes (Koes *et al.*, 1989). RNAse analysis revealed that different subsets of CHS genes are activated during different developmental stages in flower, in seedlings illuminated with UV, and in UV-illuminated cell suspension cultures. Only two genes (CHS-A and J) are expressed during normal plant development. The majority (90%) of the total CHS mRNA pool in floral tissues is transcribed from CHS-A and the rest (10%) from CHS-J. CHS-A and CHS-J, are also induced in young seedlings in response to UV illumination along with two other genes (CHS-B and CHS-G). The composition of UV induced mRNA pool differs considerably between seedlings and suspension cells. Transcript levels of CHS-J in both tissues are very similar, but the CHS-A gene is poorly expressed in cell suspension cultures as compared to seedlings. CHS genes C, D, E, F, H, I, K, & L are not detectable in any of the above tissues.

In alfalfa, mRNA accumulation for the CHS2 gene was shown to peak within 2

h after inoculation with the compatible fungus *Phoma medicaginis*, followed by a decrease in transcript levels to near the limits of detection by 72 h (Junghans *et al.* 1993). In contrast, other CHS genes were initially expressed at low levels, and increased in activity during the entire 72 h time course.

CHS catalyzes the key step in the biosynthesis of flavonoids. Flavonoids represent a class of secondary metabolites that are synthesized in different parts of the plant where they serve diverse functions such as flower pigmentation, protection against UV light and defence against pathogens. From the above examples of complex differential expression patterns of genes encoding this enzyme, it appears that the requirement for the flexibility of expression of CHS in relation to its multiple physiological roles has been met in different plant species by differential regulation of specific CHS transcripts. Existence of highly polymorphic CHS multigene families in legumes as compared to non-legumes, has been taken to indicate that multiple genes in former have evolved in relation to adaptation of the CHS enzyme for the synthesis of isoflavonoid phytoalexins characteristic of the legumes (Ryder *et al.*, 1987).

Chapter 3

PLANT DEFENSE MULTIGENE FAMILIES. I DIVERGENCE OF *FUSARIUM SOLANI* -INDUCED EXPRESSION IN PISUM AND *LATHYRUS*.

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ABSTRACT

The defense response in plants challenged with pathogens is characterized by the activation of a diverse set of genes. Many of the same genes are induced in the defense responses in a wide range of plant species. Because studies usually focus on a single host species, little data are available regarding changes in defense gene expression patterns as species diverge. Abscisic acid (ABA) and Salicylic acid (SA) were tested for their ability to elicit defence responses in pea pod tissue. ABA has little effect on PR10 expression, and salicylic acid appears to suppress both resistance and PR10 expression. Experiments in cultivated peas (*Pisum sativum*) demonstrate that PR10 gene expression is strongest in response to *Fusarium solani*, as compared to that by chemical inducers. PR10 expression was assayed in four pea ecotypes (*P. sativum*, *P. humile, P. elatius* and *P. fulvum*) and two *Lathyrus* species (*L. sativus* and *L. tingitanus*) which exhibited a range of infection phenotypes with *F. solani*. . We show that resistance was characterized by a strong induction of PR10 genes at 8 hr post-inoculation, while susceptibility was proportionally correlated with later or weaker induction. Most of the PR10 expression can be accounted for by two members of this multigene family, designated PR10.1 and PR10.2.

INTRODUCTION

Molecular and genetic evidence support a two-tiered mechanism of induced plant defense in which resistance genes carry out signal transduction leading to the activation of defense genes (Dangl, 1995). While many studies have examined the expression of genes associated with the defense response of plants to pathogens, these studies typically focus on a single host cultivar or ecotype, or on differential lines isogenic for a single resistance locus. Little is known about whether patterns of defense gene expression are conserved in more divergent taxa. It is well known that protein coding sequences tend to be more highly conserved than non-coding sequences such as intron or promoter regions. Yet, if regulatory regions have more freedom to diverge, then their expression patterns would be expected to evolve rapidly as well. That is, even among closely-related species, or among ecotypes of a given species, the developmental or environmental contexts in which a gene is expressed may be quite varied.

Genes associated with inducible defense responses include those encoding enzymes of the phenylpropanoid pathway which are involved both in lignin production and synthesis of antimicrobial phytoalexins (Dixon and Paiva, 1995) and a growing list of "pathogenesis related (PR) proteins" (Van Loon and Van Kammen, 1970). While the functions of some of the PR-proteins remain unknown (Van Loon *et al.*, 1994), it has been shown that some encode hydrolytic enzymes such as chitinases and β-1,3 glucanases (for reviews, see Bol *et al.*, 1990; Boller, 1987; Bowles, 1990).

Considering the large number of defense genes, along with the fact that most of these genes are present as multigene families, (Harrison *et al.*, 1995; Cramer *et al.*, 1989; Koes *et al.*, 1989; van Tunen *et al.*, 1988; Corbin *et al.*, 1987; Douglas *et al.*, 1987), the divergence of expression patterns for these genes could affect host/pathogen compatibility. Understanding how the regulation of defense genes contributes towards evolution of host/pathogen compatibility first requires a knowledge of the degree to which defense gene expression is conserved in ecotypes within species and in closely-related species.

Pisum and *Lathyrus* are members of the family Leguminosae, tribe Fabeae (=Vicieae) within the order Fabales (Waines, 1975). *Pisum* consists of the cultivated *P. sativum* and three wild taxa, *P. humile, P. elatius* and *P. fulvum* (Palmer *et al.*, 1985). *Pisum* species can be distinguished on the basis of morphologic, cytogenetic and molecular genetic data (Marx, 1977). While *P. sativum, P. humile* and *P. elatius* have been known to form spontaneous hybrids (Ben-Ze'ev and Zohary, 1973), crosses between *P. fulvum* and other *Pisum* taxa result in seed set only when *P. fulvum* is the male parent. Additional data from electrophoretic patterns of albumin and globulin (Waines, 1975) and chloroplast DNA variation in *Pisum* (Palmer *et al.*, 1985) have led taxonomists to consider *P. fulvum* to be a distinct species and *P. sativum* to be an aggregate of *P. humile, P. elatius* and *P. sativum.* Within this aggregate, *P. humile* is considered to be the closest wild ecotype and the direct progenitor of cultivated pea.

Pod endocarp tissue as well as seedling tissue from garden pea, *Pisum sativum,* is susceptible to infection with *Fusarium solani* f. sp. *pisi.* However, both tissues

inhibit the growth of *F. solani* f. sp. *phaseoli*, leading to the formation of pinpoint lesions (Christenson and Hadwiger, 1973). In addition to differences in pathogen growth, host responses such as an increase in phenylalanine ammonia lyase (PAL) activity and *de novo* synthesis of the phytoalexin pisatin, changes in host chromatin, and RNA synthesis (Hadwiger and Adams, 1978) are not only more rapid but also greater in intensity in response to the incompatible *F. solani* f. sp. *phaseoli* (Teasdale *et al.*, 1974). A marked increase in the rate of protein synthesis is also observed in endocarp tissue inoculated with *F. solani* f. sp. *phaseoli* over the water treated control whereas *F. solani* f. sp. *pisi* treated tissue only shows a slight increase (Christenson and Hadwiger, 1973). Treatment with RNA and protein synthesis inhibitors within five hours post inoculation suppresses resistance to the incompatible *F. solani* f. sp. *phaseoli*, whereas later treatments have no effect on resistance (Hadwiger, 1975; Teasdale *et al.*, 1974).

Although the endocarp inoculation system offers conditions which are not typical of those existing in the field, the host/Pathogen compatibility has been observed to be unaltered in pod tissue, with differences only in the intensity of symptoms (Hadwiger *et al.*, 1970). Using this assay, the bean pathogen *F.solani* f. sp. *phaseoli* is inhibited while the pea pathogen *F. solani* f. sp. *pisi* germinates and grows on the pod tissue (Teasdale *et al.*, 1974). Pod endocarp tissue is almost sterile and the senescence is slow compared to other plant parts. It provides a large, uniform surface for inoculation on which all the cells are uniformly challenged.

Differential screening of a cDNA library (Riggleman *et al.*, 1985) prepared

from endocarp tissue treated with *F. solani* f. sp. *phaseoli* was used to isolate "disease resistance response (Drr) cDNAs" (Fristensky *et al.*, 1985). Members of the Drr49 multigene family encode a 17 kD intracellular protein whose mRNA is induced by the elicitor chitosan, as well as *F. solani* f. sp. *phaseoli.* According to the nomenclature of Van Loon *et al.*, (Van Loon *et al.*, 1994) this multigene family will henceforth be referred to as PR10. PR10 homologues have subsequently been identified as PcPR1 in parsley (Somssich *et al.*, 1988), pathogenesis-related STH-2 in potato (Matton and Brisson, 1989), PvPR1 and PvPR2 in bean (Walter *et al.*, 1990), AoPR1 in asparagus (Warner *et al.*, 1993), and alfalfa (Esnault *et al.*, 1993); stress-induced SAM22 and H4 in soybean (Crowell *et al.*, 1992); the major birch pollen allergen *Betv*I (Breitender *et al.*, 1989) and abscisic acid (ABA)-responsive ABR17 and ABR18 in pea (Iturriaga *et al.*, 1994). While the function of PR10 is not yet known, a ribonuclease isolated from *Ginseng* has recently been reported to share between 60-70% sequence identity with parsley PR10 (Moiseyev *et al.*, 1994).

We have used the PR10 multigene family in peas to study the conservation of defense gene expression patterns. Initially, expression in response to salicylic acid (SA), chitosan, abscisic acid (ABA) or *F. solani* were compared to determine if chemical inducer elicit the same responses as *F. solani.* PR10 expression was assayed and compared to that of chitinase and chalcone synthase (CHS) in four pea taxa (*P. sativum, P. humile, P. elatius* and *P. fulvum*) and two *Lathyrus* species (*L. sativus* and *L. tingitanus*) which exhibited a range of infection phenotypes with *F. solani.* We show that resistance was characterized by a strong induction of PR10 genes at 8 hr.

post-inoculation, while susceptibility was proportionally correlated with later or weaker induction. Most of the PR10 expression appears to be accounted for by two members of this multigene family, designated PR10.1 and PR10.2.

MATERIALS AND METHODS

Plant material and fungal strains

Wild accessions of *Pisum* (*P. humile* 713, *P. elatius* 721 and *P. fulvum* 706) used in this study were obtained from N. O. Polans, Northern Illinois University, U.S.A. *Lathyrus sativus* L720060 and *L. tingitanus* Nc 8f-3 were kindly provided by C. Campbell, Agriculture Canada Research Station, Morden, Canada. *P. sativum* c.v. Alaska was purchased from W. Atlee Burpee and Co., Warminister, PA. Strains of *Fusarium solani* f. sp. *pisi* and *F. solani* f.sp. *phaseoli* were obtained from American Type Culture Collection (Accession numbers 38136 and 38135 respectively). Cultures were grown and maintained on Potato Dextrose Agar (PDA) plates supplemented with a few milligrams of finely chopped pea leaf and stem tissue.

All the *Pisum* and *Lathyrus* plants were grown in growth rooms in pots in 2:1:1::soil:sand:peat mix under a day/ night cycle of 16/8 hours with temperatures of 22 /15 °C, respectively. The average light intensity using 1/3 0-lux wide spectrum to 2/3 cool white was 340 μ e m^{-2} sec^{-1}.

Pod inoculation procedure

Immature pods (less than 2 cm in length; five pods per treatment) having no

developed seed were harvested from the plants, slit longitudinally along the suture lines, placed with the freshly opened side up on a sterile petri-dish and 50 µl of a 10^6 macroconidia/ml suspension spread evenly on the pod. The plates were then incubated at room temperature under continuous florescent light and samples of the pods halves harvested at 8 and 48 hours. Pods treated with sterile distilled water served as controls.

Application of chemicals on pods

Immature pea pods (1 g /treatment) were harvested and treated as for pathogen inoculations except that instead of inoculum, chemicals were applied on the exposed surface. All the treatments were applied in a total volume of 10 µl/pod at the following concentrations: Chitosan [1 mg/ml] (Bentech Labs Inc., Clackamas, OR, USA, kindly provided by D. F. Kendra, Northrup King), Abscisic acid (Sigma)[100 µM dissolved in 10% methanol] and Salicylic acid (Sigma) [50 mM].

Staining and light microscopy

Thin sections of endocarp tissue were excised from the surface of inoculated pods using a razor blade, and were stained with 0.1% cotton blue (or trypan blue) in lactophenol (Plant Pathologist's Pocketbook, 1983) [Anhydrous lactophenol 67% v/v; Distilled water, 20% v/v; cotton blue 0.1 g w/v] for about 5 minutes. Excess stain was removed using blotting paper. The tissue section was then covered with 1-2 drops of high resolution immersion oil. Either a 100 X or 63 X oil-immersion lens was used

for observation under a photomicroscope (Carl Zeiss model # 63953). Pods were scored for resistance at 8 h.p.i. according to the criteria in Table 3. Five pods per treatment were examined. At least five fields on each pod were examined for scoring. Results from each of the pods were averaged.

DNA extraction and Southern blotting

Pea hypocotyls and young leaves were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. One ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1.25% SDS] was added per 100 mg of tissue and incubated at 65 °C for 20 min. KOAc was added to a final concentration of 3 M, the samples were kept on ice for 20 min then centrifuged at 12,000 g for 15 min. The supernatant was extracted twice with an equal volume of TE (10 mM Tris Cl pH 8.0, 1 mM EDTA)-equilibrated phenol. DNA was precipitated with 1 volume of isopropanol, reprecipitated with 2.5 vol. ethanol and 0.1 vol. 3 M NaOAc (pH 5.2), then the pellet dried and resuspended in TE.

For Southern blotting, 15 µg of genomic DNA from each species was digested with *Eco*RI, electrophoresed through 0.8% agarose in 1X 0.04M Tris acetete, 0.002 M EDTA (TAE) buffer (Ausubel *et al.*, 1994), blotted onto Zeta probe GT membrane and UV crosslinked using the auto-crosslink mode of UV Stratalinker 1800 from Stratagene (1200 microjoules for 30 seconds). The blot was then probed with PR10.1 probe prepared according to the method described in the "probes" section, except that 15 pg of pUC18 plasmid was included in the labelling reaction to detect the λ/*Hin*d

Table 3. Scoring key for spore germination and hyphal growth using the pod inoculation procedure.

SCORE	OBSERVATION UNDER A LIGHT MICROSCOPE (8 hpi)	APPEARENCE OF PODS (48 hpi)
-	Less than 10% spores germinating; Germination tube less than 1/4th the size of the spore.	light brown lesions; no maceration
+	More than 50% spores germinating; Germination tube between 1/4 to 1/2 X the length of the spore.	Pinhead size dark brown lesions; little or no maceration of tissue
++	More than 50% spores germinating; Germination tube ~1/2-1 X the length of the spore.	Pinhead size dark brown lesions; little or no maceration of tissue
+++	More than 50% spores germinating; Germination tube ~1-2 X the length of the spore.	Larger than pinhead size dark brown lesions; little or no maceration of tissue
++++	More than 50% spores germinating; Germination tube ~2-3 X the length of the spore.	Large coalescing lesions; tissue macerated
+++++	More than 50% spores germinating; Germination tube more than 3 X the length of the spore.	Large coalescing lesions; tissue macerated

III, pUC18/*Hinf* I marker.

