Analysis of *cis*-Regulatory Elements in Differential Expression of the *PR10* Multigene Family in Pea (*Pisum sativum*)

by

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ABSTRACT

Zhang, Yongping, Ph.D., The University of Manitoba, September 1999. Analysis of *cis*-Regulatory Elements in Differential Expression of the *PR10* Multigene Family in Pea (*Pisum sativum*). Major Professor: Dr. Brian Fristensky.

Genomic copies of *PR10.1* and *PR10.3*, two members of the *PR10* multigene family in *Pisum sativum*, were screened for *cis*-regulatory elements associated with differential expression upon fungal and chemical challenges. Gel shift assays revealed that nuclear proteins from fungus-treated tissues specifically bound two major binding regions in both *PR10.1* and *PR10.3*. Deletion analysis of the *PR10.1* promoter region from -284 to 79 identified two binding sequences, PDA1 and PDA2. PDA1 reacted with all tested nuclear extracts while PDA2 was only bound by extracts from pods treated with the non-pathogenic fungus *Fusarium solani* f. sp. *phaseoli* (Fsph) or salicylic acid (SA). Competition assays with oligonucleotides identified two distinct binding sites, PDA2a and PDA2b within PDA2. Similarly, analysis of the *PR10.3* promoter from -621 to -196 identified a specific binding sequence, PDC1, from -544 to -461. PDC1 reacted strongly with Fsph and SA treatments and weakly with the pathogenic fungus *F. solani* f. sp. *pisi* (Fsp) treatment. Database comparisons of oligonucleotide frequencies between *PR10* genes and other defense genes, and between defense genes and genes not associated with defense, identified 4 conserved motifs, which were present in PDA1, PDA2 and PDC1.

Expression of *PR10.1* and *PR10.3* was investigated in a time course up to 48 h after challenges. The highest binding activities occurred 2 - 4 h after challenge, while *PR10.1* mRNA accumulation did not peak until 8 - 12 h.p.i. *PR10.3* was not expressed in pea pods with any treatment, but was expressed in healthy roots. *PR10.1* expression remained strong up to 48 h.p.i. with the Fsph treatment, while expression declined after 12 h with the Fsp treatment. These data suggest that PDA2 could play a role in fungus-induced gene expression. The different expression patterns between *PR10.1* and *PR10.3* suggested that there is distinct defferent gene expression regulation among members of the *PR10* multigene family in pea.

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LIST OF ABBREVIATIONS

BSA:	Bovine serum albumin
bp:	Base pair
CA:	Cold acclimatization
CDS:	Coding sequence
CH:	Treatment of chitosan (1 mg/ml)
CHS:	Chalcone synthase
CH8TP:	Cytoplasmic proteins from pea pods treated with CH 8 hours
DIG:	Digoxigenin
DMSO:	Dimethyl sulfoxide
DTT:	Dithiothreitol
EDTA:	Ethylenediamine-tetraacetic acid, sodium salt
FB:	Freezing buffer
Fsp:	Treatment of *Fusarium solani* f. sp. *pisi*
Fsp6TP:	Cytoplasmic proteins from pea pods treated with Fsp 6 hours
Fsph:	Treatment of *Fusarium solani* f. sp. *phaseoli*
Fsph6TP:	Cytoplasmic proteins from pea pods treated with Fsph 6 hours
GA:	Gum arabic
GDE:	Genomic Data Environment
h:	Hour
H:	Split healthy pea pods (wounding control)

h.p.i.:	Hour post-inoculation
HTP:	Cytoplasmic proteins from healthy pea pods (healthy control)
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt
KBB:	KCl binding buffer
LB:	Luria broth
LIEB:	Low ionic strength electrophoresis buffer
MES:	4-morpholineethanesulfonic acid
min:	Minute
MW:	Molecular weight
PAGE:	Poly acrylamide gel electrophoresis
PAL:	Phenylalanine ammonia-lyase
PCR:	Polymerase chain reaction
PDA:	Potato dextrose agar
PEG:	Polyethylene glycol
PH:	Fsph treatment following heat-shock of 40 ^{0}C 2 minutes
PH2TP:	Cytoplasmic proteins from pea pods treated with PH 2 hours
Ph-CHX:	Treatment combination of Fsph and cycloheximide
PLPh:	Plumule treated with Fsph under light
PLW:	Plumule treated with water under light
PMSF:	Phenylmethylsulfonyl fluoride
Poly dI-dC:	Polydeoxyinosinic-deoxycytidylic acid
PR:	Pathogenesis-related

RS:	Resuspension solution
RT-PCR:	Reverse transcription PCR
SA:	Treatment of salicylic acid (50 mM)
SA36TP:	Cytoplasmic proteins from pea pods treated with SA 36 hours
SA-Fsph:	Treatment combination of SA and Fsph
SC-Fsph:	Treatment combination of sodium chloride and Fsph
SC-SC:	Twice treatment of sodium chloride
SDS:	Lauryl sulfate, sodium salt
TEMED:	N,N,N´,N´-Tetramethylethylenediamine
TMV:	Tobacco mosaic virus
UPP:	Unsplit healthy pea pods (healthy control)
W:	Water treatment (parallel control)

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1. INTRODUCTION

Plants are frequently subject to infection by pathogens. During defense responses to pathogens, plants synthesize many different pathogenesis-related (PR) proteins. PR proteins are plant proteins induced only by pathogens or related signals and are believed to play a role in pathogen restriction and disease resistance. So far, eleven PR protein families were defined in plants (Van Loon *et al.*, 1994). *PR10* is one of eleven PR multigene families in plants and occurs in a wide range of species. Although the function of the *PR10* gene family is not known, it was found that accumulation of *PR10* gene transcripts in pea pods was closely associated with many challenges, including both compatible and incompatible subspecies of *Fusarium solani* (Fristensky *et al.*, 1985). The incompatible pathogen induced more expression of the *PR10* transcripts than the compatible pathogen. Differential expression of the *PR10* genes has also been reported in many plant species, such as pea (Daniels *et al.*, 1987; Chiang and Hadwiger, 1990), parsley (Somssich *et al.*, 1988), bean (Walter *et al.*, 1996), potato (Matton & Brisson, 1989; Constabel & Brisson, 1995) and birch (Swoboda *et al.*, 1995).

Cis-regulatory elements have been identified in many PR genes. *STH-2* in potato contains a positive regulatory element between -135 and -52 and a possible negative element between -52 and -28 (Matton, *et al.*, 1993). An ethylene-responsive element in *PRB-1b* was identified in tobacco (Sessa *et al.*, 1995). In the *osmotin* (*PR5*) gene from salt-adapted tobacco, three upstream regulatory elements were identified: G-

sequence, AT-sequence and PR-sequence, which are responsive to salinity and drought (Liu, *et al.*, 1995). Van de Rhee and Bol (1993) reported that *PR1a* gene expression in tobacco is highly regulated by four TMV-inducible elements located from -902 and 29 and no element by itself is responsive to TMV challenge or salicylate treatment. Recently the as-1 like *cis*-element in *PR1a* and its DNA-binding protein, similar to TGA1a, were isolated. Their interactions are responsible for fungal and SA elicitation in tobacco (Strompen *et al.*, 1998). Després *et al.*, (1995) reported that a region of 50-bp promoter sequence was necessary for the elicitor responsiveness of *PR10a* in transgenic potato plants. No promoter analysis of the *PR10* genes has been reported in peas. Although there are five known members of the *PR10* multigene family in peas, only two members, *PR10.1* and *PR10.3*, previously named *DRR49a* and *DRRG49c*, respectively (Fristensky, 1995; Chiang and Hadwiger, 1990), are available in genomic clones. Previous research in this laboratory showed that individual members of the *PR10* multigene family were differentially expressed when challenged with fungi or chemical elicitors. Presumably differential expression patterns of *PR10.1* and *PR10.3* in peas may be positively or negatively mediated by specific *cis*-regulatory elements. Investigation of *cis*-regulatory elements in the interaction between the *PR10* genes and external challenges will add to our understanding of the mechanisms by which plants respond to stress and help us look into methods to increase plant resistance at the molecular level.

When studying *cis*-regulatory elements, the most common approach is to clone the target promoter DNA into an expression cassette upstream from a reporter gene,

such as β-glucuronidase (GUS). Based on the activity of the reporter gene in a foreign plant, the function of *cis*-regulatory elements is indirectly evaluated. Theoretically, however, *cis*-elements can be located anywhere in a gene. Although the majority of *cis*-elements have been found in upstream promoter regions, they have also been found in introns (Mascarenhas *et al.*, 1990), coding sequences (Yamamoto *et al.*, 1997) and downstream regions (Sessa *et al.*, 1995b; Chinn *et al.*, 1996). The expression of a promoter-reporter gene construct may thus be different from the expression of the unmodified complete gene in the native plants.

This research was initiated using the pea *PR10* multigene family as a model to identify *cis*-regulatory elements and to analyze their biological functions in terms of differential gene expression in native pea plants. To avoid missing any potential *cis*-regulatory elements, the entire ranges of both *PR10.1* and *PR10.3* have been investigated under various stress conditions. Two major protein-binding regions in both *PR10.1* and *PR10.3* were found upstream and downstream of the coding sequences, respectively. Deletion of upstream regions in both *PR10.1* and *PR10.3* revealed three *cis*-regulatory elements. Biological function of the *cis*-elements was evaluated by comparing DNA/protein binding assays with differential gene expression of *PR10.1* and *PR10.3* in native pea plants upon challenge. That binding activities correlated with gene expression suggested that the *cis*-elements play a role in the incompatible pathogen/plant interaction in pea.

2. LITERATURE REVIEW

2.1. Disease resistance responses in plants

Unlike animals, plants do not have a systemic, multicellular immune system. Growing in unsheltered environments, plants are always exposed to unpredictable environmental conditions. Thus, plants have developed alternatives to adapt to external challenges. Except for the ever-changing weather, microorganisms are the most important external challenges. Though there are numerous microorganisms in the field, most are non-pathogenic to plants. In other words, a given plant species can resist most microorganisms. This so called non-host resistance results from an incompatible interaction between a resistant host and an avirulent pathogen. A small number of the potential pathogens become pathogenic to a limited range of plants, pathogenesis resulting from a compatible interaction between a susceptible host and a virulent pathogen.

2.1.1. *Agents inducing disease resistance response*

There are many different stresses that elicit resistance responses in plants. The stresses can be sorted into two major groups: biological and non-biological. Non-biological stresses include mechanical, chemical and environmental stresses. Some typical examples of stress-responsive genes include: a turgor-responsive gene in

Brassica napus (Stroeher *et al.*, 1995); a wound-induced transcript from *Asparagus* (Warner *et al.*, 1992); ethylene and methyl jasmonate induced plant defense genes (Sessa *et al.*, 1995a; Xu *et al.*, 1994); a sugar-responsive α-amylase gene in rice (Lu *et al.*, 1998); salicylic acid responsive genes (Delaney *et al.*, 1994; Chen *et al.*, 1995); chitosan-triggered expression (Hadwiger, 1984); dark-induced PR genes (Eyal *et al.*, 1992; Sessa *et al.*, 1995b); genes responsive to salinity or drought (Schaeffer *et al.*, 1995); UV light induction (Hadwiger *et al.*, 1971; Green *et al.*, 1995); effect of heat-shock on disease resistance response (Hadwiger *et al.*, 1983; Schweizer *et al.*, 1995); temperature-dependent defense response (Malamy *et al.*, 1992); cold acclimation induced genes (Guy and Haskell, 1987); three genes responsive to osmotic stress in potato (Zhu *et al.*, 1995); ozone-induced PR transcripts (Eckey-Kaltenbach *et al.*, 1997); and plant defense genes were activated even by air pollutants (Bahl *et al.*, 1995).

Biological stresses include pathogenic and non-pathogenic living organisms, including fungi (Hadwiger *et al.*, 1992; Yoder *et al.*, 1993), bacteria (Hadwiger *et al.*, 1984; Pastuglia *et al.*, 1997), viruses (Albrecht *et al.*, 1992; Bendahmane *et al.*, 1995; Naderi *et al.*, 1997), viroid (Vera *et al.*, 1993); nematodes (Ogallo and McClure, 1995; Rahimi *et al.*, 1996), and phytophagous insects (Fernandes, 1998). In a favorable environment, pathogens are the most important stress. The reason why most microorganisms are non-pathogenic to plants could be that plants have diverse active defense responses upon external challenges (see details below).

2.1.2. *Plant defense responses*

A plant defense response is an active phenomenon. Once plants encounter potential pathogens, either virulent or avirulent, a series of physiological or physical changes occur. These changes include: resistant barrier formation in cell walls, such as lignification of cell walls (Ride *et al.*. 1989; Smit *et al.*, 1997) and papilla deposition (Bayles *et al.*, 1990; Yokoyama *et al.*, 1991); accumulation of antimicrobial phytoalexins (Hadwiger *et al.*, 1971; Smith *et al.*, 1996); elicitation of pathogenesis-related proteins (Bowles *et al.*, 1990); induction of enzyme synthetic pathways (Lamb *et al.*, 1989); release of defense-related enzymes, such as chitinase (Rasmussen *et al.*, 1992), peroxidase (Vera *et al.*, 1993), chalcone synthase (Epping *et al.*, 1990), and glucanase (Rezzonico *et al.*, 1998); and synthesis of inhibitors of pathogenic enzymes (Matti *et al.*, 1997). These collectively constitute the disease resistance responses in plants.

Many studies have shown that plant disease resistance responses require protein synthesis, indicating that defense is an active response. When protein synthesis inhibitors were applied to pea pods before inoculation with *F. solani* f. sp. *phaseoli*, an incompatible pathogen, the resistance responses were blocked, enabling the fungus to grow (Teasdale *et al.*, 1974). Newly transcribed mRNAs were eliminated in pea pods heat-shocked at 40 °C for 2 h before inoculation with incompatible fungi (Hadwiger and Wagoner, 1983a). In contrast, if pea pods were inoculated with an incompatible pathogen prior to a compatible pathogen, growth of both fungi was suppressed

(Hadwiger and Wagoner, 1983b). Chitosan, a deacetylated derivative of chitin, is a component of fungal cell walls. This compound mimics the incompatible pathogen in inducing defense responses including phytoalexin production and elicitation of a hypersensitive response in pea pods (Hadwiger and Beckman, 1980). The application of chitosan on pea pods prior to inoculation with an compatible pathogen can protect pea tissues from fungal invasion for at least two weeks after pathogenic challenge. These results suggest that pea plants possess an active defense response system to protect themselves from external fungal challenges. In this model, susceptible hosts fail to resist compatible pathogens either because the defense system is not triggered effectively or because the pathogen suppresses the plant defense response.

During defense responses to exogenous stresses, plants synthesize many different defense-related proteins (for review see Bowles, 1990). One category includes the proteins that directly change the properties of the cell wall and thereby affect the defense status of plants. Examples of these proteins are cell wall structural proteins like hydroxyproline-rich glycoproteins (Cassab and Varner, 1988), glycine-rich proteins (Condit and Meagher, 1986) and the wide range of enzymes involved in construction and modification of cell wall components such as lignin (Lagrimini *et al.*, 1987), callose (Kauss, 1990), suberin and wall-bound phenolics. Another category of defense-related proteins either have antimicrobial activities or are involved in synthesis of antimicrobial products. These proteins include enzyme inhibitors like amylase and proteinase inhibitors (Ryan *et al.*, 1990), toxic proteins like lectins (Chrispeels and Raikhel, 1991) and thionins (Vignutelli *et al.*, 1998), and hydrolases such as

endoproteinase (Vera & Conejero, 1988), chitinase (Shinshi *et al.*, 1995) and glucanase (Rezzonico *et al.*, 1998). Proteins that are activated during plant defense responses are referred to as pathogenesis-related (PR) proteins or defense proteins. PR proteins are a heterogeneous group of proteins which are induced in plants by diverse stimuli. For most practical purposes, the term "PR proteins" is synonymous with "defense proteins". These are simply umbrella terms for anything that is activated by pathogens or related elicitors (see more details in next section).

2.2. PR proteins and their genes

2.2.1. *PR gene families in plants*

Currently PR proteins are defined as a group of proteins encoded by host plants but induced only in pathological or related situations. PR proteins are believed to play a role in pathogen restriction and disease resistance in plants (Antoniw *et al.*, 1980; Van Loon *et al.*, 1994). Pathological situations include both compatible and incompatible interactions with fungi, bacteria, viruses, nematodes, phytophagous insects and herbivores. Related situations are stress conditions such as those provoked by wounding or chemical elicitation that mimic the effect of pathogen infection (*e.g.*, chitosan, salicylic acid) but do not include abiotic stresses (*e.g.*, drought, cold acclimation, salinity, anaerobiosis, hormones, UV-light and heat-shock) (Nagao *et al.*, 1986; Eckey-Kaltenbach *et al.*, 1997).

The term "PR proteins" was first used in 1970 to describe proteins in tobacco plants exhibiting the hypersensitive response to tobacco mosaic virus (Van Loon and Van Kammen, 1970). Since then, many members of different PR protein groups have been found in plant species with various pathogen challenges. So far, based on similarities in amino acid sequences, serological relationship and enzymatic or biological activity, eleven PR protein families have been designated in higher plants (for review see Van Loon *et al.*, 1994) (Table 1). PR protein genes have invariably been found in multiple copies (multigene families) in the higher plants. *PR10* is one of eleven PR multigene families in plants and has been described from a wide range of plant species.

Table 1. Recognized families of pathogenesis-related (PR) proteins*.

Family	Type member	Biological function	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	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

* Adapted from Van Loon *et al.*, 1994.

2.2.2. *PR10 genes*

Multigene families encoding PR10 proteins have been reported in many plant species (Table 2). Both the *PR10* genes and their proteins from different species share certain properties. All *PR10* genes share extensive sequence similarity, which is not restricted to specific regions but extends throughout the entire coding sequences. The *PR10* genes are activated by pathogens in both monocotyledonous and dicotyledonous species, suggesting that they play an important role in plant disease resistance. The size of all PR10 proteins is about 17 kDa. Pea, parsley and potato PR10 polypeptides show nearly identical hydrophilic profiles. All the proteins are slightly acidic (Walter *et al.*, 1990). Unlike tobacco PR1 proteins, PR10 proteins reported so far do not contain a signal peptide (hydrophobic residues), suggesting they are not secreted through the cell memebrane and therefore biological functions intracellularly.

Table 2. Cloned *PR10* genes in plants.

Gene	Host	Clone	GenBank accession	Reference
AoPR1	*Asparagus officinalis*	genomic	X64452	Warner *et al.*, 1993 & 1994
Betv1	*Betula verrucosa*	cDNA	X15877	Breitender *et al.*, 1989
Betv1-sc1, -sc2, -sc3	*Betula verrucosa*	cDNA		Swoboda *et al.*, 1995
SAM22	*Glycine max*	cDNA	X60043	Crowell *et al.*, 1992
H4	*Glycine max*	cDNA	X60044	Crowell *et al.*, 1992
LlPR10.1a (Ypr10.1a)	*Lupinus luteus*	genomic	AF002277	Sikorski *et al.*, 1998
LlPR10.1b (Ypr10.1b)	*Lupinus luteus*	genomic	AF002278	Sikorski *et al.*, 1998
PR1-1 (PcPR1-1)	*Petroselinum crispum*	genomic	U48862	Somssich *et al.*, 1988; Rushton *et al.*, 1996
PR1-2 (PcPR1-2)	*Petroselinum crispum*	genomic	U48863	Rushton *et al.*, 1996
PR1-3 (PcPR1-3)	*Petroselinum crispum*	cDNA	X12573	Somssich *et al.*, 1988
PR2 (PcPR2)	*Petroselinum crispum*	genomic	X55736	van de Löcht *et al.*, 1990; Korfhage *et al.*, 1994
Ypr10c (PR10c)	*Phaseolus vulgaris*	genomic	X96999	Walter *et al.*, 1996
DRR49a (PR10.1)	*Pisum sativum*	cDNA genomic	X13383 U31669	Fristensky *et al.*, 1988 Culley *et al.*, 1995
DRR49b (PR10.2)	*Pisum sativum*	cDNA	M81249	Fristensky *et al.*, 1988
DRRG49c (PR10.3)	*Pisum sativum*	genomic	J03680	Chiang *et al.*, 1990
ABR17 (PR10.4)	*Pisum sativum*	cDNA	Z15128	Iturriaga *et al.*, 1994
ABR18 (PR10.5)	*Pisum sativum*	cDNA	Z15127	Iturriaga *et al.*, 1994
RH2 (PR10.3)	*Pisum sativum*	cDNA	S74512	Mylona *et al.*, 1994
STH2 (PR10a)	*Solanum tuberosum*	genomic	M29041	Matton *et al.*, 1993
STH21	*Solanum tuberosum*	genomic	M29042	Matton *et al.*, 1993

2.2.3. *PR10 function in defense response*

The induction of the ***PR10*** genes during pathological or related stress situations suggests that the ***PR10*** genes play a role in plant defense responses. Although many other PR genes demonstrate antifungal functions in laboratory (Yun *et al.*, 1997) or field experiment (Grison *et al.*, 1996), the function of the ***PR10*** genes is not known. The sequence similarity between *PR10* in parsley and a ribonuclease isolated from *Panax ginseng* calli (Moiseyev *et. al.*, 1994) hints that ***PR10*** may have a ribonuclease-like function. The ribonuclease from ginseng has a molecular weight of 18 kDa, which is close to that of PR10 proteins (17 kDa). Walter *et al.*, (1996) identified considerable spatiotemporal similarities between *Ypr10* in bean and ribonuclease genes in bean, which, together with the significant sequence similarity to the ginseng ribonuclease, support the hypothesis of a ribonuclease-like function for PR10 proteins and allow the prediction of possible biological roles. In birch, pollen allergens that belong to the PR10 class of proteins also demonstrate ribonuclease activity *in vitro* (Swoboda *et al.*, 1996). A ribonuclease-like function for ***PR10*** homologs from different plant species would be significant for two reasons (Constabel and Brisson, 1995). One relates to the parallel function between the host/pathogen interaction and self-incompatibility in plants, in which ribonucleases play a role (Murfett *et al.*, 1992). If PR10 proteins indeed function as an RNase, they are likely to be specific for certain RNA substrates or require a specific activation of their enzymatic activity. It is also possible that such

RNase-like activities specifically destroy the RNA substrates related to pathogen development.

2.2.4. *PR10 expression*

Generally speaking, expression of the *PR10* genes is related to pathogenesis and either directly induced by pathogens or elicitors derived from pathogens or pathogen/plant interactions. The expression of three members of the *Bet v I* multigene family, *PR10* homologues in birch, was observed in response to both compatible and incompatible bacterial and fungal pathogens in birch suspension cultures. When birch leaves were challenged with *Taphrina betulina* (pathogenic), all three genes were expressed above the control level in the pathogen treatment, but not in the non-pathogen treatment with *F. solani* (non-pathogenic) (Swoboda *et al.*, 1995). The transcripts of *PcPR1* and *PcPR2*, *PR10* homologues in parsley, were elevated in the cultured cells by the treatment with a fungal elicitor (Somssich *et al.*, 1988). *STH-2* (*PR10a* in potato) was induced dramatically upon treatment with a fungus-derived elicitor, reaching a peak at 24 h.p.i. and sustained up to 72 h.p.i. (Matton and Brisson, 1989). Pea *PR10* genes were expressed not only in pea pods, demonstrating non-host resistance to *F. solani* f. sp. *phaseoli*, but also activated during the race-specific resistance response to *Pseudomonas syringae* pv. *pisi* (Daniels *et al.*, 1987).

PR10 gene expression in response to pathogens is usually limited to local induction. Unlike many of the PR genes in tobacco like *PR1a*, *PR-2* or *PR-5* (Bol *et*

al., 1990; Uknes *et al.*, 1993), the systemic induction of the *PR10* genes in response to pathogen or elicitor treatments has not been observed. *In situ* hybridization of *Phytopthora megasperma* f. sp. *glycinea*-infected parsley leaves showed heavy accumulation of *PcPR1* and *PcPR2* transcripts around infection sites as early as 4 h.p.i (Schmelzer *et al.*, 1989). A chimeric GUS reporter gene driven by the *Asparagus PR10* (*AoPR1*) promoter was expressed strongly at the wounding and pathogen invasion sites in transgenic tobacco (Warner *et al.*, 1993). Soybean *PR10* (*SAM22*) transcript accumulated predominantly in the roots of young seedlings. No expression was seen in hypocotyls or leaves (Crowell *et al.*, 1992). Mylona *et al*, (1994) reported that pea *RH2* (*PR10.3*) was not expressed in leaves or stems, but was exclusively expressed in roots, particularly in the root epidermis where the plant roots are constantly under physical "stresses" like gravel in soil. Many members of the *Ypr10* gene family, including *Ypr10c* (*PR10c*), were strongly expressed in healthy bean roots while in leaves *Ypr10* transcription was very low (Walter *et al.*, 1996).

