Available online at www.sciencedirect.com
ScienceDirect
Plant Science 172 (2007) 45–56
PLANT SCIENCE
www.elsevier.com/locate/plantsci

The use of chemical genomics to detect functional systems affecting the non-host disease resistance of pea to *Fusarium solani* f. sp. *phaseoli*

Sierra Hartney, Jennifer Carson, Lee A. Hadwiger *

Department of Plant Pathology, Washington State University, Pullman, WA 99164-6430, United States

Received 30 May 2006; received in revised form 17 July 2006; accepted 18 July 2006
Available online 22 August 2006

Abstract

Non-host resistance is more durable than race-specific resistance and may involve more signaling systems than race-specific resistance. An array of chemicals capable of specifically inhibiting/affecting most of the vital systems of the plant cell was employed to evaluate a range of systems vital in promoting non-host resistance in the *Fusarium solani* f. sp. *phaseoli*/pea endocarp interaction. The parameters measured included pisatin synthesis, hypersensitive discoloration, fungal growth, PR gene induction, and DNA damage. Specific inhibitors of protein phosphatases 1 and 2A (calyculin A, okadaic acid, cantharidin and endothall) and two kinase inhibitors (staurosporine and K-252a from *Nocardiopsis* sp.) were comparable to fungal challenge in inducing pisatin accumulation. These treatments could often break non-host resistance to a bean pathogen, *F. solani* f. sp. *phaseoli*. At low concentrations the treatments transiently enhanced resistance to the pea pathogen, *F. solani* f. sp. *pisi*. Nitric oxide and superoxide-generating compounds, salicylic acid, methyl salicylate, and jasmonic acid implicated, as effectors in other systems had no major detectable effect. Thus the broad array of inhibitors delineated cellular functions associated with non-host disease resistance in pea and tentatively excluded some signaling systems reported in other systems.

Keywords: Non-host disease resistance; Pisatin; Pathogenesis-related (PR) genes; *Fusarium solani*; DNA damage; Phosphatase and kinase inhibitors

1. Introduction

Plants possess a strong stable immunity called non-host resistance that affords them protection against most of the plant pathogenic organisms in their environment. The exceptions are the true pathogens that have successfully established a strategy for by-passing this immunity. The non-host resistance response of pea endocarp tissue to the bean pathogen, *Fusarium solani* f. sp. *phaseoli* was utilized to investigate components of plant immunity. The development, biochemistry and molecular biology of this resistance response can be accurately monitored within a 6 h window [1], because without a cuticle barrier, signaling between host and pathogen is rapid. The development of a susceptible response to a true pea pathogen, *F. solani* f. sp. *pisi*, can similarly be monitored. The initiation or cessation of fungal growth can be viewed directly since the resultant growth involves minimal, if any, tissue penetration or physical wounding [2]. The cuticle-free surface also enables the use of a ''chemical genomics'' approach. Chemical genomics has been defined as the genomic response of a biological system to chemical compounds, the objective being to use low molecular weight compounds to identify a response gene family and elucidate the function of these genes and those in related families. In the case of pea defense there is a large number of genes activated by fungal challenge [3]. In this report we have extended the definition to ''using small molecules to modify or disrupt the functions of genes/proteins to complement the data derived from other genomic tools''.

The chemicals in this study were selected to determine which of the plant's vital processes are involved in developing resistance and were less informative of the actual gene families. The parameters utilized to follow the immune response were: fungal growth, DNA damage, activation of plant defense genes, development of hypersensitive discoloration and phytoalexin production. The pea phytoalexin, pisatin, is a small antifungal isoflavonoid. Pisatin initially accumulates to high concentrations

* Corresponding author. Tel.: +1 509 335 3751; fax: +1 509 335 9581.
E-mail address: chitosan@wsu.edu (L.A. Hadwiger).

0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2006.07.014

in a resistance reaction as opposed to a susceptible reaction where initially low concentrations continue to accumulate and eventually exceed those of the non-host resistance reaction [1]. Compounds whose effects were monitored included putative effectors such as salicylic acid and jasmonic acid and those that affect kinases, phosphatases, topoisomerases, methylases, acetylases, DNA replication, nucleases, and protein synthesis. DNA-specific compounds were also assessed (Table 1).

Previous research on the pea endocarp/*Fusarium* system have identified two signals, chitosan and DNase, released by the pathogen that can each independently induce the resistance response against the pea pathogen [1,6]. The orderly progression of events starts with the release of these elicitors. Within 20 min alterations occur in the plant nuclei that affect sedimentation velocity [7]. Within 3 h the transcription of some of the pathogenesis-related (PR) genes has increased and DNA fragmentation is evident, as are activities of increased enzyme product of the PR gene, phenylalanine ammonia lyase [3,6,8]. The growth of the bean pathogen subsides at 5–6 h and the accumulation of the isoflavonoid pisatin commences at 6 h [1]. The growth of the true pea pathogen *F. solani* f. sp. *pisi*, though minimal at 6 h, resurges within 18 h. After 18 h cell death and a yellow green pigmentation becomes evident [1], that exists as a component of a defined lesion or of a broader tissue coloration. In the pea endocarp this pigmentation appears to result from an increase in and polymerization of phenolics. This hypersensitivity response (HR) does not share the lesion-drying symptoms typical of the hypersensitive response accompanying resistance to bacterial pathogens on plant leaves.

Phytoalexin production and PR gene activation have been correlated with both race-specific resistance and non-host resistance [4,5,9–11]. Recently, the silencing of genes in isoflavone biosynthetic pathways has been shown to disrupt disease resistance in soybean cotyledons [12]. Pisatin degradation by the pathogen is associated with increased virulence, but is not the sole determinant of pathogenicity [13,14]. Some of the defense genes in this system have been cloned and characterized, however more than 200 clones identified as being up regulated by plus/minus hybridization analyses were obtained suggesting a number of functions associated with the interaction remain unknown [3].

Five pea genes were chosen to follow PR gene induction following phosphatase inhibitor treatment. Each of the selected genes has been identified as playing a role in the defense response of pea. The pea HMG A gene has been the most extensively studied. The nuclear protein coded by this gene recognizes PR gene promoter sequences containing long stretches of adenine (A) and thymine (T). HMG A, is a nuclear protein with ‘‘AT hooks’’, and is regarded as an architectural transcription factor [16,17]. HMG A reportedly can positively or negatively regulate transcription by attaching to the AT-rich regions of genomic DNA [18]. The enzyme products of the

Table 1
A list of compounds with reported inhibitor or effector potential that were screened on pea pod tissue for effects on non-host resistance, hypersensitive responses, and pisatin accumulation

Inhibitor/effector	Function
Salicylic acid (SA), jasmonic acid (JA), methyl salicylate	PR gene inducer [28]
Catechol	Metabolite of salicylic acid [29]
Benzothiadiazole (BTH)	SA-like function [30,31]
Methotrexate	Produces reactive oxygen species [32]
N-Acetyl-cysteine	Increases free radical scavengers [33]
Citrulline, L-arginine	Substrates for NO synthesis [34]
Sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine (SNAP)	Releases NO/NO donors [35]. NO is involved in cell to cell signaling [15,36].
N^G-Nitro-L-arginine (NNA)	Competitive inhibitor of NO synthase [15]
Diphenyl iodonium (DPI)	NO synthase inhibitor [37]
Okadaic acid, calyculin A, endothall, cantharidin	Ser/Thr protein phosphatase 1 and 2A inhibitors [38–41]
Staurosporine	Phospholipid/calcium kinase inhibitor [42], Ser/Thr protein kinase inhibitor [43]
Rescovitine, olomucine	Cyclin-dependent kinase inhibitor [44]
K-252a	CaM kinase inhibitor [45] Ser/Thr kinase inhibitor [46]
Genistein	Tyrosine-specific kinase inhibitor [47]
Teniposide, sobuzoxane, aurintricarboxylic acid	Topoisomerase II inhibitors [48–50]
Distamycin A	Competes with HMG A for AT-binding [18,51]
Apicidin	Histone demethylation inhibitor [52]
Trichostatin, *n*-butyric acid	Histone deacetylase inhibitors [53]
Antipain	Inhibits aldehyde proteases [54]
Cycloheximide	Inhibits eukaryotic protein synthesis [55]
Mimosine, cyclophosamide	Inhibit DNA replication [56]
Melphalin, carmustine	DNA alkylating agents [57,58]
Mitoxanthrone	Intercalates into AT-rich DNA, inhibits DNA synthesis [59]
N-Ethylmaleimide	Nuclease-inhibiting DNA alkylating agent [60]
Caspase I–II, I–III, I–IV inhibitors	Involved in programmed cell death [61,62]
4,6-Diamido-2-phenylindole (DAPI)	Binds AT-rich regions of DNA [63]
Cisplatin	Produces DNA–protein crosslinks in DNA [64]
Amphoterin B	Destroys fungal membrane permeability [65]

phenylalanine ammonia lyase (PAL) gene and the chalcone synthase (CHS) gene catalyze steps in the pisatin synthesis pathway [19]. The mRNA from the disease resistance response (DRR) gene, DRR206d is sustained at high levels in peas when resistance is induced by an incompatible pathogen. A homolog of DRR206d has been shown to be associated with lignan/lignin synthesis [20,21]. The gene product from PR10 (DRR49a) accumulates in peas that successfully resist *F. solani* f. sp. *phaseoli* and has homology with a plant gene coding for RNase [22,23]. Finally, the pea ubiquitin gene was used as a control. Pisatin production depends on two of these gene products and its accumulation is an indicator of the induction of the host response [24].