RNA extraction and Northern blotting

RNA was extracted from pods treated with fungus or water-treated controls at 8 and 48 h.p.i. Eight and 48 hours post inoculation (h.p.i.) were selected as the "early" and "late" time points respectively, for RNA extraction for several reasons. Eight h.p.i. was the original time used to prepare the library in the isolation of the PR10 clone, and the expression of PR10 in previous studies (Fristensky *et al.*, 1985) had been shown to peak at this time. Also, inhibition of spore germination was observed at that point. Eight h.p.i. was also the time used for disease scoring. Forty eight h.p.i. was selected since both pathogens managed to grow sufficiently well by this time on most host species. RNA was extracted using a combination of the small-scale procedure for rapid isolation of plant RNAs by Verwoerd *et al.*, 1989 and the phenol-chloroform method for RNA extraction (Ausubel *et al.*, 1994). Briefly, tissue was ground to a fine powder in liquid nitrogen, then mixed with hot (80 °C) extraction buffer [(1:1) phenol: (0.1 M LiCl, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS)] to make a loose slurry (2-3 ml per g of tissue). One-half volume of chloroform was added and the suspension was mixed by vortexing. After centrifugation for 15 minutes at 975 g, the aqueous phase was removed to a fresh tube. One third volume of an 8 M solution of LiCl was added, then RNA collected by centrifugation for 10 min after overnight incubation at 4 °C. The RNA pellet was dissolved in 250 µl of Diethyl pyrocarbonate (DEPC) treated, sterile distilled water, reprecipitated with 0.1

volume of 3 M NaOAc pH 5.2 and 2.5 vol. of ethanol on ice for 20 min, centrifuged 20 min at 13,000 rpm (15,000 g), and the pellet redissolved in DEPC-treated sterile distilled water.

Ten micrograms total RNA was denatured using formaldehyde denaturation protocol (Ausubel *et al.*, 1994) for RNA gel blot analysis and separated on 1.2% agarose-formaldehyde gels, blotted onto nylon membrane (Zeta-probe) using conditions recommended by the manufacturer and hybridized with ^{32}P labelled, random primed probe in 0.25 M Na_2HPO_4, pH 7.2 and 7% SDS at 65°C. Filters were washed twice with 20 mM Na_2HPO_4, pH 7.2 and 5% SDS at 65 °C for 20 min.

Recombinant plasmids

pI49KS and pI176KS consist of the pI49 (PR10.PS.1, GB::X13383) and pI176 (PR10.PS.2; GB::M18249) cDNAs, respectively (Fristensky *et al.*, 1988), recloned between the *Sal* I and *Hind* III sites of Bluescript KSm13+. pCC2 contains the PR10.PS.3 gene on a 3 kb *Sal* I fragment (Chiang and Hadwiger, 1990) subcloned into pUC18. DC-CHIT-26 is a pea basic chitinase gene cloned between the CaMV 35S promoter and the NOS terminator in pBI121 (Clontech) (Chang *et al.*, 1995). pCHS2KS is the 1.6 kb pea chalcone synthase *Eco*R I fragment from pCHS2 (Harker *et al.*, 1990) recloned into Bluescript KSm13+.

Preparation of probes

All the probes were synthesized using ^{32}P-α dCTP using the random primed

DNA labelling system from GIBCO-BRL. Conserved PR10 probe was prepared from a PCR fragment amplified from pCC2 using conserved primers (oC49+3:cttactccaaaggttatt and oC49-5:taaggaacttctcctttac) which amplify all known PR10 genes in pea. The amplified band was isolated from agarose gel using Prep-A-Gene DNA purification matrix from Bio-Rad (Hercules, U.S.A.)

Chitinase probe was prepared by digesting DC-CHIT-26 with *Hind* III and *Eco* RI to release the chitinase coding sequence along with CaMV 35S promoter and NOS terminator. The insert was gel-purified using Prep-A-Gene DNA purification matrix.

Chalcone synthase probe was made by labelling total pCHS2KS circular plasmid.

Preparation of subfamily-specific probes

Probes specific for individual PR10 genes were generated by making use of a conserved *Bam*H1 restriction site near the 3' end of the protein coding region (140 bp 5' from the translational stop codon) of both the PR10.1 and PR10.3 genes. A second *Bam*HI site was present in the polylinker at the 3' end of the insert in PR10.1 plasmid allowing the isolation of roughly a 1 kb fragment containing the 3' coding sequence and 3' flanking DNA. In the PR10.3 clone (pCC2), a second *Bam*HI site was present in the insert at 480 bp 3' of the stop codon, allowing the isolation of a 716 bp *Bam*HI fragment containing the 3' end of the coding sequence and 3' flanking DNA. Both the fragments were separated by gel electrophoresis, cut from the gel and recovered from the gel slice using the Prep-A-Gene kit from Bio-Rad. The recovered fragment

were labelled according to the method described in the preceding section.

Preparation of Markers

Marker PR10.1 was a mixture of equimolar amounts of pI49KS (PR10.1) digests with *Pst* I (3343, 426), *Hin*d III (3769) and *Hin*d III/*Xho* I double digest (2943, 826). The numbers in parentheses represent the size in base pairs of fragments released. The underlined fragments represent the bands that light up using PR10 non-discriminating probe.

Marker PR10.2 was prepared by mixing equal amounts of the following pI176KS (PR10.2) digests: *Pst* I (3336, 427), *Hin*d III (3763), *Pvu* II (2519, 1244) and *Hin*d III/Xho I double digest (2943, 820).

Marker M was prepared by mixing separate digests of lambda DNA with *Hin*d III and pUC19 with *Hinf* I.

One nanogram of each DNA marker was denatured in formaldehyde as described above prior to loading on formaldehyde gels.

RESULTS

Effect of chemical treatments on the growth of *F. solani* on *P. sativum* pod tissue

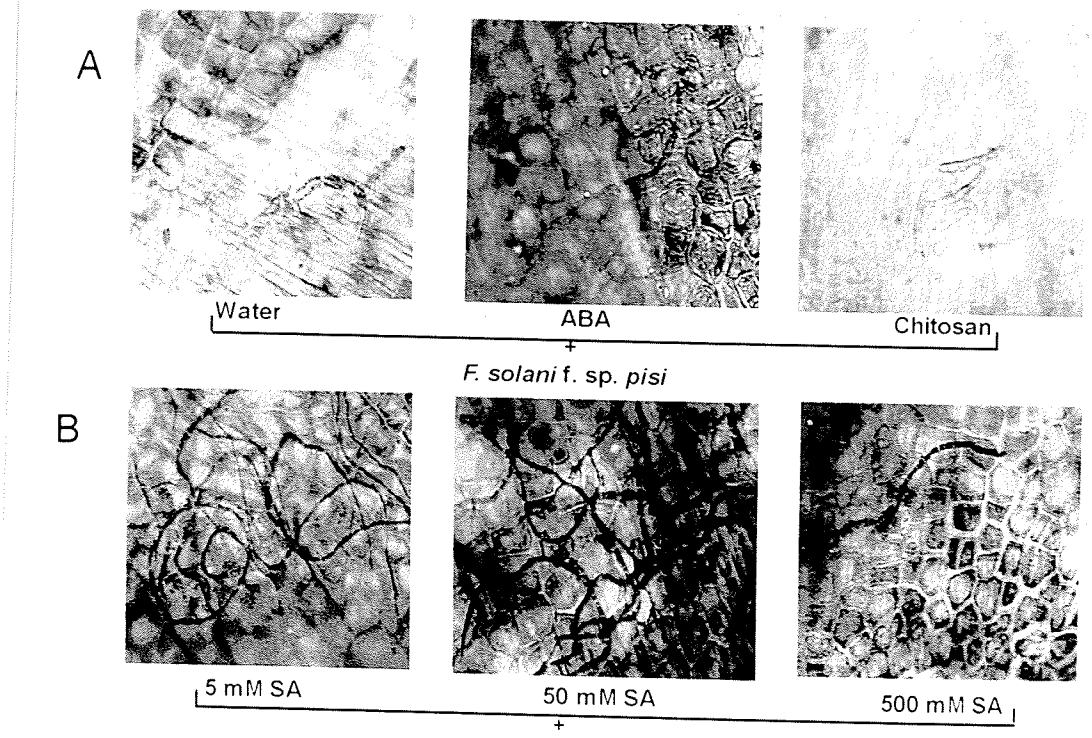
Pisum sativum pod tissue completely inhibits the growth of *F. solani* f. sp. *phaseoli* and partially inhibited *F. solani* f. sp. *pisi* at 12 h.p.i. (Teasdale *et al.*, 1974). Chitosan, salicylic acid (SA) and abscisic acid (ABA) were tested for their ability to induce resistance to *F. solani* f. sp. *pisi* growth on *P. sativum* pod tissue. Chitosan, a

component of *F. solani* cell walls, has previously been shown to protect *P. sativum* tissue against *F. solani* f. sp. *pisi* (Hadwiger and Beckman, 1980). It was included in the experiments for comparative purposes. Salicylic acid has been shown to be a component of the signal-transduction pathway leading to systemic acquired resistance in several plant-pathogen interactions (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). ABA, which induces a different subfamily of PR10 genes (ABR17 and ABR18) in *P. sativum* (Iturriaga *et al.*, 1994), was also tested for its ability to protect against the pathogen.

Treatments were applied on pea endocarp tissue along with the compatible pathogen *F. solani* f. sp. *pisi,* and the effect on growth of the pathogen was observed every 4 hours, up to 48 hours, using a light microscope. Control pods were treated with sterile distilled water immediately prior to inoculation with the fungus.

In the control pods treated with sterile distilled water immediately before treatment with the compatible fungus *F. solani* f. sp. *pisi*, the germ tubes at 12 h.p.i. were between 1-2 times the length of the spores (Fig. 1A). Spore germination was inhibited when chitosan was applied along with *F. solani* f. sp. *pisi* macroconidia (Fig. 1A). ABA demonstrated no enhancing effect on the germination and hyphal growth of the pathogen. The germ tubes did not differ significantly in length from the water control (marked with arrows in Fig. 1A). A hypersensitive response was visible in pods with both treatments (Fig. 2). Salicylic acid (50 mM) appeared to suppress the ability of pea tissue to inhibit spore germination (Fig.1 B). At 4 h.p.i., not much difference could be detected between the control and SA treated pods (Data not

Figure 1. Germination and growth of *Fusarium solani* f. sp. *pisi* macroconidia on *Pisum sativum* endocarp at 12 hours post inoculation. **A.** Pod endocarp was treated with water, 100 µM abscisic acid (ABA) or 1 mg/ml chitosan immediately prior to inoculation. **B.** Pod tissue treated as in **A**, with different concentrations of salicylic acid (SA).


A
Water
ABA
Chitosan
+
F. solani f. sp. pisi
B
5 mM SA
50 mM SA
500 mM SA
+
F. solani f. sp. pisi

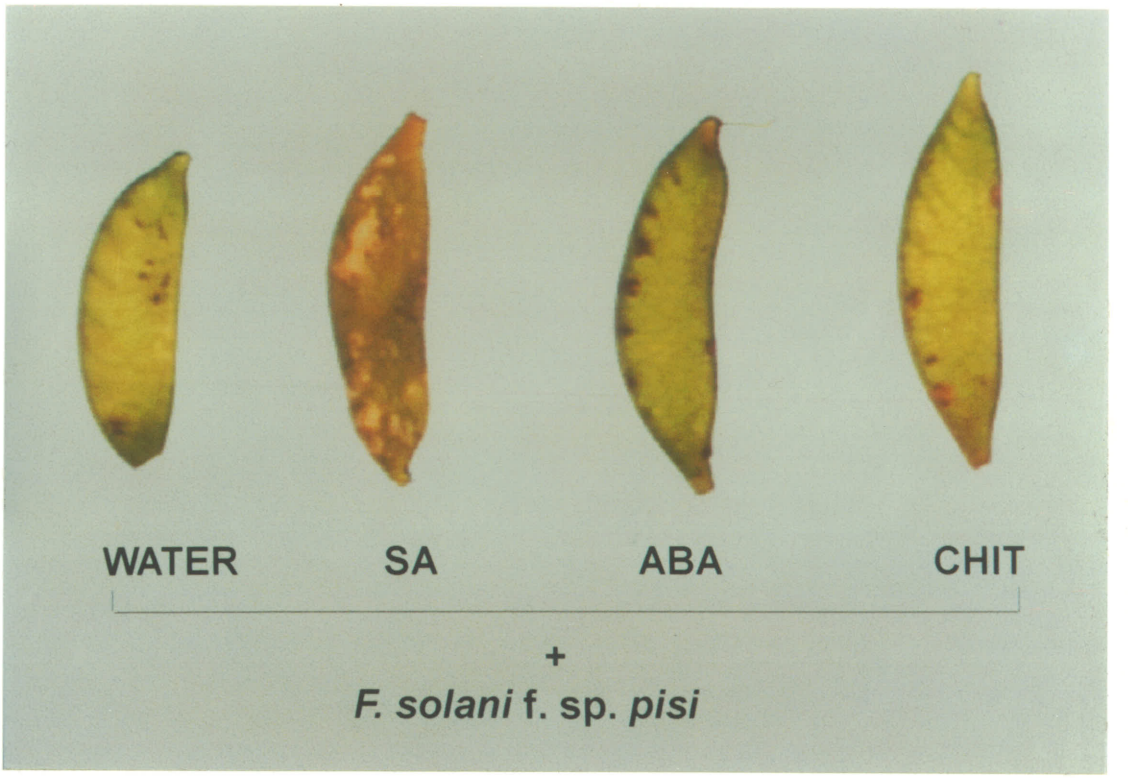
shown). Within 8 h.p.i., there was a dramatic increase in the growth of the fungal hyphae on the SA-treated pods (Data not shown). By 12 h.p.i., the fungus was observed to proliferate dramatically on *P. sativum* pod tissue (Fig. 1A). The fungus could be detected visually on the SA treated pod tissue within 20 hours. By 48 hours the whole pod was covered with fungal mycelia and appeared macerated (Fig. 2). In contrast, the fungus was not visible on control pods by 48 h.p.i. although dark brown lesions could be seen clearly.

Parallel controls were also set up to test the effect of the chemical inducers on pod tissue. There were no apparent differences in the appreance of water, salicylic acid, abscisic acid or chitosan-treated pods.

To test if SA was directly enhancing the growth of the fungus, a drop of the macroconidial suspension was mixed with same amount of SA on a glass slide and the germination of the spores monitored every 4 hours. Inoculum mixed with equal amount of water served as control. No appreciable enhancement of growth was observed by direct contact of SA with spores. A separate control was performed using a sterile Whatman filter paper inside a petri plate. The paper was saturated with 1 ml of sterile water or SA and then 1 ml of *F. solani* f. sp. *pisi* inoculum spread on the filter paper. No significant enhancement of pathogen growth was observed.

Enhancement of hyphal growth was also observed when SA (5 mM) was applied to the pod tissue followed immediately by *F. solani* f. sp. *pisi* macroconidia, (Fig. 1B). As with 50 mM SA, fungus was visible on the pods within 20 hours. Five hundred mM SA did not appear to enhance the growth of the fungus by 12 hours (Fig.

Figure 2. Visual appearance of *P. sativum* pods 48 hours after the treatments described in Fig. 1. SA = 50 mM salicylic acid, CHIT = chitosan.