In addition to pathogen stress, *PR10* expression may also be related to host development. As mentioned above, *RH2* expression was restricted to pea roots. *In situ* expression studies during post-embryonic development showed that *RH2* was expressed in the protoderm of a globular pea embryo, exclusively in cells that give rise to the radicle. This observation strongly supports the conclusion that the expression of *RH2* more or less coincides with the transition of protoderm into epidermis and therefore is regulated by a developmental cue (Mylona *et al*, (1994). Stigma- and vascular-specific expression of the *PR10a* gene was reported in transgenic

potato plants. Strong vascular-specific expression was observed in tissues treated with the potato pathogen *Phytophthora infestans*. In healthy untreated plants, however, *PR10a* was expressed exclusively in the stigma, with more PR10a protein in the stigmata of fully open rather than unopened flowers, indicating that *PR10a* expression is developmentally regulated (Constabel and Brisson, 1995). In *Asparagus*, *AoPR1* transcript was also detected in mature pollen grains (Warner *et al.*, 1993), which is consistent with the functional homology between *AoPRI* and *Bet v I*, the pollen allergen gene in birch (Swoboda *et al.*, 1994). Further studies revealed that the spatial and temporal pattern of *AoPR1* expression was remarkably similar to the genes encoding the enzymes of the phenlypropanoid pathway (Warner *et al.*, 1994).

2.3. General gene expression regulation

Every eukaryotic cell contains thousands of genes, only a fraction of which are expressed at any given time. The specific group of expressed genes changes as cells progress through various stages of development or are exposed to different environmental circumstances. There are about 60,000-80,000 different structural genes alone in a plant cell (Okamuro and Goldberg, 1989). How does the plant cell decipher the genome and regulate the expression of such a large number of genes during the course of development? The gene expression process leading to production of the functional proteints in a differentiated cell consists of five major steps: (1) differential gene transcription; (2) nuclear RNA modification, splicing, and turnover; (3) selective

RNA transport from the nucleus to the cytoplasm; (4) cytoplasmic mRNA turnover; (5) translation, post-translational processing, compartmentalization, and protein turnover. Each of these processes plays an important role in establishing the expressed state of a gene. Although the regulation of gene expression is complex, all gene expression is initiated from gene transcription, that is, conversion of information from genomic DNA to mRNA. In eukaryotes, transcription initiation on genes encoding mRNAs mainly depends on the presence of RNA polymerase II and *cis*-regulatory elements, usually located in the flanking regions of coding sequences (Maniatis *et al.*, 1987).

Besides the RNA polymerases, upstream proximal promoters and distal enhancers are critical to the control of gene expression. Promoters are regulatory elements immediately upstream from the transcription start site and usually comprise conserved core DNA motifs, such as the TATA-box (TATAAA), the CAT-box (CCAAT) and the Y-box (ATTGG) (Nussinov, 1990). In contrast, regulatory elements controlling gene expression from a greater distance from the RNA start site are referred to as distal enhancers (Marriott and Brady, 1989). Transcriptional enhancers have been found upstream as well as downstream from the transcription initiation site of protein-coding regions and are able to exert control in an orientation-independent fashion on the promoter. Many enhancers are now known to be binding sites for nuclear proteins and to be involved in both negative and positive regulation. Interactions between *cis*-regulatory elements in enhancers and DNA-binding proteins play critical roles in controlling the differentiation and development of eukaryotic

organisms and in regulating their metabolism. There has, therefore, been considerable interest in how *cis*-regulatory elements communicate with their cognate promoters and influence promoter functions (Kustu *et al.*, 1991). The structural assembly of the transcription preinitiation complex on the target gene may be rather simple with only a limited number of regulatory elements. However, many genes have been described as containing multiple DNA binding sites for *trans*-acting factors which may influence transcription either positively or negatively (Wasylyk, 1988). This scenario probably reflects a complex spatial and temporal regulation of gene activity.

2.4. *Cis*-regulatory element

"*Cis*" in Latin means "on the same side as" or "linked". Therefore "*cis*-regulatory element" refers to physical linkage of regulatory sequences to the regulated gene. There are several different names for *cis*-regulatory elements in the literature, such as *cis*-acting elements, *cis*-elements, *cis*-acting sequences, DNA elements, regulatory elements, transcription elements, etc. They all refer to the same concept, whereby a *cis*-regulatory DNA element must be recognized and bound by a corresponding *trans*-regulatory protein factor and together both play an important role in promoter function (Krajewska, 1992). Every time a *cis*-regulatory element is identified, we can infer the existence of a corresponding *trans*-regulatory factor. Therefore *cis*-regulatory elements and *trans*-regulatory factors are partners and they are equally important in the regulation of gene expression.

2.4.1. *Location of cis-regulatory element*

Theoretically, *cis*-regulatory elements can be located anywhere in the vicinity of a gene. Most *cis*-regulatory elements are located in the proximal promoter regions (within 500-bp relative to the transcription start site), but they have also been found in introns (Mascarenhas *et al.*, 1990), protein-coding regions (Sessa *et al.*, 1995b; Yamamoto *et al.*, 1997) and downstream regions (Sessa *et al.*, 1995b; Chinn *et al.*, 1996). Some *cis*-regulatory elements in the distal enhancers, however, are as far as 10-kb away from encoding sequences (Wasylyk, 1988; Marriott and Brady, 1989).

2.4.2. *Identification of cis-regulatory elements*

The most popular methods to identify *cis*-regulatory elements are gel retardation and DNA footprinting. Gel retardation assays, or gel mobility shift assays, are used to search for DNA-binding proteins, which may serve as *trans*-regulatory factors, in nuclear extracts from a target plant (Ausubel *et al.*, 1998). Certain nuclear proteins specifically recognize a fixed DNA sequence pattern (motif). When a labelled DNA probe is bound by a nuclear protein to form a DNA/protein complex, its mobility is retarded in non-denaturing polyacrylamide gel electrophoresis (PAGE). In this case, a shifted band indicates that a DNA-binding protein binds to the DNA probe. We can roughly determine the protein-binding region of the DNA probe through the effects of nested deletions on gel retardation assay.

After detection of a protein-binding region, one can determine the special binding site or DNA motif. DNA footprinting is applied to pinpoint the precise DNA sequence recognized by a DNA-binding protein (Metzger and Heumann, 1994; Ausubel *et al.*, 1998). When a DNA motif is bound by a nuclear protein, the nucleotide sequence is generally protected from cutting by DNase I. With deliberately controlled partial digestion of the DNA/protein complex and the control free probe (without nuclear extract) by DNase I, blank areas appear on the DNase I sequencing gel of the DNA-protein reaction, while a ladder of bands (with no blanks) is generated in the control reaction. One blank region (footprint) suggests one DNA motif which is bound by a protein. In this way, different motifs are identified for corresponding DNA-binding proteins in nuclear extracts and a DNA footprint map can be generated (for a good example see Manzara *et al.*, 1991).

Once *cis*-regulatory elements are identified by gel retardation assays and/or DNA footprinting their biological functions can be evaluated by means of a reporter gene (*e.g.*, the *E. coli* uida gene or "GUS" gene) in transgenic plants. Examples include elements such as Box I (CACGTG) in *prxC2* (Kawaoka *et al.*, 1994), three *cis*-elements in *osmotin* (Liu *et al.*, 1995), the GCC-box (TAAGAGCCGCC) in *Chn48* (Shinshi *et al.*, 1995), the AT-rich sequence (TAAAATACT) in *PsChs1* (Seki *et al.*, 1996) and the as-1-like box (ACGTCATCGAGATGACGGCC) in *PR1a* (Strompen *et al.*, 1998). However, some promoter elements or regions have been examined solely by means of an expression cassette with a reporter gene (van de Rhee *et al.*, 1993; Raventós *et al.*, 1995) or gel retardation assays alone (Howley and Gatehouse, 1997).

For example, transcriptional activity of the bean *Ypr10c* promoter was investigated only by GUS fusion gene expression in transgenic tobacco, leading to identification of organ-specific, dark-dependent and SA or glutathione-inducible promoter regions (Walter *et al.*, 1996). In some cases, *cis*-regulatory elements were identified by *in vivo* DNA footprinting following gel retardation assays and RNA expression studies in native plants. For example, two *cis*-elements which cover an 11-bp inverted repeat and are essential for fungal elicitation in parsley were identified by *in vivo* DNA footprinting in the *PcPR1* promoter region from -240 to -130 (Meier *et al.*, 1991). An abscisic acid-responsive element, GRA (CACTGGCCGCCC), was identified in *rab17* from maize by *in vivo* DNA footprinting (Busk *et al.*, 1997). Table 3 lists more examples of *cis*-regulatory elements or promoter regions which have been identified in stress-related genes in plants (see the column "Study method").

2.4.3. *Cis-regulatory elements in stress-related genes*

Cis-regulatory elements in plants are assumed to have specific functions in regulating gene expression. These *cis*-regulatory elements can be tissue- or development-specific (Van der Meer *et al.*, 1990; Faktor *et al.*, 1997; Suzuki *et al.*, 1995; Ohtsubo *et al.*, 1997) or have specificity for stimuli such as dark or light (Walter 1996; Yamamoto *et al.*, 1997), pathogen species or races (Constabel and Brisson, 1992), flooding or drought (Joshee *et al.*, 1998; Schaeffer *et al.*, 1995), heat-shock or cold acclimatization (Nagao *et al.*, 1986; Graumann *et al.*, 1994), chemical

elicitors or heavy metals (Rastogi *et al.*, 1997; Mhiri *et al.*, 1997), hormones (Liu and Lam, 1994; Busk and Pagès, 1998), sugar (Lu *et al.*, 1998), UV-light (Murakami *et al.*, 1997) and wounding (Pastuglia *et al.*, 1997; Vignutelli *et al.*, 1998).

Cis-regulatory elements have been identified in many stress-related genes in plants. For example, the ethylene-responsive elements, GCC-box (TAAGAGCCGCC) in *Chn48* and *PRB-1b*, and ERE (ATTTACCACCTATTTCAAA) in *GST1*, were identified and their corresponding *trans*-regulatory factors, EREBP, CEBP-1 and AtEBP, were isolated from tobacco, carnation and *Arabidopsis*, respectively (Ohme-Takagi *et al.*, 1995; Sessa *et al.*, 1995a; Maxson *et al.*, 1996; Büttner *et al.*, 1997). In the *osmotin* gene from salt-adapted tobacco, three upstream regulatory elements have been identified: G-sequence, AT-sequence and PR-sequence, which are responsive to salinity and drought (Liu, *et al.*, 1995). Van de Rhee and Bol (1993) reported that *PR1a* gene expression in tobacco is coordinately regulated by four TMV (tobacco mosaic virus)-inducible elements located from -902 and 29, such that no element by itself is responsive to TMV challenge or salicylate treatment. Recently the as-1 like *cis*-element, (ACGTCATCGAGATGACGGCC) in *PR1a*, and its DNA-binding protein, TGA1a-like, were identified in tobacco. Their interactions are responsible for fungal and SA elicitation (Strompen *et al.*, 1998).

Table 3. *Cis*-regulatory elements or promoter regions in stress-related genes in plants (1 of 4).

Gene[a]	Host	Regulatory element/region[b]	Protein factor	Study method	Elicitor	Reference
α-Amy2	wheat, barley	box 2 (ATTGACTTGACCGTCATCGG), box 3 (TTTTTCGTAACAGAGTCTGGT)	ABF1, ABF2	gel retardation	gibberellin, abscisic acid	Rushton *et al.*, 1995
AoPR1	*Asparagus*	-982 to 1 contains three H-box, one G-box, and a sequence (ATTTGACCG)	not isolated	reporter gene	wounding	Warner *et al.*, 1993 & 1994
Chitinase	*Arabidopsis*	-1128 to -590 (positive), -590 to -384 (negative)	not isolated	reporter gene	fungus	Samac & Shah, 1991
Chn48	tobacco	GCC-box (TAAGAGCCGCC)	EREBPs	gel retardation, reporter gene	ethylene	Ohme-Takagi *et al.*, 1995; Shinshi *et al.*, 1995
CHN50	tobacco	(GATTTGGTCAGAAAGTCAGTCC)	not isolated	gel retardation, reporter gene	fungus	Fukuda *et al.*, 1994
chsA	*Petunia*	-142 to 81, direct repeats $(TACPyAT)_n$	not isolated	gel retardation, reporter gene	UV, flower-specific	van der Meer *et al.*, 1990
chs15	bean	H-box [CCTACC(N_7)CT]	KAP-1, KAP-2	gel retardation	fungus, glutathione	Yu *et al.*, 1993
CHS15	soybean	G-box (CACGTG), H-box [CCTACC(N_7)CT]	G/HBF-1 (bZIP protein)	gel retardation, reporter gene	bacterium, tissue-specific	Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997
gln2	tomato	PR-box core (GCCGCC)	Pti4/5/6	gel retardation	fungus	Zhou *et al.*, 1997
gst1 (prp1-1)	potato	-402 to -130	not isolated	reporter gene	fungi, symbionts	Strittmatter *et al.*, 1996
GST1	carnation	ERE (ATTTACCACCTATTTCAAA)	CEBP-1	gel retardation	ethylene	Maxson *et al.*, 1996
gyrA	mammalian	Y-box (ATTGG), CAT-box (CCAAT)	CspB	gel retardation	cold shock	Graumann *et al.*, 1994

Table 3. *Cis*-regulatory elements or promoter regions in stress-related genes in plants (2 of 4).

Gene	Host	Regulatory element/region	Protein factor	Study method	Elicitor	Reference
hsr203J	tobacco	1.4-kb promoter	not isolated	reporter gene	bacterium	Pontier *et al.*, 1994
HVA22	barley	ABRE 1 (CCGCGTAGGCAC), ABRE 2 (GCACGTGTCGG)	not isolated	gel retardation, DNA footprint, reporter gene	abscisic acid, cycloheximide	Shen *et al.*, 1993
NiR	spinach	-230 to -200	NIT2-like	gel retardation, DNA footprint	nitrate	Rastogi *et al.*, 1997
nopaline synthase	tobacco	as-1 (CTGACGTAAGGGATGACGCAC), nos-1 (TGAGCTAAGCACATACGTCAG)	ASF-1	gel retardation, reporter gene	auxin	Liu & Lam, 1994
Osmotin (PR5)	tobacco	G-box-like (CAAGTGTCACGTT), AT-1-like (AATTATTTTATG), PR-box (TAAGA/CGCCGCC)	not isolated	gel retardation, DNA footprint, reporter gene	salinity, abscisic acid	Liu *et al.*, 1995
PAL-1	parsley	box P	BPF-1	gel retardation	fungus, UV	da Costa e Silva *et al.*, 1993
Ppc1	maize	(GTGCCCTT)	MNF1	gel retardation	light	Morshima, 1998
Ppc1 *Gap1*	*Mesembrya-nthemum*	-977 to -487 (for *Ppc1*), -735 to -549 (for *Gap1*)	not isolated	gel retardation, reporter gene	salinity, drought	Schaeffer *et al.*, 1995
PR1a	tobacco	-184 to -172, -68 to -51	not isolated	gel retardation, DNA footprint	virus	Hagiwara *et al.*, 1993
PR1a	tobacco	-902 to -691 (element 1), -689 to -643 (element 2), -643 to -287 (element 3), -287 to 29 (element 4)	not isolated	reporter gene	virus, salicylate	van de Rhee *et al.*, 1993

Table 3. *Cis*-regulatory elements or promoter regions in stress-related genes in plants (3 of 4).

Gene	Host	Regulatory element/region	Protein factor	Study method	Elicitor	Reference
PR1a	tobacco	as-1-like box (ACGTCATCGAGATGACGGCC)	TGA1a-like	gel retardation, reporter gene	fungus, SA	Strompen *et al.*, 199
PR1 (PcPR1)	parsley	-240 to -130	not isolated	DNA footprint	fungus	Meier *et al.*, 1991
PR1-1 (PcPR1-1), PR1-2 (PcPR1-2), PR1-3 (PcPR1-3)	parsley	W-box [(T)TGAC(C)]	WRKY1, WRKY2, WRKY3	gel retardation	fungus	Rushton *et al.*, 1996
PR2 (PcPR2)	parsley	(CTAATTGTTTA)	homeodomain protein	gel retardation, reporter gene	fungus, bacterium	van de Löcht *et al.*, 1990; Korfhage *et al.*, 1994
PR-5	tobacco	-1364 to -718	not isolated	reporter gene	virus	Albrecht *et al.*, 1992
PRB-1b	tobacco	GCC-box (TAAGAGCCGCC)	not isolated	gel retardation, reporter gene	ethylene	Sessa *et al.*, 1995a
PRms	maize	(AATTGACC)	not isolated	reporter gene	fungus	Raventós *et al.*, 1995
prxC2	tobacco	-307 to -99 (positive), box 1 (CACGTG)	TFHP-1	gel retardation, DNA footprint, reporter gene	wounding	Kawaoka *et al.*, 1994
psaDb (PSI-D)	tobacco	-170 to +24, +1 to +861	not isolated	reporter gene	light	Yamamoto *et al.*, 1997
PsChs1	pea	AT-rich sequence (TAAAATACT)	not isolated	gel retardation, DNA footprint, reporter gene	fungus	Seki *et al.*, 1996
PSPAL1	pea	box II (TCAACAAACCAC), box IV (TAATTAAT)	not isolated	reporter gene	fungus, UV	Murakami *et al.*, 1997

Table 3. *Cis*-regulatory elements or promoter regions in stress-related genes in plants (4 of 4).

Gene	Host	Regulatory element/region	Protein factor	Study method	Elicitor	Reference
pwsi18	rice	three G-box, a MEF-2 sequence	not isolated	reporter gene	water stress	Joshee *et al.*, 1998
rab16B	rice	motif I (AGTACGTGGC), motif III (GCCGCGTGGC)	not isolated	reporter gene	abscisic acid	Ono *et al.*, 1996
rab17	maize	CACTGGCCGCCC (GRA)	not isolated	DNA footprint, reporter gene	abscisic acid, water stress	Busk *et al.*, 1997
Shpx6a, *Shpx6b*	*Stylosanthes*	G-box (CACGTG), MJ-box (CCCTATAGGG)	not isolated	reporter gene	fungus, wounding, methyl-jasmonate	Cuttis *et al.*, 1997
STH2 (PR10a)	potato	-155 to -105 (positive), -52 to -28 (negative)	PBF-1, PBF-2	gel retardation, reporter gene	fungus, wounding	Matton *et al.*, 1993; Després *et al.*, 1995
synthesized DNA	*Arabidopsis*	GCC-box (TAAGAGCCGCC)	AtEBP	gel retardation, DNA footprint	ethylene	Büttner *et al.*, 1997
Ypr10c (PR10c)	bean	2.5-kb promoter	not isolated	reporter gene	fungus, dark, SA, glutathione	Walter *et al.*, 1996

Note: "a" the gene in bracket is other names used in literature (see Appendix I for detail); "b" underlined sequence is either direct repeats or inverted repeats.

Table 3 lists typical stress-responsive *cis*-regulatory elements or promoter regions in plants. Some *cis*-regulatory elements confer inducibility by a single elicitor, while others mediate responsiveness to a broad range of external challenges (see the column Elicitor). For instance, the *cis*-regulatory elements in *PcPR1* (Meier *et al.*, 1991), *PcPR2* (van de Löcht *et al.*, 1990), *PRms* (Raventos *et al.*, 1995) and *PsChs1* (Seki *et al.*, 1996) appear to be induced only by fungal challenges. The *cis*-regulatory elements in *AoPR1* (Warner *et al.*, 1993) and *prxC2* (Kawaoka *et al.*, 1994) are activated by wounding only. The elements, ERE (ATTTACCACCTATTTCAAA) in *GST1* (Maxson *et al.*, 1996) and GCC-box (TAAGAGCCGCC) in *Chn48* (Shinshi *et al.*, 1995) and *PRB-1b* (Sessa *et al.*, 1995a), appear to be solely ethylene-inducible. A *cis*-element or promoter region in *HVA22* (Shen *et al.*, 1993), nopaline synthase gene (Liu and Lam, 1994), *NiR* (Rastogi *et al.*, 1997), *Ppc1* (Morshima *et al.*, 1998), *PR1a* (Hagiwara *et al.*, 1993) and *pwsi18* (Joshee *et al.*, 1998) apper to be exclusively responsive to abscisic acid, auxin, nitrate, light, virus and flooding, respectively. In contrast, the genes *gst1* (Strittmatter *et al.*, 1996), *osmotin* (Liu *et al.*, 1995), *PR1a* (Strompen *et al.*, 1998), *PR2* (Korfhage *et al.*, 1994), *Shpx6a* and *Shpx6b* (Curtis *et al.*, 1997), *STH-2* (Matton *et al.*, 1993) and *Ypr10c* (Walter *et al.*, 1996) contain regulatory elements or promoter regions which are not only activated by many different elicitors but also display tissue/development-dependent features.

Interestingly, several conserved DNA sequence motifs are observed in the promoter regions of these stress-related genes (see the column Regulatory

element/region in Table 3). Typical examples include: the G-box (core consensus CACGTG) in *AoPR1* (Warner *et al.*, 1993), *prxC2* (Kawaoka *et al.*, 1994), *CHS15* (Dröge-Laser *et al.*, 1997), *Shpx6a* and *Shpx6b* (Curtis *et al.*, 1997); the H-box (core consensus CCTACC) in *AoPR1* (Warner *et al.*, 1993), *chs15* (Yu *et al.*, 1993), and *CHS15* (Faktor *et al.*, 1997); the W-box [core sequence (T)TGAC(C)] in three members of the *PcPR1* multigene family in parsley (Rushton *et al.*, 1996); the as-1 motif (repeat core sequence TGACG) in tobacco *nopaline synthase* (Liu and Lam, 1994), tobacco *PR1a* (Strompen *et al.*, 1998) and CaMV 35S gene promoter (Fang, *et al.*, 1989); and particularly the PR-box (formerly GCC-box, core consensus GCCGCC) (Shinshi *et al.*, 1995; Liu *et al.*, 1995; Sessa *et al.*, 1995a). Table 4 summarizes 21 defense-related genes that contain the PR-box or PR-box-like motifs in their promoter regions. It was recently reported that three transcription proteins recognize and bind to the *cis*-element (PR-box) which is present in the plant disease resistance gene, *Pto* kinase, in tomato and many PR genes. The expression of these PR genes was specifically enhanced upon *Pto/avrPto* recognition in transgenic tobacco and therefore there may have interactions between disease resistance genes and plant PR genes (Zhou et al., 1997).

Table 4. Occurrence of PR-box in plant defense-related genes*.

Plant	Gene	Protein encoded	Reference
A. thaliana	*CHA2*	basic chitinase	Samac *et al.*, 1990
A. thaliana	*PAL3*	phenylalanine ammonia-lyase	Wanner *et al.*, 1995
B. napus	*Bp10*	ascorbate oxidase	Albani *et al.*, 1992
N. tabacum	*chi-v*	class V chitinase	Melchers *et al.*, 1994
L. esculentum	*CHN*	basic chitinase	cited by Hart *et al.*, 1993
N. tabacum	*CHN14*	basic chitinase	van Buuren *et al.*, 1992
N. tabacum	*CHN17*	basic chitinase	Shinshi *et al.*, 1990
N. tabacum	*CHN50*	basic chitinase	Fukuda *et al.*, 1991
N. tabacum	*GLA (gln2)*	basic β-1,3-glucanase	Ohme-Takagi and Shinshi, 1990; Sperisen *et al.*, 1991
N. tabacum	*GLB*	basic β-1,3-glucanase	Sperisen *et al.*, 1991
N. plumbaginifolia	*gn1*	β-1,3-glucanase	Castresana *et al.*, 1990
N. plumbaginifolia	*gn2*	basic β-1,3-glucanase	Gheysen *et al.*, 1990
N. tabacum	*OPL*	neutral PR-5	Sato *et al.*, 1996
N. tabacum	*Osmotin*	basic PR-5	Liu *et al.*, 1995
N. tabacum	*prb-1b*	basic PR-1	Meller *et al.*, 1993
N. tabacum	*PRP1*	basic PR-1	Payne *et al.*, 1989
P. vulgaris	*CH5B*	basic chitinase	Broglie *et al.*, 1989
S. commersonii	*pOSML13*	basic PR-5	Zhu *et al.*, 1995
S. commersonii	*pOSML81*	basic PR-5	Zhu *et al.*, 1995
S. tuberosum	*STPRINPSG*	protease inhibitor	Y. Choi *et al.*, (DDBJ/EMBL/GenBank Z12824
S. tuberosum	*WIN2*	wound inducible (PR-4-like)	Stanford *et al.*, 1988

* Adapted from Zhou *et al.*, 1997.