The importance of phosphatase and kinase functions was noted in the initial screening of pea responses based initially on the monitoring of phytoalexin production, fungal growth, and hypersensitive coloration and subsequently on DNA damage assessment, PR gene and HMG A gene expression.

Many of the compounds utilized affect major cell functions in both plant and animal systems. This strategy provides an alternative to gene targeting in plants, as the pea is recalcitrant to gene transformation techniques [15]. This report reiterates the importance of phosphorylation and maintenance of PR gene activation and its associated protein-synthesis potential in the non-host resistance of peas. The treatments included individual effectors/inhibitors alone, effectors/inhibitors in combination with *F. solani* f. sp. *phaseoli*, effectors/inhibitors with chitosan and chitosan alone and in combination with the standard elicitation by *F. solani* f. sp. *phaseoli* macroconidia or crab shell chitosan.

2. Materials and methods

2.1. Array of compounds

All of the compounds, except BTH (a gift from Roy Navarre), were obtained from Sigma–Aldrich (U.S.A.) or Calbiochem (EMD Biosciences Inc., San Diego, CA, U.S.A.). Reported targets of the inhibitor/effector compounds are summarized in Table 1. These compounds were applied both as single treatments and in combination with standard elicitation by *F. solani* f. sp. *phaseoli* macroconidia or crab shell chitosan (dissolved in dilute acetic or citric acid and neutralized to pH 6.0; Vanson Halosource, Redmond, WA, U.S.A.). The marketed definitions by suppliers of the inhibitor compounds often indicate specific targets; however some of the inhibitors used in this study are known to have secondary effects. The attribution to a given target was resolved in part by utilizing multiple inhibitors for similar processes.

Immature pods of uniform size (3 cm length) from greenhouse grown *Pisum sativum* L. cv. Lance were used in this research [8]. Fifty microliters of each treatment was distributed with a smooth glass rod to the surface of pod halves (four per treatment) and incubated at 22 °C for 24 h. Each of the array compounds was applied to the pea pods in half-fold dilutions typically starting at 1 μg/μl and extending to as low as 0.0015 μg/μl with or without *F. solani* f. sp. *phaseoli* macroconidia (3×10^7 macroconidia/ml) and with chitosan at two concentrations. Pod tissue was assayed 24 h post-treatment for pisatin accumulation, hypersensitive response, fungal growth, and general condition of the tissue. Compounds showing an initial effect on one or more of the parameters were assayed two additional times. *F. solani* f. sp. *phaseoli* or *F. solani* f. sp. *pisi* macroconidia (3×10^7 macroconidia/ml) were combined with the treatment just prior to application. For DNA damage and gene expression assays, the incubation time was terminated at 5 h by immediately freezing the intact pod halves in liquid nitrogen.

2.2. Fungal material

Fungal cultures of *F. solani* f. sp. *phaseoli* strain W-8 (ATCC no. 38135) and *F. solani* f. sp. *pisi* strain P-A (ATCC no. 38136) were maintained on potato dextrose agar supplemented with 3 g whole pea pods at room temperature to provide macroconidia for experiments. Macroconidia were harvested by gently scraping fungal spores from agar plates.

2.3. Pisatin assay

To measure pisatin production, treated pods at 24 h post-inoculation were placed in 5 ml hexane. After 24 h the pisatin-containing hexane layer was decanted off and the hexane volatilized away at room temperature in a ventilation hood. The pisatin was recovered from the dried residue with 95% ethanol and quantitated in a spectrophotometer at 309 nm (1.0 OD unit at 309 nm = 43.7 μg/ml pisatin). The samples were also qualitatively assayed for the characteristic 320–230 nm pisatin UV spectrum.

2.4. Microscopic assay of fungal growth and hypersensitivity

Growth of *F. solani* f. sp. *phaseoli* and *pisi* on the pea endocarp was evaluated as follows: hyphae were stained with cotton blue and visible growth was estimated using the length (57 μm) of the macroconidia proper as a measure. Fungal growth of the hyphal tip extending less than half the length of the macroconidia was termed germinated (GT); no growth was termed zero (0); growth three times the macroconidia length was termed fair growth (F); growth seven times the length was termed good growth (G); growth greater then seven times the length within 24 h was termed massive growth (M). The growth of a minimum of 50 macroconidia on the pea pod endocarp surface was recorded for each assay. The yellow green color of the hypersensitive response in the presence of the array compounds with the fungi was visually compared in the light microscope to the *F. solani* f. sp. *phaseoli*-induced hypersensitive reaction.

The phosphatase inhibitor action directly on *F. solani* f. sp. *phaseoli* and *pisi* macroconidia was evaluated in 96 well plates in Vogel's media [27]. Two replications of concentration gradients for each compound started at 10 or 100 μg/ml and extended to 10 half-fold dilutions. The growth of ~50 macroconidia in each well was evaluated at 24 and 48 h.

2.5. Nucleic acid extraction

RNA and DNA were extracted using the method described in [25]. Briefly, 0.6 g tissue was pulverized in liquid N_2 and 2 ml of buffer #1 (5 M Na percholate, 0.5 M Tris base, 2.5% SDS and 0.02% polyvinylpyrrolidone). Following centrifugation at 11,000 × g at 4 °C, the supernatant was removed and the nucleic acids precipitated with 2.5 volumes of ethanol. After centrifugation, the pellet was dissolved in sterilized distilled water and subjected to a phenol–chloroform extraction. The nucleic acids were precipitated from the aqueous phase (2.5 volumes ethanol, −20 °C) and the 10,000 × g pellet was redissolved in sterilized distilled water and combined with an equal volume of 4 M $LiCl_2$. The RNA was precipitated at −20 °C for 3 h and pelleted, leaving the DNA in solution. The RNA was solubilized in sterilized distilled water and reprecipitated in 0.25 volume 3.0 M potassium acetate and 2.5 volumes ethanol and stored at −80 °C prior to quantification. The DNA was redissolved in sterilized distilled water and precipitated with ethanol in preparation for DNA damage assessment.

2.6. DNA damage assessment

To resolve DNA damage caused by single strand nicks, a 6 μg aliquot of DNA was dissolved in water and combined with 1% melted chromatin grade agar (50 μl total; BioRad Inc., Hercules, CA, U.S.A.). The solidified 50 μl agar gel mold was suspended in alkaline buffer (30 mM NaOH, 4 mM EDTA) overnight to allow the diffusion of smaller segments of single stranded DNA into the buffer [26]. The DNA was precipitated from the diffusate (0.5 volume 2 M potassium acetate and 2.5 volumes 95% ethanol) and electrophoretically separated on a 1% agarose gel.

2.7. Quantitation of gene-specific RNA content with RT-PCR

In preparation for RT-PCR, RNA from the pea pods was transcribed into DNA using a reverse transcriptase kit from Invitrogen (Carlsbad, CA, U.S.A.).

Several primers were constructed to follow the accumulation of RNA specific, via real-time PCR, for HMG A and the PR genes of interest (Table 2). The first strand primer (5′-TATGACACGCGTCGACTAGC $(T)^{17}$-3′) was used during first strand synthesis to ensure that only RNA messages were being amplified by binding to the poly-A tail. The AdPrime primer (5′-TATGACACGCGTCGACTAGC-3′) recognizes and binds the sequence on the first strand primer, ensuring that the DNA product is generated from the extracted pea RNA. All of these primers bind the 3′ end of the sequences and work with the 5′ AdPrime to amplify a portion of the gene of interest in order to confirm its presence in the extracted RNA solution.

2.8. Polymerase chain reaction (PCR) and electrophoresis

PCR was performed to analyze the first strand synthesis as well as to optimize the functionality of the primer pairs. The PCR mixture included 2 μl first strand synthesis DNA, 2 μl 10× buffer (Invitrogen), 1.2 μl 2.5 mM dNTPs (Promega, Madison, WI, U.S.A.), 0.8 μl 50 mM $MgCl_2$ (Invitrogen), 0.6 μl 20 μM AdPrime primer (an add on extention contruction of non-homologous sequence on an oligo dT primer), 0.6 μl 20 μM of the other primer of interest, 12.6 μl ddH_2O, and 0.2 μl Taq polymerase (Invitrogen). The thermocycler program was set for 1 cycle at 94 °C for 4 min; 50 cycles of 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 20 s; 1 cycle of 72 °C for 7 min. cDNA from both the first strand synthesis and the PCR were separated by gel electrophoresis to verify product size.