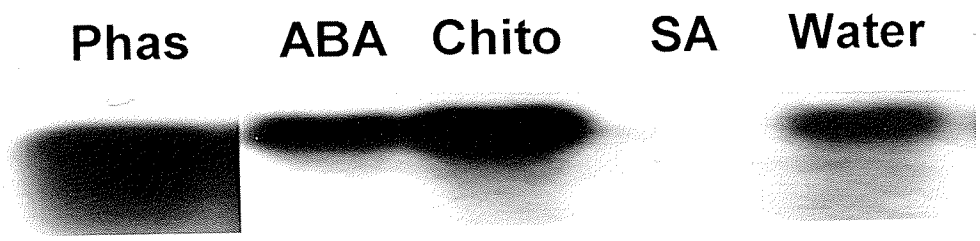

WATER
SA
ABA
CHIT
+
F. solani f. sp. pisi

1B). However, the fungus could be seen on the pod by 48 hours (data not shown). In parallel experiments, pods pretreated with water, 5, 50 or 500 mM SA were inoculated with *F. solani* f. sp. *phaseoli*. Both in the water control and in SA treatments, no spore germination was seen. acid.

Gene expression in response to chemical treatments

RNA extracted from the pods treated with the above mentioned chemicals only (minus the pathogen) for 8 hours was used for analyzing PR10 transcript accumulation. Some accumulation of PR10 transcript was seen in the water control in an overexposed autoradiogram (Fig. 3). It is however not possible to say with these data if this is a basal level of transcript present in the pods or a response to possible wounding resulting from splitting the pods. The levels of the PR10 transcripts were increased significantly above water control by chitosan treatment, which on pea pod tissue, was observed to inhibit pathogen germination and growth (Fig. 3). This accumulation was, however, lower than that observed with *F. solani* f. sp. *phaseoli*. ABA treatment, which did not inhibit the growth of the pathogen relative to control, also did not have any significant effect on the PR10 transcript accumulation. PR10 transcript levels decreased below water treated control in response to 50 mM SA treatment. Transcripts for this gene were not detectable at 8 hours in the SA treated pods. Thus, the fungus was, by far, the strongest inducer of PR10. In view of these results, *F. solani*, rather than chemical treatments, was chosen for further studies on PR10 expression.

Figure 3. Expression of PR10 mRNA 8 hr. after treatment of *P. sativum* pod endocarp tissue with *F. solani* f. sp. *phaseoli* (phas), 100 µM abscisic acid (ABA), 1mg/ml chitosan (chito), 50 mM salicylic acid (SA), or water. Ten µg of RNA was loaded per lane, and the blot was probed with PR10.1 probe. The autoradiogram was overexposed to allow visualization of the basal level of expression seen in the water control, for comparison with other treatments.


Phas
ABA
Chito
SA
Water

Compatibility between *Pisum* and *Lathyrus* tissues and *F. solani*

Compatibility between *F. solani* and different ecotypes of *Pisum* and *Lathyrus* was assayed using the pod inoculation procedure. Within 48 h.p.i., both *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* exhibited heavy mycelial growth on pods which made it difficult to score fungal growth accurately on all species tested (except *F. solani* f. sp. *phaseoli* on *P. sativum*). Scoring was therefore done at 8 h.p.i., at which time a wide range of reactions was seen, from complete inhibition of spore germination to prolific hyphal growth.

Wild *Pisum* and *Lathyrus* permitted more hyphal proliferation than domestic pea (Table 4). The delay in hyphal growth at 8 h.p.i. was less pronounced in these ecotypes than in *P. sativum* (Fig. 4). The closest relative of garden pea, *P. humile* inhibited both pathogens, albeit more weakly than *P. sativum*, (Table 4). Germ tubes at 8 h.p.i. were about 1/4-1/2 the size of the spores (Fig. 4B and H). *P. elatius* and *P. fulvum* were even more permissive to hyphal growth of both the pathogens, with scores of ++ and +++ respectively (Table 4, Fig. 4C, D, I and J). Both *Lathyrus* species allowed extensive growth of both pathogens with germ tubes more than twice the length of the spore (Fig. 4E, F, K and L) within 8 h.p.i.

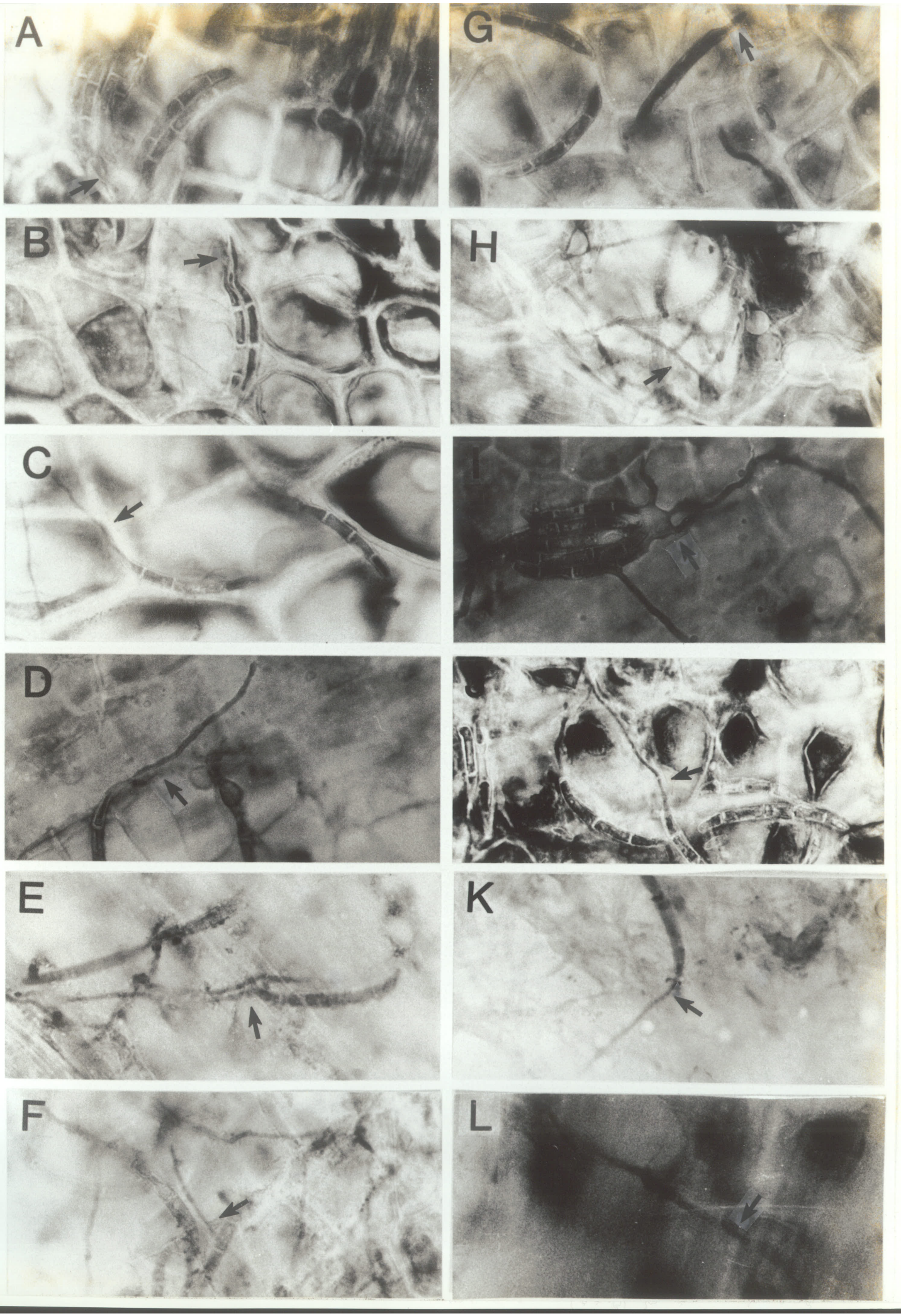
PR10 is present as a multigene family in *Pisum* and *Lathyrus*

In order to confirm the presence of homologous PR10 sequences in *Pisum* and *Lathyrus* species, the *P. sativum* PR10.3 gene was used as a probe in a DNA gel blot

Table 4. Extent of hyphal proliferation on different host species using pod inoculations. Interactions were scored as described in Table 1. ■ = hyphal growth too dense to score.

	F. solani f. sp. *phaseoli*		*F. solani* f. sp. *pisi*	
	8 h.p.i.	48 h.p.i.	8 h.p.i.	48 h.p.i.
P. sativum	-	-	+	■
P. humile	+	■	+	■
P. elatius	++	■	++	■
P. fulvum	+++	■	+++	■
L. sativus	++++	■	++++	■
L. tingitanus	+++++	■	+++++	■

Figure 4. Light micrographs of *Fusarium solani* f. sp. *phaseoli* (**A-F**) and *F. solani* f. sp. *pisi* (**G-L**) macroconidia on the endocarp tissue of *Pisum* and *Lathyrus* species at 8 hours post inoculation. Germ tubes are marked with arrows. Panels **A-F** show f. sp. *phaseoli* s on *P. sativum* (**A**), *P. humile* (**B**), *P. elatius* (**C**), *P. fulvum* (**D**), *L. sativus* (**E**) and *L. tingitanus* (**F**). Panels **G-L** show f. sp. *pisi* on *P. sativum* (**G**), *P. humile* (**H**), *P. elatius* (**I**), *P. fulvum* (**J**), *L. sativus* (**K**) and *L. tingitanus* (**L**). Interactions are arranged in increasing order of susceptibility from top to bottom.


A
G
B
H
C
I
D
J
E
K
F
L

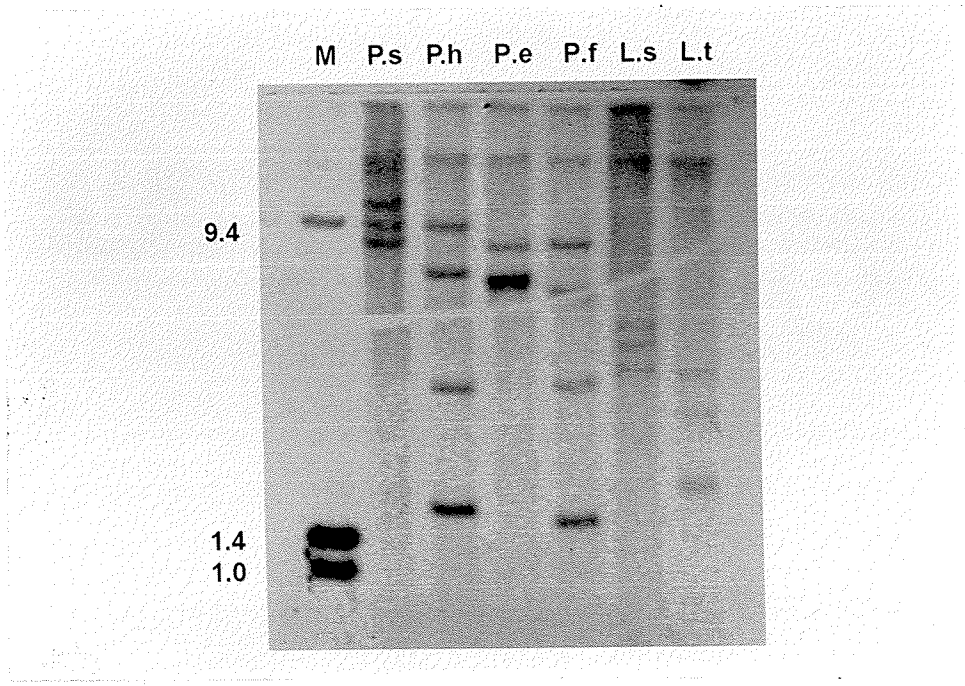
of *Pisum* and *Lathyrus* species (Fig. 5). Band patterns in all species were consistent with 3-5 gene copies per haploid genome, demonstrating the existence of PR10 multigene families in each species. The variation in banding patterns between species indicates sequence divergence either within the coding region or in DNA flanking the PR10 genes. *P. sativum* and its closest relative, *P. humile*, share a common 9.4 kb band, while an 8.0 kb band is common to all *Pisum* species except *P. humile*. *P. humile* and *P. fulvum* share a 3.4 kb band. The 6.5 kb band of *P. elatius* appears in greater stoichiometric intensity, which could be due to a duplication of that region in *P. elatius*. There are no bands that appear to be conserved between *Pisum* and *Lathyrus* species. Finally the decreased band intensity seen in the *Lathyrus* lanes suggests that *Pisum* and *Lathyrus* PR10 genes have diverged substantially.

The conservation of bands within *Pisum*, but not between *Pisum* and *Lathyrus*, is consistent with the fact that between-species divergence has been more recent than the divergence of *Pisum* and *Lathyrus*. The interfertility between *Pisum* species, although partial (Waines, 1975), may also have contributed to the observed interspecific band conservation.

Divergence of gene expression patterns

Gel blots using RNA from *F. solani*-challenged pod tissue were hybridized with pea PR10, chalcone synthase (CHS) and chitinase probes. Some accumulation of PR10 mRNA was observed in the water-treated pod tissue (Fig. 3). The same filters were sequentially stripped and reprobed to maintain consistency between experiments.

Figure 5. Genomic DNA gel blot analysis of *P. sativum* (**P.s**), *P. humile* (**P.h**), *P. elatius* (**P.e**), *P. fulvum* (**P.f**), *L. sativus* (**L.s**) and *L. tingitanus* (**L.t**) genomic DNA using ^{32}P-labelled PR10.3 probe. Fifteen micrograms of *Eco* RI-digested genomic DNA was loaded in each lane. **M** = Lambda/*Hin*d III, pUC19/*Hin*f I marker.


M
P.s
P.h
P.e
P.f
L.s
L.t
9.4
1.4
1.0

Equal loading of RNA was confirmed by staining the gel with ethidium bromide [0.5 µg/ml](Data not shown).

P. sativum

In *P. sativum*, which is resistant to *F. solani* f. sp. *phaseoli*, PR10 mRNA was present at high levels within 8 h.p.i. (Fig. 6) but decreased in abundance by 48 h.p.i. A similar pattern was observed with CHS and chitinase genes but the signal was much weaker than that of PR10 (Fig. 6).

In contrast, *P. sativum* inhibits the germination of *F. solani* f. sp. *pisi* spores at 8 h.p.i. although by 48 h.p.i., the fungus is observed to grow uninhibited. At 8 h.p.i., PR10 was observed to be induced to a high level in response to this pathogen (Fig. 7). However, unlike that with *F. solani* f. sp. *phaseoli*, expression of PR10 was maintained at high level up to 48 hour. CHS mRNA was much less abundant than PR10 but exhibited the same pattern at both time-points (Fig .7). Chitinase mRNA was also detectable within 8 h.p.i. and its level rose by 48 h.p.i.

P. humile

P. humile which partially inhibited both pathogens (Table 2), also expressed PR10 to high levels at 8 h.p.i. in response to *F. solani* f. sp. *phaseoli*, albeit lower than that in *P. sativum* (Fig. 6). CHS and chitinase mRNA were barely detectable in *P. humile* at 8 h.p.i. but appeared by 48 h.p.i.

In response to *F. solani* f. sp. *pisi*, PR10 transcript was abundant at 8 h.p.i.,

Figure 6. Expression of PR10, CHS and chitinase (CHIT) mRNA in pod tissue of *Pisum* and *Lathyrus* species inoculated with *F. solani* f. sp. *phaseoli* for 8 or 48 hours. Gel blots of total RNA (5 µg per lane) were probed with ^{32}P-labelled PR10, CHS and chitinase probes. **P.s** = *P. sativum*, **P.h** = *P. humile*, **P.e** = *P. elatius*, **P.f** = *P. fulvum*, **L.s** = *L. sativus* and **L.t** = *L. tingitanus*. **PR10 -1, -2, -3** = PR10.1, PR10.2 and PR10.3 markers, as described in the methods section. **h.p.i.** = hours post inoculation. The relationships between taxa, as described in the introduction, are represented in a cladogram. The extent of hyphal growth at 8 hpi, as described in Table 4, is represented graphically.