2.4.4. *Cis-regulatory elements in PR10* genes

So far promoter functions of five *PR10* genes have been investigated. A sequence from -240 to -130 relative to the transcription start site in *PcPR1*, a *PR10* homologue in parsley, was essential for fungal elicitation (Meier *et al.*, 1991). Recently the W-box [core sequence (T)TGAC(C)] in three members of the *PcPR1* multigene family has been identified and three sequence-specific DNA-binding proteins, WRKY1, 2 and 3, were isolated. The interaction between the W-box and these DNA-binding proteins was demonstrated to be responsible for fungal elicitor perception leading to *PcPR1* gene activation in parsley (Rushton *et al.*, 1996). In *PcPR2*, another *PR10* gene family in parsley, an 11-bp DNA motif (CTAATTGTTTA) in a 125-bp region within the promoter was required for fungal or bacterial elicitor-mediated expression (van de Löcht *et al.*, 1990; Korfhage *et al.*, 1994). A wounding-responsive promoter, from -982 to 1, in *AoPR1* from *Asparagus*, was studied and several putative DNA motifs were proposed, such as the G-box, the H-box and a 9-bp sequence (ATTTGACCG) that is also found in *PcPR1* (Warner *et al.*, 1993 and 1994). The promoter of *PR10c* (*Ypr10c*), a member of the *PR10* family in bean, displayed organ-specific, dark-dependent and SA or glutathione-inducible functions. No specific *cis*-regulatory elements were identified (Walter *et al.*, 1996).

The most detailed promoter study of *PR10* genes fosused on the *PR10* multigene family in potato, *STH-2* and *STH-21*. Matton *et al.*, (1993) examined 1015-

bp of 5′-flanking sequence in transgenic potato and found two possible regulatory regions. A positive *cis*-regulatory element associated with fungal elicitation or wounding was located between -155 to -52 and a possible negative element between -52 and -28. Investigation of *PR10a* (*STH-2*) expression using *PR10a* promoter-GUS fusion revealed that strong *PR10a* expression was observed in many tissues or organs following elicitation by pathogenic fungus or wounding, while in healthy potato plants the gene was not expressed in any tissues, except the stigma, during normal development of the plant (Constabel and Brisson, 1995). Detailed deletion analysis of the *PR10a* promoter identified a region of 50-bp, located between positions -155 and -105, necessary for full elicitor responsiveness in transgenic potato plants. A 30-bp sequence within the region, from -135 to -105, was specifically recognized and bound by two nuclear factors, PBF-1 and PBF-2. Furthermore, phosphorylation of one factor, PBF-1, was demonstrated to be required for the activation of *PR10a*. The protein kinase inhibitor, staurosporine, was shown to block *PR10a* gene expression (Després *et al.*, 1995).

Based on these results, a working model was proposed for *PR10a* gene expression. The fungal elicitor is first perceived by the cell, possibly through an interaction with a receptor, which activates a staurosporine-sensitive protein kinase. The kinase, either directly or through a cascade of signal transduction, stimulates the DNA binding activity of the transcription factor PBF-1 by phosphorylation. This finally results in the initiation of *PR10a* transcription.

3. MATERIALS AND METHODS

3.1. Treatment of pea pod

3.1.1. *Pea pod*

Garden pea seed, *Pisum sativum* L. cv. Alaska, was purchased from W. Atlee Burpee and Co., Warminister, PA, USA. Plants were grown in growth rooms with a soil-sand-peat mix of 2-1-1 under a day/ night cycle of 14/10 h and a temperature cycle of 22 °C/15 °C. Immature pea pods 1-2 cm in length (2-3 days after flowering) were used for inoculation.

3.1.2. *Fungal inoculum*

Fusarium solani f. sp. *pisi* and *F. solani* f. sp. *phaseoli* were obtained from the American Type Culture Collection (Accession numbers 38136 and 38135, respectively). Cultures were grown and maintained on 3.9% potato dextrose agar (PDA) (DIFCO Laboratories, Detroit, MI, USA) plates. Conidia were stored at -70 °C.

Fungi were cultured on PDA plates under continuous light for about 7 days. The macroconidia were collected by washing the plates with sterile water and centrifuged for 5 min at 1,000 g. Pellets were suspended in sterile water and spore concentration was measured using a haemacytometer. Spore suspensions were diluted

to 10^6 spores/ml (working solution) before inoculation.

3.1.3. *Fungal and chemical treatment*

Five grams of fresh immature pea pods were used for each treatment. The pods were slit longitudinally along the suture lines and placed on a sterile petri-dish with the open endocarp facing upwards. The fungal suspension was applied evenly on the pod endocarp. The pods were then incubated at room temperature under continuous light for 0 to 48 h. The treated pods were rinsed with sterile water and briefly dried with paper towels. Pods treated with sterile water served as controls. In addition, healthy controls included split pods (H, wounding) and intact pods (UPP). Samples were frozen in liquid nitrogen after treatment and were used immediately or stored at -70 °C for later use (Table 5).

Chemical treatments were applied as following: 50 mM pH 6.7 salicylic acid (sodium salt, Sigma) and 1 mg/ml pH 6.5 chitosan (Bentech Labs Inc., Clackamas, OR, USA).

Table 5. Time course for different challenges on immature pea pods (*P. sativum*).

Elicitor	Time course (hour post-inoculation)									
	0.5	1	2	4	6	8	12	24	36	48
F. solani phaseoli (Fsph)	*	*	*	*	*	*	*	*	*	
F. solani pisi (Fsp)	*	*	*	*	*	*	*	*	*	
Salicylic acid (SA)					*		*	*	*	*
Chitosan (CH)		*	*	*	*	*	*			
Sterile water (W)			*	*	*			*		

Note: For a given elicitor, only time points marked with "*" were sampled.

3.2. Preparation of pea nuclear extracts

3.2.1. *Nuclei from pea pod*

All steps involving preparation and handling of nuclei were carried out on ice or at 4 °C, using solutions pre-cooled at 4 °C. Nuclei were prepared as in (Tautvydas, 1971; Watson and Thompson, 1986), with modifications as described below.

Five grams of treated pea pods were pulverized under liquid N_2 and transferred to 50-ml plastic tubes (Corning Inc, Corning, NY, USA) containing 10 ml of 4% gum arabic (GA, Sigma) in filter-sterilized resuspension solution (RS: 5 mM MES buffer, 4 mM MgAc, 0.15 M sucrose, and 5 mM 2-mercaptoethanol, pH 6.0, fresh weekly) supplemented with 0.2 mM PMSF (diluted from a stock of 0.5 M in DMSO). The suspension was first filtered through two layers each of 100-μm and 50-μm nylon meshes (Spectrum/Mesh, Spectrum Medical Industries Inc., CA, USA). The filtrate was then filtered through one layer each of 30-μm and 10-μm nylon meshes under light pressure. The final filtrate was centrifuged at 300 g for 10 min. In some experiments, the supernatant containing the cytoplasmic fraction was saved for extraction of cytoplasmic proteins (see Section 3.2.4.). The pellet was resuspended in 10 ml of 8% GA and applied to the top of two GA-Percoll (Pharmacia) gradient tubes (10 ml each of 8%, 10% and 12% of GA, and 15 ml of 60% of Percoll, all solutions in RS). The gradient tubes were centrifuged at 1,000 g for 15 min in a swinging

bucket rotor. The nuclear band at the interface between 12% GA and 60% Percoll was collected. The nuclear suspension was then washed twice with 20 ml of RS and centrifuged at 1,000 g for 15 min and 700 g for 10 min, respectively. Finally the nuclear pellet was resuspended either in 1 ml of freezing buffer [100 mM NaCl, 50 mM Hepes pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 50% glycerol, 1 mM DTT, 1 mM EDTA pH 8.0, 0.5 mM PMSF, 0.5 μg/ml leupeptin (Sigma) and 50 μg/ml antipain (Sigma)] and stored at -70 °C until use, or in 1 ml of the extraction buffer [0.47 M NaCl, 0.3% Triton X-100 (Fisher Scientific), 45% glycerol, 50 mM HEPES pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 10 mM DTT, 1 mM EDTA pH 8.0, 1 mM PMSF, 0.5 μg/ml leupeptin and 50 μg/ml antipain] for immediate nuclear extraction.

3.2.2. *Examination of quality of pea nuclei*

The purified pea nuclear suspension in either RS or freezing buffer was mixed with staining solution (0.2 mM Acridine Orange and 10 mM EDTA in RS) at a ratio of 9:1. The stained nuclei were examined and counted in a haemacytometer under 16 x 10 amplification of the fluorescent microscope ZEISS-MC63 (ZEISS, Germany). Intact nuclei are roughly round and emit green fluoresce. The concentration of nuclei was calculated as following:

$$K \text{ (nuclei/ml)} = A \times B \times C \times 10^4.$$

Where A is the number of nuclei in one field of sight under the fluorescent microscope at 160x magnification; B is the dilution factor for nuclear suspension; C

(estimated) is the ratio of the big square (containing 16 middle squares) on haemacytometer to one field of sight of the fluorescent microscope (since the scale on haemacytometer can not be seen under fluorescence, this ratio must be first estimated under normal light); 10^4 is the given value for changing the volume of the big square (0.1 μl) of haemacytometer to 1 ml.

For example: A=45; B=100; C=1.3 (under amplification 16 x10)

$$K = 45 \times 100 \times 1.3 \times 10^4 = 5.9 \times 10^7 \text{ nuclei/ml.}$$

High quality nuclear preparations contain more than 5×10^7 intact nuclei/ml.

3.2.3. *Extraction of nuclear proteins*

Purified nuclei in extraction buffer were shaken gently on a Deluxe Mixer (Scientific Products, McGaw Park, IL, USA) for 30 min at 4 ^{0}C. If the nuclei were stored in freezing buffer at -70 ^{0}C, 180 μl of lysis buffer (2.5 M NaCl, 2% Triton X-100, 20% glycerol, 50 mM Hepes pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 10 mM DTT, 1 mM EDTA pH 8.0, 1 mM PMSF, 0.5 μg/ml leupeptin and 50 μg/ml antipain) was added to each 1 ml nuclear suspension to convert the freezing buffer to an extraction buffer. The lysate was then centrifuged at 10,000 g for 15 min at 4 ^{0}C and the supernatant was dialyzed (Spectrapor 1, MW cut-off 6,000 - 8,000 D) three times against 200 ml of the dialysis buffer (40 mM NaCl, 20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 0.2 mM DTT, 1 mM EDTA pH 8.0, 2 mM Tris-HCl pH 8.0) for a total of more than 4 h. If necessary, the nuclear extract was

concentrated by immerging the dialysis bag in PEG_{8000} for 2 h and then collected. The concentration of nuclear protein was measured with Bio-Rad Protein Assay Agents (Bio-Rad Laboratories, Hercules, CA, USA) (see Section 3.2.5.). Aliquots of 200 µl were made and stored at -70 °C (Table 6).

3.2.4. *Total cytoplasmic protein of pea pods*

The cytoplasmic supernatant obtained after centrifugation at 300 g (Section 3.2.1.) was saved and centrifuged again at 12,000 g for 20 min. The proteins in the supernatant were precipitated twice with ammonium sulphate, the first time with 50% saturation at 4 ^{0}C and the second time with 80%. The pellet was resuspended in dialysis buffer. The rest of the protocol was the same as described in Section 3.2.3.

3.2.5. *Measurement of protein concentration*

Concentrations of the nuclear proteins and the total cytoplasmic proteins were measured by Bio-Rad Protein Assay Agents, according to the manufacturer´s instruction. A standard curve was prepared with a series of concentrations of BSA in dialysis buffer. The samples were diluted in the dialysis buffer and mixed with the ready-to-use agents. After reacting at room temperature for 10 min, the mix was measured by spectrophotometry at OD_{595nm}. Concentration of proteins was derived from a standard curve.

Table 6. List of the nuclear extracts from immature pea pods (*P. sativum*).

Extract	Treatment	Note
UPP	unsplit healthy control	nuclear protein
H2	split healthy 2 h.p.i.	nuclear protein
H2TP	split healthy 2 h.p.i.	cytoplasmic protein
W2	Sterile water 2 h.p.i.	nuclear protein
W6	Sterile water 6 h.p.i.	nuclear protein
W24	Sterile water 24 h.p.i.	nuclear protein
CH8	chitosan 8 h.p.i.	nuclear protein
CH8TP	chitosan 8 h.p.i.	cytoplasmic protein
Fsp2	*F. solani pisi* 2 h.p.i.	nuclear protein
Fsp6	*F. solani pisi* 6 h.p.i.	nuclear protein
Fsp24	*F. solani pisi* 24 h.p.i.	nuclear protein
Fsph0.5	*F. solani phaseoli* 0.5 h.p.i.	nuclear protein
Fsph1	*F. solani phaseoli* 1 h.p.i.	nuclear protein
Fsph2	*F. solani phaseoli* 2 h.p.i.	nuclear protein
Fsph4	*F. solani phaseoli* 4 h.p.i.	nuclear protein
Fsph6	*F. solani phaseoli* 6 h.p.i.	nuclear protein
Fsph8	*F. solani phaseoli* 8 h.p.i.	nuclear protein
Fsph24	*F. solani phaseoli* 24 h.p.i.	nuclear protein
Fsph6TP	*F. solani phaseoli* 6 h.p.i.	cytoplasmic protein
SA6	salicylic acid 6 h.p.i.	nuclear protein
SA36	salicylic acid 36 h.p.i.	nuclear protein
SA36TP	salicylic acid 36 h.p.i.	cytoplasmic protein

3.3. Preparation of DNA probes

3.3.1. *PEG preparation of plasmid DNA*

Five ml of LB medium containing 50 μg/ml ampicilin was inoculated with a single colony of the bacterium harboring the target plasmid DNA and incubated overnight by shaking at 240 rpm and 37 °C. The culture was harvested by centrifuging for 5 min at 4 °C in microcentrifuge tubes. The pellet was resuspended in 250 μl of lysozyme solution [50 mM D-glucose, 25 mM Tris-HCl, 10 mM Na-EDTA, pH 8.0, adding lysozyme (Boehringer Mannheim) to 2 mg/ml before use] and seated on ice for 5 min. 500 μl of alkaline SDS solution (0.2 N NaOH, 1% SDS, fresh weekly) was added and mixed by gentle inversion and then incubated on ice for 5 min. Following addition of 375 μl of 3 M NaAc pH 5.0 and incubating on ice for 20 min, the tube was centrifuged for 15 min at 4 °C and the supernatant was saved. One volume of isopropanol was added and centrifuged for 20 min at 4 °C. The pellet was resuspended in TE (25 mM Tris-HCl, 10 mM EDTA, pH 7.0). Ten mg/ml of RNase A in H_2O was added to a final concentration of 50 μg/ml and incubated at 65 °C for 10 min. Thirty percent PEG_{8000} in 1.8 M NaCl was added to a final concentration of 9% PEG and set at 4 °C for at least 4 h. The suspension was centrifuged for 15 min and the pellet was resuspended in TE. The DNA suspension was then extracted once with phenol:$CHCl_3$:isoamyl alcohol (25:24:1) and once with $CHCl_3$:isoamyl alcohol (24:1).

One-half volume of 7.5 M ammonium acetate and one volume of cold isopropanol were added and the tube set at -20 °C for 30 min then spun for 20 min and rinsed once with 70% cold ethanol alcohol. The pellet was dried briefly and resuspended in 10 mM Tris-HCl, 1 mM EDTA. The quantity of DNA was determined by spectrophotometry and agarose gel electrophoresis. The DNA preparations were stored at -20 °C until use.

3.3.2. *Fast mini-preparation of plasmid DNA*

This protocol was used in regular screening of recombinant plasmid DNA clones.

Five ml of LB medium containing 50 μg/ml ampicilin was inoculated with a single colony of the bacterium harboring the target plasmid DNA and incubated overnight by shaking at 240 rpm and 37 °C. One and half ml of the culture was harvested by centrifuging for 2 min at 4 °C in a microfuge tube. The pellet was resuspended in 370 μl of STET (8% sucrose, 0.5% triton X-100, 50 mM EDTA, 10 mM Tris-HCl pH 8.0) and 25 μl of 10 mg/ml of lysozyme and 5 μl of 10 mg/ml of RNase A were added. After incubation at room temperature for 5 min, the tube was heated at 90 °C for 45 seconds and centrifuged at room temperature for 8 min. The pellet was pulled out using a toothpick and discarded. To the supernatant, 40 μl of 3 M NaAc pH 5.0 and 400 μl of pre-cooled isopropanol were added and centrifuged immediately for 10 min. After rinsing with 70% ethanol alcohol and briefly drying, the

DNA pellet was resuspended in 20 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0).

3.3.3. *Recombinant plasmid constructs*

All the original plasmids were available in this laboratory. pKX contains a genomic copy of *PR10.1* (GB::U31669) (Culley *et al.*, 1995), which was recloned into the *Sal*I/*Hin*dIII sites of pBluescript KSm13$^+$. pCC2 contains a genomic copy of *PR10.3* (GB::J03680) (Chiang & Hadwiger, 1990). pCHS2KS was made by cloning the 1.6-kb *Eco*RI fragment of genomic pea chalcone synthase (GB::X63333) from pCHS2 (Harker *et al.*, 1990) into pBluescript KSm13$^+$. All the other constructs were derived from pKX or pCC2 by either deletion or subcloning fragments into pBluescript KSm13$^+$ (see detail in Table 9).

3.3.4. *DNA probe design*

Using the programs BACHREST in GDE (Smith *et al.*, 1994) and then DIGEST (Fristensky *et al.*, 1982), restriction sites within the genomic sequences of *PR10.1* and *PR10.3*, including promoter region, coding sequence and downstream region, were identified. Seven restriction enzymes were selected (Figure 1A) and six primary DNA probes were designed for *PR10.1* (Figure 2A). The sequence of *PR10.3* was cut by six restriction endonucleases (Figure 1B) and seven major probes were

generated (Figure 2B).

3.3.5. *Isotope-labelling of DNA probes*

The target plasmid DNA (1-5 μg) was digested with restriction enzymes and completed digestion was verified on an agarose gel. The remaining DNA was precipitated with ethanol and resuspended in distilled H_2O. End-labelling was performed in 1 x GIBCO REact buffer 2, a total volume of 20 μl by filling in 3′-recessive ends with 1.0 unit of Klenow large fragment, appropriate labelled nucleotide and 25 μM (final) each of the other three unlabelled dNTP nucleotides. After incubation at room temperature for 30 min, the labelled DNA was run on a high concentration agarose gel, 1.5% to 3.5%, depending on the size of the probe. The radioactive band was cut out and probe DNA was isolated with the Prep-A-Gene kit (Promega). Alternatively, the radioactive band was cut out and frozen in a microcentrifuge tube at -70 °C for more than 30 min. The frozen gel was centrifuged at top speed for 5 min at room temperature and the supernatant containing the labelled probe was collected. One μl of the probe was taken to measure the incorporated radioactivity.

Figure 1. *PR10.1* and *PR10.3* genomic sequences from pea (*P. sativum*). The coding sequence (CDS) is shown in lowercase letters. Uppercase: upstream or downstream regions. Italic uppercase: intron. Single underline: the restriction sites used to generate six primary DNA probes for *PR10.1* (Figure 2A) and seven probes for *PR10.3* (Figure 2B). Double underline: *Alu*I sites which flank the fragment AA424 (probe A4) used in deletion analysis for *PR10.1*. Italic lowercase: primers for *PR10.1* (oS49a+8 and oS49a-7) and for *PR10.3* (oS49c+4 and oS49c-5) used in the gene expression assay (Figure 19). Numbers on the left side indicate GenBank positions in *PR10.1* (GB::U31669) and *PR10.3* (GB::J03680). Numbers on the right side indicate positions relative to the translation start site.

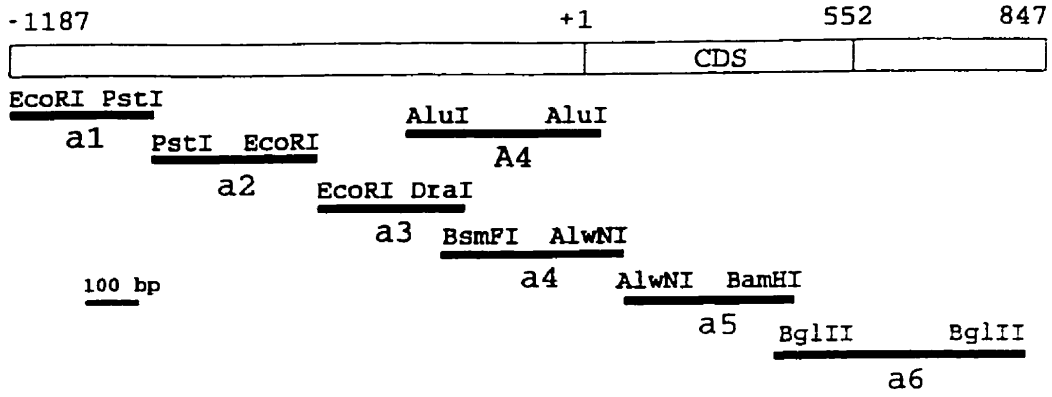
A. *PR10.1*

B. *PR10.3*

Figure 1. *PR10.1* (A) and *PR10.3* (B) genomic sequences from pea (*P. sativum*).

Figure 2. DNA probes for *PR10.1* and *PR10.3*. A) Six primary DNA probes for *PR10.1*: a1, a2, a3, a4, a5, a6 and their relative locations and scaled sizes. The probe A4, which covers part of a3 and a4, was specifically used in deletion analysis. B) Seven primary DNA probes for *PR10.3*: c1, c2, c3, c4, c5, c6, c7 and their relative locations and scaled sizes.

A. *PR10.1*



B. *PR10.3*

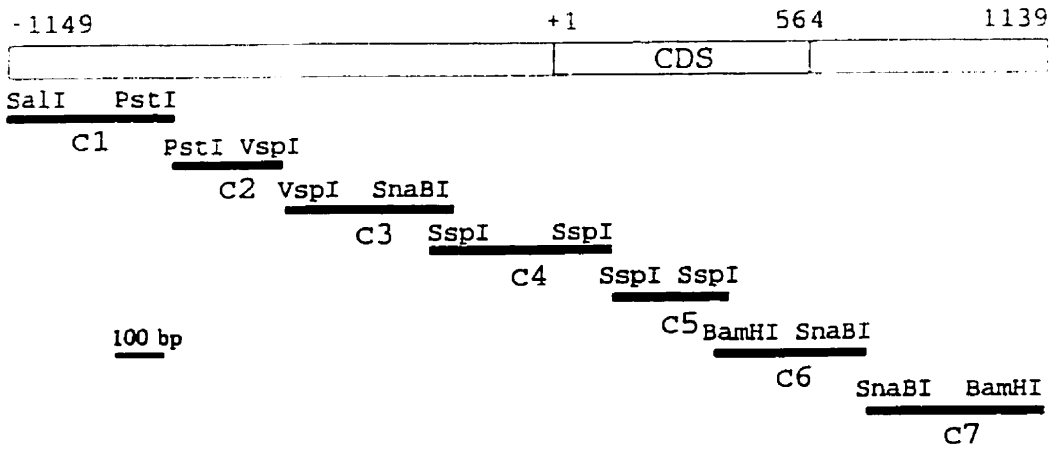


Figure 2. DNA probes for *PR10.1* and *PR10.3*.

3.4. Gel retardation assay

The published protocol for the gel retardation assay (Data *et al.*, 1989; Ausubel *et al.*, 1998) was modified as described below.

3.4.1. *Non-denaturing PAGE*

Glass plates were washed with detergent and then cleaned with 95% ethanol. One side of one plate was siliconized with 4% (v/v) dimethyldichlorosilane in chloroform and the other side was marked. Gel cassettes were assembled using a spacer of 0.75 or 1.5 mm thick. Instant gel mix was prepared by adding 100 µl of 30% ammonium persulfate and 34 µl of TEMED to 40 ml of low ionic gel mix (6.7 mM Tris-HCl pH 7.9, 3.3 mM NaAc pH 7.9, 1 mM EDTA pH 8.0, 2.5% glycerol and 79/1 ratio of acrylamide/bisacrylamide, fresh monthly). The electrophoresis unit was assembled and cooling system set up at 10 ^{0}C. A pump was set up with 2 heads to recirculate the low ionic electrophoresis buffer (6.7 mM Tris-HCl pH 7.9, 3.3 mM NaAc pH 7.9, 1 mM EDTA) between the lower and the upper reservoirs. The native PAGE gel was run at a constant voltage of 100 V for more than 1 h before loading the samples.

3.4.2. *Gel retardation assay*

All reaction components were prepared in 1 x KCl binding buffer (KBB: 20 mM HEPES pH 8.0, 16 mM KCl, 1 mM EDTA pH 8.0, 10% glycerol, 1 mM DTT, 1 mM Tris-HCl pH 8.0). One μg poly dI-dC (Sigma) and 5 μg nuclear extract were added to a 0.5-ml microcentrifuge tube and incubated for 5 min at room temperature. DNA probe (10,000 cpm) and 1 μl 0.1% bromophenol blue were added to a final volume of 10 μl. The reaction mix was further incubated at room temperature for 20 min and then loaded on the non-denaturing PAGE gel.