2.9. RT-PCR

Following first strand synthesis, 3 μl of the first strand solution are combined with 1 μl of AdPrime, 1 μl of the gene-specific primer of choice, 12.5 μl of SYBR Green Supermix (BioRad Inc., Hercules, CA, U.S.A.), and 7.5 μl distilled water. This mixture was centrifuged briefly before being placed in PCR tubes (Midwest Scientific, St. Louis, MO, U.S.A.). These tubes allow for visualization of the fluorescence by the iQ iCycler PCR instrument (BioRad Inc.). The iQ iCycler was programmed to run the PCR protocol as well as a melt curve from 60 to 95 °C. The RT-PCR product was also run on a 1.5% agarose gel with a 1 kb standard to allow for product visualization. RT-PCR data was obtained from three separate RNA extractions.

3. Results

The chemicals and their reported inhibitor or effector actions on vital cell processes screened on pea pod tissue for effects on non-host resistance, pisatin-inducing ability, and hypersensitive response are listed in Table 1. The results of the initial screening of inhibitor/effector compounds on the induced accumulation of pisatin are presented in Table 3. The accumulations are expressed as percentage of pisatin induced by treatment with *F. solani* f. sp.

Table 2
Primers for genes used in RT-PCR

Gene	Accession number	Primer name	Primer sequence (5′–3′)
HMG A	X99373	HMB-559F	CCTAATGCACCACCGAAGACTC
PAL	D10003	PAL-4072F	AGTTGAAGACCCTCTTGCCA
CHS	D88261	CHSY-UNI	TACATGATGTACCAACAAGG
DRR206d	U11716	DRR206dV1588	CTAGTCTTTTATTTTCATGACA
PR10 (DRR49a)	U31669	PR10 1513	AGCATAGTTGGTGGTGTTGG
Ubiquitin	L81142	Ubiq2174	GTGAATGTTGCGTAGCCATC

Table 3
The initial screening of inhibitor/effector action on pisatin production, *Fusarium solani* f. sp. *phaseoli* growth (Fsph), and the hypersensitive response (HR) following the treatment of the pea pod tissue

Inhibitor/effector	Low concentration (μg/ml)	Pisatin (% Fsph)	High concentration (μg/ml)	Pisatin (% Fsph)	Fsph		HR	
					Low concentration	High concentration	Low concentration	High concentration
Okadaic acid	0.5[a]	87[a]	40[a]	2[a]	0[b]	M[b]	+[b]	−[b]
Calyculin A	0.15	46	10	286	GT	G	+	−
Endothall	15	144	500	110	0	M	+	−
Cantharidin	15	130	250	202	0	0	+	+
Staurosporine	6	136	100	153	0	G	+	+
Rescovitine	125	0	500	16	0	0	+	+
Olomucine	10	4	500	13	0	0	+	+
K-252a	25	14	800	74	0	0	+	+
Genistein	7	0	60	0	P	GT	+	+
NNA	50	0	400	0	0	0	+	+
DPI	60	5	1000	0	nd	nd	nd	nd
SNAP	150	0	600	4	0	0	+	+
Na nitroprusside	60	0	500	0	G	G	+	+
Citrulline	250	6	1000	0	F	0	+	+
L-Arginine	250	0	1000	0	0	0	+	+
N-Acetyl-cysteine	62	0	1000	0	P	P	+	+
Antipain	30	15	1000	0	nd	nd	nd	nd
Mitoxanthrone	62	0	1000	9	0	0	+	+
DAPI	62	0	1000	14	0	0	+	+
Distamycin A	30	2	500	35	0	0	+	+
Trichostatin	6	0	100	7	0	GT	+	+
n-Butyric acid	15	5	250	0	0	0	+	+
Teniposide	12	0	400	0	0	0	+	+
Sobuzoxane	500	10	1000	2	0	0	+	+
Aurintricarboxylic	31	0	250	23	0	0	+	+
Apicidin	62	9	500	21	0	0	+	−
Cycloheximide	3	15	200	0	P	F	−	−
Cisplatinum	6	0	200	0	0	P	+	+
Melphalin	250	6	1000	0	0	0	+	+
N-Ethylmaleimide	7	0	500	2	0	0	+	+
Caspase I–II	15	0	1000	3	0	F	+	+
Caspase I–III	7	0	500	5	0	0	+	+
Caspase I–IV	250	0	1000	0	0	0	+	+
Methotrexate	62	3	1000	0	G	F	+	+
Mimosine	31	20	500	0	F	G	+	+
Cyclophosamide	31	0	500	10	GT	0	+	+
Salicylic acid	30	0	1000	0	0	0	+	+
Methyl salicylate	7	0	500	0	0	0	+	+
Catechol	62	0	500	0	0	F	+	+
Jasmonic acid	15	0	250	0	P	0	+	+
BTH	15	0	1000	8	0	0	+	+

[a] The surfaces of four pea pod halves (0.6 g) were treated with 50 μl of the indicated treatment. Pisatin was extracted 24 h post-treatment and its quantity was expressed as percentage of that induced by the *F. solani* f. sp. *phaseoli* (Fsph) macroconidia (4×10^5/ml) inoculated control. These data were the most significant and except from comprehensive dose response treatments typically including a half-dilution series of seven concentrations. The concentrations selected for the table were those most closely representing the induction range.

[b] The same dilutions of compounds were combined with macroconidia applied to the pod and assayed for HR and fungal growth. Fungal growth of 50 propagules/treatment was evaluated after 24 h (see Section 2). GT: germ tube is visible; M: massive growth; G: good growth; F: fair growth; P: poor growth; 0: no detectable growth; +: visible HR; −: no detectable HR; nd: not determined.

phaseoli alone and indicate how alterations in certain vital cellular functions were associated with specific processes affecting the development of non-host resistance in pea pod tissue. Some of the compounds that effectively promoted pisatin accumulations at their higher concentrations allowed growth of *F. solani* f. sp. *phaseoli* at rates greatly higher than water-treated tissue. This suggests that an attempt to block a single process may have a narrow window of effects on the total non-host resistance response. Pisatin-inducing ability assessed for cantharidin and endothall alone or in combination with *F. solani* f. sp. *phaseoli* or chitosan, due to their dramatic influence, is presented in Fig. 1 and is an example of the analyses repeated and presented as supplemental data for all of the compounds tested. Based on the initial screen of the hypersensitive response, fungal growth, and pisatin production, the phosphatase inhibitors (okadaic acid, calyculin A, cantharidin, and endothall), and kinase inhibitors (staurosporine and K-252a) were reassessed to both verify concentrations effective in pisatin production (Table 4) and their

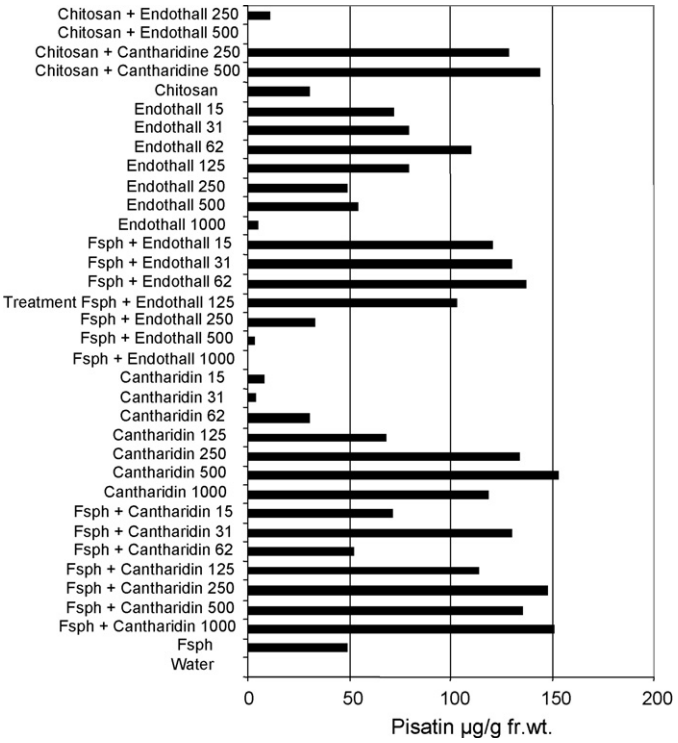

Fig. 1. The effect of cantharidin and endothall on pisatin production of pea pod tissue (0.6 g) treated with 50 μl of the indicated concentrations expressed in μg/ml. *Fusarium solani* f. sp. *phaseoli* (4×10^5 macroconidia/ml) were included in "Fsph" treatments. Chitosan concentration was 1 mg/ml. Pisatin was assayed after 24 h. The format of this bar graph for these two compounds was that used for the other compounds in the first screening.

subsequent potential to alter the non-host resistance in pea pod tissue (Table 5). Serine/threonine phosphatase inhibitors, primarily those specific for protein phosphatases 1 and 2A, enhanced the accumulation of pisatin as shown in Table 3, often at very low concentrations (e.g. calyculin A at 0.6 μg/ml). Except for the cantharidin treatment, the pisatin-inducing treatments applied at higher concentrations were accompanied by significant growth of the bean pathogen, *F. solani* f. sp. *phaseoli*, and termed "breaks resistance" in Table 5. The protein kinase inhibitors staurosporine and K-252a, inhibitors of phospholipid/calcium kinase, Ser/Thr protein kinase, and CaM kinase respectively were also effective in inducing pisatin (Tables 1 and 4). Staurosporine, but not K-252a, was also able to break resistance to *F. solani* f. sp. *phaseoli* (Table 5).