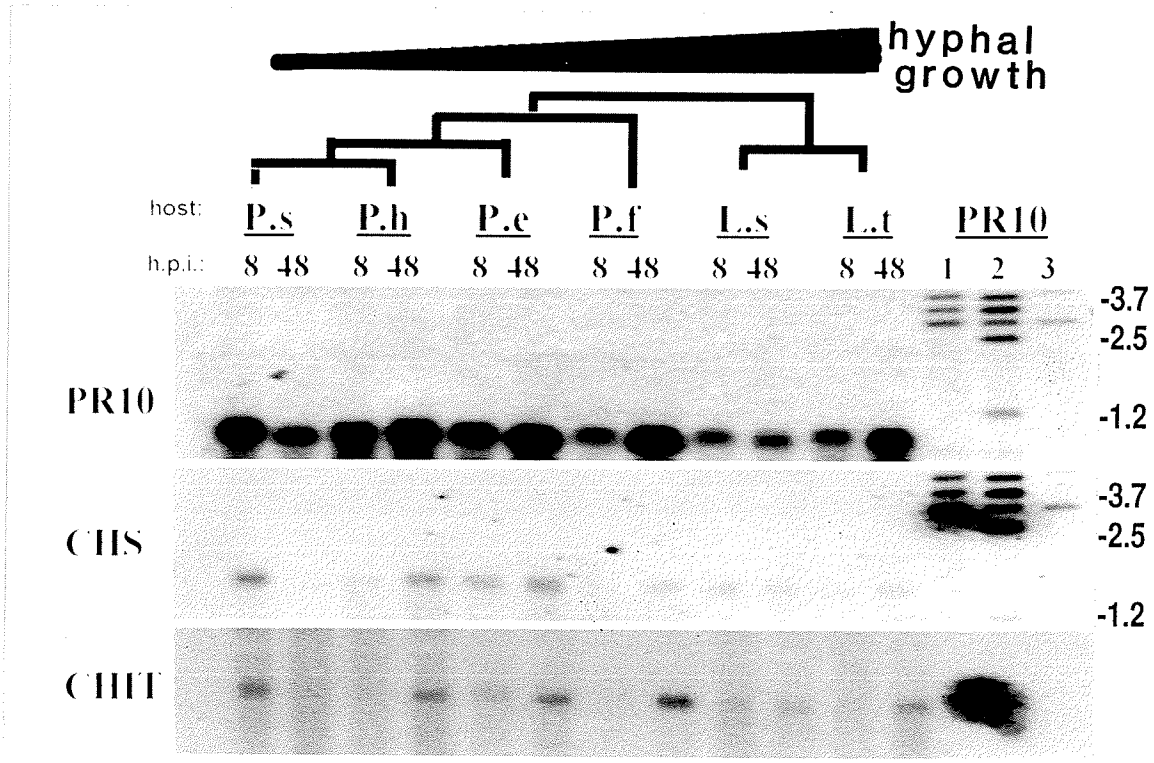
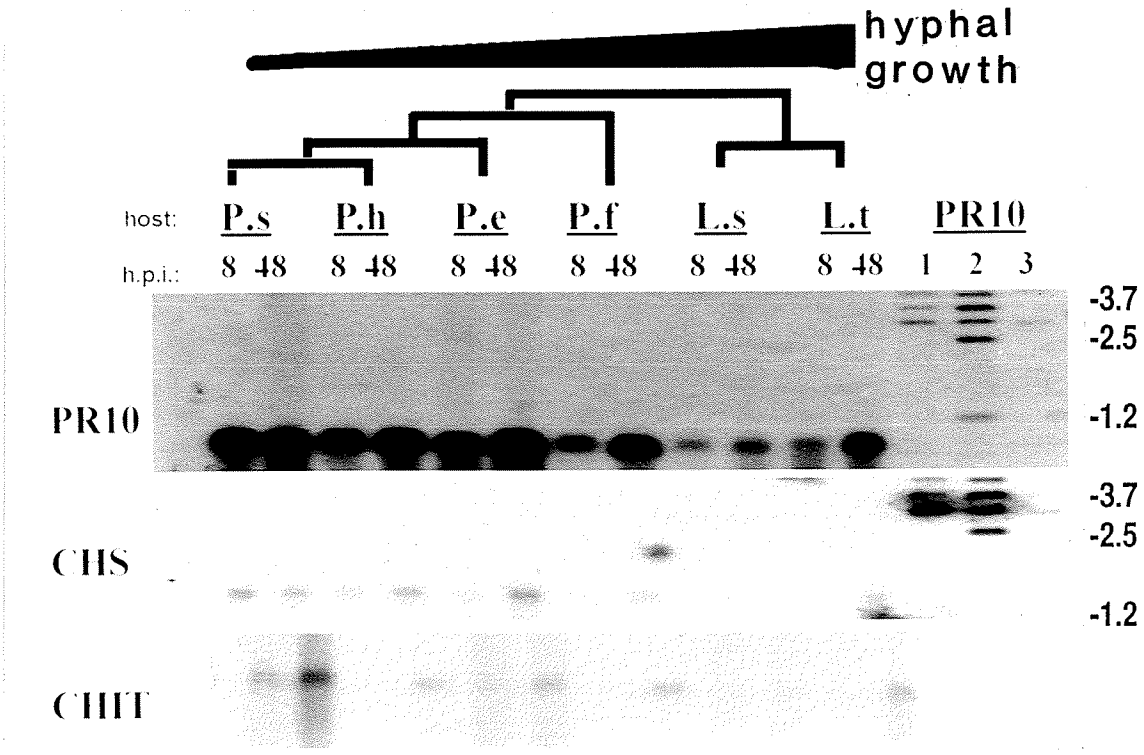

hyphal growth
host:
P.s
P.h
P.e
P.f
L.s
L.t
PR10
h.p.i.:
8 48 8 48 8 48 8 48 8 48 8 48 1 2 3
PR10
CHS
CHIT
-3.7
-2.5
-1.2
-3.7
-2.5
-1.2

Figure 7. Expression of PR10, CHS and chitinase mRNA in pod tissue of *Pisum* and *Lathyrus* species inoculated with *F. solani* f. sp. *pisi* for 8 and 48 hours. All other experimental conditions and annotations are the same as in Fig. 6.


hyphal growth
host:
P.s
P.h
P.e
P.f
L.s
L.t
PR10
h.p.i.:
8 48 8 48 8 48 8 48 8 48 8 48 1 2 3
PR10
CHS
CHIT
-3.7
-2.5
-1.2
-3.7
-2.5
-1.2

accumulating to higher levels by 48 h.p.i. (Fig. 7). Chitinase and CHS mRNAs exhibited a stronger signal at 48 h.p.i than at 8 h.p.i.

P. elatius

P. elatius allowed moderate growth of both *F. solani* f. sp. *phaseoli* and f. sp. *pisi* (Table 2). In response to both pathogens, PR10 was expressed to high levels within 8 h.p.i. with the expression increasing by 48 h.p.i (Figs. 6 and 7). A similar pattern was observed for chitinase and CHS with both pathogens although transcript abundance was much lower (Figs. 6 and 7).

P. fulvum

Both *F. solani* f. sp. *phaseoli* and f. sp. *pisi* were able to grow relatively uninhibited on *P. fulvum* (Table 2). It showed a remarkably similar expression pattern for all three genes in response to both pathogens. This pattern was characterized by very low to undetectable expression at 8 h.p.i. followed by relatively much higher transcript accumulation at 48 h.p.i. (Figs. 6 and 7).

L. sativus

L. sativus allowed both fungi to germinate and grow rapidly (Table 2). PR10 expression was somewhat greater at 48 h.p.i than 8 h.p.i, while CHS and chitinase transcripts were barely detected in response to either pathogen (Figs 6 and 7). This does not necessarily imply low expression of these genes in *L. sativus*. It is possible

that the latter two pea probes hybridize only weakly due to lack of sequence conservation between *Pisum* and *Lathyrus*.

L. tingitanus

In *L. tingitanus*, which allowed maximum fungal growth among all the tested host species (Table 2), PR10 transcript was detectable by 8 h.p.i (Figs 6 and 7), accumulating to higher levels by 48 h.p.i. CHS RNA was hardly detectable in this species (Figs 6 and 7). Chitinase was not detectable at 8 h.p.i with either pathogen but some transcript accumulation was observed at 48 h.p.i. in response to both pathogens.

Differential expression of PR10 genes

Conservation of distinct PR10 subfamilies within *Pisum* and *Lathyrus* species prompted us to question if expression patterns for PR10 subfamily members are consistent throughout *Pisum*, or whether these patterns change along with the observed changes in germination and hyphal growth. Subfamily-specific probes were therefore constructed from the C-terminal protein coding regions, extending into the 3' non-transcribed region of each gene (see Methods). These probes were then used in gel blots using RNA isolated from different host taxa inoculated with *F. solani* f. sp. *phaseoli* or f. sp. *pisi* to determine if each subfamily was active in different host taxa. The specificity of these probes was verified by the use of plasmids containing PR10.1, PR10.2 and PR10.3 sequences as internal controls on each RNA blot. In Figures 8

Figure 8. Differential expression of PR10 subfamilies in *Pisum* and *Lathyrus* species in reponse to inoculation with *F. solani* f. sp. *phaseoli* using the subfamily-specific probes derived from the 3' untranslated region of the genes as described in Methods. All other experimental conditions and annotations are the same as in Fig. 6.

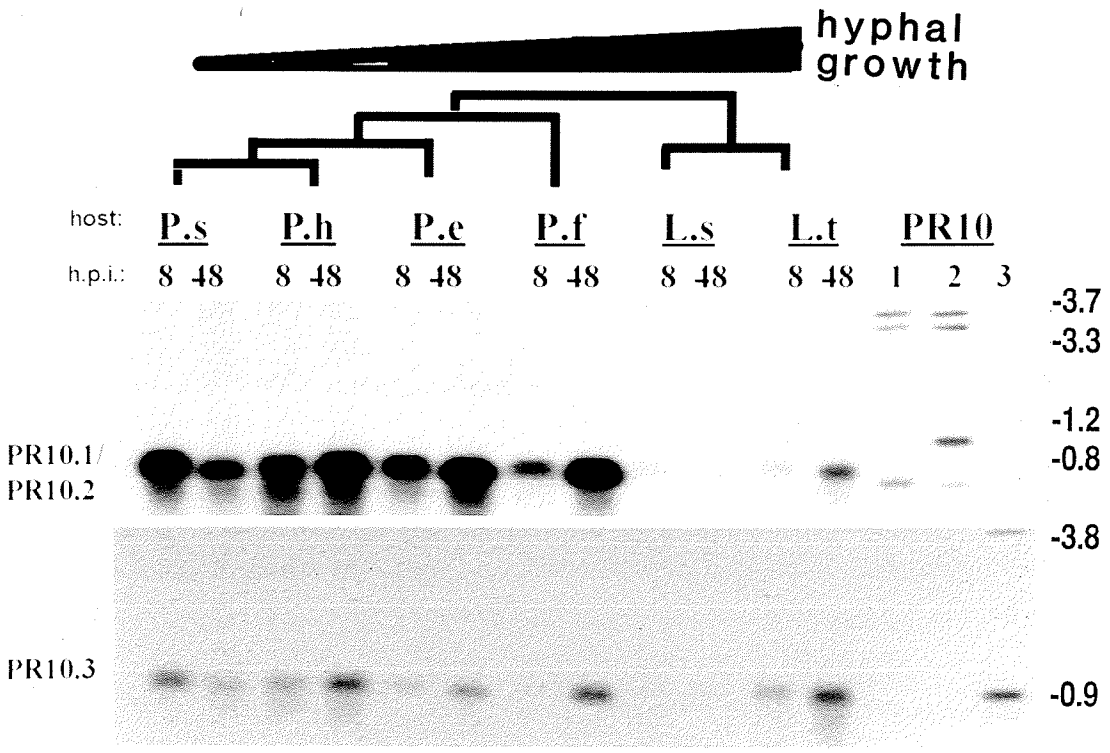
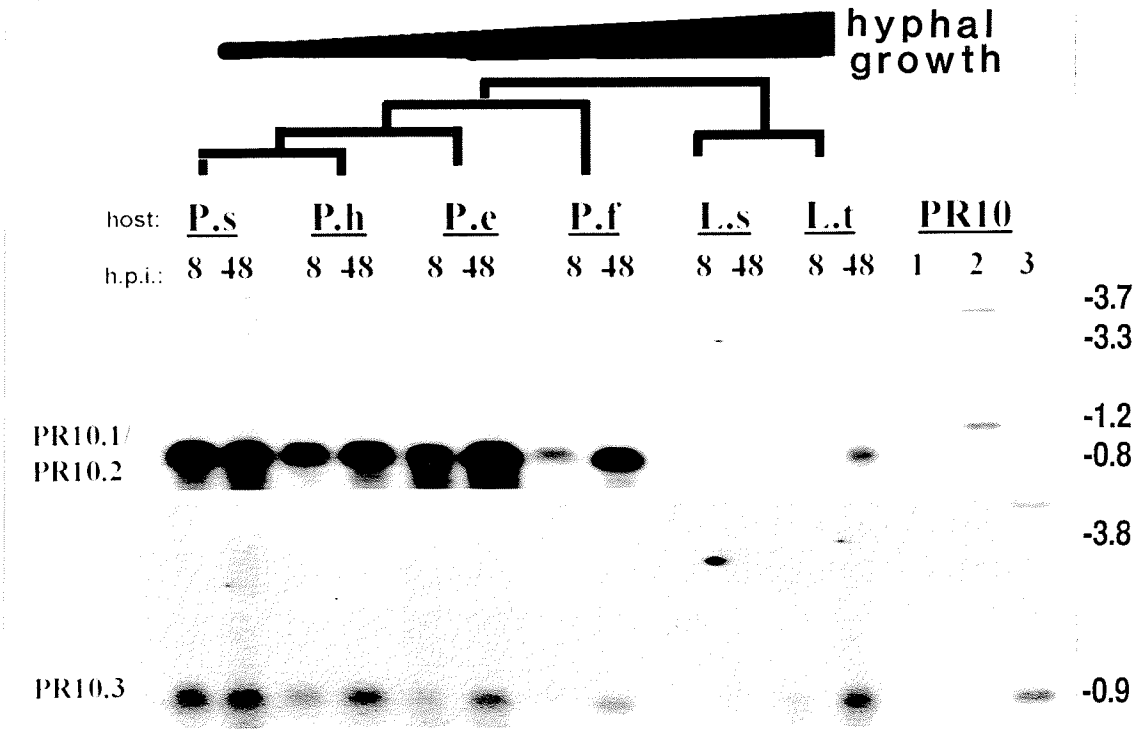

hyphal growth
host:
P.s
P.h
P.e
P.f
L.s
L.t
PR10
h.p.i.:
8 48 8 48 8 48 8 48 8 48 8 48 1 2 3
-3.7
-3.3
-1.2
PR10.1/
PR10.2
-0.8
-3.8
PR10.3
-0.9

Figure 9. Differential expression of PR10 subfamilies in *Pisum* and *Lathyrus* species in reponse to inoculation with *F. solani* f. sp. *pisi* using the subfamily-specific probes derived from the 3' untranslated region of the genes as described in Methods. All other experimental conditions and annotations are the same as in Fig. 6.


hyphal growth
host: P.s P.h P.e P.f L.s L.t PR10
h.p.i.: 8 48 8 48 8 48 8 48 8 48 8 48 1 2 3
-3.7
-3.3
-1.2
PR10.1/
PR10.2
-0.8
-3.8
PR10.3
-0.9

and 9, the PR10.1 probe hybridized to PR10.1 and PR10.2, but not to PR10.3. The PR10.3 specific probe hybridized only to the PR10.3 plasmid. The stronger signal with the PR10.1 probe as compared to the PR10.3 probe indicates that PR10.1/PR10.2 subfamily specific transcripts accumulate in greater abundance, as compared to PR10.3 transcripts, in *Pisum* and *Lathyrus* inoculated with *F. solani*. This suggests that PR10.1/PR10.2 subfamily accounts for most of the *Fusarium*-induced PR10 expression. Low signal in *Lathyrus* under higher stringency hybridization and washing conditions indicates that PR10 genes have diverged substantially and is consistent with low signal in the DNA gel blot (Fig 5).