A double dye (0.25% bromophenol blue, 0.25% xylene cyanol and 15% Ficoll) was loaded into the left- and right-most lanes and run for 5 min before loading samples. The dye was used to monitor the progress of electrophoresis. Bromophenol blue and xylene cyanol migrate approximately at the same rate as 100-bp and 400-bp DNA probes on 6% native PAGE, respectively. Each sample was loaded to the bottom of its well with a very fine pipette tip (made manually by heating and stretching regular plastic pipette tips). Two PAGE gels were run at a constant current of 35 mA for 0.75 mm-gels or at 65 mA for 1.5 mm-gels. After electrophoresis, gel cassettes were disassembled and glass plates were laid in a tray with the siliconized glass plate facing up. The siliconized plate was slowly pried from the gel using a spatula. A piece of Whatman paper was placed on the gel and the non-siliconized plate was inverted. The gel was removed from the plate by slowly peeling the paper away from the plate. After covering with plastic film, the gel was dried completely at 80 °C under vacuum using a gel drier (Bio-Rad), followed with autoradiography.

3.4.3. *Competition gel retardation assay*

The procedure was the same as that described in Section 3.4.2. except for the following: In the DNA competition assay, DNA competitors were individually mixed with target DNA probes before adding other components. Similarly, in the protein competition assay, protein competitors were mixed with target nuclear extracts before adding other components.

3.5. Investigation of gene expression

3.5.1. *RNA extraction from pea plant*

Pea tissue was frozen immediately after treatment. RNA extraction was conducted by using the RNeasy Plant Mini Kit (QIAGEN GmbH, Germany), following the manufacturer's instructions. The concentration of total RNA was measured by spectrophotometry and confirmed by electrophoresis. All samples were adjusted to the same concentration with RNase-free water. Samples were stored at -70 °C until use.

3.5.2. *Reverse transcription*

Three μg total RNA were incubated with 0.75 μg of oligo(dT) $_{12\text{-}18\ mer}$ primer

(GIBCO BRL cat. # 18418-012) at 65 °C for 15 min. Reverse transcription was performed in a volume of 30 µl at 50 °C for 30 min with a final concentration of 1 x reverse transcription buffer, 1 mM dNTP, 60 units of RNAsin and 100 units of M-MLV reverse transcriptase (all from Promega). The cDNA products were stored at -20 °C.

3.5.3. *Internal control plasmid for RT-PCR*

pI49KS was constructed by cloning the *Hin*dIII/*Sal*I fragment of pI49 (*PR10.PS.1*, GB::X13383), *PR10.1* cDNA (Fristensky *et al.*, 1988; Culley *et al.*, 1995), into *Hin*dIII/*Sal*I-digested pBluescript KSm13$^+$. pI49KSv was constructed by cloning the 585-bp *Sau*3AI fragment from pUC18 into the *Bgl*II site within *PR10.1* cDNA in pI49KS. p49cKS was made by recloning the 868-bp *Nsi*I/*Xba*I fragment from pCC2 (Chiang & Hadwiger, 1990) into *Pst*I/*Xba*I-digested pBluescript KSm13$^+$. pCC2 contains a genomic copy of *PR10.3*, including an 88-bp intron (i). More details are found in Figure 3.

Figure 3. Internal control constructs. Details were described in Section 3.5.3.

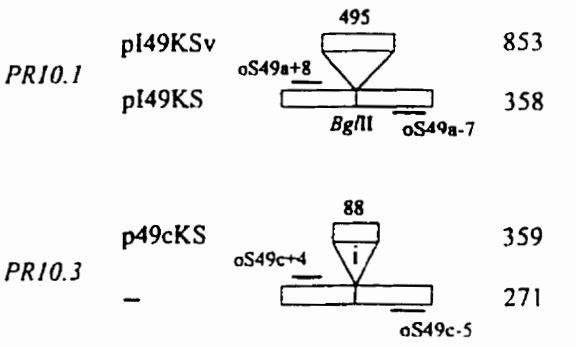


Figure 3. Internal control constructs.

3.5.4. *Oligo DNA and primers*

The following oligonucleotide DNAs and primers were used in either competition gel retardation assays or RT-PCR. All oligonucleotides, from 5´ to 3´, were synthesized by GIBCO BRL.

21S: (TGAATAGTAGATTTAAG)

22S: (CCAAATAAAATTTTCTTTT)

oS49a+8: (CTAGTTACAGATGCTGATAAC)

oS49a-7: (CATCCCCCTTAGCTTTGTCAG)

oS49c+4: (TGTTGAAGGAAACGGTGGCCC)

oS49c-5: (GATTTCCTCTTCACTAGGAAT)

3.5.5. *DIG-labelling PCR*

Ten µl of a 1:10 dilution of the cDNA product were mixed with specific primers for *PR10.1* (oS49a+8 and oS49a-7) or *PR10.3* (oS49c+4 and oS49c-5). PCR was carried out in a total volume of 25 µl by using the PCR DIG Labelling Mix from Roche (previously Boehringer Mannheim, Cat.# 1585550), following the manufacturer´s instructions. The final concentration of the reaction mix was: 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM dATP, dCTP and dGTP, 190 µM dTTP, 10 µM DIG-dUTP, 1.0 unit Taq polymerase, 0.5 µM of each primer and typically, 0.5 pM of internal control plasmid. Wherever possible,

master mixes were prepared to improve reproducibility. Fourteen cycles of PCR were performed by PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA 02172, USA): denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min.

Before amplifying the cDNA sample, the linear range of the internal control plasmid DNA was determined by the same PCR program. It was found that the linear range for both *PR10.1* and *PR10.3* was 0.1 to 3.0 pM of final concentration of the control DNA (data not shown). Therefore, 0.5 pM of the internal control was typically included in the DIG-labelling PCR. Occasionally, however, the concentration of the internal control was adjusted empirically within the linear range to avoid large discrepancies between mRNA-derived and control-derived band intensities.

3.5.6. *DIG detection*

Five μl of the DIG-integrated PCR product was electrophoresed on a 1.5% agarose gel and transferred to Hybond membrane (Amersham) following the manufacturer's instructions. After brief drying at 37 °C, the membrane was crosslinked using the auto-crosslink mode of the Stratagene UV Crosslinker. The blot was equilibrated in buffer A (100 mM Tris-HCl pH 7.0, 150 mM NaCl) for 2 min and blocked in buffer B [1% (w/v) blocking reagent (Roche Cat. # 1096176) in buffer A] for 30 min on an orbital shaker. The membrane was then incubated for 30 min in the anti-DIG-AP (Roche Cat. # 1093274) conjugate suspension which was diluted by

1:5,000, with a final concentration of 75 unit/ml. After two 15-min washing in washing buffer [buffer A plus 0.3% (v/v) Tween 20], the membrane was equilibrated in buffer C (100 mM Tris-HCl pH 9.5, 10 mM NaCl and 50 mM $MgCl_2$) for 2 min. The chemiluminescent substrate, CDP-Star (Roche Cat. # 1685627) with a final concentration of 250 μM, was added directly to the membrane which was then sealed in a plastic bag. After 15 min, the blot was either exposed to X-ray film or read directly by the Fluor-S MultiImager (Bio-Rad).

3.6. Computer analysis of DNA sequences

3.6.1. *General tasks*

Most sequence analysis programs were run from GDE (Genetic Data Environment) (Smith *et al.*, 1994).

3.6.2. *Selecting potential conserved motifs in binding sequences*

The process of identifying DNA motifs in binding sequences, which are conserved in other gene promoters, included three steps: 1) selecting potential conserved motifs in binding sequences among plant defense-related genes; 2) creating different promoter datasets; 3) searching for conserved DNA motifs in binding sequences against these datasets.

Plant defense-related genes were retrieved by keyword (such as plant, genomic, gene, defense, resistance, PR, "-" cDNA, *etc.*) searches using ENTREZ (Schuler *et al.*, 1996). The promoter region of each gene was extracted by FEATURES (Fristensky, 1993) and saved individually in XLAND format (Levy *et al.*, 1998). The XLAND program was originally designed to generate a sequence "landscape" or sequence pattern ratio landscape between two sequences or two datasets (one as target and the other as source). The program first tabulates the frequencies of all possible sequence patterns within a target sequence which occur in a source sequence. For a simplified example, assume that the target sequence is (AGCT) and the source sequence is (AGCTAGAG). After reading both sequences, XLAND generates an intermediate result like this:

possible pattern in target	A	G	C	T	AG	GC	CT	AGC	GCT	AGCT
frequency in source	3	3	1	1	3	1	1	1	1	1

These frequency numbers are used to plot the sequence landscape. A peak in the landscape of the target sequence means a conserved DNA pattern in the source sequence. The higher the peak, the longer the conserved sequence (see an actual example in Figure 12). Therefore, when searching for potential conserved motifs in binding sequences (target sequence), XLAND was applied to generate sequence landscape of each target sequence with each source sequence (defense genes). Different landscapes, which were generated by individual defense genes as sources against the same binding sequence (target), were manually aligned and compared to select potentially conserved DNA motifs (see column "Motif" in Table 14). Each binding region (PDA1, PDA2 and PDC1 in Results) was compared with each of 49 defense gene promoters listed in Table 13, using XLAND.

Motifs conserved among defense genes show up as peaks in the landscape that occur at the same location in the binding region, when many defense genes are compared to the binding region.

3.6.3. *Creating promoter datasets*

In addition to plant defense-related genes, genes not associated with plant defense were retrieved by keyword (plant, gene, genomic, "-" defense, "-" cDNA, etc.) searches using ENTREZ. The promoter regions of all retrieved genes, defined as those sequences upstream from the transcription start site, were extracted by FEATURES. The following datasets were created in XLAND format: Defense Genes including two subdatasets, *PR10* Genes and Non-*PR10* Genes; and Non-Defense Genes including two subdatasets, Pea Genes and Non-Pea Genes (refer to Table 13 and 14 for details).

Dataset											
Defense Gene						Non-Defense Gene					
PR10		Non-*PR10*		Total		Pea		Non-Pea		Total	
No.[a]	Size[b]	No.	Size	No.	Size	No.	Size	No.	Size	No.	Size
11	8 kb	38	43 kb	49	51 kb	29	26 kb	137	116 kb	166	142 kb

Note: "a" number of genes in dataset; "b" size of dataset in kilobase (kb).

3.6.4. *Searching conserved motifs in binding sequences against datasets*

The purpose of XLAND in this research was to determine whether DNA motifs in the protein-binding sequences in pea *PR10* are conserved in other genes' promoters.

Therefore, XLAND was applied to compare all three binding sequences as targets with each of the datasets as sources and automatically retrieve the frequencies of the potential motifs which occur in the datasets. The motifs most highly conserved between pea *PR10* binding sequences and selected datasets are shown in Table 14. Frequencies of each motif are expressed as the number of occurrences per 10-kb in each dataset.

4. RESULTS

4.1. Screening for protein-binding sequences

PR10 was originally identified as a gene activated in pea pods by *Fusarium solani* (Riggleman *et al.*, 1985). In order to screen for protein-binding sequences, nuclear extracts were prepared from pods treated with W2 (2 h.p.i. with water), Fsph2 (2 h.p.i. with *F. solani* f. sp. *phaseoli*), Fsp2 (2 h.p.i. with *F. solani* f. sp. *pisi*), CH8 (8 h.p.i. with chitosan) and SA36 (36 h.p.i. with salicylic acid). Six DNA probes from *PR10.1* (Figure 2A) and seven probes from *PR10.3* (Figure 2B) were screened at least twice with pea nuclear extracts in gel retardation assays. The results showed that 3 probes each from *PR10.1* and *PR10.3* exhibited binding activity with nuclear extracts (Figure 4 and Table 7).

To broaden the range of conditions tested, nuclear or cytoplasmic extracts were prepared from pods treated with water, *Fusarium* or elicitor over a 48 h time course. Both positive and negative reactions in the original tests were further examined to ensure that all potential binding sequences in *PR10.1* and *PR10.3* were found. The results of the extended survey with all the nuclear and cytoplasmic extracts available were similar to the results with the original nuclear extracts. The positive probes exhibiting a shifted band in the original test, a3, a4, a6, c2, c3 and c7, were positive with a majority of extracts and negative with some extracts in the extended survey.

Figure 4. Gel retardation assay to screen for protein-binding DNA sequences in *PR10.1* and *PR10.3*. The figure shows the results of gel retardation assays between six positive probes (a3, a4, a6, c2, c3 and c7) and five representative nuclear extracts (CH8, chitosan 8 h treatment; Fsp2, *F. solani* f. sp. *pisi* 2 h; Fsph2, *F. solani* f. sp. *phaseoli* 2 h; SA36, salicylic acid 36 h; and W2, water 2 h). Arrow←: shifted band. A) for *PR10.1* and B) for *PR10.3*. More details are included in Table 7.

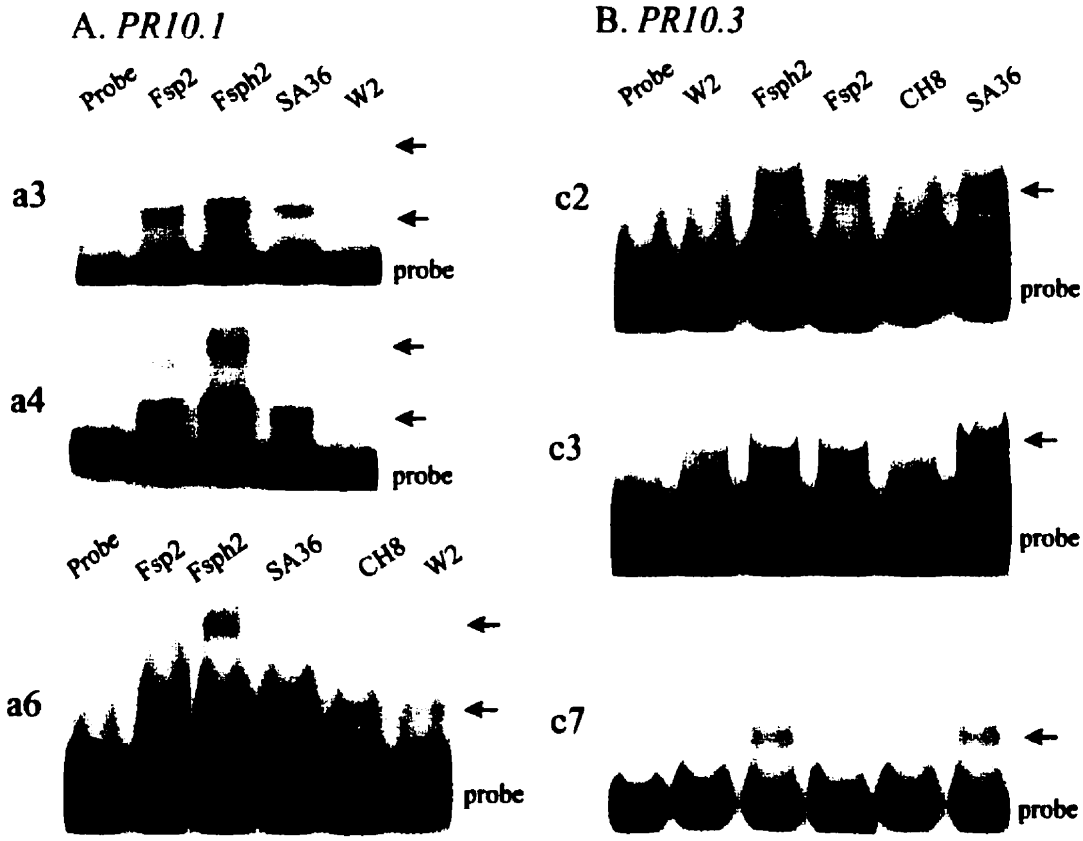


Figure 4. Screening of protein-binding sequences in *PR10.1* and *PR10.3*.

Table 7. Screening for protein-binding probes from *PR10.1* and *PR10.3*.

Probe	Location	Nuclear extract				
		W2	Fsph2	Fsp2	CH8	SA36
a1	-1187 to -883	-*	-	-	-	-
a2	-882 to -549	-	-	-	-	-
a3	-548 to -237	±1	2	2	N	1
a4	-288 to 79	±1	2	2	N	1
a5	80 to 407	-	-	-	-	-
a6	367 to 832	±1	2	2	±1	1
c1	-1149 to -831	-	-	-	-	-
c2	-830 to -622	-	1	1	-	1
c3	-621 to -196	±1	1	1	-	1
c4	-252 to 195	-	-	-	-	-
c5	196 to 446	-	-	-	-	-
c6	420 to 743	-	-	-	-	-
c7	744 to 1135	-	1	1	-	1

*: "-" no binding band; "1" one band; "2" two bands; "±" weak band; "N": not tested.

Table 8. Extended survey for primary probes from *PR10.1* and *PR10.3*.

Nuclear Extract	*PR10.1*						*PR10.3*						
	a1	a2	a3	a4	a5	a6	c1	c2	c3	c4	c5	c6	c7
Probe	-*	-	-	-	-	-	-	-	-	-	-	-	-
BSA	-	-	-	-	-	-	-	-	-	-	-	-	-
H2	-	-	±1	±1	-	±1	-	-	±1	-	-	-	-
W2	-	-	±1	±1	-	±1	-	-	±1	-	-	-	-
W6						±1		-	±1				
W24						±1		-	±1				
CH8	-	-			-	±1	-	-	-	-	-	-	-
Fsp2	-	-	2	2	-	2	-	1	1	-	-	-	1
Fsp6						-		-	-				-
Fsp24						-		-	-				-
Fsph2	-	-	2	2	-	2	-	1	1	-	-	-	1
Fsph4						2		-	1				1
Fsph6						1		-	1				1
Fsph24						1		-	-				-
SA36	-	-	1	1	-	1	-	1	1	-	-	-	1
H2TP						1							-
CH8TP						-							-
Fsp6TP						-							-
Fsph6TP						1							-
SA36TP						-							-

*: "-" no visible binding band; "1" one band; "2" two bands; "±" weak band; "blank" not tested.

The negative probes (absence of binding band) in the original test, a1, a2, a5, c1, c4, c5 and c6, were negative to all tested extracts in the extended survey (Table 8). However, the strength and the number of the shifted bands varied in the different DNA/protein reaction combinations. Of the positive probes, a3, a4 and a6 from *PR10.1*, all exhibited two shifted bands with Fsph2 and Fsp2, and one band with all the other nuclear extracts (Figure 4A). All the positive probes, c2, c3 and c7 from *PR10.3*, exhibited only a single shifted band (Figure 4B). In both genes, positive probes were located in two major binding regions, one in the upstream promoter region and the other downstream of the coding sequence. The presence of multiple bands at several locations indicated that there could be several different protein-binding sequences or *cis*-regulatory elements in *PR10.1* and *PR10.3*.

4.2. DNA competition gel retardation assay

Competition assays were used to demonstrate binding specificity between the nuclear extracts and the DNA probes. Four different kinds of DNA competitors were included: non-labelled probe; related DNA which partially or fully covered the target probe; non-related DNA which does not overlap the target probe; and homologous DNA. DNA competitors were prepared in the same molar concentration (pM). Figure 5B showed the binding activities between the Fsph2 nuclear extract from pods, which were treated with *F. solani* f. sp. *phaseoli* for 2 h, with the a4 probe and various unlabelled competitors. The non-labelled a4 (cold probe) at 40-fold molar excess completely out-competed the labelled a4 probe. The a3 fragment, which partially overlaps a4, competed

partially at 40-fold excess and eliminated the shifted band at 200-fold. pKX containing the entire *PR10.1* gene, including a3 and a4, completely eliminated the bound band. The more distant BB fragment, however, was not able to compete78 with the specific probe and the shifted band was as strong as with no competitor. pCHS2KS, the pea chalcone synthase gene, which shares sequence similarity with *PR10* and is also pathogen-inducible (Harker *et al.*, 1990), dramatically reduced the binding activity. Similar results were obtained when a3 was used as the labelled probe and the unlabelled a3, a4, pKX, pCHS2KS and BB as competitors (Figure 5C). It should be noted that a 200-fold excess of a3 was required to eliminate the bound band when a3 was used as aprobe (Figure 5C). In general, though, shifted bands can be eliminated by the specific DNA competitors demonstrated that the binding activity between the probe (a4 or a3) and the nuclear extract (Fsph2) was highly specific, indicating that a *cis*-regulatory element is recognized by a nuclear protein factor appeared in Fsph2.

Figure 5. DNA competition assay for *PR10.1*. (A) Schematic representation of the relative locations of the probes and DNA competitors for *PR10.1*. The labelled probes were a4 (B) and a3 (C). The nuclear extract was Fsph2. Other competitors: chs, chalcone synthase genomic DNA (GB::X63333); BB, a more distant promoter fragment of *PR10.1* which does not overlap the a3/a4 binding region; pKX, plasmid DNA harboring genomic *PR10.1* (GB::U31669). ck, assay mix without any competitor DNA; arrow←, shifted band. The inferred binding region is boxed.

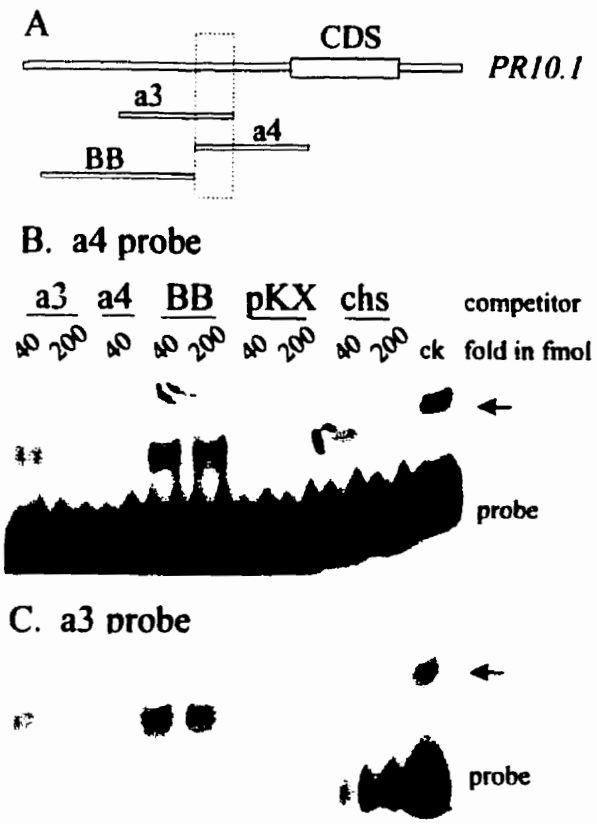


Figure 5. DNA competition gel retardation assay for *PR10.1*.

4.3. Protein competition gel retardation assay

Specific DNA-binding proteins may constitute a very small portion of the total nuclear proteins. The majority of proteins in the nuclear extracts are non-specific proteins (mostly histone). Non-specific proteins would cause non-specific binding activities in the binding assay. As a further check on the specificity of DNA-binding proteins in nuclear extracts, protein competitors were used in protein competition gel shift assays. Elimination of shifted bands by competitor proteins would meas that the binding activities are non-specific, otherwise the binding would be considered to be specific. Two protein competitors were used, BSA and histone (Sigma), both of which are from animals. SA36 and a6 were used as the nuclear extract and the labelled probe, respectively. The results showed that neither of the competitor proteins eliminated or reduced the binding activities, compared to the normal gel shift assay (Figure 6) (histone data not shown). This suggests that specific recognition occurred between the a6 probe and the SA36 nuclear extract.

Figure 6. Protein competition gel retardation assay. The labelled probe was A4-2 (covering PDA2, see Figure 7). The nuclear extract was Fsph2. Arrow←: the shifted band; prb, probe; +/-, presence or absence of a reaction component; Numbers, μg/reaction; BSA, bovine serum albumen; dI.dC, double strands poly(dI-dC)·poly(dI-dC) sodium salt, 2 μg/reaction; All the components were mixed with the Fsph2 nuclear extract before adding the labelled probe.

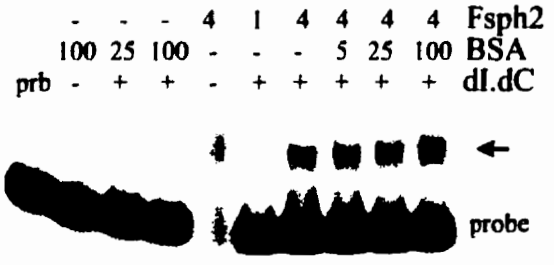


Figure 6. Protein competition gel retardation assay.

4.4. Plasmid constructs containing primary positive probes

To allow a more precise analysis of binding regions, positive DNA probes a3, a4, and a6 from *PR10.1*, and c2, c3, and c7 from *PR10.3*, were cloned into pBluescript KSm13$^+$ (Strategene). Since a3 and a4 can compete with each other in the competition gel retardation assay (Figure 5), the binding sequence may be in the overlap region. *Alu*I fragment A4 in *PR10.1* covers part of the a3 probe and almost the entire sequence of a4, and it particularly covers the overlap region between a3 and a4 (Figure 2A). pD49A4 was constructed by inserting the A4 fragment into the *Eco*RV sites of pBluescript KSm13$^+$. Recombinant plasmid constructs with both orientations of the insert were obtained. pD49A6 was made by inserting the a6 probe, *Bgl*II fragment, into the *Bam*HI sites of the vector. Similarly, the plasmid constructs with both orientations of the insert were selected (Table 9). pD49C2, pD49C3 and pD49C7 were constructed by inserting c2 (*Pst*I/*Vsp*I fragment), c3 (*Vsp*I/*Sna*BI fragment) and c7 (*Sna*BI/*Bam*HI fragment) into *Sma*I/*Pst*I, *Eco*RV/*Hin*dIII and *Bam*HI/*Sma*I sites of pBluescript KSm13$^+$, respectively (Table 9). Some termini needed partial or full end-filling before religation.