Table 4
A comparison of the pisatin-inducing potential of phosphatase and kinase inhibitors at lower effective concentrations

Treatment	Concentration (μg/ml)	Pisatin (μg/g fr.wt.)	S.E.	*t* grouping[a]
Calyculin A	2	193	11	A
Okadaic acid	1	227	5	A
Cantharidin	100	145	13	B
Endothall	100	154	30	B
K-252a	50	43	9	C
F. solani f. sp. *phaseoli*	4×10^6	207	10	A
Water	0	0	0	D
Chitosan	1000	46	7	C

Mean data is based on three replications of 0.3 g pod tissue. Pisatin was extracted after 24 h. Treatment vol. = 25 μl. SAS analysis provided an *F* value of 37 and $Pr > F = < 0.001$.

[a] Treatments in the same *t* group are similar and those in different groups are statistically different.

Table 5
Effects of phosphatase and kinase inhibitors, selected on the basis of pisatin induction potential, on the pea tissue's resistance to *F. solani* f. sp. *phaseoli*

Treatment	Effect of inhibitor on non-host resistance[a]
Okadaic acid	0.5–10 μg/ml, breaks resistance; 0.01–0.2 μg/ml, resistance was maintained
Calyculin A	0.3–10 μg/ml, breaks resistance; 0.03–0.15 μg/ml, resistance was maintained
Cantharidin	0.01–1000 μg/ml, resistance was maintained
Endothall	0.25–1000 μg/ml, breaks resistance; 0.01–0.03 μg/ml, resistance was maintained
Staurosporine	25–100 μg/ml, breaks resistance; 1 μg/ml, resistance was maintained
K-252[a]	25–400 μg/ml, resistance was maintained
Distamycin A	300–100 μg/ml, resistance was maintained

[a] The pod surfaces of four pod halves (0.6 g) were treated with 50 μl of the indicated treatment. This data is a portion of the comprehensive assays of each individual inhibitor/effector compound applied in treatments in half-dilution series of seven concentrations (see Section 2). The growth of >50 propagules of *F. solani* f. sp. *phaseoli* (1×10^6 macroconidia/ml) applied with the compound was examined cytologically by cotton blue staining 24 h post-inoculation.

3.1. Pisatin induction

Minimal, but still effective, concentrations derived from the initial screening data were utilized in a three-replication experiment comparing four phosphatase inhibitors and a kinase inhibitor. These comparisons indicated the higher potency of calyculin A and okadaic acid compared with the other inhibitors shown in Table 4. Accumulations of pisatin that occurred following inoculations with *F. solani* f. sp. *phaseoli* are initially more rapid than those following *F. solani* f. sp. *pisi* inoculations and in general are characteristic distinctions of susceptibility and compatibility. Pisatin was not detected in water-treated pod tissue. Treatment of the pea pods with 0.6–10 μg/ml of calyculin A resulted in high levels (>100 μg/g fr.wt.) of pisatin accumulation. However, the net effect of high concentrations of calyculin A at the tissue surface was to break resistance to *F. solani* f. sp. *phaseoli* allowing fungal growth to persist (Table 5). The lower concentrations of calyculin A (0.03–0.15 μg/ml) were less disruptive and the non-host resistance was retained. Comparatively higher concentrations of the chromatin-altering distamycin A were capable of inducing pisatin without breaking resistance (Table 5).

3.2. Cytological assessment

Cytological examination of inoculated pea pod tissue indicated that both okadaic acid (0.01–0.06 μg/ml) and calyculin A (0.03–0.07 μg/ml) treatments induced transient resistance to *F. solani* f. sp. *pisi*. The photographic illustration of how the high and low concentrations of calyculin A affected fungal growth is presented in Fig. 2. A higher concentration of

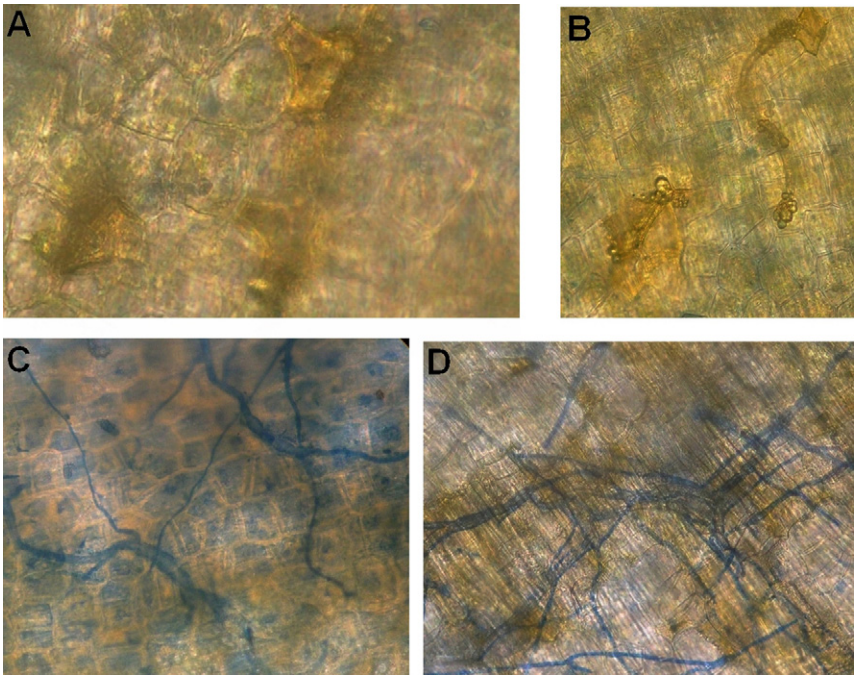

Fig. 2. Growth of *F. solani* f. sp. *phaseoli* (3×10^6 macroconidia/ml) and *F. solani* f. sp. *pisi* (3×10^6 macroconidia/ml) on pea endocarp tissue 24 h after inoculation in water or with calyculin A. (A) *F. solani* f. sp. *pisi* with calyculin A (0.03 μg/ml) (growth of the three macroconidia was totally suppressed. The positive HR is indicated by a yellow pigmentation accumulation). (B) *F. solani* f. sp. *phaseoli* (the growth of three macroconidia was suppressed in this water treatment). The morphological distortion of hyphal tips and the very adjacent yellow green pigment deposit is indicative of a typical non-host resistance response. (C) *F. solani* f. sp. *phaseoli* with calyculin A (2.5 μg/ml) (the fungal growth indicates that resistance has been broken). (D) *F. solani* f. sp. *pisi* (typical growth of the true pathogen 24 h post-treatment with water).

calyculin A, 2.5 μg/ml, diminishes resistance to *F. solani* f. sp. *phaseoli* (Fig. 2C). The resistance against *F. solani* f. sp. *pisi* that is generated by calyculin A at 0.03 μg/ml (Fig. 2A) became diminished after 3 days resulting in a continuation of fungal growth (Table 6). The normal non-host resistance and susceptibility responses are shown in Fig. 2B and D, respectively. Staurosporine (25–100 μg/ml) was able to break resistance; however cantharidin, K-252a, and distamycin A were capable of inducing high levels of pisatin (Table 3) without detectable alteration of fungal growth (Table 5). These results implicate a potential role of phosphatase inhibitors in initiating or diminishing the plant's defense response.

3.3. Phosphatase inhibitor action directly on the fungus

The growth of both *F. solani* f. sp. *phaseoli* and *pisi* are inhibited directly by calyculin A, starting at 10 μg/ml. Okadaic acid did not inhibit either at 10 μg/ml. Endothall did not inhibit either at 100 μg/ml the highest level used. Cantharidin inhibited *F. solani* f. sp. *phaseoli* starting at 25 μg/ml and *F. solani* f. sp. *pisi* at 12 μg/ml. Thus the fungal growth that was inhibited when lower levels of these inhibitors were applied to the pea tissue appeared to be indirect and dependent on the plant's induced response (Table 6).