Expression patterns seen with PR10 subfamily-specific probes (Fig. 8 & 9) agreed with results using non-specific PR10 probes (Fig. 6 & 7).

DISCUSSION

Exogenous application of SA to tobacco and cucumber and several other plant species leads to the induction of various PR-proteins and also protects plants against subsequent attack by a broad range of viral, bacterial and fungal pathogens (see Malamy and Klessig, 1992). In this study, SA treatment enhanced the growth of *F. solani* on *P. sativum* tissue. Controls indicate that this enhancement of fungal growth on pod tissue was not due to a direct effect of SA either fungal growth or on pod viability (data no shown). Application of SA also decreased the levels of PR10 transcript below control levels. This correlation could be evidence that, at least in pea pod tissue, SA actively suppresses the defense response. The correlation between

PR10 mRNA accumulation and inhibition of spore germination agrees with the data from other chemical treatments. Chitosan, which induces PR10 expression, has also been shown to induce resistance to spore germination and growth of the compatible pathogen *F. solani* f. sp. *pisi* (Hadwiger and Beckman, 1980; Fig. 1 and 3). ABA had no detectable effect either on PR10 accumulation or pathogen growth.

Pod endocarp tissue from *P. sativum* inhibited the germination of macroconidia of *F. solani* f. sp. *phaseoli*. *P. humile*, which is most closely-related to *P. sativum*, exhibited a phenotype more similar to *P. sativum* than the other two wild species with a relatively strong inhibition to germination of *F. solani* f. sp. *phaseoli* spores. *Lathyrus* species, which are further diverged from *Pisum*, were more permissive to hyphal growth. A similar increase in compatibility was seen in the interaction with the pea pathogen, *F. solani* f. sp. *pisi*. Complexity of experiments prevented analysis of gene expression in more than one accession of each ecotype, these data do not rule out variation of defense response within each ecotype. Nonetheless, it is worth noting that the divergence of interaction phenotype appears to be gradual because neighbouring taxa always had the most similar scores.

Changes in the interaction phenotype across taxa were accompanied by divergence of expression patterns for PR10, CHS and chitinase genes. In our experiments, the DNA markers loaded on the PR10 and CHS blots gave signals of comparable intensity, although the CHS and chitinase mRNA bands were much less intense than those of PR10. Previously, Chang *et al.*, (1995) used the same basic chitinase probe to detect chitinase induction in response to *F. solani*, although their

study did not compare transcript levels of PR10 with chitinase. CHS expression has not previously been studied in this system. However, in light of the low relative levels of CHS transcript seen in this work, it is interesting to note that CHS mRNA was not detectable in either compatible or incompatible interactions of *Arabidopsis* with *Pseudomonas syringae* pathovars (Dong *et al.*, 1991). While there are some similarities between the PR10 pattern of expression and those of CHS and chitinase, there are also many apparent differences, in the timing and levels of respective transcript accumulation (Fig. 6 & 7). Thus, while some regulatory pathways may be shared among these gene families, our data do not point to a strict coordinate regulation.

Resistance was accompanied by expression of defense genes at 8 h.p.i. In *P. sativum, P. humile* and *P. elatius*, significant accumulation of PR10 occurs within 8 h.p.i. All the three species show marked inhibition of spore germination of both pathogens at this time (Fig. 4). In the other ecotypes which do not demonstrate suppression of germ tube growth to this extent at 8 h.p.i., there is no significant PR10 mRNA accumulation at 8 hours.

All taxa except *P. sativum* show a similar pattern of expression of PR10 genes during infection with either *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli*. This pattern is characterized by a weak signal in the first 8 h.p.i., followed by a stronger induction by 48 hours. In contrast, *P. sativum* shows a high accumulation of PR10 transcript at 8 hours after infection with either pathogen, followed by a decline in transcript levels by 48 hours in case of *F. solani* f. sp. *phaseoli*, but similar levels of

expression at both time points after infection with *F. solani* f. sp. *pisi*. These results parallel the observation that on *P. sativum* tissue inoculated with *F. solani* f. sp. *phaseoli*, hyphal growth was completely suppressed, whereas on tissue inoculated with *F. solani* f. sp. *pisi*, growth is initiated, but is halted, to resume at later times.

Pea PR10 hybridized to multiple bands in the *Eco* RI digested genomic DNA from wild *Pisum* and *Lathyrus*, indicating that PR10 exists as a multigene family in these taxa. RNA gel blot analysis using PR10 subfamily-specific probes showed that PR10.1/2 subfamily transcript increased greatly in response to *F. solani* while that of PR10.3 subfamily was barely detectable. Mylona *et al.*, (1994) have independently cloned the pea PR10.3 cDNA while isolating genes expressed in root epidermis and root-hairs. PR10.3 (referred to as RH2 by Mylona *et al.*, 1994) transcript was far more abundant in roots than transcripts detected using PR10.1-specific oligonucleotides. Further, inoculation of roots with *Rhizobium leguminosarum* bv. *viciae* did not have any detectable effect on the already high PR10.3 transcript accumulation, but caused a slight increase in accumulation of PR10.1 transcript over control levels. These observations imply specialized roles for the two gene families, such that the PR10.1/PR10.2 subfamily is primarily pathogen-induced, while PR10.3 appears to be root-specific. Because our probes could not discriminate between PR10.1 and PR10.2 transcripts, we can not rule out the possibility of further specialization between PR10.1 and PR 10.2.

While differential expression has been observed in several defense multigene families (Koes *et al.*, 1989; Ryder *et al.*, 1987; Shufflebottom *et al.*, 1993), one point

that has received little attention is that amplification and deletion of family members over the course of evolution would affect the differential expression patterns for a given gene family. Differences were observed in the banding patterns in the blot of different host species probed with PR10. These differences could be due to amplification and deletion of some gene copies and subsequently sequence divergence in the coding region or flanking DNA. Preliminary sequence data indicate that all wild *Pisum* ecotypes have homologues of PR10.1 and PR10.3 and all but *P. elatius* have homologues of PR10.2 (unpublished results). Differential expression patterns must be changing frequently as speciation proceeds. Our results suggest that this may be occurring in the case of the PR10 multigene family. Figure 6 shows that the pattern of PR10, CHS and chitinase mRNA accumulation in *P. Sativum* and its closest relative *P. humile* is quite different in response to *F. solani* f. sp. *phaseoli*. *P. sativum* accumulates relatively high levels of all the three transcripts at 8 hr followed by a decline in the levels by 48 h. The pattern is reversed in the *P. humile* which has high accumulation of defence transcripts at 48 hr. Most importantly, it is apparent that expression patterns of neither PR10, nor CHS, nor chitinase, are well conserved, even between *P. sativum* and its closest relative, *P. humile*.

We recognize that the demonstrating a causal link between the changes in basic compatibility between plant and pathogen and the evolution of defense multigene families is beyond the scope of any single study such as this. While at present we can only speculate as to whether the evolution of defense gene regulation in general is as dynamic as that seen in this study, it is commonly accepted that regulatory regions of

genes evolve more rapidly than protein coding sequences. For example, 3' untranslated regions of the gene are often used as gene-specific probes due to their characteristic lack of conservation, relative to translated regions (Dean *et al.*, 1985). Since dozens of genes may be involved in the defense response, and most of these are present as multigene families, the precise set of genes activated in response to a given pathogen, and their patterns of regulation, could vary enormously, within and between species. As a consequence, the phenotypic diversity of plant populations, with respect to their response to pathogens, may be greater than revealed by typical gene expression studies.

Chapter 4

DIFFERENTIAL EXPRESSION OF THE PR10 MULTIGENE FAMILY IN CLOSELY-RELATED *PISUM* TAXA

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ABSTRACT

The pathogenesis-related PR10 multigene family in *P. sativum* is comprised of atleast five genes: PR10.1, PR10.2, PR10.3 (formerly pI49, pI176 and drrg49-c, respectively), ABR17 and ABR18 (ABA-responsive). We have compared transcript accumulations of specific members of this gene family in pod tissue of closely-related *Pisum* ecotypes inoculated with *Fusarium solani* f. sp. *phaseoli* or *F. solani* f. sp. *pisi*. A combination of cDNA synthesis followed by polymerase chain reaction (PCR) and chemiluminescent detection was used for high sensitivity and specificity of detection. Each of the four tested *Pisum* species demonstrated a unique pattern of expression by accumulating different combinations of PR10 transcripts. ABR17 and ABR18 mRNAs accumulated in all the four ecotypes following fungal inoculation. PR10.3 mRNA was not detected in any of the ecotypes in response to any of the treatments, suggesting that the expression of this gene may be limited to roots. A time course of transcript accumulation in *P. sativum* in response to *F. solani* revealed that PR10.1 and ABR18 accumulate to high levels within 4-8 hours post inoculation. PR10.2 and ABR17 appear later and are induced only weakly. Salicylic acid, abscisic acid and chitosan, in general, were not as effective in inducing PR10 in *P. sativum* pods as fungal pathogens.

INTRODUCTION

Plants often respond to pathogen challenge by inducing the synthesis of pathogenesis-related (PR) proteins (Van Loon *et al.* 1994). The first gene encoding the PR10 family within this group was isolated from pea tissue expressing non-host resistance to *F. solani* f. sp. *phaseoli* (Riggleman *et al.* 1985). Subsequently, homologs of this gene have been independently cloned from many dicotyledonous (Somssich *et al.* 1988, Matton and Brisson 1989, Walter *et al.* 1990, Crowell *et al.* 1992, Breitender *et al.* 1989) and a monocotyledonous species (Warner *et al.* 1993). In most cases, this gene has been shown to be induced either with pathogens and their elicitors or some stress condition like wounding (Matton and Brisson 1989; Warner *et al.* 1993) or cytokinin starvation (Crowell *et al.* 1992). In parsley, PR10 transcript accumulation occurs around the infection site (Schmelzer *et al.* 1989) following attempted invasion by *Phytopthora megasperma.* At least some of these genes are also expressed in unstressed asparagus and potato plants during normal development (Warner *et al.* 1994, Constabel and Brisson, 1995). Evolutionary conservation of the protein coding sequence across monocotyledonous as well as dicotyledonous plant species coupled with correlative evidence of accumulation at a critical time and location in response to pathogens suggest that these genes play an important role in the defence response.

No direct evidence is available regarding the function of PR10. Although similarity of the parsley PR10 homolog with a putative ribonuclease from *Ginseng* (Moiseyev *et al.* 1994) suggests that these genes may have a ribonuclease function,

such an activity has not yet been demonstrated for any of the PR10 proteins identified so far.

PR10 genes exist as multigene families in all the species from which they have been cloned so far. In the garden pea, *P. sativum*, at least five PR10 homologs are known (Table 5.). PR10.1 and PR10.2 (previously pI49 and pI176, respectively) were identified by differential expression of a fungus-induced cDNA library and their transcripts accumulate in response to fungal challenge and treatment with the elicitor chitosan (Fristensky *et al.* 1985, Tewari *et al.*, manuscript submitted). PR10.3 (formerly Drrg49-c) was isolated from a genomic library using PR10.1 as a probe but has not yet been shown to be induced in response to pathogens (Chiang and Hadwiger 1990). Two distinct PR10 homologs (designated ABA-responsive ABR17 and ABR18) were cloned from cultured embryos of pea undergoing normal seed desiccation (Iturriaga *et al.*, 1994) and their products are enhanced in abundance by exogenously supplied abscisic acid in the culture medium.

Most defense-related proteins induced in plants in response to pathogens are encoded by multigene families, including phenylalanine ammonia-lyase (Cramer *et al.* 1989), chalcone synthase (Koes *et al.*, 1989), chalcone isomerase (Van Tunen *et. al.* 1988) hydroxyproline-rich glycoproteins (Corbin *et al.*, 1987), 4-coumarate CoA ligase (Douglas *et al.*,1987) β-1,3 glucanase (Ward *et al.*, 1991), PR1 (Rigden and Coutts, 1988), peroxidase (Harrison *et al.* 1995) and many others. Existence of multiple copies of identical or closely-related genes allow for the possibility of differential expression wherein specific members of a gene family may be induced in response to

Table 5. Cloned PR10 genes from *P. sativum.*

Gene	old designation	Clone	Genbank accession	Refrence
PR10.1	pI49/drr49a	cDNA	X13383	Fristensky *et al.*, 1988
	pKX	genomic	U31669	Culley *et al.*, 1995
PR10.2	pI176/drr49b	cDNA	M81249	Fristensky *et al.*, 1988
PR10.3	drrg49-c	genomic	J03680	Chiang and Hadwiger, 1990
ABR17	-	cDNA	Z15128	Iturriaga *et al.*, 1994
ABR18	-	cDNA	Z15127	Iturriaga *et al.*, 1994

different signals. While differential expression of defense genes has typically been studied with respect to developmental stages or tissue specificity, it has been proposed that differential expression of multigene families may be the molecular basis of the characteristic property of phenotypic plasticity in plants (Smith 1990). Plants are continually challenged with pathogens belonging to diverse kingdoms, each unique in its characteristics. On the other hand, only subtle differences may exist between individual races of a pathogen. Differential expression of defense related genes by the host plants, such that an identical or slightly different protein becomes available in response to different signals may provide the required versatility in responding to different challenges.

Pisum is a relatively recently evolved genus consisting of four ecotypes (Waines, 1975; Ben-Ze'ev and Zohary 1973). *P. sativum* is the cultivated "garden pea" while the other ecotypes viz. *P. humile*, *P. elatius* and *P. fulvum* are wild species. All these *Pisum* ecotypes, although morphologically and cytologically distinct, are at least partially interfertile. Our previous studies (Tewari *et al.*, manuscript submitted) showed that PR10 is present as a small multigene family of 2-4 genes in the wild ecotypes of pea as well. This system therefore provides an opportunity to study the extent of conservation of defense multigene families in divergent taxa that are not yet fully reproductively isolated. It also permits us to examine whether structurally similar genes in different species exhibit similar patterns of expression.

We have previously shown (Tewari *et al.*, manuscript submitted) that mRNA hybridizing to the PR10.1/PR10.2 subfamily-specific probe accounts for the major

portion of PR10 mRNA accumulating in *Pisum* ecotypes in response to the fungal pathogen, *Fusarium solani*. However, the probe used in this analysis could not distinguish between individual gene members because of high sequence similarity between the genes. Here we report a detailed analysis of differential accumulation of specific PR10 transcripts in response to different signals using gene-specific primers for each member of the PR10 family.