Table 9. Recombinant constructs.

Construct	Insert	Location[a]	Source	Vector
pKX	PR10.1	-1187 to 848	*PR10.1*	pBSKSm13+[b]
pD49A6+/-	BB425	367 to 832	*PR10.1*	pBSKSm13+
pD49A4+/-	AA424	-360 to 64	*PR10.1*	pBSKSm13+
pD49A4v	BH197	-360 to -192	*PR10.1*	pBSKSm13+
pD49A4d	BH150	-360 to -237	*PR10.1*	pBSKSm13+
pD49A4b	BH102	-360 to -287	*PR10.1*	pBSKSm13+
pCC2	PR10.3	-1149 to 1140	*PR10.3*	pUC19
pD49C2-	VP209	-830 to -622	*PR10.3*	pBSKSm13+
pD49C3+	BV426	-621 to -196	*PR10.3*	pBSKSm13+
pD49C7-	BB302	744 to 1135	*PR10.3*	pBSKSm13+
pD49C3L9	c3L9	-621 to -290	*PR10.3*	pBSKSm13+
pD49C3L11	c3L11	-621 to -410	*PR10.3*	pBSKSm13+
pD49C3R1	c3R1	-544 to -196	*PR10.3*	pBSKSm13+
pD49C3R2	c3R2	-498 to -196	*PR10.3*	pBSKSm13+
pD49C3R3	c3R3	-471 to -196	*PR10.3*	pBSKSm13+
pD49C3R4	c3R4	-464 to -196	*PR10.3*	pBSKSm13+
pD49C3R5	c3R5	? to -196	*PR10.3*	pBSKSm13+
pD49C3R6	c3R6	-452 to -196	*PR10.3*	pBSKSm13+
pD49C3R7	c3R7	? to -196	*PR10.3*	pBSKSm13+
pD49C3R8	c3R8	-448 to -196	*PR10.3*	pBSKSm13+
pD49C3R9	c3R9	-331 to -196	*PR10.3*	pBSKSm13+

"a": Insert location relative to the translation start site; "b": all in pBluescript Ksm13 except for pCC2, where the insert was subcloned into pUC19; "?": not available.

4.5. Deletion analysis to narrow down binding region in *PR10.1*

Specific binding regions in *PR10.1* were found both upstream and downstream of the coding sequence. To narrow the focus of this study, deletion in the overlapping region between the probes a3 and a4 were analyzed, since the region appeared to contain specific binding sequences.

4.5.1. *Construction of pD49A4⁺ derivatives*

There are three restriction sites within the insert of pD49A4⁺: *Vsp*I, *Dra*I and *Bsm*FI. Since there is more than one site for each of these endonucleases in pD49A4⁺, partial digestion was used to generate the desired fragments. The pD49A4⁺ DNA was first cut at the unique *Eco*RI site at one end of the insert, then the DNA was partially digested by *Vsp*I, *Dra*I or *Bsm*FI individually. Resulting fragments were cut out from agarose gel and purified. Three pD49A4⁺-derived plasmids were constructed by religating the purified fragments following partial or full end-filling. These new constructs, pD49A4v, pD49A4d and pD49A4b, contained different size of the inserts. Three smaller probes, A4-1, A4-2 and A4-3, were produced by cutting the plasmids with *Bam*HI and *Hin*dIII (Table 10).

Table 10. List of DNA probes.

Probe	Other name	Location[a]	Carrier plasmid[b]	Source
a1	PE311	-1187 to -883	pKX	*PR10.1*
a2	EP334	-882 to -549	pKX	*PR10.1*
a3	DE312	-548 to -237	pD49A4$^{+/-}$	*PR10.1*
a4	AB367	-284 to 79	pD49A4$^{+/-}$	*PR10.1*
a5	BA287	80 to 407	pKX	*PR10.1*
a6	BB425	367 to 832	pD49A6$^{+/-}$	*PR10.1*
A4	AA424	-360 to 64	pD49A4$^{+/-}$	*PR10.1*
A4-1	BH197	-360 to -190	pD49A4v	*PR10.1*
A4-2	BH150	-360 to -237	pD49A4d	*PR10.1*
A4-3	BH102	-360 to -285	pD49A4b	*PR10.1*
21S	NA	-284 to -267	oligo DNA	*PR10.1*
21M	NA	-284 to -256	oligo DNA	*PR10.1*
21L	NA	-284 to -237	oligo DNA	*PR10.1*
22S	NA	-255 to -237	oligo DNA	*PR10.1*
c1	PS319	-1149 to -831	pCC2	*PR10.3*
c2	VP209	-830 to -622	pD49C2^{-}	*PR10.3*
c3	BV426	-621 to -196	pD49C3^{-}	*PR10.3*
c4	S450	-252 to 195	pCC2	*PR10.3*
c5	S251	196 to 446	pCC2	*PR10.3*
c6	BB324	420 to 743	pCC2	*PR10.3*
c7	BB302	744 to 1135	pD49C7^{-}	*PR10.3*
L9	c3L9	-621 to -290	pD49C3L9	*PR10.3*
L9-1	c3L9-1	-439 to -360	pD49C3L9	*PR10.3*
L9-2	c3L9-2	-621 to -440	pD49C3L9	*PR10.3*
L11	c3L11	-621 to -410	pD49C3L11	*PR10.3*
R1	c3R1	-544 to -196	pD49C3R1	*PR10.3*
R4	c3R4	-464 to -196	pD49C3R4	*PR10.3*
R4A	c3R4A	-464 to -440	pD49C3R4	*PR10.3*
R6	c3R6	-452 to -196	pD49C3R6	*PR10.3*
R8	c3R8	-448 to -196	pD49C3R8	*PR10.3*
R9	c3R9	-331 to -196	pD49C3R9	*PR10.3*

"a": Relative location in reference to the translation start site;
"b": "+" the insert is in the same orientation of the gene; "-" the insert is in the opposite orientation of the gene; "+/-" both orientations of constructs are available.
"NA": not applicable.

4.5.2. *Binding sequences in* PR10.1 *promoter region*

Deletion probes A4-1, A4-2 and A4-3, covering the a3/a4 overlap region, were constructed as illustrated in Figure 7. These probes were incubated with a wide range of nuclear extracts as summarized in Table 11. A4-1 reacted with almost all nuclear extracts tested. A4-3 did not react with any nuclear extracts. The A4-2 probe reacted only with the nuclear extracts from *F. solani* f. sp. *phaseoli* or salicylic acid treatments, Fsph2 and SA36, respectively (Figure 8A, summarized in Figure 7). Since A4-1 completely overlapped both A4-2 and A4-3, the different binding results between A4-1 and A4-2 revealed that there were at least two distinct binding sequences in the A4-1 fragment, designated here as PDA1 (Pea Defense gene A element 1) and PDA2 (Figures 7 and 9A). The A4-1 probe contains both PDA1 and PDA2, A4-2 contains only PDA2, while A4-3 does not contain any binding sequences. Since PDA2 exclusively reacted with the nuclear extracts treated with *F. solani* f. sp. *phaseoli* or salicylic acid, it appears to contain *cis*-regulatory elements inducible in pea defense responses.

In order to confirm that the binding activities associated with PDA2 are highly specific, competition gel shift assays, similar to those presented in Figure 5, were performed with several DNA competitors. The results showed that the binding activity was eliminated by *PR10.1* genomic clone pKX, A4-1 and A4-2, but not A4-3, which does not cover PDA2 (data not shown). These results indicate that PDA2 is specifically bound by some protein factors related with fungal treatments.

Figure 7. Deletion analysis of the probe A4 from *PR10.1*. The probes A4-1, A4-2 and A4-3 were generated from A4 and examined in gel shift assays. Numbers represent the sequence location relative to the translation start site. Prb is abbreviation for "probe". Fsph represents Fsph2 and SA36. Ck stands for all the tested nuclear extracts except Fsph and SA treatments (for more detail see Tables 8 and 11). "+" refers to presence of a shifted band; "-", no shifted band was seen. The two binding sequences revealed by this experiment are designated PDA1 and PDA2.

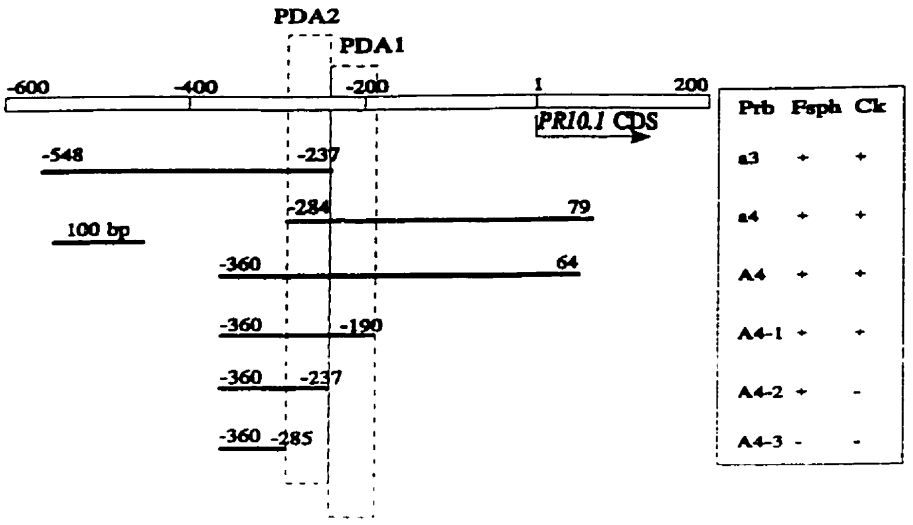


Figure 7. Deletion analysis of the A4 probe from *PR10.1*.

Table 11. Extended survey for deleted probes from *PR10.1* and *PR10.3*.

Extract	A4 (*PR10.1*)				c3 (*PR10.3*)			
	A4	A4-1	A4-2	A4-3	c3	R1	R4	R9
Probe	-*	-	-	-	-	-	-	-
BSA	-	-	-	-	-	-	-	-
UPP		-	-			-	-	-
H2	+	++	-	-	+			
W2	+	++	-	-	+			
W6		+	-	-	-			
W24		+	-	-	-			
CH8		++	-	-	-	-	-	-
Fsp2	++	++	-	-	++	+	-	-
Fsp6			-	-	-	-	-	-
Fsp24			-	-	-	-	-	-
Fsph0.5		-	-			-	-	-
Fsph1		-	-			-	-	-
Fsph2	++	+++	+++	-	++	++	-	-
Fsph4		+++	+	-	+++	++	-	-
Fsph6		+	-	-	++	-	-	-
Fsph24		+	-	-	-	-	-	-
SA6		-	-			-	-	-
SA36	++	+++	++	-	++	++	-	-
H2TP		+	-	-				
Fsp6TP		+	-	-				
Fsph6TP		+	-	-				
SA36TP		+	+	-				

*: "-" no visible band; "+++" strong band; "++" moderate band; "+" weak band.; "blank" not tested.

To pinpoint the binding sites within PDA2, DNA footprinting was attempted, but no footprints were obtained. As an alternative strategy, as previously reported (Korfhage *et al.*, 1994), competition gel shift assays were performed with several double stranded synthetic oligonucleotides as competitors to identify protein-binding sites. Results are summarized in Figure 8. The specific binding band was competed out by the competitors 21M and 21L but not 21S. The competitor 22S eliminated the binding band only at high concentration (200-fold molar excess) (Figure 8C). Since both 21M and 22S eliminated the binding band, there should be two binding sites within PDA2, one in 21M and the other in 22S (renamed PDA2b). Since 21S was part of 21M and there was no binding site within 21S, the binding site in 21M must be within the sequence (AATTTTGTGAGT), named PDA2a. It is highly possible that the same protein factor bound to two different sites, with strong binding with a site in PDA2a and weak binding with the other site in PDA2b. With computer analysis, it was found that there were a pair of 8-bp inverted repeats and a pair of 8-bp direct repeats in PDA2a and PDA2b, each of which harbors one leg of the repeats, respectively (Figure 17B). Thus, it was believed that one of such repeats should be related with the binding activity which occurred in both PDA2a and PDA2b (see detail in Section 5.2.).

Figure 8. Binding sequences in PDA2 from *PR10.1*. The labelled probe was A4-2 which covers PDA2. A) gel shift assays between A4-2 and six nuclear extracts (CH8, chitosan 8 h treatment; Fsp2, *F. solani* f. sp. *pisi* 2 h; Fsph2, *F.* solani f. sp. *phaseoli* 2 h; SA36, salicylic acid 36 h; W2, water 2 h; and H2, split pea pods 2 h). B) Location of DNA competitors 21L, 21M, 21S and 22S, and two inferred binding sequences PDA2a and PDA2b. C) The competition gel shift assay between A4-2 and the Fsph2 nuclear extract. Arrow→: the shifted band; ck: the normal gel shift assay without any competitors. The lanes missing the shifted band indicate that the specific binding was blocked by the DNA competitors.

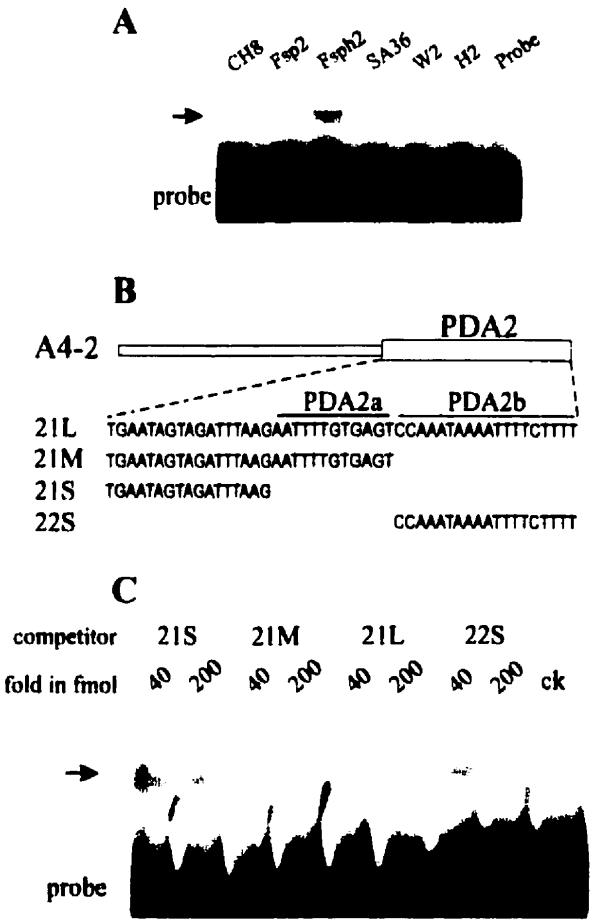


Figure 8. Binding sequences in PDA2 from *PR10.1.*

4.6. Deletion analysis to narrow down binding region in *PR10.3*

There were also two major binding regions in *PR10.3*, one in the upstream promoter region, the other downstream of the coding sequence. pD49C3$^+$, which contains the c3 probe and covers the upstream binding region in *PR10.3*, was selected and analyzed further. Nested deletion analysis was applied within the entire range of the c3 insert.

4.6.1. *Construction of pD49C3$^+$ derivatives*

pD49C3$^+$ was deleted by Erase-a-Base System (Promega) following the manufacture's instructions. One set (right, R) of nested deletions was from *ClaI*/*ApaI* sites on pD49C3$^+$. The other set (left, L) was from *PstI*/*Eco*RI sites. Two clones from the left deletion: L9 and L11, and eight clones from the right deletion: R1, R2, R3, R4, R6, R8 and R9, were selected and sequenced from the T7 primer on pBluescript KSm13$^+$ (Table 9).

Table 12. Deletion analysis for *PR10.3* promoter region (c3).

Probe	Location	Nuclear extract						
		W2	Fsph2	Fshp4	Fsp2	Fsp6	CH8	SA36
L9	-621 to -290	-*	+++	+++	+	-	-	+++
L11	-621 to -410	-	++	++	+	-	-	++
L9-2	-621 to -440	-	+	++	+	-	-	++
L9-1	-439 to -360	-	-	-	-	-	-	-
R1	-544 to -196	-	+	++	+	-	-	++
R4	-464 to -196	-	-	-	-	-	-	-
R6	-452 to -196	-	-	-	-	-	-	-
R8	-448 to -196	-	-	-	-	-	-	-
R9	-331 to -196	-	-	-	-	-	-	-
R4A	-464 to -440	-	-	-	-	-	-	-

*: "+++" one strong band; "++" one moderate band; "+" one visible band.; "-" no visible band.

4.6.2. *Binding sequences in* PR10.3 *promoter region*

A total of ten probes, L9, L9-1, L9-2, L11, R1, R4, R4A, R6, R8 and R9, were prepared from the pD49C3^{+}-derived plasmids (Table 10). These DNA probes were tested in gel retardation assays with at least five nuclear extracts, Fsph2, Fsp2, SA36, CH8 and W2, as summarized in Figure 10B and Table 12. All nuclear extracts except CH8 and W2 showed bound bands with the probes L9, L11, L9-2 and R1, but not with the probes L9-1, R4, R6, R8, R9 and R4A. Quantitatively, however, Fsph2 and SA36 exhibited much stronger bands than Fsh2. Figure 10A shows an example of the binding reactions between the L9-2 probe and the tested nuclear extracts. As shown in the figure, L9-2 had a strong shifted band with both Fsph2 and SA36, a weak band with Fsp2, and no band with CH8 and W2. The binding region defined by these deletions in *PR10.3* is designated PDC1 (Pea Defense gene C element 1) (Figures 9B and 10B).

Figure 9. Binding regions in *PR10.1* and *PR10.3*.

A. Binding region PDA1 and PDA2 in *PR10.1*;

B. Binding region PDC1 from *PR10.3*.

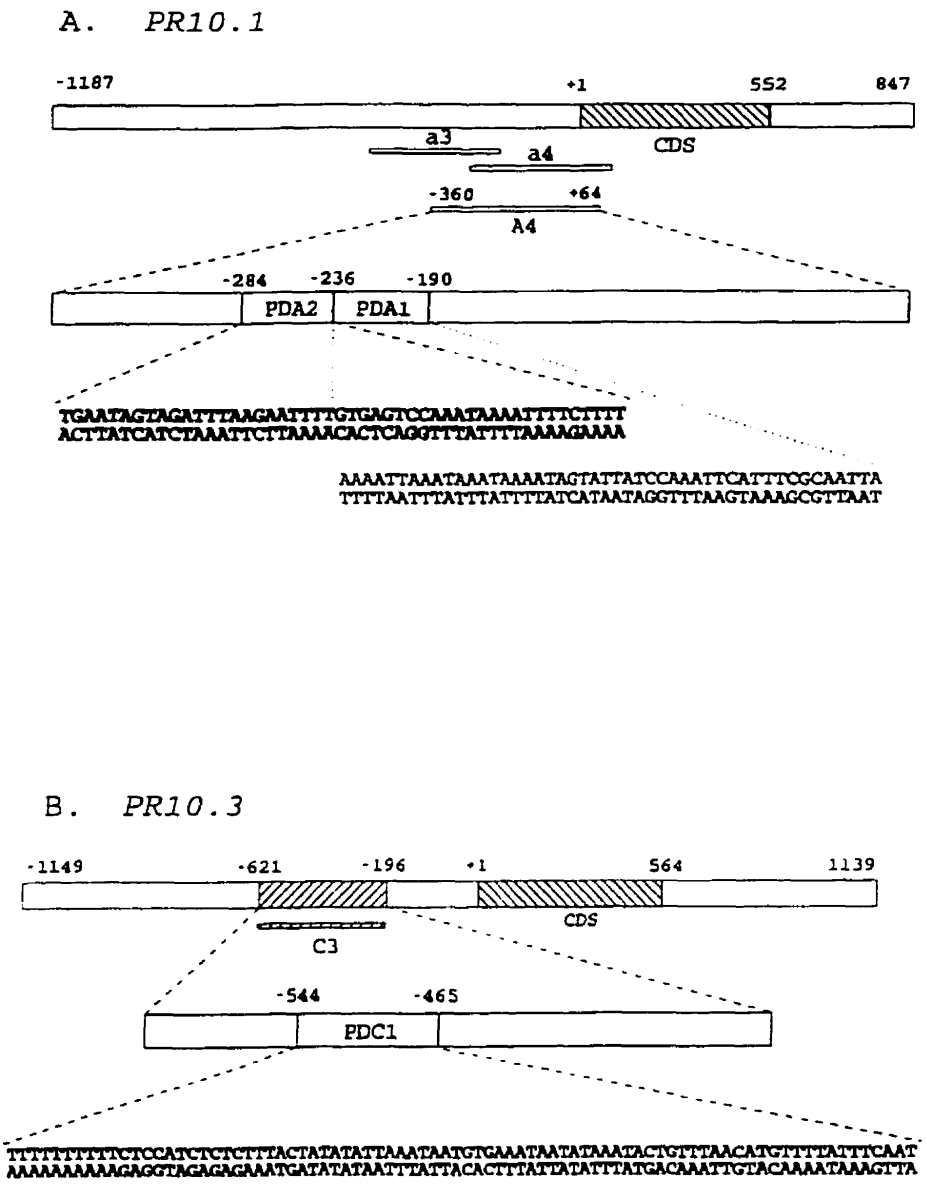


Figure 9. Binding sequences in *PR10.1* and *PR10.3*.

Figure 10. Deletion analysis of the probe c3 from *PR10.3*. A) Gel retardation assays between the probe L9-2 and the nuclear extracts. Arrow←, the shifted band. B) Summary of c3 deletion analysis. Numbers represent sequence locations relative to the translation start site. GRA, qualitative results between the individual probe and the nuclear extracts (see Table 12); +/-, presence or absence of the shifted band. The shifted bands are present in the probes L9, L11, L9-2 and R1, but absent in L9-1, R4, R6, R8, R9 and R4A, indicating that the binding sequence is located in the range from -544 to -465 (boxed with dashed line), which is assigned PDC1.

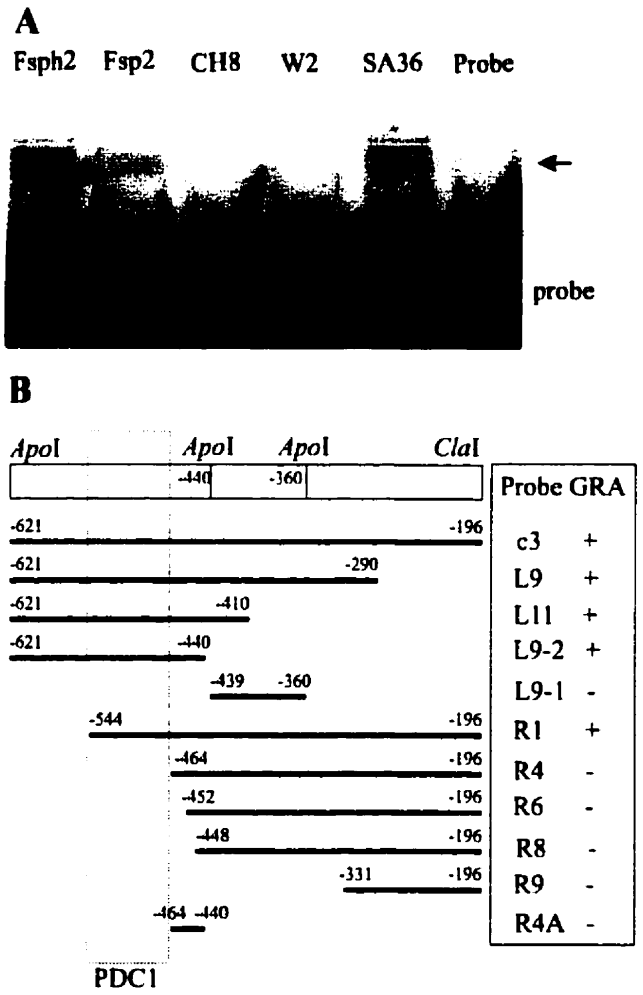


Figure 10. **Deletion analysis of the c3 probe from *PR10.3*.**

4.7. Effect of treatments of nuclear extract on binding reaction

4.7.1. *Phosphorylation and dephosphorylation*

Post-translational modifications of nuclear protein factors, such as phosphorylation or dephosphorylation, are often required for DNA binding activity or activation of gene expression. It was reported, for example, that phosphorylation was crucial for the nuclear factor PBF-1 to bind to the promoter DNA *in vitro* and to activate the potato *PR10a* gene in pathogen-treated plants (Després *et al.*, 1995). The protein kinase inhibitor staurosporine was found to completely block the transcriptional activation by fungal elicitors, indicating that protein phosphorylation is involved in the signal transduction pathway leading to *PRms* expression (Raventós *et al.*, 1995). Dephosphorylation enhanced the interaction between an ethylene-responsive element in *PRb-1b* and tobacco nuclear extract (Sessa *et al.*, 1995a). The expression of the *PR-1* gene in tobacco is also mediated by protein dephosphorylation (Conrath *et al.*, 1997).