3.4. Inhibitors with low level pisatin induction

A second function of the inhibitor array was its ability to assess other metabolic processes that may have lesser importance in the non-host resistance of peas. Compounds with little or no potential to induce pisatin (<30% of Fsph induced pisatin) include: salicylic acid, methyl salicylate, catechol, jasmonic acid, benzo(1,2,3)thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH), hydrogen peroxide, methotrexate, *N*-acetyl-L-cysteine, citrulline, L-arginine, sodium nitroprusside dehydrate, N^G-nitro-L-arginine, N^W-nitro-L-arginine, diphenyl iodium, mimosine, genistein, rescovitine, olomucine, teniposide, sobuzoxane, *N*-ethylmaleimide, cycloheximide, cisplatinum, carmustine, cyclophosamide, melphalin, mitoxanthrone, DAPI, trichostatin, sodium butyrate, amphotericin B and caspase inhibitors II, III and IV (Table 3). Some of these compounds had an observable influence on the accumulation of pisatin when combined with chitosan or the *F. solani* f. sp. *phaseoli* inoculum in the initial screen (data not shown).

3.5. Treatment combinations

The induction of pisatin by *F. solani* f. sp. *phaseoli* was enhanced by certain concentrations of endothall, cantharidin, staurosporine, olomucine, apicidin, N^G-nitro-L-arginine, sodium nitroprusside, antipain, carmustine, and sobuzoxane. Alternately, the *F. solani* f. sp. *phaseoli* induction of pisatin was suppressed by salicylic acid, methyl salicylate, citrulline, L-arginine, methotrexate, okadaic acid, endothall, K-252a, aurintricarboxylic acid, cycloheximide, cisplatinum, melphalin, mitoxanthrone, DAPI, distamycin A, sodium nitroprusside, *N*-acetyl-L-cysteine, and carmustine (data not shown). The induction effect on pisatin accumulation appeared to be additive when calyculin A was applied at 0.01–0.07 μg/ml (Table 7) with the fungal spore suspension.

Table 6
A comparison of the effects of two phosphatase inhibitors on the growth of the bean pathogen, *F. solani* f. sp. *phaseoli*, and the pea pathogen, *F. solani* f. sp. *pisi*, on pea pod tissue

Treatment	Treatment concentration[a] (μg/ml)	*F. solani* f. sp. *phaseoli*		*F. solani* f. sp. *pisi*	
		24 h growth[b]	HR[c]	24 h growth	HR
H_2O only		GT	+	GT–G	+
okadaic acid	10	M	+	M	–
	2	G	+	G	+
	1	G	+	F	+
	0.5	F	+	G	+
	0.25	0	+	G	+
	0.125	F	+	F	+
	0.06	0	+	GT–0***	+
	0.03	0	+	0**	+
	0.01	0	+	0*	+
Calyculin A	10	G	+	G	+
	5	M	+	M	+
	2.5	P	+	G	–
	1.2	G	+	G	–
	0.6	P	+	G	–
	0.3	F	+	G	+
	0.15	0	+	F	+
	0.07	0	+	P***	+
	0.03	0	+	P	+

[a] Fifty microliters of the indicated treatment was applied to the pod surface of four pea pod halves along with 1×10^5 macroconidia/ml of the fungus.
[b] Growth of 50 propagules/treatment was analyzed 24 h after inoculation. GT: germtube is visible; M: massive growth; G: good growth; F: fair growth; P: poor growth; 0: no growth (see Section 2). The asterisks indicate the relative level of growth resumed after 3 days.
[c] +: the yellow-green discoloration of a hypersensitive-like response (HR) was detected in the vicinity of the fungal spore; –: no HR.

Chitosan-promoting accumulations of pisatin were enhanced by certain concentrations of inhibitors, especially methyl salicylate, citrulline, L-arginine, cantharidin, staurosporine, rescovitine, K-252a, okadaic acid, DAPI, distamycin A, *n*-butyric acid, N^G-nitro-L-arginine, *N*-acetyl-L-cysteine, teniposide, and sobuzoxane and alternately were suppressed by concentrations of salicylic acid, catechol, endothall, aurintricarboxylic acid, apicidin, cycloheximide, melphalin, mitoxanthrone, and sodium nitroprusside (Fig. 1; data not shown).

Table 7
Effect of calyculin A and calyculin A plus *Fusarium* f. sp. *phaseoli* (Fsph) on pisatin accumulation

Treatment[a]	Concentration (μg/ml)	Treatment only pisatin (μg/g fr.wt.)	Treatment plus Fsph pisatin (μg/g fr.wt.)
Calyculin A	10	189 ± 1	201 ± 10
Calyculin A	5	157 ± 10	196 ± 3
Calyculin A	2.5	176 ± 6	189 ± 5
Calyculin A	1.25	153 ± 31	273 ± 6
Calyculin A	0.6	107 ± 31	271 ± 10
Calyculin A	0.3	81 ± 40	257 ± 16
Calyculin A	0.15	31 ± 26	205 ± 22
Calyculin A	0.07	10 ± 8	248 ± 10
Calyculin A	0.03	10 ± 3	258 ± 9
Calyculin A	0.01	13 ± 10	144 ± 35
Water	0	0 ± 0	66 ± 29[b]

[a] The exposed pod surface of two replications of 0.3 g pea pods were treated with 12 μl of the indicated concentrations of calyculin A. Pods were harvested and extracted following 24 h incubation at 22 C. SAS analysis indicated an *F* value of 27.58 and $Pr > F = < 0.0001$.
[b] *F. solani* f. sp. *phaseoli* pisatin induction was 66 ± 29 μg/g fr.wt.

3.6. Breaking non-host resistance

In addition to the phosphatase and kinase inhibitors, various concentrations of some of the other compounds can enable the growth of *F. solani* f. sp. *phaseoli* on the pea pod surface. These include methotrexate, sodium nitroprusside, *N*-acetyl-L-cysteine, mimosine, and cycloheximide (data not shown).

3.7. DNA damage assessment

The DNA of the entire pea pod was extracted from treated and untreated pea pods at a 6 h time point and examined by taking 6 μg of DNA from each treatment and entrapping it in 1% agarose molds that were subsequently submerged in an alkaline buffer to allow the small and moderate sized DNA fragments, which become single stranded, to leach out into the buffer. Minor DNA fragmentation was associated with the phosphatase inhibitors endothall and calyculin A and fungal treatments of pea pods, shown in Fig. 3. *F. solani* f. sp. *phaseoli* and *pisi* treated pods released the most DNA, suggesting the most extensive DNA damage. Endothall at 60 and 30 μg/ml were next in terms of possible damage above that of the water control. The calyculin A 0.1 μg/ml treatment also showed DNA damage greater than the water control. These DNA fragments are detectable from tissue treated with pisatin-inducing concentrations of phosphatase inhibitors, except for the higher concentration treatment of okadaic acid (125 μg/ml). This okadaic acid treatment so severely altered the structural integrity of the pod tissue that it may have interfered with the DNA fragmentation process.

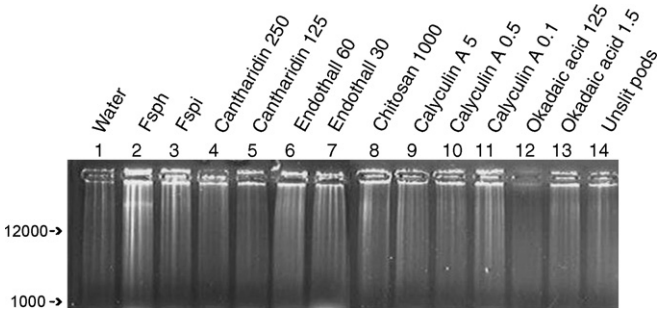

Fig. 3. DNA damage assessment based on the separation of small DNA fragments released from 6 μg extracted genomic DNA retained in agarose gel molds. Genomic DNA was extracted from tissue treated 6 h with: water (lane 1), *F. solani* f. sp. *phaseoli* (Fsph; 1×10^6 macroconidia) (lane 2), *F. solani* f. sp. *pisi* (Fspi; 1×10^6 macroconidia) (lane 3), cantharidin, 250 μg/ml (lane 4), 125 μg/ml (lane 5), endothall 60 μg/ml (lane 6), 30 μg/ml (lane 7), chitosan 1 mg/ml (lane 8), calyculin A 5 μg/ml (lane 9), 0.5 μg/ml (lane 10), 0.1 μg/ml (lane 11), okadaic acid 125 μg/ml (lane 12), 1.5 μg/ml (lane 13), and untreated, unsplit (lane 14). DNA damage above that of the water control caused by *F. solani* f. sp. *phaseoli* and *pisi*, endothall at 60 and 30 μg/ml, and calyculin A treatments was observed. Similar results were obtained in repeated experiments.