MATERIALS AND METHODS

Plant Material and treatments

Wild accessions of *Pisum* (*P. humile* 713, *P. elatius* 721 and *P. fulvum* 706) used in this study were obtained from N. O. Polans, Northern Illinois University, U.S.A. *P. sativum* c.v. Alaska was purchased from W. Atlee Burpee and Co., Warminister, PA. Strains of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* were obtained from American Type Culture Collection (Accession numbers 38136 and 38135 respectively). Cultures were grown and maintained on potato dextrose agar (PDA) plates supplemented with few milligrams of finely chopped pea leaf tissue.

All the *Pisum* and *Lathyrus* plants were grown in growth rooms in pots in 2:1:1::Soil:Sand:Peat mix under a day/ night cycle of 16/8 hours with temperatures of 22 /15 °C respectively. The average light intensity using 1/3 0-lux wide spectrum to 2/3 cool white was 340 μ e m^{-2} sec^{-1}.

Pathogen inoculation and chemical treatments

Immature pods (less than 2 cm in length; five pods per treatment) having no developed seed were harvested from the plants, slit longitudinally along the suture lines and placed the freshly opened side up on a sterile petri-dish. For pathogen inoculations 50 µl of a 10^6 macroconidia/ml suspension of either *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli* were spread evenly on the pod. The macroconidial suspension was prepared from the one week old culture of the respective pathogen on the PDA plates. 10 ml of sterile distilled water was poured over the cultures under aseptic conditions, swirled gently and poured into a sterile tube. The concentration of macroconidia was determined using a haemocytometer and appropriate dilutions carried out to get a final concentration of 10^6 macroconidia/ml. The plates were then incubated at room temperature under continuous florescent light and tissue harvested at appropriate times. Pods treated with sterile distilled water served as controls.

For the chemical treatments, pods were harvested as for pathogen inoculations and the given chemical applied on the exposed surface. All the treatments were applied in a total volume of ten µl/pod half at the following concentrations: Chitosan, 1 mg/ml; ABA, 100 µM; and SA, 50 mM.

RNA extraction

Pod endocarp tissue from *Pisum* species after a given treatment was frozen in liquid nitrogen and RNA extracted by the method of Verwoerd et. al. (1989) using the modifications described in (Tewari *et al.*, chapter 3).

Reverse transcription

Two µg of total RNA was incubated with 0.5 µg oligo $(dT)_{12-18}$ primer (Gibco BRL cat. # 18418-012) at 65 °C for 5 min. Reverse transcription was carried out in a 30 µl final volume at 50 °C for 30 min. The mixture for reverse transcription contained final concentrations of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, 64 units of RNAsin (Gibco BRL), 12 units of AMV-RT (Promega), 1 mM each of dATP, dCTP, dGTP and dTTP.

Preparation of internal controls for PCR

Internal controls for primers were prepared by inserting randomly cleaved *Sau*3AI pieces of pUC18 plasmid internal to the primer binding sites of the cloned PR10.1, PR10.2 and PR10.3 plasmids. Those clones were chosen in which appropriate sized fragments were inserted such that the product amplified by the internal control plasmid and the respective cDNA could be easily resolved on an agarose gel. It was also ensured that each internal control had a different sized insert to distinguish between each of the PR10 genes (see Table 6; Fig 10 legend for the details on the construction of internal standards).

DIG labelling of cDNA using PCR

Ten µl of a 1:10 dilution of the cDNA synthesized using the method described above was used in the PCR reaction with specific primers (see Table 6 for sequences) for PR10.1 (oS49a+8 and oS49a-7), PR10.2 (oS49a+8 and oS49b-7), PR10.3 (oS49c+4

Table 6. Sequences of the primers used in the RT-PCR assay

Gene	Primer name	Sequence	Position	Length of PCR Product	
				Control	cDNA
PR10.1	oS49a+8	5'-ctagttacagatgctgataac	67	851	323
	oS49a-7	5'-catccccttagctttgtcag	430		
PR10.2	oS49a+8	5'-ctagttacagatgctgataac	67	574	316
	oS49b-7	5'-gcagcatcacctttgtgtaa	383		
PR10.3	oS49c+4	5'-tgttgaaggaaacggtggccc	132	368	263
	oS49c-5	5'-gatttcctcttcactaggaat	395		
ABR17	oSABR17+4	5'-ggtgatcaagaagaagcacaa	99	-	322
	oSABR17-5	5'-tttggcttttgtttcatcacg	423		
ABR18	oSABR18+1	5'-atgataccacctctaccgtcc	23	-	400
	oSABR18-5	5'-cttagctttgccttcctcaac	423		

and oS49c-5), ABR17 (oSABR17+4 and oSABR17-5) and ABR18 (oSABR18+1 and oSABR18-5). Preliminary sequence data shows that the respective genes cloned from wild *Pisum* have the same primer sequence as those of *P. sativum.* PCR was carried out in a 25 µl total volume and internal controls for PR10.1, PR10.2, PR10.3, which migrate to a smaller distance on agarose gel (see construction of internal controls above), were included in the PCR reaction. Since internal controls are not available for ABR17 and ABR18, cloned ABR17 and ABR18 plasmids served as external controls, which were amplified in separate tubes.

PCR was carried out using the PCR ELISA (DIG labelling kit from Boehringer Mannheim (Cat. # 1636 120) following manufacturer's instructions. The final concentration of the reaction mix was : 1X PCR buffer [10 mM Tris-HCl, 50 mM KCl (pH 8.3)], 1.5 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP, 190 µM dTTP and 10 µM DIG-dUTP, 0.625 U Taq polymerase, 10 pmole of each primer. Wherever possible, master-mixes were prepared to improve the reproducibility of experiments. Fourteen cycles of PCR were carried out: denaturation at 94 °C, 1 min; annealing at 50 °C, 1 min; extension at 72 °C, 1.5 min.

DIG Detection

Five µl of the DIG labelled PCR product was separated on a 1.5% agarose gel and transferred to Hybond membrane (Amersham) following instructions from the manufacturer. The transferred DNA was crosslinked to the membrane using the auto-crosslink mode of a UV crosslinker from Stratagene. The blot was equilibrated in

Buffer A [100 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 0.3% Tween 20] for 1 minute and blocked in buffer B [1% (w/v) blocking reagent (Boehringer Mannheim cat. # 1096 176) in buffer A] for 3 hours on an orbital shaker. A 1:1000 dilution of anti-DIG-POD conjugate in buffer B was prepared (final 150 U of anti-DIG-POD/ ml of buffer) near the end of three hours. This dilution was added and the membrane incubated for 30 min. This was followed by two 15-min washes in buffer A. The membrane was then equilibrated in buffer C [100 mM Tris-HCl pH 9.5, 10 mM NaCl and 50 mM $MgCl_2$] for 2 min. A chemiluminescent substrate [luminol mixed with starting solution, H_2O_2 (Boehringer Mannheim Cat. # 1500 708) was then added to the blot for 1 min. in a plastic bag. The solution was discarded and the blot exposed to an X-ray film.

RESULTS

Specificity of primers

The specificity of each primer pair was tested with each of the five internal standards. All the primer pairs detected only the respective sequences for which they were designed at low [100 a mole (1 amole= 10^{-15} moles)] template concentrations (data not shown). At higher concentrations [1 f mole (1 fmole= 10^{-12} moles), however, ABR17-specific primer also detected the ABR18 plasmid. To circumvent the problem of unspecific amplification of ABR18 cDNA by the ABR17 primer, differences in the sequences of the two cDNAs was utilized to distinguish the transcripts. Digestion with *Eco*RV was carried out to confirm that ABR18 transcript was not amplified by

the ABR17 primers in the RT-PCR assay (data not shown). The legitimate PCR product when ABR17 cDNA amplifies using oSABR17+4 and oSABR17-5 primers should yield a 249 and a 70 bp fragment upon digestion with *EcoR*V. The product obtained if ABR18 cDNA falsely amplifies with oSABR17+4 and oSABR17-5 primers should yield a 195 and a 124 bp fragment upon *EcoR*V digestion.

Linearity of the RT-PCR assay

It was first important to test if the ratio of signal intensities of the detected bands represent the ratio of RNA amounts present in the beginning, since at higher number of cycles, transcripts which are present in low abundance are over-represented, whilst those present in high levels will reach a plateau and hence be relatively under-represented. When all other reagents are in molar excess over PCR product, it is possible to obtain a linear relationship between template input and the output signal by limiting the amount of template and the number of PCR cycles. A dilution series of the cloned DNA plasmids, ranging from 10 amoles to 1000 amoles, was made. This series was subjected to 10, 14 and 17 cycles of PCR and a standard curve constructed (data not shown). Fourteen cycles of PCR was found to be sufficient in maintaining the range of assay linear without compromising the sensitivity of detection.

Differential transcript accumulation in wild *Pisum* species

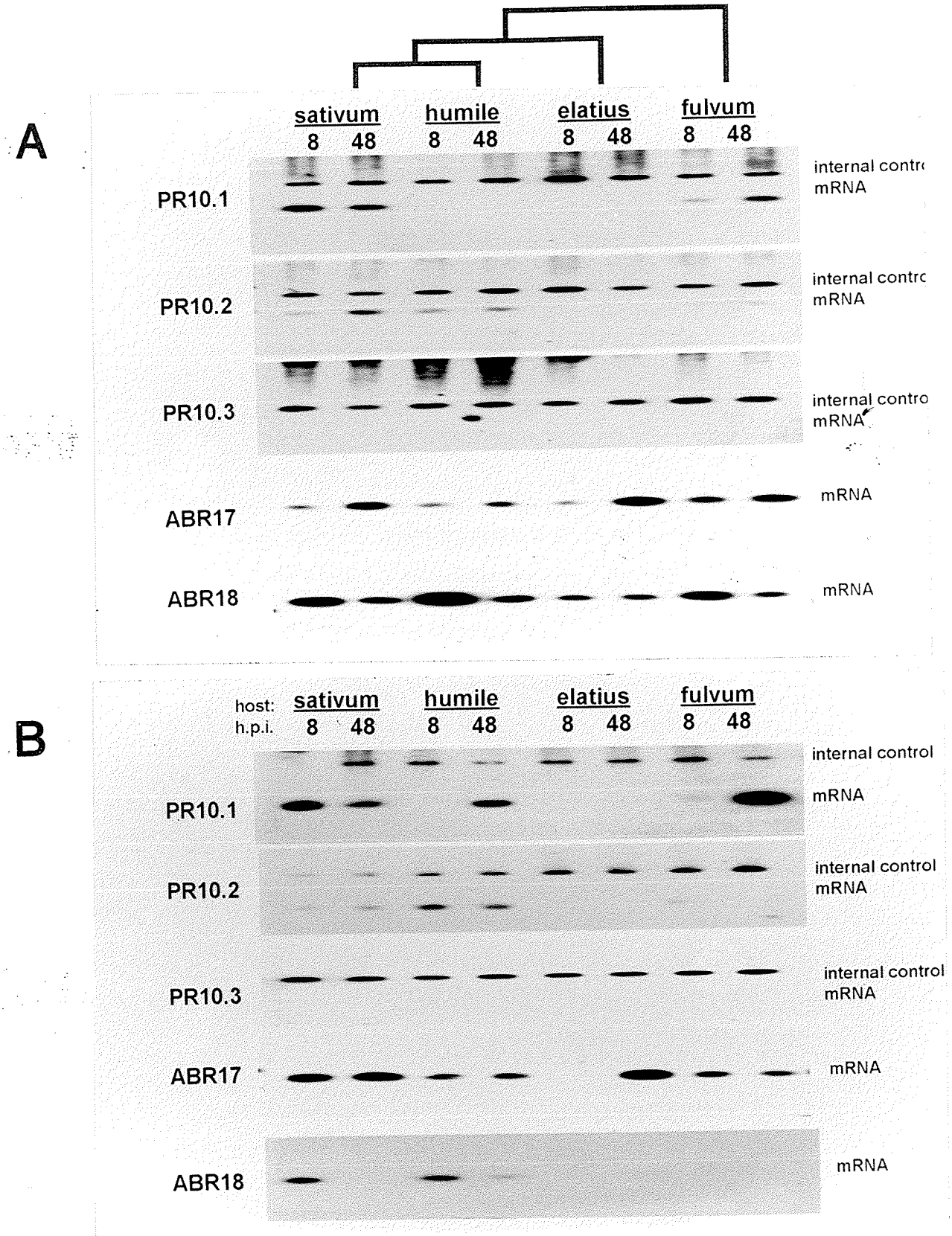
Having demonstrated that at least some of the same PR10 genes are conserved in wild pea species, we were interested in knowing if the expression patterns for these

genes were also conserved across the genus. RNA was isolated from *P. sativum, P. humile, P. elatius* and *P. fulvum*, pod tissue treated separately with a spore suspension of *F. solani* f. sp. *phaseoli* or *F. solani* f. sp. *F. solani* f. sp. *pisi*. RT-PCR was performed using specific primers for PR10 -1, -2, -3, ABR17 and ABR18, as described in Methods. We have not attempted to determine the precise numbers of RNA molecules present at a given time but rather ascertain the relative levels of transcripts. The PR10-1, -2, and -3 signals are directly comparable since they were assayed in a single experiment. Analysis of ABR17 and ABR18 transcript accumulation (Figs. 10-12) was performed separately from the other PR10 genes and hence the signals may not be directly comparable due to differences in exposure times. However, those filters are shown where the external standards give signals of comparable intensity.

PR10.1

PR10.1 transcript accumulated in both *P. sativum* and *P. fulvum* in response to challenge with either *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli*, although the kinetics of accumulation were different in each species (Fig. 10). Further, *P. fulvum* showed differences in relative abundance of PR10.1 mRNA upon challenge with the two pathogens. *P. humile* accumulated this transcript in response to inoculation with *F. solani* f. sp. *pisi* only and not with f. sp. *phaseoli*. PR10.1 mRNA was not detectable with either pathogen in *P. elatius*. Although the pattern of accumulation (Fig. 10) was similar in response to both pathogens, *P. fulvum* accumulated much higher levels of this transcript with *F. solani* f. sp. *pisi*.

Figure 10. Differential accumulation of specific PR10 mRNAs in *Pisum* species.
(A) In response to *Fusarium solani* f. sp. *phaseoli*
(B) In response to *Fusarium solani* f. sp. *pisi.*
c-DNA synthesized from reverse-transcription of 2 µg of RNA isolated from fungus-treated pod tissue from *P. sativum, P. humile, P. elatius* and *P. fulvum* was amplified using digoxigenin (DIG)-dUTP in polymerase chain reaction (RT-PCR). The product was blotted onto hybond membrane after running on 1.5% agarose gel and detected using a chemiluminescent substrate. Internal controls for RT-PCR were created by inserting Sau 3AI fragments from pUC18 into the conserved Bgl II site of the PR10 genes in pI49KS, pI176KS and p49cKS, which contain cDNAs for PR10.1, PR10.2, and an 868 bp NsiI/XbaI fragment of the PR10.3 genomic clone, respectively. Table 6 lists the sizes of the PCR products amplified from control plasmids or from cDNA. Since the conserved Bgl II site is internal to the gene specific priming sites in each gene, amplification of each clone with a given primer pair will generate a distinct PCR product, larger than that generated by RT-PCR from an mRNA population. **h.p.i.**=hours post inoculation


A
sativum
humile
elatius
fulvum
8 48 8 48 8 48 8 48
PR10.1
PR10.2
PR10.3
ABR17
ABR18
internal contr
mRNA
internal contro
mRNA
internal contro
mRNA
mRNA
mRNA
B
host:
h.p.i.
sativum
humile
elatius
fulvum
8 48 8 48 8 48 8 48
PR10.1
PR10.2
PR10.3
ABR17
ABR18
internal control
mRNA
internal control
mRNA
internal control
mRNA
mRNA
mRNA

PR10.2

This member of the gene family was expressed in *P. sativum* and its closest relative *P. humile* upon challenge with either pathogen (Fig 10). Some mRNA was also detectable in *P. fulvum* inoculated with *F. solani* f. sp. *phaseoli*. No signal was seen in *P. elatius*.