To confirm the effect of phosphorylation or dephosphorylation in this study, the SA36 nuclear extract was treated as following before adding the a6 DNA probe. For investigation of phosphorylation effects, SA36 was treated with different combinations of $MgCl_2$ ATP/GTP (enhancing phosphorylation) and sodium fluoride (inhibitor of phosphatase) (Després *et al.*, 1995). Since nuclear extracts usually contain protein kinases, it is only necessary to add extra $MgCl_2$ ATP/GTP to enhance protein phosphorylation. To examine dephosphorylation, the nuclear extract was treated with alkaline phosphatase

(Roche, previously Boehringer Mannheim) and/or staurosporine (inhibitor of protein kinase, Sigma). The results showed that the binding of the pea SA36 nuclear extract to the a6 probe was not changed detectibly by protein phosphorylation or dephosphorylation (Figure 11). A similar result was achieved when the Fsph2 nuclear extract and the c3 probe were investigated (data not shown). These results suggest that the control of DNA binding by phosphorylation state is not a characteristic shared by all DNA-binding protein factors.

4.7.2. *Heat, detergent and proteinase treatments*

In the binding assay of the Fsph2 nuclear extract with the A4-2 probe, the nuclear extract was treated with SDS from 0.002% to 0.1% (final concentration) for 5 min before the probe was added. SDS concentrations as low as 0.002% eliminated binding activities. Heat treatment was performed five nuclear extracts (Fsph2, Fsph4, SA36, CH8 and UPP) and two DNA probes (A4-2 and A4-1). The nuclear extracts were heated at 70 °C for 5 min before adding the probes. Compared to normal binding assay, the heat treatment eliminated all the shifted bands. Proteinase K was used to treat the Fsph2 nuclear extract for 15 min at 37 ^{0}C before the A4-2 probe was added to the binding reaction mix. Proteinase K at a concentration of 0.1 μg/μl was able to eliminate the bound band (data not shown).

Figure 11. Effect of protein phosphorylation or dephosphorylation on binding reactions. The SA36 nuclear extract was treated before gel retardation was performed. Equal amounts of nuclear extract were used in each lane. All the treatment mixtures were incubated at 37 °C for 15 min before the a6 probe was added. Two different probes and nuclear extracts were examined at least twice (only one combination data showed). prb: free probe lane; arrow: the shifted band.

A) Phosphorylation. SA36 was treated with (+) or without (-) 20 mM $MgCl_2$ and 2 mM ATP/GTP (MgATP), 50 mM sodium fluoride (NaF), 5 nM staurosporine (ST). All the concentrations were final.

B) Dephosphorylation. SA36 was treated with (+) or without (-) 10 unit alkaline phosphatase, 5 nM staurosporine (ST), 50 mM sodium fluoride (NaF).

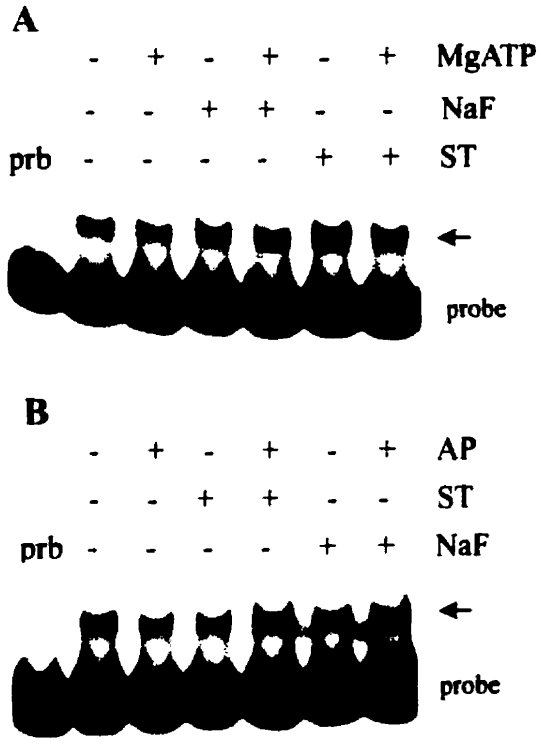


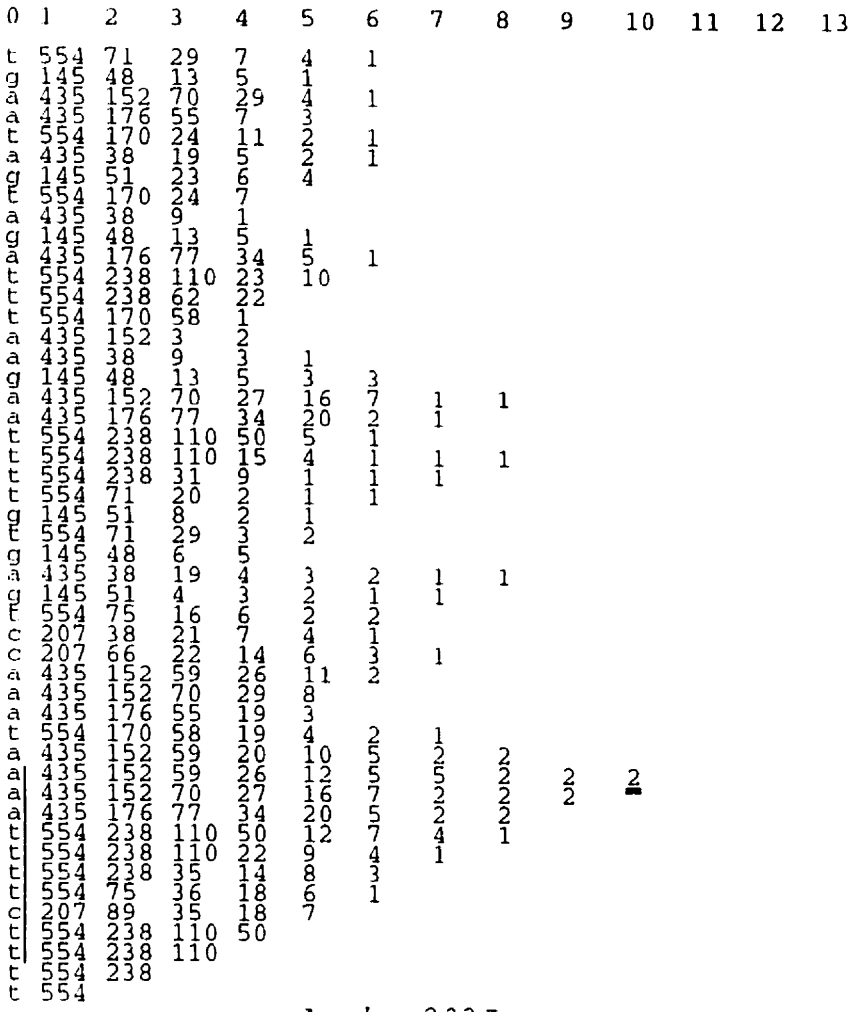
Figure 11. Effect of protein phosphorylation and dephosphorylation.

4.8. Conserved DNA motifs in binding sequences

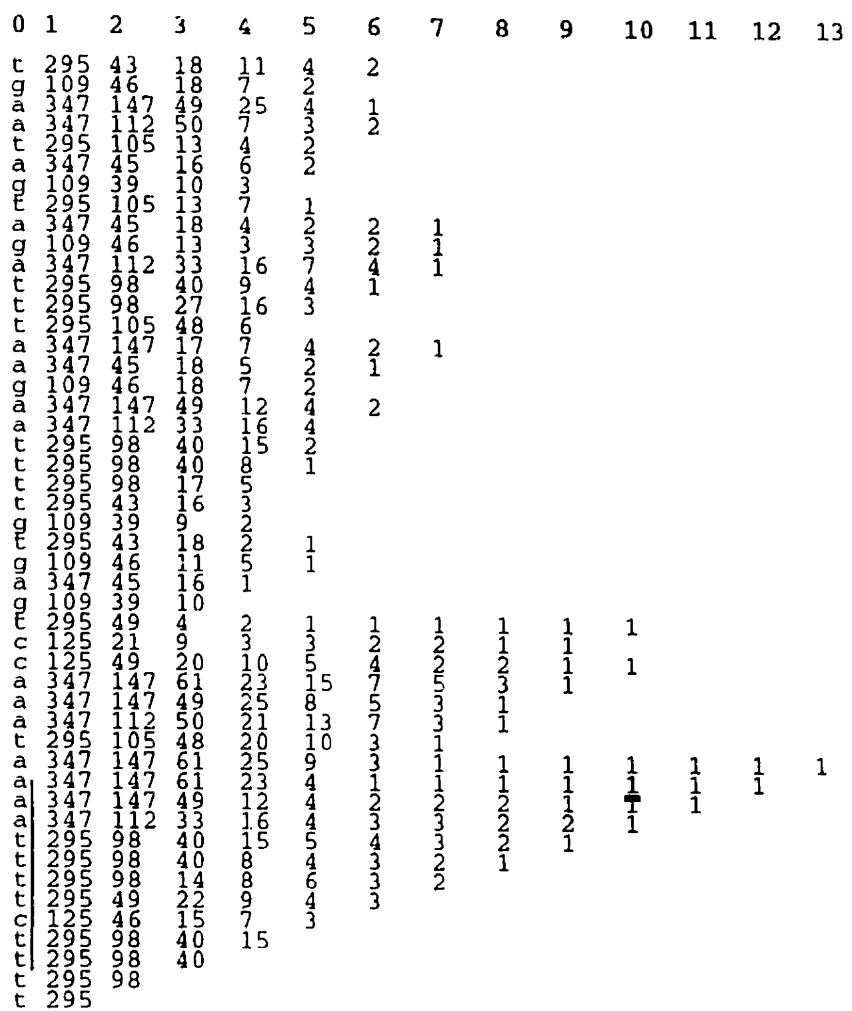
The XLAND program (Levy *et al.*, 1998) was applied to identify DNA motifs in pea *PR10.1* and *PR10.3* which are conserved in other defense gene promoters. Promoters used in the study were taken from a variety of plant defense gene families such as *PR1, PR2, PR5, PR10, PAL, osmotin* and the hypersensitive response gene *hrs203J*, for which promoter sequences were available in GenBank. A total of 147 sequence landscapes were generated by XLAND, with three binding sequences (PDA1, PDA2 and PDC1) against each of 49 plant defense-related gene promoters. Figure 12 shows landscape examples of PDA2 against the tobacco *hrs203J* gene (Pontier *et al.*, 1994) and the potato *STH-21* gene (Matton *et al.*, 1993). Forty-nine sequence landscapes for each binding sequence were manually aligned and compared. Ten DNA motifs in PDA1, PDA2 and PDC1 which are conserved among some of the defense gene promoters tested, were selected.

We wished to determine whether these DNA motifs in PDA1, PDA2 and PDC1 are unique to pea *PR10* genes, shared among *PR10* genes in other species, among defense genes, or commonly found in both defense genes and in genes not associated with defense responses. Therefore, two major promoter datasets were established: Defense Genes including two subdatasets, *PR10* Genes and Non-*PR10* Genes; and Non-Defense Genes including two subdatasets, Pea Genes and Non-Pea Genes. There was no overlap between datasets; each gene was represented only once across all the datasets (Table 13).

Figure 12. DNA sequence landscapes generated by XLAND. The target sequence and the source sequences are respectively PDA2 from *PR10.1* and the promoter regions of *hrs203J* (incompatible fungus-inducible gene in tobacco, GB::X77136)(A) and *STH-21* (*PR10b* in potato, GB::M29042)(B). The column "0", DNA sequence of PDA2 from 5′ to 3′ (top to bottom); Column 1 to 13, the length (bp) of DNA sequence patterns in PDA2; The numbers, frequencies of DNA sequence patterns occurred in the *hrs203J* (A) and *STH-21* (B) promoters; The double-underlined numbers "1" (B) and "2" (A) in Column 10 indicate that the *STH-21* and *hrs203J* promoters have 1 and 2 copies of the 10-bp DNA sequence (AAATTTTCTT) (a line at side), respectively. Notably, this 10-bp sequence contains the PDA2-2 DNA motif (AAATTTTC) which is conserved among defense-related genes (see Figure 15).



A. *hrs203J*



B. *STH-21*

Figure 12. DNA sequence landscapes generated by XLAND.

Table 13. List of genes (promoters) used in conserved motif analysis (in GenBank FEATURES format).

[Note: Automated extraction of genomic sequences 5′ to transcription start sites was performed by the FEATURES program (Fristensky, 1993) with the Feature Expressions listed in the table]

Defense Genes (49)

PR10 Genes (11)

AF002277:1..2088
AF002278:1..1372
J03680:1..1150
M29041:1..1477
M29042:1..876
U31669:1..1123
U48862:1..840
U48863:1..1082
X55736:1..790
X64452:1..1041
X96999:1..2449

Non-PR10 Genes (38)

AF017277:1..100
AJ001627:1..690
D10661:1..1493
D10662:1..1889
D76437:1..1354
J03679:1..1520
L77080:1..887
M59196:1..379
M63634:1..1646
M83314:1..1337
S68111:1..2051
U11716:1..1432
U89895:1..964
X05959:1..1451
X06361:1..407
X06930:1..335
X12572:1..840
X12737:1..902
X14065:1..715
X17680:1..401
X17681:1..416
X52555:1..701
X52556:1..306
X54325:1..73
X56012:1..478
X66942:1..850
X69794:1..1706
X72927:1..1062
X72928:1..1357
X76982:1..1309
X76983:1..653
X77136:1..1341
X78337:1..789
X96600:1..201
Y08844:1..994
Z15117:1..1555
Z26333:1..1239
Z48728:1..528

Non-Defense Genes (166)

Pea Genes (29)

AJ222771:1..905
D88261:1..785
L20976:1..1189
L36637:1..672
L41046:1..753
M31713:1..671
M37217:1..896
M64619:1..641
M73805:1..1018
M93051:1..894
U22971:1..848
U28925:1..1140
X00806:1..1085
X02433:1..628
X02982:1..1203
X03074:1..1034
X06398:1..561
X16082:1..812
X51594:1..684
X54844:1..733
X57665:1..583
X58024:1..671
X59015:1..1198
X69213:1..774
X78580:1..1339
X90996:1..1418
Y13322:1..998
Z18288:1..1397
Z23097:1..804

Non-Pea Genes (137)

Arabidopsis

D26508:1..974
D83257:1..1754
L15229:1..424
L22568:1..809
M17130:1..405
M17131:1..368
M17132:1..605
M17133:1..608
M58380:1..571
M58381:1..558
U01843:1..764
U02069:1..73
U08315:1..354
U12126:1..138
U33932:1..903
U53856:1..1352

Brassica

AB000970:1..236
AF016009:1..695
AF016010:1..658
AF016011:1..691
AF036386:1..92
AF052241:1..2074
AF055707:1..745
D13987:1..893
D88192:1..3284
D88193:1..2110
M64632:1..2152
M83332:1..1454
M83334:1..314
M95835:1..330
U14665:1..1338
U55032:1..2073
U76555:1..995
U77666:1..1832
U86642:1..818

Maize

AB001387:1..966
J01238:1..221
L05934:1..2582
L26305:1..101
L29418:1..1153
M13377:1..350
M13379:1..526
S42508:1..626
S59780:1..685
S94466:1..1650
U09989:1..1446
U20450:1..1318
V01472:1..459
X05068:1..1758
X12564:1..187
X12872:1..801
X15596:1..2505
X53514:1..1710
X55314:1..1291
X55726:1..1543
X67203:1..1461
X68678:1..1062
Y09238:1..81
Z11488:1..211
Z26824:1..693
Z34465:1..464

Parsley

A22706:1..371
M77494:1..474
M77495:1..474
Z33878:1..1566
Z54183:1..5001

Potato

D17332:1..254
L22576:1..921
M63642:1..201

Rice

AF013581:1..606
D16685:1..2502
D49551:1..1588
D50307:1..135
D86744:1..1527
L10346:1..263
L19434:1..1497
M36469:1..639
U07338:1..1056
U12171:1..1275

Tobacco

AJ223328:1..631
AJ223329:1..1111
AJ223330:1..542
D11111:1..845
D11396:1..316
D11469:1..668
D11470:1..1594
D42070:1..1090
D49526:1..1675
D49804:1..553
D83696:1..349
J04972:1..245
L10114:1..366
M16896:1..349
M21397:1..437
M21398:1..979
M94204:1..344
X14059:1..965

Tomato

AF004878:1..300
AF004879:1..204
D11112:1..3351
L22189:1..1018
L25128:1..2896
L26529:1..421
L34171:1..2402
L40938:1..189
L41253:1..512
M14443:1..191
M14444:1..203
M32605:1..294
M32606:1..347
M63642:1..201
U03391:1..414
U04336:1..779
U28795:1..1424
U28796:1..1366
U42444:1..1677
U42445:1..1639
U59317:1..258
U64789:1..2271
U64790:1..187
U70675:1..373
U70676:1..420

Wheat

D13795:1..992
D16415:1..1238
D37944:1..1637
D37945:1..1298
D38111:1..2010
D87064:1..848
D87065:1..850
L75802:1..1790
M16842:1..145
M22208:1..1260
M22209:1..501
M95500:1..88
U08287:1..2834
U19774:1..568
U51307:1..596

XLAND was applied again to generate sequence landscapes of all ten DNA motifs against each of the four datasets and automatically retrieve frequencies of the 10 DNA motifs which occurred in the datasets. Out of ten motifs, at least four conserved motifs had higher presence frequency (at least more than 2-fold) in one dataset category than the compared dataset. These include PDA1-1 (AAATAAATA), PDA2-1 (ATAAAATT), PDA2-2 (AAATTTTC) and PDC1-2 (TTTTATTT) (Table 14). Statistically, the random occurrence frequency of a sequence pattern follows this formula:

$$f\ (n\text{-mer}) = 1 / 4^n$$

Where n is the bp number of the sequence pattern. Therefore, 7-bp, 8-bp and 9-bp sequences would be expected to be represented once per 16-kb, 66-kb and 262-kb, respectively.

PDA1-1 was most conserved in pea genes promoters, having 4.3 repeats/10-kb, and only 0.9 repeats/10-kb in non-pea genes. The conserved motif (AAATAAATA) was aligned for PDA1-1 among pea genes (Figure 13). PDA1 had the following long matched sequences with certain pea genes: the 12-bp sequence (AAATAAATAAAA) in *PsCHS2* (An *et al.*, 1993), *Lox1.3* (Knox *et al.*, 1994) and *GS2* (Tjaden *et al.*, 1995); the 14-bp sequence (AAATTAAATAAATA) in *gdcT* (Vauclare *et al.*, 1998); and the 15-bp sequence (AATTAAATAAATAAA) in *as1* (GN::Y13321). Notably, all four motifs in the PDA1 binding sequence were conserved among pea genes, since these motifs had 2.7-fold higher average frequencies in pea genes than in non-pea genes.

Table 14. Occurrence frequencies of DNA motifs in defense and non-defense genes.

Motif	Dataset											
	Defense Gene						Non-Defense Gene					
	PR10 (8 kb)[a]		Non-*PR10* (43 kb)		Total (51 kb)		Pea (26 kb)		Non-Pea (116 kb)		Total (142 kb)	
	Repeat	No./10k	Repeat	No./10k	Repeat	No./10k	Repeat	No./10k	Repeat	No./10k	Repeat	No./10k
PDA1-1 (AAATAAATA)	2[b]	2.5[c]	9	2.1	11	2.1	11	**4.3**[d]	10	**0.9**	21	1.5
PDA1-2 (ATAAAATA)	4	5.0	20	4.6	24	4.7	11	4.3	24	2.1	35	2.5
PDA1-3 (AAATTCA)	4	5.0	12	2.8	16	3.1	8	3.1	20	1.7	28	2.0
PDA1-4 (TTCATTT)	5	6.3	19	4.4	24	4.7	15	5.8	34	2.9	49	3.5
PDA2-1 (ATAAAATT)	5	**6.3**	12	**2.8**	17	3.3	5	1.9	19	1.6	24	1.7
PDA2-2 (AAATTTTC)	1	1.3	8	1.8	9	**1.8**	1	0.4	10	0.9	11	**0.8**
PDA2-3 (AAATAAAA)	7	8.8	28	6.4	35	6.8	17	6.6	51	4.4	68	4.8
PDC1-1 (TATAAATA)	5	6.3	30	6.9	35	6.8	9	3.5	40	3.4	49	3.5
PDC1-2 (TTTTATTT)	5	**6.3**	7	**1.6**	12	2.3	10	3.9	45	3.9	56	3.9
PDC1-3 (ATTTCAA)	4	5.0	17	3.9	21	4.1	5	1.9	28	2.4	33	2.3

Note: "a" the number in round bracket is the size of dataset in kilo-basepair (kb); "b" frequencies (absolute repeat numbers) of DNA motifs occurred in dataset; "c" frequency ratio (relative repeat numbers) of DNA motifs occurred in dataset after normalization (frequencies/10-kb); "d" underlined numbers were used to identify DNA motifs which occur more than twice as often in one dataset versus the other dataset.

Figure 13. Conserved consensus PDA1-1 (*AAATAAATA*) in pea gene promoters. The first column (LOCUS), the GenBank entry name; The second column (GENE), the gene name; The third column (LOCATION), the beginning of the fragment (the last column), referring to the transcription start site (the number in round bracket refers to the beginning of the fragment in GenBank entry); The last column (MATCHED SEQUENCE), the fragments containing the PDA1-1 conserved motif (*italic*); Underlined, the sequence matched with the PDA1 binding sequence; Double underlined, sequences of the PDA1-1, PDA1-2, PDA1-3 and PDA1-4 motifs in the PDA1 binding sequence, respectively.

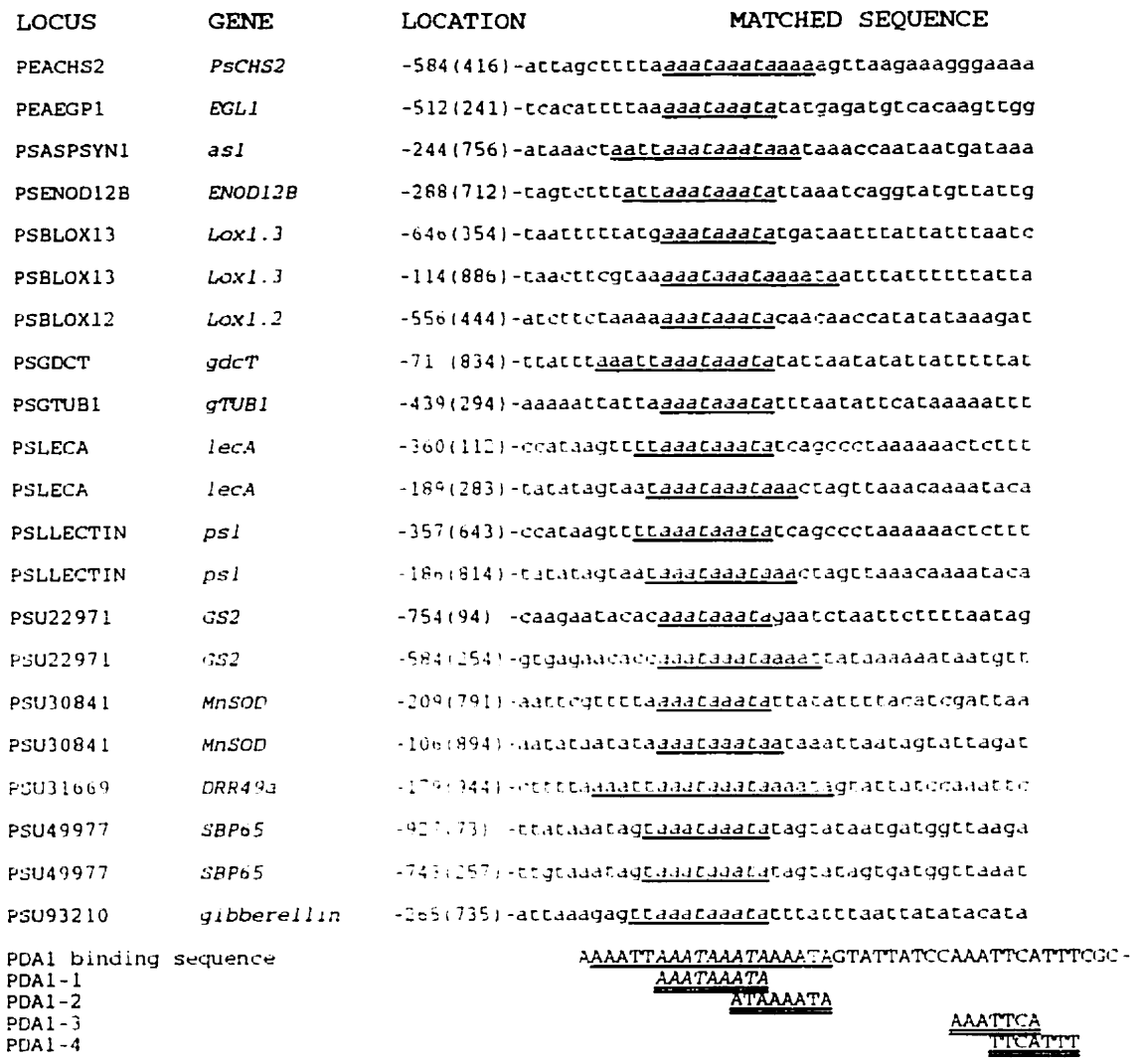


Figure 13. Conserved consensus PDA1-1 (*AAATAAATA*) among pea genes.