Table 8
RT-PCR detectable levels of expression of pea defense genes and HMG A

Treatments	Concentration (μg/μl)	PAL	PR 10	CHS	DRR206	HMG A	Ubiquitin
Water		1	1	1	1	1	1
Calyculin A	0.003	2.6	1.6	5.3	0.1	1.2	0.5
Okadaic acid	0.0028	4.7	0.7	4.1	0.4	1.0	−0.3
Fsph 2×10^6 spores/ml		0.4	2.0	0.1	0.2	1.4	0.7
Pisi 2×10^6 spores/ml		0.9	1.8	0.5	1.3	0.7	−0.8
Chitosan	1.0	1.4	5.5	5.5	0.1	−2.2	1.0

Expression of the defense genes, PAL, PR 10, CHS, and DRR206 compared with the water treatment were changed (fold changes, derived from Ct values, within 5 h) by chitosan, the compatible fungus (Pisi) and incompatible fungus (Fsph). Chitosan rapidly suppressed the transcription of HMG A.

3.8. PR gene induction

RNA was extracted from challenged tissue at a 5.5 h window to monitor PR genes. The relative RNA accumulations of five pea genes in treated pea pods was followed with RT-PCR analyses, utilizing the pea ubiquitin gene as a reference. Each of the treatments as well as the water control was assayed for each gene. Melt curves of the RT-PCR products were also determined to ensure that the RT-PCR product was indeed the DNA of interest (data not shown). The effects of calyculin A, okadaic acid, chitosan, *F. solani* f. sp. *phaseoli*, and *F. solani* f. sp. *pisi* on the activities of five pea genes are presented in Table 8. Messenger RNA accumulations specific for the five pea genes at this time point were evaluated by using gene-specific primers to synthesize a first strand of DNA under uniform conditions. Different concentrations of calyculin A and okadaic acid were associated with major increases in the gene products such as chalcone synthetase and phenylalanine ammonia lyase over those of the water-treated control in the first 5 h. The HMG A transcription factor gene experienced only a minor change within 5 h. A low level of change was obtained with the ubiquitin gene.

4. Discussion

A major benefit visualized from the employment of this inhibitor array study was to further implicate or exclude signaling pathways from the pea system that are potential signaling chemicals studied in other systems. Signals and signaling disrupting chemicals including, reactive O_2^-, H_2O_2, nitric oxide, salicylic acid, jasmonic acid, catechol, methyl salicylate, and BTH, had no apparent major effect on the pisatin production in peas. Chemicals, including methotrexate and *S*-nitroso-*N*-acetyl penicillamine, that generate reactive oxygen species and nitric oxide, respectively, and citrulline and arginine that are precursors to signaling agent NO likewise did not have a significant role in the disease resistance response of pea.

At higher concentrations phosphatase inhibitors disrupt non-host resistance. This suggests that phosphatase inhibitors target processes that initially affect resistance, yet the inhibitor persistence in tissue eventually negatively affects processes vital for both resistance and normal metabolic processes. Currently there is no evidence that fungal-derived phosphatase and kinase inhibitors are involved in this fungal/plant interaction. The mechanisms of how these alien compounds complement or detract from the natural responses induced by chitosan or the *F. solani* f. sp. *phaseoli* inoculum are not known. The biotic inducers, chitosan and Fsph DNase, both produced by *F. solani* f. sp. *phaseoli* are more efficient inducers of a more stable disease resistance [66–68]. We reported earlier that the biotic elicitors released from this formae speciales target nuclear DNA causing subtle but detectable alterations of the pea genomic DNA [6,8]. Furthermore, certain DNA-specific chemicals are potent inducers of PR genes and pisatin accumulation [6]. Also TUNEL assays indicated fungal-induced DNA strand breaks within the nucle of adjacent pea cells. The breaks are also associated with fragmentation of genomic DNA, nuclear distortion observed with fluorescent tags, and PR gene induction [6,69]. All of these alterations were observed following treatment with the pathogens or an array of various DNA-influencing compounds including the biotic elicitors, Fsph DNase, and chitosan. The chromatin isolated from pea tissue treated with fungi or pisatin-inducing polyamines changed sufficiently to expose new attachment sites for DNA-specific marker compounds [8]. All these previous results point to the genomic DNA or associated nuclear proteins as targets for stimulating the defense response. Although in the current study indicates that was minor DNA fragmentation detected following induction by the most active inhibitors, the diversity of the potential actions suggest a more complicated effect than simply DNA alteration. The remarkably potent induction of phytoalexin production by extremely low concentrations of inhibitors such as calyculin A suggests that the phosphorylation state of cellular proteins can also be central in initiating the general non-host defense response.

The serine/threonine phosphatase inhibitors are reportedly specific for protein phosphatases 1 and 2A; both of which have been found in pea tissue [70,71]. Individual phosphatase inhibitors have been previously shown to enhance PAL activity and isoflavonoid production in soybeans [72,73]. They can also mimic elicitor action in inducing rapid hyperphosphorylation of specific proteins [74]. The silencing of phosphatase synthesis can result in the activation of plant defense responses in other plants [75]. The observation that resistance and phytoalexin production in pea can be manipulated by both phosphatase and kinase inhibitors suggests a complex of actions on multiple proteins rather than simply a control of the phosphorylation state of a central protein. Targets of kinase and phosphatase inhibitors could be

nuclear kinases and phosphatases that either enhance or detract from protein's ability to reside in sites within the vicinity of nucleosomes or particular promoter regions.

The mechanism responsible for the minor DNA damage that occurs with the inhibitor treatments is not as apparent as that from other elicitors particularly the Fsph DNase [6,69,76]. The low level inhibitor-caused damage indicated by the recovered DNA fragments represents a very early alteration of host DNA even though the fragments comprise only a small part of the entire genomic DNA. This damage however, appears to be the only effect consistent with the previously reported elicitors of this system. The phosphatase inhibitors endothall and calyculin A showed the most DNA damage above the water control and thus provide some commonality of action on chromatin that may initiate the transcription of PR genes and accumulation of pisatin. Chromatin structure can be changed with the phosphorylation state of proteins that are associated with DNA [75,77,78]. Chromatin changes associated with gene transcription have been more intensely studied in other systems. Recent reports on nucleosome assembly and gene activation may provide valuable insights into the activation of the plant defense response. Such reports indicate that the repression of certain genes requires appropriate nucleosome assembly [79,80]. The reverse side of the picture is that disassembled nucleosomes require reassembly to maintain suppression. Most importantly, in the absence of reassembly, transcription activators are not required for gene activation. In the pea system there are multiple possibilities for the disassembly of chromatin structure: (1) single strand excision via Fsph DNase, (2) DNA conformational changes via DNA-specific agents, (3) competition for histone sites on DNA by chitosan, and (4) exclusion of a transcription factor from a promoter site via alteration of phosphorylation of HMG A [1,6,8,17,18,78]. Each action could participate in the activation of defense gene transcription, albeit somewhat non-specifically. However, in nature where the plant is challenged by a wide variety of pathogens, some non-specificity may be required.

In recognizing that the conformation of chromatin is becoming an intensively studied approach to understand gene activation in eukaryotic organisms, these results suggest that the phosphorylated states of chromatin proteins such as HMG A and the histones be given renewed attention [81]. This study also draws attention to the phosphatase inhibitor's potential to both alter the non-host resistance by breaking resistance to the growth of a bean pathogen and temporarily suppress growth of a pea pathogen. Additionally, optimal inhibitor concentrations can enhance PR gene transcription within the early hours and very significantly enhance phytoalexin accumulation within 24 h.

Acknowledgments

The authors thank Kari Druffel and Marian Silies for RT-PCR instruction, Katie Murray for graphs, Kurt Schroeder for statistical advice and the Washington Sea Grant Program grant R/B-44 for support of this research. The first two authors provided near equivalent efforts in acquiring data. Agriculture Research Center paper no. 0398.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2006.07.014.