PR10.3

The transcript for this gene was not detected in any of the host species upon infection with either *F. solani* f. sp. *phaseoli* or *F. solani* f. sp. *pisi* (Fig. 10) although the internal control amplifies using the PR10.3 specific primer.

ABR17

All host species accumulated ABR17 mRNA when inoculated with either *F. solani* f. sp. *phaseoli* or *F. solani* f. sp. *pisi* (Fig. 10). In *P. sativum* tissue inoculated with *F. solani* f. sp. *pisi*, strong signal was seen at both 8 and 48 hours. Only a weak signal was obtained when this host was inoculated with *F. solani* f. sp. *phaseoli*. *P. humile* and *P. fulvum* gave signals of comparable intensity at both time-points in response to inoculation with either pathogen. *P. elatius* responded to both pathogens by low to undetectable signal at 8 hours followed by strong accumulation by 48 hours.

ABR18

High levels of ABR18 mRNA were detected in the *F. solani* f. sp. *phaseoli* -

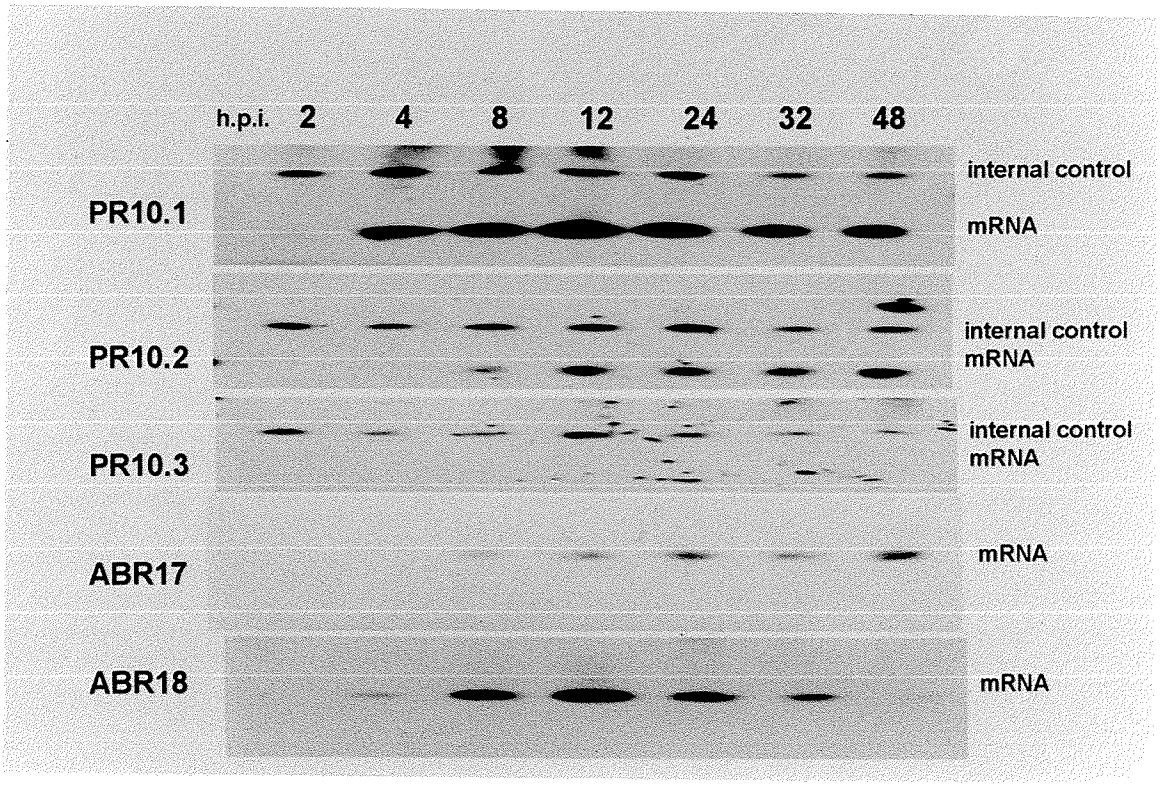
treated pod tissue of all the host taxa tested (Fig. 10A). The mRNA for this gene was detected only in *P. sativum* and *P. humile* pods treated with *F. solani* f. sp. *pisi* (Fig. B). Except in *P. elatius*, where similar levels of mRNA were present at both time points tested (8 and 48 hours), in all other species in which ABR18 transcript was detectable, there was very high transcript accumulation at 8 hours followed by a decline in levels by 48 hours.

Detailed time course of PR10 transcript accumulation in *P. sativum*

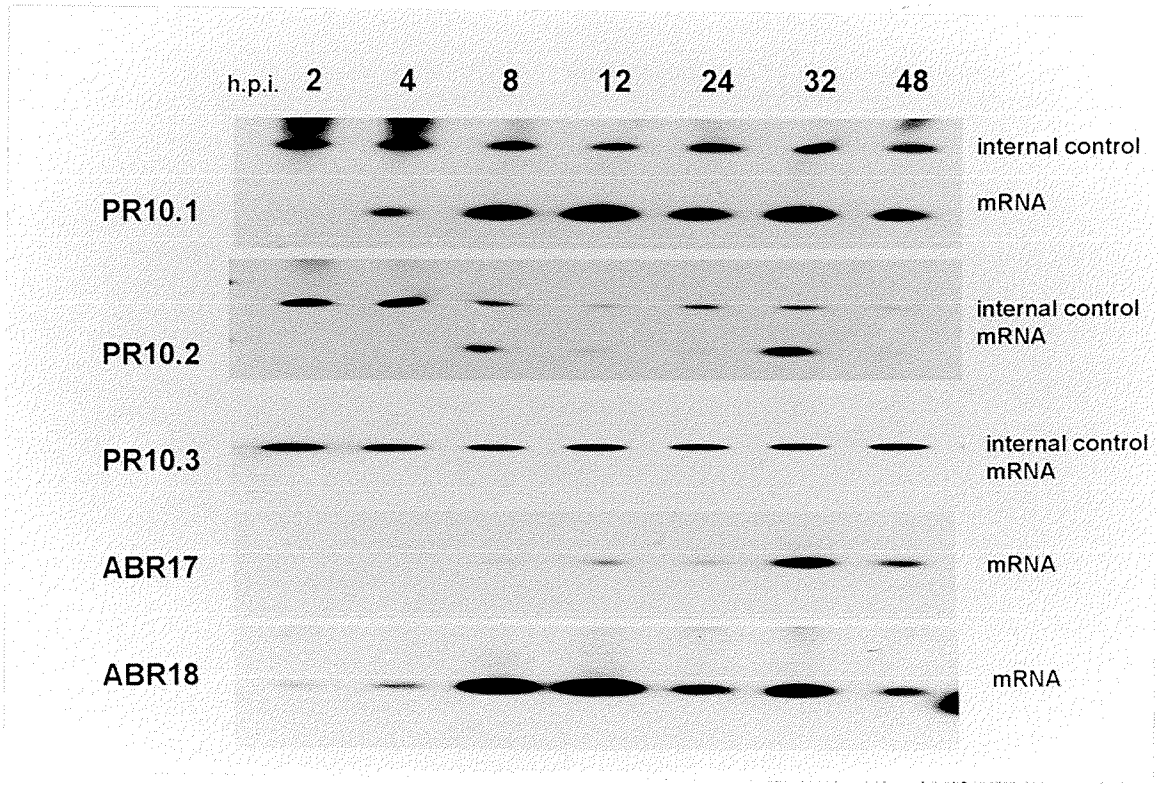
Our previous results showed that *P. sativum* was the only *Pisum* ecotype which was differentially susceptible to the two *formae specialis* of *F. solani* (Tewari *et al.*, manuscript submitted). *P. sativum* was therefore chosen for detailed analysis of PR10 transcript accumulation in response to *F. solani* f. sp. *pisi* (compatible) and *F. solani* f. sp. *phaseoli* (incompatible). Pod tissue from *P. sativum* was inoculated with either *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli* and RNA extracted at different time points. Figure 11. shows the time course of PR10.1, PR10.2, PR10.3, ABR17 and ABR18 transcript accumulation in response to the inoculations. In *F. solani* f. sp. *phaseoli* treated tissue (Figure 11A), PR10.1 transcript could be detected in the autoradiogram as early as 2 hours after inoculation (this does not reproduce very well in the picture). Transcript levels increased sharply within 4 hours, reaching a peak around 12 hours. PR10.2 transcript was not detectable until 8 hours. The signal was also much weaker than that of PR10.1. PR10.3 transcript was not detected under these conditions. ABR17 transcript was only weakly induced and also became detectable

Figure 11. Timecourse of accumulation of specific PR10 mRNAs in *P. sativum.*
(A) In response to *Fusarium solani* f. sp. *phaseoli*
(B) In response to *Fusarium solani* f. sp. *pisi.*
c-DNA synthesized from reverse-transcription of 2 µg of RNA isolated from fungus-treated pod tissue for the indicated time was amplified using digoxigenin (DIG)-dUTP in polymerase chain reaction (RT-PCR). The product was blotted onto hybond membrane after running on1.5% agarose gel and detected using a chemiluminescent substrate. Internal controls were prepared as described in figure 10. **h.p.i.**=hours post inoculation

A


h.p.i. 2 4 8 12 24 32 48
PR10.1
internal control
mRNA
PR10.2
internal control
mRNA
PR10.3
internal control
mRNA
ABR17
mRNA
ABR18
mRNA

B


h.p.i. 2 4 8 12 24 32 48
PR10.1
internal control
mRNA
PR10.2
internal control
mRNA
PR10.3
internal control
mRNA
ABR17
mRNA
ABR18
mRNA

only at 8 hours. ABR18 transcript accumulation, although transient, was high at 8 and 12 hours.

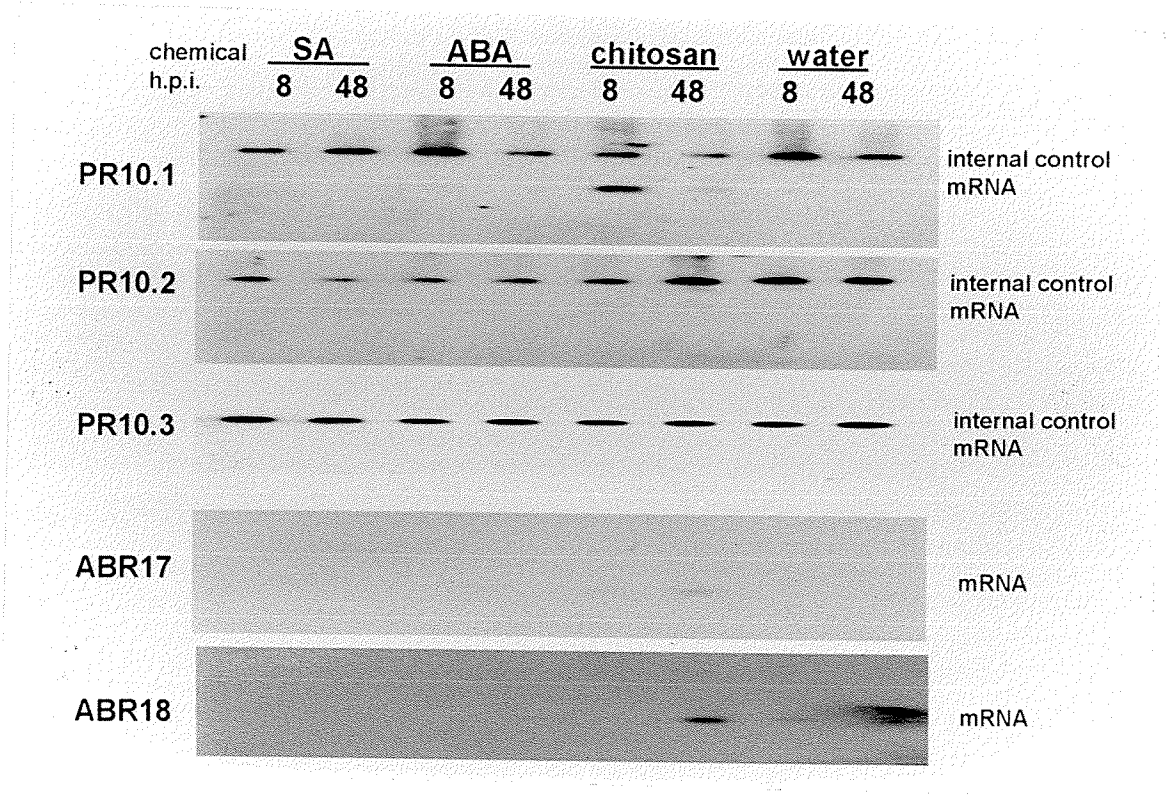
F. solani f. sp. *pisi* treated tissue (compatible), showed an overall similar expression characteristics with a few key differences (Fig.11B). PR10.1 mRNA appeared only at 4 hours post inoculation and increased sharply by 8 hours, after which the level remained steady till 32 hours. As in case of *F. solani* f. sp. *phaseoli* treated tissue, PR10.2 signal was weaker and delayed. Two peaks were observed, one at 8 hour and the other at 32 hours. PR10.3 was not detected with this pathogen either. ABR17 transcript became detectable at 8 hours and peaked at 32 hours after which the levels were seen to decrease. ABR18, in contrast, was detectable as early as 2 hours, peaked between 8 to 12 hours, then declined gradually by 48 hours.

Differential transcript accumulation in *P. sativum* in response to chemical treatments

Messenger RNA for any of the genes was not detectable either in water control or pods treated with SA (Figure 12). Upon chitosan treatment, PR10.1 and ABR18 transcripts were seen to be elevated at 8 hours and 48 hours respectively. PR10.2 and ABR17 transcripts were barely detected following treatment with this elicitor. PR10.3 was not detected.

Following ABA treatment, a very faint signal was obtained for PR10.1 and ABR17 (Figure 12). No signal was observed for PR10.2, PR10.3 or ABR18.