Figure 14. Conserved consensus PDA2-1 (*ATAAAATT*) in defense-related genes. The first column (LOCUS), the GenBank entry name; The second column (GENE), the gene name; The third column (LOCATION), the beginning of the fragment (the last column), referring to the transcription start site (the number in round bracket refers to the beginning of the fragment in GenBank entry); The last column (MATCHED SEQUENCE), the fragments containing the PDA2-1 conserved motif (*italic*); Underlined, the sequence matched with the PDA2 binding sequence; Double underlined, the sequences of the PDA2-1, PDA2-2 and PDA2-3 motifs in the PDA2 binding sequence, respectively.

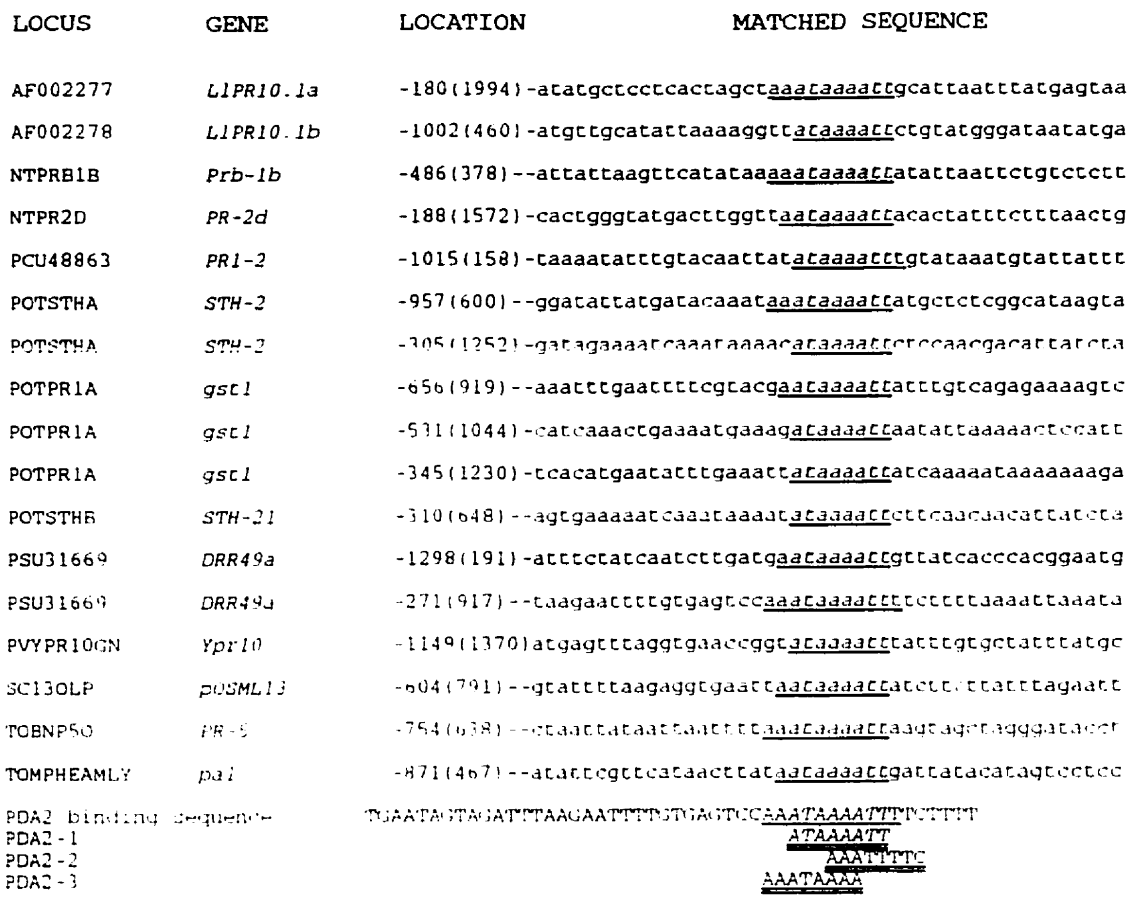


Figure 14. Conserved consensus PDA2-1 (*ATAAAATT*) in defense-related genes.

PDA2 contained several partly overlapping motifs which were conserved among defense gene promoters (Figures 14 and 15). PDA2-1 occurred at the rate of 6.3 repeats/10-kb in *PR10* genes, compared to 2.8 repeats/10-kb in non-*PR10* genes and 1.7 repeats/10-kb in non-defense genes. The motif (ATAAAATT) was aligned for PDA2-1 among defense genes (Figure 14). PDA2-1 occurred two or more times in some defense gene promoters: twice in *DRR49a* (*PR10.1*) and *STH-2* (*PR10a*) (Matton *et al.*, 1993), three times in *gst1* (*prp1-1*) (Taylor, *et al.*, 1990; Strittmatter *et al.*, 1996). A related motif (AAATAAAATT), which is 2-bp longer than PDA2-1, was present among five PR genes: *DRR49a*, *PR-5* (Sato *et al.*, 1996), *Prb-1b* (Eyal *et al*, 1992), *STH-2* and *LlPR10.1a* (Sikorski *et al.*, 1998).

PDA2-2, which partially overlaps PDA2-1, was conserved in defense gene promoters, occurring at 1.8 repeats/10-kb, compared to only 0.8 repeats/10-kb in non-defense genes (Table 14). The motif (AAATTTTC) was found among eight defense genes (Figure 15). PDA2-2 was represented as two copies in *hrs203J* (Pontier *et al.*, 1994) and a 13-bp matched sequence in *STH-21* (Matton *et al.*, 1993), (AAAATTTTCTTTT).

PDC1-2 was also conserved among *PR10* genes, having 6.3 repeats/10-kb, with only 1.6/10-kb in non-*PR10* genes and 3.9/10-kb in non-defense genes (Table 14). The PDC1-2 motif (TTTTATTT) was shared among defense genes (Figure 16). *hrs203J* and *osmotin* (Raghothama *et al.*, 1993) had two copies of PDC1-2. *Ypr10* (Walter *et al.*, 1996) had three copies of PDC1-2 within less than 700-bp promoter sequence.

Figure 15. Conserved consensus PDA2-2 (*AAATTTTC*) in defense-related genes. The first column (LOCUS), the GenBank entry name; The second column (GENE), the gene name; The third column (LOCATION), the beginning of the shown fragment (the last column), referring to the transcription start site (the number in round bracket refers to the beginning of the fragment in GenBank entry); The last column (MATCHED SEQUENCE), the fragments containing the conserved PDA2-2 motif (*italic*); Underlined, the sequence matched with the PDA2 binding sequence; Double underlined, the sequences of the PDA2-1, PDA2-2 and PDA2-3 motifs in the PDA2 binding sequence, respectively.

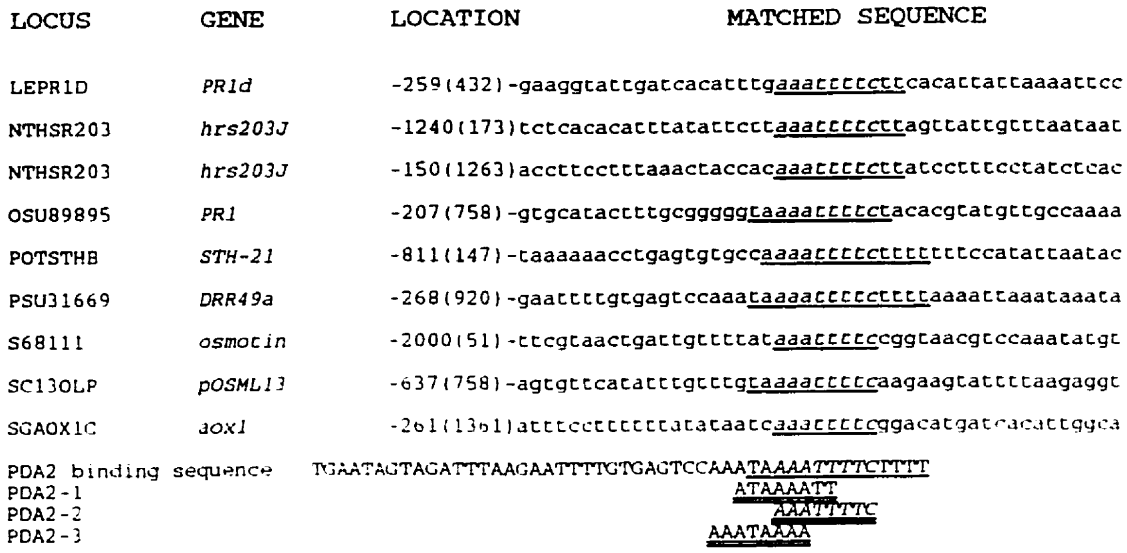


Figure 15. Conserved consensus PDA2-2 (*AAATTTTC*) in defense-related genes.

Figure 16. Conserved consensus PDC1-2 (*TTTTATTT*) in defense-related genes. The first column (LOCUS), the GenBank entry name; The second column (GENE), the gene name; The third column (LOCATION), the beginning of the shown fragment (the last column), referring to the transcription start site (the number in round bracket refers to the beginning of the fragment in GenBank entry); The last column (MATCHED SEQUENCE), the fragments containing the conserved motif PDC1-2 (*italic*); Underlined, the sequence matched with the PDC1 binding sequence; Double underlined, the sequences of the PDC1-1, PDC1-2 and PDC1-3 motifs in the PDC1 binding sequence, respectively.

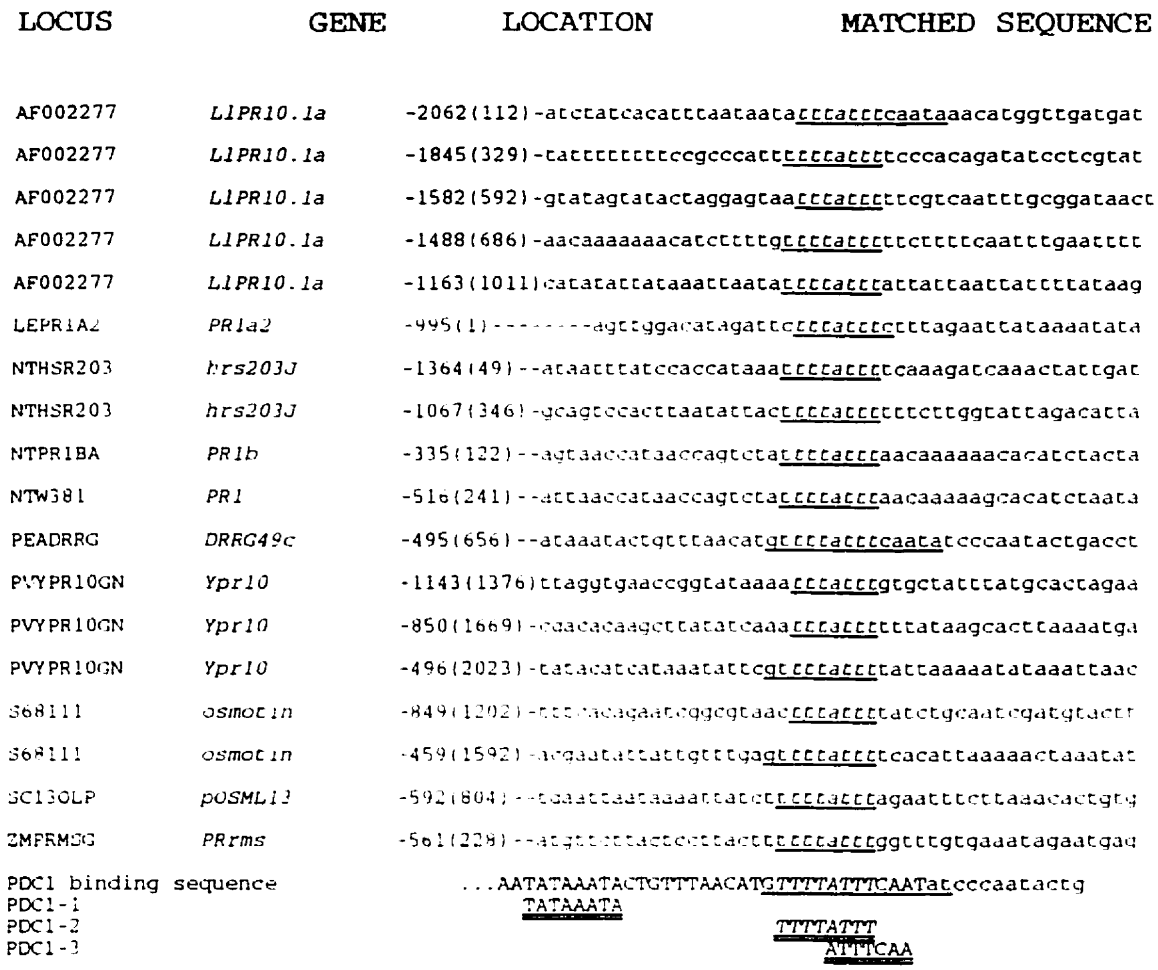


Figure 16. Conserved consensus PDC1-2 (*TTTTATTT*) in defense-related genes.

LlPR10.1a had five repeats of PDC1-2 in a 900-bp promoter region and one of the copies had a 12-bp matched sequence with PDC1-2 and its adjacent sequence. In contrast, *L1PR10.1b* (Sikorski *et al.*, 1998), which was in the same multigene family as *L1PR10.1a*, did not have any matched sequence with PDC1-2.

4.9. Characteristics of three binding sequences

Cis-regulatory elements frequently contain certain sequence characteristics, such as inverted repeats or direct repeats. For instance, a fungus-specific *cis*-acting element contains a direct repeat of (GTCAG) separated by three nucleotides (Fukuda & Shinshi, 1994). Promoter activity of a flower-specific and UV-inducible element is associated with the direct repeats (TACPyAT) (van der Meer *et al.*, 1990). A 38-bp *cis*-element in the pea seed storage gene *legA* contains two 17-bp direct repeats (Howley *et al.*, 1997). All of the following *cis*-elements contain inverted repeat sequences (underlined): the G-box (CCACGTGG) in *Arabidopsis* (McKendree *et al.*, 1990) and in maize (Pla *et al.*, 1993); the AT-1 element (AATATTTTTATT) in pea (Datta *et al.*, 1989); the RY repeats (CATGCATG) in numerous legume seed-protein genes (Dickinson *et al.*, 1988); (TGAGTCA) in rice (Kim and Wu, 1990); CRE (TGACGTCA) in yeast (Nehlin *et al.*, 1992); the heat-shock element consensus (CTNGAANNTTCNAG) in plants (Hawkins, 1991); the ROS-box (TATATTTCATGTAATATA) in *Agrobacterium* (D´Souza-Ault *et al.*, 1993); the MJ-box (CCCTATAGGG) immediate upstream of a G-box in *Shpx6a* and *Shpx6b* (Curtis

et al., 1997); the as-1-like motif (ACGTCATCGAGATGACGGCC) in tobacco *PR1a* promoter (Strompen *et al.*, 1998); finally, the well-known conserved DNA motifs, the CAT-box (CCAAT) and the Y-box (ATTGG) in the proximal promoter of eukaryotic genes are inverted repeats to each other. Both inverted repeats, which are orientation-independent, and direct repeats are believed capable of increasing the efficiency of gene transcription.

While PDA1, PDA2, and PDC1 themselves are too large to be binding sites for *trans*-acting factors, computer analysis and oligonucleotide competition assays have identified several likely binding sites (Figure 17). PDA1 contains the pea-conserved motif PDA1-1 (AAATAAATA), the AT-1-like motif (3´-TTTATTTTAT-5´) and the CHS A motif (ATAGTA), which is involved in fungal elicitation and tissue-specific expression (Ito, *et al.*, 1997; Faktor *et al.*, 1996 and 1997).

PDA2 has two 8-bp imperfect inverted repeats [AATTTTGT$(N)_8$ATAAAATT] and [AAATAAAA$(N)_0$TTTTCTTT], and one pair of 8-bp imperfect direct repeats [AATTTTGT$(N)_{12}$AATTTTCT]. PDA2 also has the CHS A motif and the AT-1-like motif (3´-TTTATTTTAA-5´), the same as that found in PDA1. The *PR10*-conserved motif PDA2-1 (ATAAAATT) and the defense-gene-conserved motif PDA2-2 (AAATTTTC) are both located in PDA2.

PDC1 has one 8-bp perfect inverted repeat [TGAAATAA$(N)_{20}$TTATTTCA] and the TATA-box like motif PDC1-1 (TATAAATA). In addition, PDC1 contains the *PR10*-conserved motif PDC1-2 (TTTTATTT) and the defense-gene-conserved motif PDC1-3 (ATTTCAA).

Figure 17. Characteristics of the elements from *PR10.1* and *PR10.3*. All the sequences were numbered from the translation start site. →←, direct repeat; ←→, inverted repeat; bold, active sequence region where binding sites are expected. A). PDA1 element: lowercase, PDA1-1 (pea conserved); underlined, AT-1 like motif; italic, CHS A motif. B). PDA2 element: lowercase, PDA2-1 (*PR10* conserved); top lined, PDA2-2 (defense gene conserved); underlined, AT-1 like motif; italic, CHS A motif. C). PDC1 element: lowercase, PDC1-2 (*PR10* conserved); underlined, PDC1-3 (defense gene conserved); boxed, TATA-box like motif.

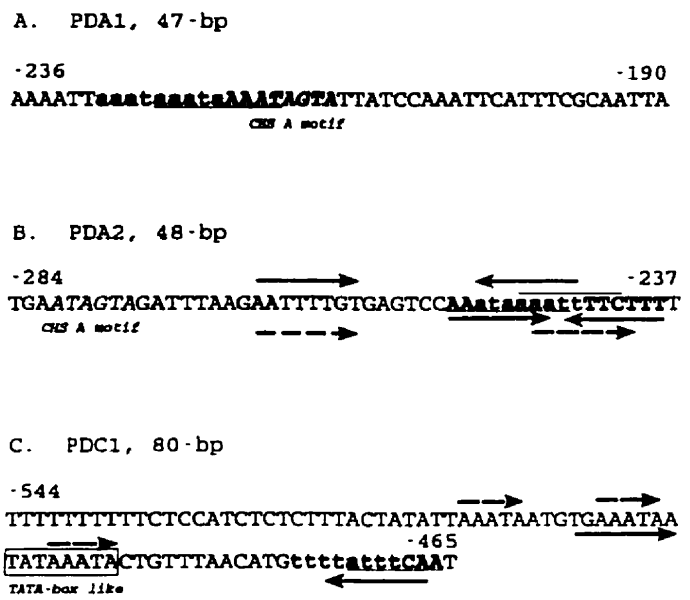


Figure 17. Characteristics of the elements from *PR10.1* and *PR10.3*.

4.10. Correlation of binding activity and *PR10* expression in pea

4.10.1. *Time course for binding activity in* PR10.1 *and* PR10.3

To elucidate the relationship between DNA/protein binding activity and *PR10* expression in pea, time courses for the binding activity were examined. The labelled probes were A4-2 from *PR10.1*, which contains the PDA2 binding sequence, and c3 and R1 from *PR10.3*, which contain the PDC1 binding sequence. The tested nuclear extracts were from three independent treatments: *F. solani* f. sp. *phaseoli, F. solani* f. sp. *pisi* and sterile water. Results showed that only *F. solani* f. sp. *phaseoli* challenge induced binding reactions with A4-2 (*PR10.1*). There were no binding activities within the range of time courses with *F. solani* f. sp. *pisi* treatment and water control. The binding peak related to the challenge of *F. solani* f. sp. *phaseoli* was at 2 h.p.i. and a weak binding activity at 4 h.p.i. After 6 h.p.i. the binding activity was not detectable (Figure 18A). Although the binding time course in *PR10.3* was similar to *PR10.1*, the binding activity of R1 (*PR10.3*) with Fsph4 was stronger than Fsph2 and R1 also had a weak binding reaction with Fsp2 (Figure 18B). The weak binding band between the c3 probe and water control at 2 h.p.i. was not related with the PDC1 binding sequence, since nested deletions of c3 had no binding reactions with water control (see details in Table 12 and Figure 10).

Figure 18. Time course for binding activity in *PR10.1* and *PR10.3*. A) *PR10.1*. The labelled probe was A4-2 which contains the PDA2 binding sequence. Lane 1 (from left) was free probe (prb); Lane 2 to 4, the pea nuclear extracts with water treatment at 2, 6 and 24 h.p.i.; Lane 5 to 9, *F. solani* f. sp. *phaseoli* treatment (Fsph) at 1, 2, 4, 6 and 24 h.p.i.; Lane 10 to 12, *F. solani* f. sp. *pisi* treatment (Fsp) at 2, 6 and 24 h.p.i. B) *PR10.3*. The labelled probes were c3 for the first 4 lanes from left and R1 for the rest of the lanes, both of which contain the PDC1 binding sequence. The nuclear extracts are the same as A, arrangement as indicated. The arrow shows the shifted band.

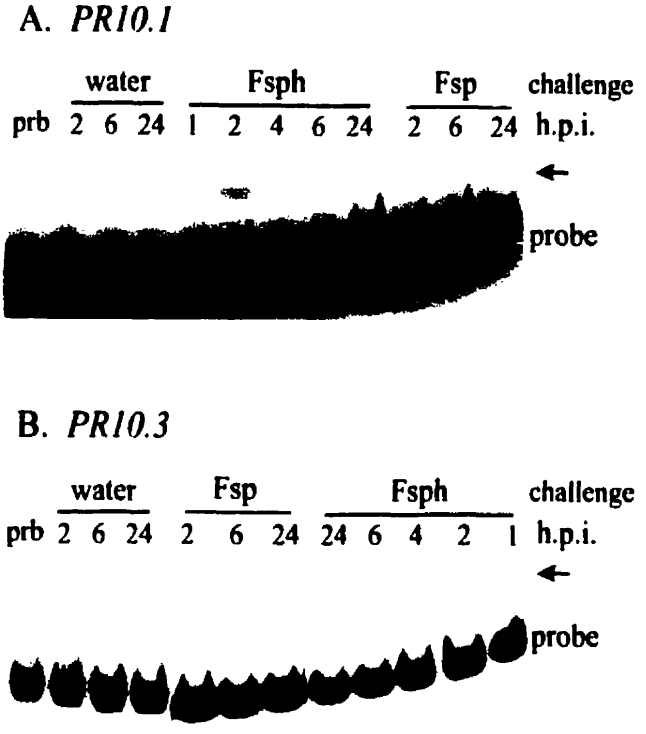


Figure 18. Time course for binding activity in *PR10.1* and *PR10.3*.

4.10.2. *Expression of* PR10.1 *and* PR10.3 *in pea*

Immature pea pods from *P. sativum* Alaska were individually treated with *F. solani* f. sp. *phaseoli, F. solani* f. sp. *pisi* and sterile water for times ranging from 0 to 48 h.p.i. Total RNA was extracted from each sample and RT-PCR was performed. *PR10.1* expression upon the fungal treatments was stronger than that in the water control from 0 to 48 h.p.i. At most times after inoculation with the incompatible fungus *F. solani* f. sp. *phaseoli*, expression of *PR10.1* was stronger than in pods inoculated with the compatible fungus *F. solani* f. sp. *pisi*. The *PR10.1* expression level in *F. solani* f. sp. *phaseoli* challenge was sustained after 12 h.p.i., while the *PR10.1* expression level in *F. solani* f. sp. *pisi* challenge gradually decreased (Figure 19A). In contrast, *PR10.3* was not expressed in pea pods treated with either *F. solani* f. sp. *phaseoli* or *F. solani* f. sp. *pisi* (Figure 19B).

To confirm the lack of *PR10.3* expression, healthy pea roots, buds and pods were prepared and the gene expression of *PR10.1* and *PR10.3* was investigated. Figure 20 showed that *PR10.3* was expressed exclusively in pea roots, but not in buds or pods. It was found that *PR10.1* was also strongly expressed in healthy roots, but very weakly in healthy buds and pods. These results are consistent with reports that *PR10.3* is exclusively expressed in root hair and root epidermal tissues from both healthy pea root and *Rhizobium* treated root (Mylona *et al,* 1994). Recently it was observed in this laboratory that *PR10.3* was also expressed exclusively in the roots of transgenic

Brassica when it was integrated with the GUS reporter gene (B. Fristensky, unpublished data). These results demonstrated that *PR10.1* was indeed induced strongly by fungal challenges in pea pods and also suggests that there might exist a down-regulation with *PR10.3* expression in aerial parts of pea plants.