References

[1] L.A. Hadwiger, J.M. Beckman, Chitosan as a component of pea *Fusarium solani* interactions, Plant Physiol. 66 (1980) 205–211.
[2] L.A. Hadwiger, W. Wagoner, Effect of heat shock on the mRNAdirected disease resistance response of peas, Plant Physiol. 72 (1983) 553–556.
[3] R.C. Riggleman, B. Fristensky, L.A. Hadwiger, The disease resistance response in pea is associated with increased levels of specific mRNAs, Plant Mol. Biol. 4 (1985) 81–86.
[4] L.A. Hadwiger, D.M. Webster, Phytoalexin production in five cultivars of peas differentially resistance to three races of *Pseudomonas syringae* pv. *pisi*, Phytopathology 74 (1984) 1312–1314.
[5] L.A. Hadwiger, C.C. Chiang, D. Horovitz, Expression of disease resistance response genes in near-isogenic pea cultivars following challenge by *Fusarium oxysporum* race 1, Physiol. Mol. Plant Pathol. 40 (1992) 259–269.
[6] J.J. Choi, S.J. Klosterman, L.A. Hadwiger, A comparison of the effects of DNA-damaging agents and biotic elicitors on the induction of plant defense genes, nuclear distortion, and cell death, Plant Physiol. 125 (2001) 752–762.
[7] L.A. Hadwiger, M.J. Adams, Nuclear changes associated with the host-parasite interaction between *Fusarium solani* and peas, Physiol. Plant Pathol. 12 (1978) 63–72.
[8] L.A. Hadwiger, A. Jafri, S. Von Broembsen, R. Eddy Jr., Mode of pisatin induction: increased template activity and dye-binding capacity of chromatin isolated from polypeptide-treated pea pods, Plant Physiol. 53 (1973) 52–63.
[9] A. Parsons, L.A. Hadwiger, Photoactivated psoralens elicit defense genes and phytoalexin production in the pea plant, Photochem. Photobiol. 67 (1998) 438–445.
[10] C.H. Daniels, B. Fristensky, W. Wagoner, L.A. Hadwiger, Pea genes associated with non-host disease resistance to Fusarium are also active in race-specific disease resistance to Pseudomonas, Plant Mol. Biol. 8 (1987) 309–316.
[11] J.A. Bailey, J.W. Mansfield, Phytoalexins, John Wiley and Sons, New York, 1982, pp. 253–289.
[12] S. Subramanian, M.Y. Graham, O. Yu, T.L. Graham, RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*, Plant Physiol. 137 (2005) 1345–1353.
[13] J.P. Morrissey, A.E. Osbourn, Fungal resistance to plant antibiotics as a mechanism of pathogenesis, Microbiol. Mol. Biol. Rev. 63 (1999) 708–724.
[14] C.C. Wasmann, H.D. VanEtten, Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea, Mol. Plant Microbe Interact. 9 (1996) 793–803.
[15] Y. Zhang, M.J. Tessaro, M. Lassner, X. Li, Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance, Plant Cell 15 (2003) 2647–2653.
[16] S.J. Klosterman, L.A. Hadwiger, Plant HMG proteins bearing the AT hook motif, Plant Sci. 162 (2002) 855–866.
[17] R. Reeves, L. Beckerbauer, HMG I/Y proteins: flexible regulators of transcription and chromatin structure, Biochim. Biophys. Acta 1519 (2001) 13–19.
[18] R. Reeves, Molecular biology of HMG A proteins: hubs of nuclear function, Gene 277 (2001) 63–81.

[19] Q. Wu, H.D. VanEtten, Introduction of plant and fungal genes in to pea (*Pisum sativum* L.) hairy roots reduces their ability to produce pisatin and affects their response to a fungal pathogen., Mol. Plant Microbe Interact. 17 (2004) 798–804.
[20] L.B. Davin, H.-B. Wang, A.L. Crowell, D.L. Bedgar, D.M. Martin, S. Sarkanen, N.G. Lewis, Stereoselective bimolecular phenoxy radical coupling by an auxillary (dirigent) protein without an active center, Science 275 (1997) 362–366.
[21] D.E. Culley, D. Horovitz, L.A. Hadwiger, Molecular characteristics of disease-resistance response gene DRR206-d from *Pisum sativum* (L.), Plant Physiol. 107 (1995) 301–302.
[22] D.E. Culley, S.M. Brown, A. Parsons, L.A. Hadwiger, B.W. Fristensky, Cloning and sequencing of disease-resistance response gene DRR49a from *Pisum sativum*, Plant Physiol. 109 (1996) 722–727.
[23] G.P. Moiseyer, J.J. Beintema, L.I. Fedoreyeva, G.I. Yakovlev, High sequence similarity between ribonuclease from ginseng calluses and fungus-elicited proteins from parsley indicates pathogenesis-related proteins are RNAses, Planta 193 (1994) 470–472.
[24] L.A. Hadwiger, Changes in plant metabolism associated with phytoalexin production, Neth. J. Plant Pathol. 74 (1968) 163–169.
[25] W. Wagoner, D.C. Loschke, L.A. Hadwiger, Two-dimensional electrophoretic analysis of in vivo and in vitro synthesis of proteins in peas inoculated with compatible and incompatible *Fusarium solani*, Physiol. Plant Pathol. 20 (1982) 99–107.
[26] R. Drouin, S. Gao, G.P. Holmquist, Agarose gel electrophoresis for DNA damage analysis, in: G.P. Pfeifer (Ed.), Technologies for Detection of DNA and Mutations, Plenum Press, New York, 1996 (Chapter 3).
[27] H.J. Vogel, A convenient growth medium for *Neurospora*., Microb. Genet. Bull. 13 (1956) 43.
[28] M. Heil, R.M. Bostock, Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses, Ann. Bot. 89 (2002) 503–512.
[29] S. Oikawa, I. Hirosawa, K. Hirakawa, S. Kawanishi, Site specificity and mechanism of oxidative DNA damage induced by carcinogenic catechol, Carcinogenesis 22 (2001) 1239–1245.
[30] J. Gorlack, S. Volratha, G. Knauf-Beiter, G. Hengy, U. Beckhove, K.-H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann, J. Ryals, Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat, Plant Cell 8 (1996) 629–643.
[31] A. Kohler, S. Schwindling, U. Conrath, Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding and infiltration of water into leaves requires the NPR1/NIMI gene in Arabidopsis, Plant Physiol. 128 (2002) 1046–1056.
[32] D.C. Phillips, K.J. Woollard, H.R. Griffiths, The anti-inflammatory actions of methotrexate are critically dependent upon the production of reactive oxygen species, Br. J. Pharmacol. 138 (2003) 501–511.
[33] M. Nakamura, S. Nakaskhima, Y. Katagiri, Y. Nozawa, Involvement of tyrosine phosphorylation in inhibition of fMLP-induced PLD activation by *N*-acetyl-L-cysteine in differentiated HL60 cells, J. Leukocyte Biol. 63 (1998) 781–789.
[34] D. Voet, J.G. Voet, Biochemistry, John Wiley and Sons Inc., New York, 1995, pp. 1290–1291.
[35] F.J. Azula, E.S. Alzola, M. Conde, M. Trueba, M.M. Macarulla, A. Marino, Thrombin-stimulated phospholigase C activity is inhibited without visible delay by a rapid increase in the cyclic GMP levels induced by sodium nitroprusside, Mol. Pharmacol. 50 (1996) 367–379.
[36] H.-S. Park, J.W. Yu, J.-H. Cho, M.-S. Kim, S.-H. Huh, K. Ryoo, E.-J. Choi, Inhibition of apoptosis signal-regulating kinase 1 by nitric oxide through a thiol redox mechanism, J. Biol. Chem. 279 (2003) 7584–7590.
[37] Y.X. Wang, X. Chang, C.C. Pang, Vascular pharmacology of methylene blue in vitro and in vivo: a comparison with N^G-nitro-L-arginine and diphenyleneiodonium, Br. J. Pharmacol. 114 (1995) 194–202.
[38] L. Caccavelli, I. Manfroid, J.A. Martial, M. Muller, Transcription factor AP1 is involved in basal and okadaic acid-stimulated activity of the human PRL promoter, Mol. Endocrinol. 12 (1998) 1215–1227.
[39] H.J. Kim, M.H. Lee, H.J. Kim, H.I. Shin, J.Y. Choi, H.M. Ryoo, Okadaic acid stimulates osteopontin expression through de novo induction of AP-1, J. Cell. Biochem. 87 (2002) 93–102.
[40] S. Corvera, S. Jaspers, M. Paseri, Acute inhibition of insulin-stimulated glucose transport by the phosphatase inhibitor, okadaic acid, J. Biol. Chem. 266 (1991) 9271–9275.
[41] Y.M. Li, C. Mackintosh, J.E. Casida, Protein phosphatase 2A and its [^{3}H] cantharidin/[^{3}H] endothall thioanhydride binding site. Inhibitor specificity of cantharidin and ATP analogues., Biochem. Pharmacol. 46 (1993) 1435–1443.
[42] G.W. Peet, J. Li, IKB Kinases alpha and beta show a random sequential kinetic mechanism and are inhibited by staurosporine and quercetin, J. Biol. Chem. 274 (1999) 32655–32661.
[43] P. Piedras, K.E. Hammond-Kosack, K. Harrison, J.D.G. Jones, Rapid, Cf-9- and Avr9-dependent production of active oxygen species in tobacco suspension cultures, Mol. Plant Microbe Interact. 11 (1998) 1155–1166.
[44] Y.-S. Seong, C. Min, L.L. Jae, J.Y. Yang, S.Y. Kim, X. Cao, K. Dim, S.H. Yuspa, H.-H. Chung, K.S. Lee, Characterization of a novel cyclin-dependent kinase 1 inhibitor, BMI-1026, Cancer Res. 63 (2003) 7384–7391.
[45] I. Hajimohammadreza, A.W. Probert, L.L. Coughenour, S.A. Borosky, F.W. Maracoux, P.A. Boxer, K.K. Wang, A specific inhibitor of calcium/calmodulin-dependent protein kinase-II provides neuroprotection against NMDA and hypoxia/hypoglycemia-induced cell death, J. Neurosci. 15 (1995) 4093–4101.
[46] A. Heese, A.A. Ludwig, J.D.G. Jones, Rapid phosphorylation of a syntaxin during the Avr9/Cf-9-race-specific signaling pathway, Plant Physiol. 138 (2005) 2406–2416.
[47] I. Molokanova, R.H. Kramer, Mechanism of inhibition of cyclic nucleotide-gated channel by protein tyrosine probed with genistein, J. Gen. Physiol. 117 (2001) 219–234.
[48] U. Lonn, S. Lonn, U. Nylen, G. Winblad, Altered formation of DNA in human cells treated with inhibitors of DNA topoisomerase II (etoposide and tenoposide), Cancer Res. 49 (1989) 6202–6207.
[49] Y. Onishi, Y. Azuma, H. Kkizaki, Bis (2,6-dioxopiperaxine) derivatives, topoisomerase II inhibitors which do not form a DNA cleavable complex, induce thymocyte apoptosis, Biochem. Mol. Biol. Int. 32 (1994) 1115–1122.
[50] Y. Benchokroun, J. Couprie, A.K. Larsen, Aurintricarboxylic acid, a putative inhibitor of apoptosis, is a potent inhibitor of DNA topoisomerase II in vitro and in Chinese hamster fibrosarcoma cells, Biochem. Pharmacol. 49 (1995) 305–313.
[51] C. Zimmer, B. Puschendorf, H. Grunicke, P. Chandra, H. Venner, Influence of netropsin and distamycin A on the secondary structure and template activity of DNA, Eur. J. Biochem. 21 (1971) 269–278.
[52] J.-W. Cheong, S.Y. Chong, J.Y. Kim, J.I. Eom, H.K. Jeung, H.Y. Maeng, S.T. Lee, Y.H. Min, Induction of apoptosis by apicidin, a histone deacetylase inhibitor, via the activation of mitochondria-dependent caspase cascades in human Bcr-Abl-positive leukemia cells, Clin. Cancer Res. 9 (2003) 5018–5027.
[53] J.-Y. Shin, H-S. Kim, J. Park, J.-B. Park, J.-Y. Lee, Mechanism for inactivation of the KIP family cyclin-dependent kinase inhibitor genes in gastric cancer cells, Cancer Res. 60 (2000) 262–265.
[54] H. Umezawa, Enzyme Inhibitors of Microbial Origin, University of Tokyo Press, Tokyo, 1972, pp. 29–32.
[55] B.S. Baliga, A.W. Pronczuk, H.N. Munro, Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver, J. Biol. Chem. 244 (1969) 4480–4489.
[56] E.W. Oppenheim, I.M. Nasrallah, M.G. Mastri, P.J. Stover, Mimosine is a cell-specific antagonist of folate metabolism, J. Biol. Chem. 275 (2000) 19268–19274.
[57] F. Ali-Osman, G. Antoun, H. Wang, S. Rajagopal, E. Gagucas, Buthionine sulfoximine induction of gamma-L-glutamyl-L-cysteine synthetase gene expression, kinetics of glutathione depletion and resynthesis and modulation of carmustine-induced DNA–DNA cross-linking and cytotoxicity in human glioma cells, Am. Soc. Pharmacol. Exp. Ther. 49 (1996) 1012–1020.
[58] K. Zhang, M. Chew, E.B. Yang, K.P. Wong, P. Mack, Modulation of cisplatin cytotoxicity and cisplatin-induced DNA cross links in HepG2