Figure 12. Accumulation of specific PR10 mRNAs in *P.sativum* in response to chemical treatments.
Complimentary-DNA synthesized from reverse-transcription of 2 µg of RNA isolated from pod tissue treated with 50 mM salicylic acid (SA), 100 µM abscisic acid (ABA), 1 mg/ml chitosan or water for indicated times was amplified using digoxigenin (DIG)-dUTP in polymerase chain reaction (RT-PCR). The product was blotted onto hybond membrane after running on 1.5% agarose gel and detected using a chemiluminescent substrate. Internal controls were prepared as described in figure 10. **h.p.i.**=hours post inoculation


chemical
h.p.i.
SA
8
48
ABA
8
48
chitosan
8
48
water
8
48
PR10.1
internal control mRNA
PR10.2
internal control mRNA
PR10.3
internal control mRNA
ABR17
mRNA
ABR18
mRNA

DISCUSSION

We have previously shown that PR10 comprises a multigene family in *P. sativum* and its closely-related wild ecotypes (Tewari *et al.*, manuscript submitted). Sequence of PCR-generated DNAs from wild pea species indicate that the similarity between each PR10 gene from *P. sativum* and its counterpart from wild species is within a few percent of complete identity, even within the intron (Fristensky and Brown, unpublished results). The use of PCR for the detection of specific PR10 mRNAs after reverse transcription to DNA (RT-PCR) enabled us to analyze the differential accumulation patterns of this multigene family. PR10.1, PR10.2, ABR17 and ABR18 mRNA are activated following fungal infection whilst PR10.3 mRNA was not expressed with any treatment in any of the four hosts. PR10.3 was first isolated by screening of pea genomic library with the PR10.1 probe (Chiang and Hadwiger, 1990). Pathogen-inducibility has not yet been demonstrated for this gene. PR10.3 was recently also cloned independently by Mylona et al. (1994) while cloning root hair specific transcripts from *P. sativum.* They demonstrate that this PR10 homolog is developmentally expressed in pea. Transcripts for this gene were detected specifically in the root-epidermis and root hair and also in the parts of pea embryo determined to form the root. Inoculation of pea roots with *Rhizobium leguminosarum* bv. *viciae* did not have any appreciable effect on expression of PR10.3. (Mylona *et al.*, 1994). In contrast, enhanced expression of PR10.1 was seen upon pathogen challenge. Our results are consistent with these findings and taken together, these results suggest that

PR10.1 is pathogen-inducible while PR10.3 is developmentally regulated and is probably root-specific. Two other legumes, viz. soybean (Crowell et al, 1992) and bean (Walter et al. 1995) also show high expression of PR10 homologs in roots. Soybean PR10 homolog (SAM22) message accumulates predominantly in the roots of young seedlings, in cotyledons as the plants mature and the cotyledons begin to show signs of senescence. Bean PR-10 (formerly PvPR1 & PvPR2, Walter et al., 1990) transcripts accumulate in roots, senescent leaves, mature pollen and styles (Walter et al., 1995). Roots are constantly exposed to microorganisms and are also subject to wounding and stress during penetration of lateral roots through main root. The expression of PR10 in roots is thus consistent with a role of these genes in stress.

Our results show that ABR17 and ABR18 are pathogen inducible. This has not yet been shown for these genes in any of the previous studies. Strong signals were obtained in all the four tested *Pisum* species in response to fungal treatment when primers specific for ABR17 and ABR18 were used in the RT-PCR analysis.

All the host species distinguished the two pathogens used. Whereas the expression kinetics and transcript accumulation of some genes was similar in response to either pathogen inoculation, some differences were noted in at least one of the five genes studied. These differences ranged from changes in levels or pattern of accumulation to total absence of detectable mRNA for the gene. For example, no appreciable differences were seen in *P. humile* in mRNA accumulation for PR10.2, PR10.3 and ABR17 regardless of the pathogen used. PR10.1 mRNA was detected in this host at 48 hours upon challenge with *F. solani* f. sp. *pisi* but not with *F. solani* f.

sp. *phaseoli*. ABR18 mRNA was much more abundant in response to inoculation with *F. solani* f. sp. *phaseoli* as opposed to that with *F. solani* f. sp. *pisi*.

Each host species had a unique pattern of PR10 mRNA accumulation. None of the species examined had identical patterns of expression in responses to a particular pathogen. Differences were seen in the expression pattern of at least one gene. For example, upon challenge with *F. solani* f. sp. *phaseoli*, *P. sativum* expressed PR10.1, PR10.2, ABR17 and ABR18; *P. humile* expressed PR10.2, ABR17 and ABR18; *P. elatius* expressed only ABR17 and ABR18; *P. fulvum* expressed the same sets of genes as *P. sativum* although differences were detected in the intensity of signal and the pattern of accumulation at two time-points tested.

Some common features were found between expression patterns of *P. sativum* and its closest wild relative *P. humile*. Both the ecotypes induced the same set of genes, albeit with different intensities, in response to inoculation with a particular pathogen. *P. elatius*, a more primitive ecotype, was even more divergent in its expression patterns than *P. fulvum*, which is recognized as a distinct species. Expression patterns of *P. elatius* were most diverged from those of the other hosts in only expressing ABR17 and ABR18 and not PR10.1, PR10.2 or PR10.3. In the light of these results it is interesting to note that *P. elatius* shows a unique banding pattern in the genomic DNA gel blot. (Tewari *et al.*, manuscript submitted). In contrast to the appearance of more than three bands in other *Pisum* species, only two bands appeared in *P. elatius* genomic DNA using a conserved PR10 probe. A 6.5 kb band hybridizing to PR10 probe in *P. elatius* appeared much greater in stoichiometric intensity, which

could be due to a duplication of that region in *P. elatius*. In parallel with these findings, PR10.2 could not be isolated from *P. elatius* by the method used for cloning PR10 homologs from wild *Pisum* ecotypes (Tewari *et al.*, manuscript submitted). Thus changes in amino acid sequences and amplification and deletion of family members over the course of evolution may have generated a substantially different multigene family in *P. elatius*. These processes may underlie the changes in differential expression patterns for specific PR10 subsets in different *Pisum* species.

Analysis of detailed time course of accumulation of each PR10 gene in *P. sativum* revealed a characteristic expression pattern of each gene. In general, PR10.1 mRNA accumulates to relatively high levels early in the interaction (within 4 hours); PR10.2 and ABR17 transcripts appear later and are only weakly induced; ABR18 transcripts accumulate to high levels, albeit transiently; PR10.3 is not inducible by the pathogens under the conditions used. Thus, individual members of PR10 gene family are differentially expressed with respect to *timing* of expression. These results imply differences signal transduction pathways leading to the activation of at least some of the genes.

Chemical treatments, in general, were not as effective in inducing PR10 genes as fungal pathogens. These findings are in agreement with our previous results (Tewari *et al.*, manuscript submitted). No signal was detectable for any of the genes upon salicylic acid (SA) treatment. SA treatment has been shown to induce SAM22 moderately in young soybean leaves (Crowell *et al.*, 1992). Little induction of GUS activity was observed after spraying SA on intact transgenic tobacco plants

transformed with the asparagus AoPR1 promoter-GUS gene fusion (Warner *et al.*, 1994). In our experiments, treatment with abscisic acid (ABA) did not lead to dramatic accumulation of PR10 transcripts, including those of ABR17 and ABR18. This was a little surprising because these genes were originally cloned from cultured *P. sativum* embryos and their products were markedly induced by exogenously supplied ABA. These proteins are not detectable in the embryos during the early stages of development but appear during the late stages of seed development. These proteins are not, however, induced in embryos when pods are cultured in presence of ABA. Both proteins are also induced to a certain extent in culture medium without exogenously supplied ABA. These observations have led the authors to suggest that the effect of ABA is probably indirect (for example through changes in water-relations) rather than a direct regulation of these proteins through ABA (Iturriaga *et al.*, 1994). Our results support this hypothesis. Treatment of potato leaves with ABA also did not induce PR10a expression (Constabel and Brisson, 1995). These observations combined with our results showing strong signal for ABR17 and ABR18 in response to pathogens and weak to undetectable signal upon ABA treatment suggest that ABA may affect the accumulation of the ABA-responsive proteins through generalized activation of stress responses rather than directly.

Chapter 5

GENERAL DISCUSSION

Although PR10 probe from *P. sativum* hybridizes to multiple bands in the genomic DNA from wild pea ecotypes, enough sequence divergence has occurred in the coding region or in the flanking DNA to demonstrate variations in the banding patterns. Amplification, deletion and mutation of genes over the course of evolution would be expected to alter the phenotypes that depend on that gene family. For example, changes in amino acid sequences, differential regulation patterns or copy number in a defense multigene family might either increase or decrease the resistance of a host species to a given fungal pathogen. When inoculated on pod endocarp tissue, the two *Fusarium solani* forms germinated and proliferated to different extents on different *Pisum* and *Lathyrus* taxa. *P. humile*, which is most closely related to *P. sativum*, had a score more similar to *P. sativum* than the other two wild species. *Lathyrus* species, which are more distantly related exhibited a more diverged score. Thus a divergence in species from *P. sativum* was seen to coincide with a gradual increase in host - pathogen compatibility. The divergence of interaction phenotype appears to be gradual because neighbouring taxa had the most similar scores. Analysis of several accessions within a species/ecotype is required, however, before such a generalization can be confirmed.

The PR10 transcript was detectable in all the taxa in response to fungal

challenge. Our results indicated a strong correlation between rapid accumulation of PR10 mRNA and inhibition of pathogen growth. This conclusion was supported both by RNA gel blot analysis from different host taxa and the data from chemical treatments on *P. sativum* pod tissue. One interesting finding was that in contrast to the protection of pea tissue from pathogen by application of chitosan, application of salicylic acid (SA) to the pods leads to an enhanced rate of pathogen growth. This enhancement of growth coincided with the disappearance of PR10 transcript below detectable levels upon SA treatment. Evolutionary conservation of this gene family in closely related as well as widely divergent plant species and its rapid activation following pathogen challenge suggests that the product of this gene probably plays an important role in plant defense. The classical tobacco PR proteins show strong induction with salicylic acid and this metabolite of the phenylpropanoid pathway also protects the plants against subsequent pathogen attack (see Malamy and Klessig, 1992 for a review). Comparisons of induction patterns of PR10 with chalcone synthase, one of the genes in the phenylpropanoid pathway, did not reveal coordinate regulation (Figs. 6 and 7). Lack of signal for any of the genes of the PR10 family upon treatment with SA further suggests that this gene family may be a part of a distinct regulatory pathway (Fig. 12).

Northern blot analysis, using gene specific probes PR10.1/PR10.2 and PR10.3 subfamilies indicated that this gene family was differentially expressed in *Pisum* and *Lathyrus*. However, due to high degree of similarity between the genes, individual genes could not be distinguished. A combination of cDNA synthesis followed by

PCR using specific primers for all the five cloned *P. sativum* PR10 genes was therefore used to discriminate between genes.

Initially, the amplified product after electrophoresis was quantified by determining the intensity of each amplified band in the polaroid picture of the gel using image analysis. One disadvantage of this method was that it required the use of higher number of cycles for visualization of the amplified product, which compromised the linearity of amplification.

The DIG-PCR ELISA kit from Boehringer Mannheim was also tested for its effectiveness in quantifying the accumulation of specific PR10 transcripts. The main advantage in using this system was that a semi-quantitative reading could be obtained directly by using an ELISA plate reader. However, in our hands, the method was not very reproducible. The product from the same PCR tube yielded different readings in different wells.

Finally, a method was devised in which DIG-dUTP was included in the PCR reaction and the DIG-labelled product was detected by electrophoresis on an agarose gel followed by blotting onto a membrane and chemiluminescent detection on X-ray film (see Methods). The advantage to using this system is that noise due to non-specific binding of the antibody is eliminated and internal markers that amplify along with the cDNA can also be included in the PCR reaction to account for tube-to-tube variation. The disadvantage is that the bands in the autoradiogram provide only a visual estimation of amount of signal and some other technique must be used if a numerical value to quantify the signal is required.

P. sativum derived primers did not amplify the cDNA from *Lathyrus* species. Therefore, data from *Pisum* species only are shown. Each of the four tested *Pisum* ecotypes demonstrated a unique pattern of expression by accumulating different combinations of PR10 transcripts. PR10.1, PR10.2, ABR17 and ABR18 were found to be pathogen-inducible while PR10.3 mRNA was not detected in any of the four hosts using RT-PCR. Some signal was however, seen when PR10.3 probe was used in the Northern blot (Fig. 8 & 9). Although the probe is derived from the 3' end of the gene, a part of the coding region was also present in the probe. Thus cross-hybridization between genes can not be ruled out. The signal for PR10.3 seen in the RNA gel blot with may be due to cross hybridization of the PR10.3 probe with PR10.1/PR10.2 mRNA. The use of specific primers in the RT-PCR assay helped circumvent this problem. In Figs. 10-12, the internal control amplifies with the PR10.3 specific primers but no signal is seen for mRNA. Since PR10.3 was also independently cloned during isolation of root hair specific transcripts and pathogen-inducibility has not been shown for this gene, it is likely that this PR10 homolog is root specific. Further, each gene is activated to different extents in response to different signals. Each gene within the PR10 family may be specialized for specific functions.

Northern blot analysis using subfamily-specific probes (Fig. 6 & 7) suggested that the PR10.1/PR10.2 subfamily accounted for most of the PR10 transcript accumulation in response to fungal inoculation. A different result, in terms of which members are involved in the major response, was obtained using RT-PCR. For example ABR17 and ABR18 mRNA appeared to be the major component of the total

PR10 mRNA accumulating in all the four *Pisum* ecotypes in response to *F. solani* f. sp. *phaseoli*. There are at least two possible sources for this apparent inconsistency. ABR17 and ABR18 (Iturriaga *et al.*, 1994) were isolated much later than the PR10.1, PR10.2, PR10.3 and hence became available to us only recently. The signal intensities between PR10.1, PR10.2, PR10.3 and ABR17 and ABR18 are not directly comparable since the analysis of transcript accumulation of the later two genes were performed separately from the former three.

For the same reason (non availability of ABR clones) these plasmids were not loaded on the RNA gel blots and therefore it is not known if the subfamily specific probes used in the experiment cross hybridized to the ABR17 and ABR18 mRNA under the conditions used.

Another apparant inconsistency in results from two methods used is between RT-PCR (Fig. 10) and Northern blot analysis using a non-discriminating PR10 probe (Figs. 6 and 7). Ideally, the signal at each data point for all five genes should approximately add up to the signal obtained in Figs. 6 and 7. Such a visual estimation shows that most data points are consistent. However, in some data points, these signals do not appear to add up. This apparant discrepency could result from several reasons. Firstly, in the RT-PCR experiments, ABR17 and ABR18 intensities may not be comparable to those of PR10.1, PR10.2 and PR10.3 since they were assayed in different experiments. It is possible that the probe used in Northerns hybridized to some addtional sequences while the primers used in RT-PCR only amplify specific transcripts. From the Southern blot data alone, it is not possible to the copy number

for each member in each genome. For example, *P. elatius* could have more than one copies of PR10.1 or ABR17 genes. Alternatively, it may have additional genes that do not amplify with the primers used in the RT-PCR, but hybridize to the probe used in the Northerns.

Detailed analysis of mRNA accumulations in *P. sativum* showed that different transcripts accumulated with different kinetics upon challenge with pathogens. While both PR10.1 and ABR18 mRNAs accumulate to high levels within 4 hours post inoculation, those of ABR18 decline rapidly. This period is also characterized by strong inhibition of pathogen spore germination and growth (Teasdale *et al.*, 1974). The expression pattern of PR10.1 and ABR18 suggests that they may be important for expression of resistance by the plant. These two genes may be good candidates for transformation into host plants to determine if their constitutive expression enhances the resistance of plants to pathogens.

In conclusion, the PR10 multigene family is differentially expressed with respect to *timing* and extent of expression in *Pisum* ecotypes. Multigene families have the potential to generate a great deal of phenotypic diversity in the defense responses of hosts. Our results show that the patterns of differential expression of members of a multigene family can change dramatically over a short evolutionary time. While changes in the coding sequences of pathogenesis-related genes can generate some genetic diversity, the changes in the *regulatory* component have an even greater potential to do so. Further studies could aim at identifying *cis*-regulatory elements responsive to different signals using gel-shift mobility assays or DNA footprinting.

Differential binding of trans-acting factors may mediate differential expression of PR10 genes. Each gene may be activated by a unique combination of proteins binding to a unique combination of regulatory sites in each promoter. Studies on various multigene families show that differential expression is an inherent part of defense gene expression. The greatest selective advantage may exist in those genotypes that undergo frequent mutations in defense gene expression patterns.

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