Figure 19. Time course of *PR10* expression in pea (*P. sativum*) as measured by RT-PCR. For *PR10.1* (A) and *PR10.3* (B), the PCR product amplified from the internal control plasmid migrates at a higher molecular weight class than the mRNA-derived product. Curves represent relative expression signal (sample/control), as measured by chemiluminescence, averaged over three independent experiments. The X-axis represents hours post-inoculation (h.p.i.). The Y-axis represents an arbitrary scale (relative expression) normalized to the highest data point. Vertical lines indicate the standard error of the mean.

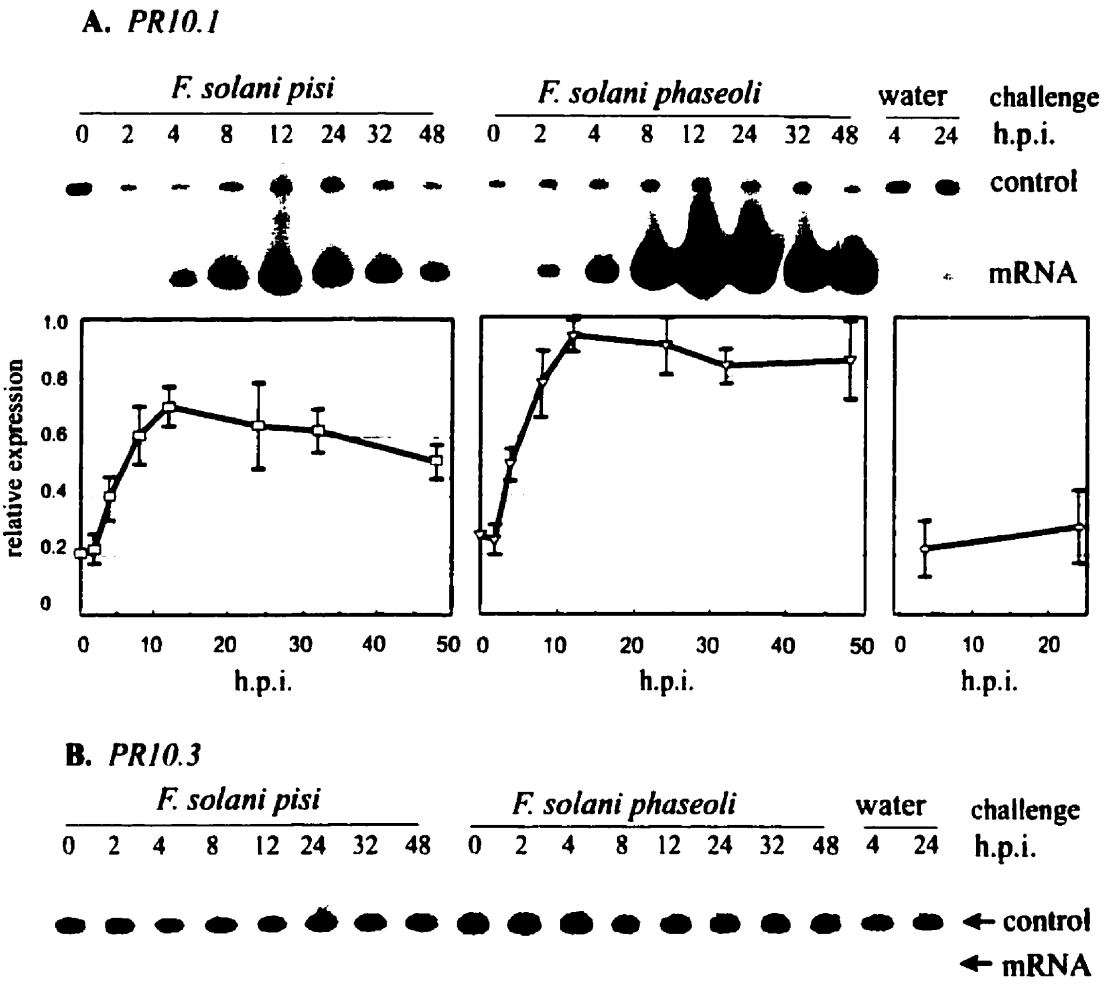


Figure 19. Time course of *PR10* expression in pea (*P. sativum*).

5. CONCLUSIONS AND DISCUSSIONS

5.1. Two major binding regions in both *PR10.1* and *PR10.3*

In this study I have investigated the DNA/protein binding activities between two pathogenesis-related genes of pea *PR10.1* and *PR10.3*, and various nuclear extracts in native pea plants. Compared to many studies in which only promoter areas were examined in a foreign plant, I personally think that a promoter study like this investigating the entire target gene in native plants is more convincing and precise. Both *PR10.1* and *PR10.3* contain two major binding regions. Not only do the proximal promoters in each gene have a binding region, but also the near downstream sequences of the genes have another major binding region. We did not observe binding activities elsewhere in the coding regions or introns of the genes as reported (Mascarenhas *et al.*, 1990; Yamamoto *et al.*, 1997). Since this study subsequently focused on the proximal promoter regions using deletion analysis, it is likely that besides the binding sequences we identified, *i.e.*, PDA1, PDA2 and PDC1, there are other binding sequences as well in two downstream regions or distal promoters. In particular, the binding sequence responsible for the higher bound band in *PR10.1* (Figure 4A) was not identified.

5.2. PDA2 contains a fungus-inducible *cis*-element

Many *cis*-elements have been reported to be associated with specific stresses, such

as virus (Van de Rhee *et al.*, 1993), fungus (Fukuda and Shinshi, 1994), wounding (Firek *et al.*, 1993), salinity or drought (Schaeffer *et al.*, 1995), UV light (Murakami *et al.*, 1997) and osmotic conditions (Lu *et al.*, 1998). It was also reported that *cis*-acting elements in *hsr203J* are differentially involved in compatible vs. incompatible plant/pathogen interactions (Pontier *et al.*, 1994). That PDA2 is bound only by nuclear extracts from pea pods treated with fungus *F. solani* f. sp. *phaseoli* and salicylic acid (Figures 8A and 18) suggests that PDA2 contains a fungus-inducible *cis*-regulatory element. There are many reports that salicylic acid is a signal that plays a central role in plant defense responses (Delaney *et al.*, 1994; Chen *et al.*, 1995), but may not be the original source (elicitor) for plant disease resistance responses (Chasan, 1995). Therefore, the induced-binding activity as well as the induction of *PR10.1* expression by *F. solani* f. sp. *phaseoli* may occur through a salicylic acid-mediated pathway. The fungal challenge is the only original elicitor for PDA2's binding specificity.

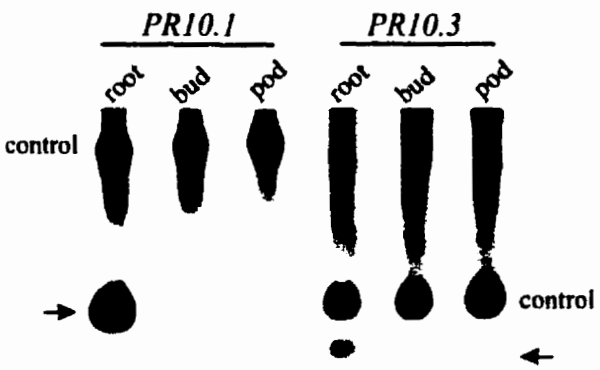
Although we were not able to pinpoint the precise binding sites in PDA2 by DNA footprinting, the protein-binding sequences were located by competition gel shift assays with synthesized oligonucleotide competitors as previously reported in *PR10* (*PcPR2*) multigene family in parsley (Korfhage *et al.*, 1994). The results suggest two binding sites within PDA2, PDA2a (AATTTTGTGAGT) and PDA2b (CCAAAtAAAATTTTcTTTT) (Figure 8B). It was observed that the binding affinity in PDA2a was much stronger than in PDA2b. We do not know yet whether there are two different protein factors binding to each independent site or if the same protein factor binds to two different sites, with stronger binding in PDA2a and weaker binding in PDA2b. Consistent with the latter

possibility is the observation that PDA2 contains one imperfect direct repeat (AATTTTGT/AATTTTCT) and one imperfect inverted repeat (AATTTTGT/ATAAAATT), each with one leg in each of PDA2a and PDA2b. We consider the alternative hypothesis, that PDA2 contains only a single binding site that overlaps the sequences we have designated as PDA2a and PDA2b to be unlikely, in view of the results in Figure 8. Each of the 21S and 22S fragments by themselves can outcompete the A4-2 probe. That is, both 21S and 22S have sequences that are necessary and sufficient for protein binding. If PDA2a and PDA2b are two distinct binding sites, then it is also likely that different proteins bind to each, since PDA2a and PDA2b are distinct sequences. A similar situation was reported by Hagiwara *et al.*, (1993) that one protein factor in tobacco bound to two independent *cis*-acting elements from *PR1a*. Presumably, if there is only one protein factor specific for PDA2 and it binds strongly to one leg of the repeats (AATTTTGT) in PDA2a, then it probably binds weakly to the other imperfect leg (AtAAAATT or AATTTTcT) in PDA2b because both the repeats are imperfect, with one unmatched nucleotide (in lowercase). Binding assays with substituted nucleotides would reveal the exact binding sequences for the fungus-inducible elements in PDA2a and PDA2b. For example, The unmatched nucleotides (t and c) in PDA2b could be replaced with matched nucleotides (C for t or G for c) to make perfect repeats. If the binding affinity in PDA2b is improved after the substitution, it could be concluded that one binding site in PDA2 is (AATTTTGT), which has one copy in each of PDA2a and PDA2b.

5.3. Binding activity correlates with gene expression

Previous studies of *PR10* gene expression in pea have not used gene-specific probes (Riggleman *et al.*, 1985; Fristensky *et al.*, 1985; Daniels *et al.*, 1987). Since there are five *PR10* genes in pea, it was important to determine whether a correlation existed between binding activity to *PR10.1* and *PR10.3* sequences, and expression of these two genes. In the incompatible interaction with *F. solani* f. sp. *phaseoli* the binding activity peaked at 2 h.p.i. and there was a reduced band at 4 h.p.i. and occasionally a very weak band at 6 h.p.i. (Figure 18A). We were not able to detect any binding activities before 2 h.p.i. (i.e. 1, 0.5, 0 h.p.i.) and after 6 h.p.i. Furthermore, there were no detectible bands between PDA2 and the nuclear extracts treated with the compatible fungus *F. solani* f. sp. *pisi* or the water control, indicating that the binding is quite specific for *F. solani* f. sp. *phaseoli* treatment. When *PR10.1* gene expression was investigated under the same circumstances, it was found that the expression peak was around 8-12 h.p.i. (Figure 19A), 6-10 h later than the binding peak. This could be explained as a lag between initiation of transcription and accumulation of the transcript, which would be testable by run-on transcription assays. In addition, we observed that *PR10.1* expression is stronger and more sustained in *F. solani* f. sp. *phaseoli* treatment than in *F. solani* f. sp. *pisi* treatment. It is possible that the binding activity with PDA2 may contribute to this increased expression level. Although the expression level of *PR10.1* in *F. solani* f. sp. *pisi* treatment is lower than the one in *F. solani* f. sp. *phaseoli* treatment, it is significantly higher than the water control. Presumably, besides PDA2 there may be other *cis*-regulatory elements

Figure 20. *PR10.1* and *PR10.3* expression in untreated pea (*P. sativum*). Roots and young buds were collected from 3-day seedlings in dark. Immature pods were collected from healthy pea plants. All the materials were frozen immediately after cutting from plants without further treatment. cDNA products were amplified and labelled by DIG-labelling PCR using gene specific primers (Figure 3). The internal control and expression bands (arrow) were detected by DIG detection kit (see Section 3.5.6.).



PR10.1 and *PR10.3* expression in untreated pea (*P. sativum*).

(enhancers) which are non-specifically involved in the mediation of *PR10.1* expression in peas. The PDA1 binding sequence is a good candidate for such elements. PDA1 could be required for basal expression of *PR10.1* or ubiquitously involved in the regulation of *PR10.1* expression in peas, since PDA1 contains the pea gene-conserved motif PDA1-1 (AAATAAATA) (see details in Section 5.6.).

5.4. PDC1 may contain a negative regulatory element

Negative regulation is equally as important as positive regulation and quite common in plant defense genes. Negative regulatory regions, from -590 to -384, were reported in the distal upstream of acidic chitinase gene in *Arabidopsis* (Samac and Shah, 1991) and from -52 to -28, in the proximal upstream of *STH-2* (*PR10a*) in potato (Matton *et al.*, 1993). The expression of phenylalanine ammonia-lyase (*PSPAL1*) in pea was deactivated by a fungal suppressor binding to the upstream *cis*-regulatory element (Murakami *et al.*, 1997). Transcriptional down-regulation was also reported in pathogenesis-related beta-1,3-glucanase genes (*PR-2d*) in tobacco cell cultures (Rezzonico *et al.*, 1998). We have shown that pea *PR10.3* is not expressed in pea pods, but exclusively expressed in healthy roots and not healthy buds or pods (Figure 20). Mylona *et al*, (1994) also reported that *RH2* (*PR10.3*) did not express in leaves or stems, but exclusively expressed in roots, particularly in the root epidermis which forms the radicle. Many members of the *Ypr10* gene family, including *Ypr10c*, were strongly expressed in healthy bean roots while in leaves *Ypr10* transcript levels were very low in young and mature stages (Walter *et al.*, 1996). One concervable explanation for expression of a stress-related gene in the roots is that the physical displacement of soil

during root growth places a stress upon growing roots. Another possibility is that roots are completely immersed in a microbe-rich environment, whereas arerial parts of the plant are a less favorable environment for microbes. The fact that *PR10.3* did not express in aerial parts of pea plants while there was a strong binding activity leads us to propose that a negative *cis*-regulatory element may mediate the down-regulation of *PR10.3* expression. Our binding results showed a strong binding activity between PDC1 and the nuclear extracts from treated pea pods (Figures 10 and 18B). In contrast to *PR10.1*, the fact that *PR10.3* did not express in pea pods may be because of down-regulation. We did not investigate the nuclear extracts from roots. If no binding activity is found between PDC1 and the root nuclear extracts, we could conclude that PDC1 contains a negative *cis*-regulatory element which is recognized by a protein factor in aerial parts of pea plants and results in suppression of *PR10.3* expression. Another hypothesis is that *PR10.3* is developmentally regulated to be non-inducible in the shoot. Developmental regulation of genes in this fashion is often the result of developmental changes in chromatin structure that render genes inaccessible to transcription factors or other DNA binding proteins. Thus, *PR10.3* has a motif that can be bound by defense-specific binding proteins, but can not be activated dure to its chromatin conformation. This hypothesis could be tested by DNAse I sensitivity assays in isolated nuclei.

5.5. Like many *cis*-elements, pea *PR10 cis*-elements are AT-rich

Some *cis*-acting elements are AT-rich. For example, the well-known TATA-box contains AT only. The AT-1 box also consists of only AT without any GC (Datta *et al.*, 1989). The AT-rich *cis*-element (TAAAATACT) was shown to be imperative for the

maximal elicitor-mediated activation of chalcone synthase I (*PsChs1*) in pea (Seki *et al.*, 1996). The AT-rich element PE1 (GAAATAGCAAATGTTAAAAATA) in *A3* gene promoter was strongly bound by the regulatory protein factor in rice and crucial for UV reception (Nieto-Sotelo *et al.*, 1994). In *PR10.1* and *PR10.3*, all three sequences PDA1, PDA2 and PDC1 are AT-rich, with average 83% AT-content. PDA1 contains not only an AT-1 box-like motif (3′-TTTATTTTAT-5′), but also a similar sequence (TAAAATAGT) with the AT-rich element in *PsChs1* (Figure 17A). PDA2 also has an AT-1 box-like motif (3′-TTTATTTTAA-5′) in its active sequence area. Both PDA1 and PDA2 contain the CHS A motif (ATAGTA), major part of which is located in the AT-rich element (Figures 17A and 17B). Apparently, a high AT content is characteristics of *cis*-regulatory elements in *PR10* genes in pea as well as other related genes. Since A-T base-pairs have less bonding strength (or lower melting temperature) than C-G pairs, AT-rich elements may help open DNA duplexes during transcription initiation and increase gene expression efficiency.

5.6. Conserved motifs in *PR10.1* and *PR10.3*

Many functional *cis*-regulatory elements contain motifs that are conserved across different species or within species. These conserved motifs frequently share a core structure and have a common biological function. The G-box and H-box (Faktor *et al.*, 1997; Dröge-Laser *et al.*, 1997; Curtis *et al.*, 1997), for instance, which are conserved in many light-responsive genes and defense-related genes (Table 3), have core motifs (CACGTG) and (CCTACC), respectively. The PR-box (Zhou *et al.*, 1997), which is conserved in a number of basic PR genes from bean, tobacco, potato, *Arabidopsis* and

tomato (Table 4), contains the core sequence (GCCGCC) and was previously referred to as the GCC-box (Shinshi *et al.*, 1995). The W-box [(T)TGAC(C)] is conserved in three members of the *PR1* (*PcPR1*) multigene family in parsley and responsible for fungal elicitor perception to *PR1* gene activation (Rushton *et al.*, 1996). Three *cis*-elements in this study, PDA1, PDA2 and PDC1, do not contain any conserved motifs previously reported in defense-related genes such as G-box, H-box, PR-box and W-box. However, after database searching by computer, we propose at least four conserved sequences in *PR10.1* and *PR10.3* which are also present in many other defense-related genes: PDA1-1 (AAATAAATA), PDA2-1 (ATAAAATT), PDA2-2 (AAATTTC) and PDC1-2 (TTTTATTT).

PDA1-1 is quite conserved in pea genes since it occurs almost 4 times more frequently in pea genes than in non-pea genes (Table 14). Furthermore, many pea genes not associated with defense response have long matched sequences with PDA1-1 and its adjacent regions (Figure 13). PDA1-1 may either be required for basal expression of *PR10.1* gene in pea or may be non-specifically responsive to general external stresses. The observation that PDA1 had the same binding reactions to all tested pea nuclear extracts, while PDA2 selectively reacted to certain challenges, would support the former hypothesis. However, according to the expression results that the expression level from both *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* treatments is much higher the water control (Figure 19A), PDA1 may respond non-specifically to general external challenges.

PDA2-1 and PDA2-2 are more conserved in *PR10* genes and defense genes than in non-defense genes (Table 14). The fact that many *PR10* genes or defense genes have one or more copies of these conserved sequences in their upstream promoters (Figures 14 and 15, respectively) suggests that there might be some common functions which relate to the

sequences. Particularly, both of these conserved sequences are located in the active sequence area in PDA2, which harbors three direct or inverted repeats (Figure 17B) and the binding sequence PDA2b (Figure 8B). Besides the binding site in PDA2a, we believe that either PDA2-1 or PDA2-2 contains a *cis*-acting element which is specifically induced by *F. solani* f. sp. *phaseoli* or salicylic acid. It would be very interesting if all *PR10* genes or defense genes which have this consensus sequence were studied together. If their expression and binding activity were all related to the same source of challenges, then it would be concluded that the conserved sequences, PDA2-1 or PDA2-2, have a fixed biological function.

PDC1-2 in *PR10.3* is conserved among *PR10* genes since it has 4-fold higher frequency in *PR10* genes than in non-*PR10* genes (Table 14). The sequence alignment showed that several *PR10* genes have one or more copies of this consensus sequence (Figure 16). After the binding and expression assays, we know that there might be a gene down-regulation in *PR10.3* (discussed in Section 5.4.). However, we do not know yet the exact location of the negative *cis*-regulatory element in PDC1. One possible location is in the active area in PDC1 (Figure 17C), which has the conserved sequence PDC1-2 and one leg of 8-bp perfect inverted repeats [TGAAATAA$(N)_{20}$TTATTTCA]. Further deletion of PDC1 or functional analysis in a transgenic plant would determine whether or not this conserved sequence is responsible for the down-regulation of *PR10.3* in aerial parts of pea plants.

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7. APPENDICES

7.1. List of genes cited in thesis

(Note: Very often gene names are changed from their original names and therefore a confusion will easily occur when reading literatures. This cross-index of genes cited in the thesis is intentionally created to clarify any confusions when reviewing this thesis.)

Gene name	Other name	Host	GenBank accession
A3		oat	L24390
α-Amy2		wheat	S05490
AoPR1		*Asparagus*	X64452
ABR17	*PR10.4*	pea	Z15128
ABR18	*PR10.5*	pea	Z15127
aox1		*Sauromatum*	S57369
as1		pea	Y13321
Bet v I		birch	X15877
Bet v I-sc1		birch	
Bet v I-sc2		birch	
Bet v I-sc3		birch	

Gene name	Other name	Host	GenBank accession
Bp10		*Brassica*	X64257
CH5B		bean	S43926
CHA2		*Arabidopsis*	AI000996
chi-v		tobacco	X77111
Chn48		tobacco	
CHN14		tobacco	X66433
CHN17		tobacco	
CHN50	*class I chitinase*	tobacco	X51599
CHS2	*chalcone synthase*	pea	AF060235
CHS15	*chalcone* synthase	soybean	X16184
DRR49a	*PR10.1*	pea	U31669
DRR49b	*PR10.2*	pea	M81249
DRRG49c	*PR10.3*	pea	J03680
EGL1		pea	L41046
ENOD12B		pea	X57232
Gap1		*Mesembrianthemum*	
gdcT		pea	AJ222771
gibberellin		pea	U93210
GLA	*gln2*	tobacco	M60402
GLB		tobacco	M60403

Gene name	Other name	Host	GenBank accession
gln2		tobacco	X53600
gn1		tobacco	
gn2		tobacco	
GS2		pea	U22971
gst1	*prp1-1*	potato	J03679
GST1		carnation	S33628
gTUB1		pea	X54844
gyrA		mammal	Z99994
H4		soybean	X60044
hrs203J		tobacco	X77136
HVA22		barley	
LlPR10.1a	*Ypr10.1a*	lupine	AF002277
L1PR10.1b	*Ypr10.1b*	lupine	AF002278
lecA		pea	Y00440
legA		pea	X02982
Lox1		pea	X78580
MnSOD		pea	U30841
NiR		spinach	X07568
OPL		tobacco	
osmotin	*PR5*	tobacco	S68111

Gene name	Other name	Host	GenBank accession
pal		tomato	M83314
PAL-1		parsley	S04463
PAL3		*Arabidopsis*	L33679
PcPR1	*PR1*	parsley	
PcPR1-1	*PR1-1*	parsley	U48862
PcPR1-2	*PR1-2*	parsley	U48863
PcPR1-3	*PR1-3*	parsley	X12573
PcPR2	*PR2*	parsley	X55736
pOSML13		tomato	X72928
pOSML81		tomato	X72927
Ppc1		maize	E01120
Ppc1		*Mesembrianthemum*	X14587
PR1		tobacco	X06362
PR1		rice	U89895
PR1	*PcPR1*	parsley	
PR1-1	*PcPR1-1*	parsley	U48862
PR1-2	*PcPR1-2*	parsley	U48863
PR1-3	*PcPR1-3*	parsley	X12573
PR1a		tobacco	X06361
PR1a2		tomato	Y08844

Gene name	Other name	Host	GenBank accession
PR1b		tobacco	X17680
PR1d		tomato	AJ001627
PR2	*PcPR2*	parsley	X55736
PR-2d		tobacco	X69794
PR-5	*PR-5d*	tobacco	D76437
PR10.1	*DRR49a*	pea	U31669
PR10.2	*DRR49b*	pea	M81249
PR10.3	*DRR49c*	pea	J03680
PR10.3	*RH2*	pea	S74512
PR10.4	*ABR17*	pea	Z15128
PR10.5	*ABR18*	pea	Z15127
PR10a	*STH-2*	potato	M29041
PR10b	*STH-21*	potato	M29042
PR10c	*Ypr10*c, *PvPR*	bean	X96999
Prb-1b		tobacco	X66942
PRms		maize	X54325
PRP1		tobacco	X14065
prp1-1	*gst1*	potato	J03679
prxC2		tobacco	JH0149
psaDb	*PSI-D*	tobacco	S37380

Gene name	Other name	Host	GenBank accession
PsChs1		pea	X63333
PsCHS2		pea	D10662
psl		pea	X66368
PSPAL1		pea	D10002
Pto		tomato	U28007
PvPR	*Ypr10c, PR10c*	bean	X96999
pwsi18		rice	
rab16B		rice	S11846
rab17		maize	
RH2	*PR10.3*	pea	S74512
SAM22		soybean	X60043
SBP65		pea	U49977
Shpx6a		*Stylosanthes*	L36112
Shpx6b		*Stylosanthes*	L77080
STH-2	*PR10a*	potato	M29041
STH-21	*PR10b*	potato	M29042
STPRINPSG		potato	Z12824
WIN2		potato	X13497
Ypr10c	*PR10c, PvPR*	bean	X96999
Ypr10.1a	*LlPR10.1a*	*Lupine*	AF002277
Ypr10.1b	*L1PR10.1b*	*Lupine*	AF002278

7.2. Index of conserved motifs cited in thesis

(Note: all sequences in this thesis are from 5′ to 3′ except wherever indicated)