cells by regulation of glutathione-related mechanisms, Mol. Pharmacol. 59 (2001) 837–843.
[59] K.R. Fox, M.J. Waring, J.R. Brown, S. Neidle, DNA sequence preferences for the anti-cancer drug mitoxanthrone and related anthraquinones revealed by DNase I footprinting, FEBS Lett. 202 (1986) 289–294.
[60] G.V. Rao, K.S. Rao, Modulation of K+ transport across synaptosomes of rat brain by synthetic pyrethroids, J. Neurol. Sci. 147 (1977) 127–133.
[61] P.P. Graczyk, Caspase inhibitors as anti-inflammatory and anti-apoptotic agents, Prog. Med. Chem. 39 (2002) 1–72.
[62] M. Enari, H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, S. Nagata, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, Nature 391 (1998) 43–50.
[63] J.A. Collins, C.A. Schandl, K.Y. Young, J. Vesely, M.C. Willingham, Major DNA fragmentation is a late event in apoptosis, J. Histochem. Cytochem. 45 (1997) 923–930.
[64] L. Li, C. Peterson, X. Lu, P. Wei, R. Legerski, Interstrand cross-links induced DNA synthesis in damages and undamaged plasmids in mammalian cell extracts, Mol. Cell. Biol. 19 (1999) 5619.
[65] K. Osaka, V.M. Ritov, J.F. Bernardo, R.A. Branch, V.E. Kagan, Amphotericin B protects cis-parinaric acid against peroxyl radical-induced oxidation: amphotericin B as an antioxidant, Antimicrob. Agents Chemother. 41 (1997) 743–747.
[66] D.F. Kendra, D. Christian, L.A. Hadwiger, Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/β-glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance, Physiol. Mol. Plant Pathol. 45 (1989) 215–230.
[67] L.A. Hadwiger, M.M. Chang, M.A. Parsons, Fusarium solani DNase is a signal for increasing expression of non-host disease resistance response genes, hypersensitivity, and pisatin production, Mol. Plant Microbe Interact. 8 (1995) 871–879.
[68] J.J. Choi, S.J. Klosterman, L.A. Hadwiger, A promoter from pea gene DRR206 is suitable to regulate an elicitor-coding gene and develop disease resistance, Phytopathology 94 (2004) 651–660.
[69] S.J. Klosterman, J. Chen, J.J. Choi, E.E. Chinn, L.A. Hadwiger, Characterization of a 20 kDa DNase elicitor from *Fusarium solani* f. sp. *phaseoli* and its expression at the onset of induced resistance in *Pisum sativum*, Mol. Plant Pathol. 2 (2001) 147–158.
[70] Y.-L. Guo, J. Roux, Partial purification and characterization of a type 1 protein phosphatase in purified nuclei of pea plumules, Biochem. J. 319 (1996) 985–991.
[71] M. Evans, T. Fawcett, D. Boulter, A.P. Fordham-Skelton, A homologue of the 65 kDa regulatory subunit of protein phosphatase 2A in early pea (*Pisum sativum* L.) embryos, Plant Mol. Biol. 24 (1994) 689–695.
[72] C. Mackintosh, G.D. Lyon, R.W. Mackintosh, Protein phosphatase inhibitors activate anti-fungal defense responses of soybean cotyledons and cell cultures, Plant J. 5 (1994) 137–147.
[73] T.J. Gianfagna, M.A. Lawton, Specific activation of soybean defense genes by the phosphoprotein phosphatase inhibitor okadaic acid, Plant Sci. 109 (1995) 165–170.
[74] G. Felix, M. Regenass, P. Spanu, T. Boller, The protein phosphatase inhibitor calyculin A mimic's elicitor action in plant cells and induces rapid hyperphosphorylation of specific proteins as revealed by pulse labeling with [^{32}P] phosphate, Proc. Natl. Acad. Sci. 91 (1994) 952–956.
[75] X. He, J.C. Anderson, O. del Pozo, Y.-Q. Gu, X. Tang, G.B. Martin, Silencing of subfamily 1 of protein phosphatase 2A catalytic subunits results in activation of plant defense responses and localized cell death, Plant J. 38 (2004) 563–577.
[76] S. Luan, Protein phosphatases in plants, Annu. Rev. Plant Biol. 54 (2003) 63–92.
[77] T. Xing, T. Ouellet, B.L. Miki, Towards genomic and proteomic studies of protein phosphorylation in plant–pathogen interactions, Trends Plant Sci. 7 (2002) 224–230.
[78] D.L. Gerhold, A.J. Pettinger, L.A. Hadwiger, Characterization of a plant stimulated nuclease from *Fusarium solani*, Physiol. Mol. Plant Pathol. 43 (1993) 33–46.
[79] M.W. Adkins, J.K. Tyler, Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions, Mol. Cell 21 (2006) 405–416.
[80] J.K. Davie, S.Y.R. Dent, No Spt6 no nucleosomes, no activator required, Mol. Cell 21 (2006) 452–453.
[81] I. Kojiro, U.K. Laemmli, Structural and dynamic functions establish chromatin domains, Mol. Cell 11 (2003) 237–248.