Theor Appl Genet (2006) 112: 1452–1457
DOI 10.1007/s00122-006-0247-9

ORIGINAL PAPER

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Differential gene expression in *Phaseolus vulgaris I* locus NILs challenged with *Bean common mosaic virus*

Received: 9 September 2005 / Accepted: 20 February 2006 / Published online: 28 March 2006

Abstract The *Phaseolus vulgaris I* locus-*Bean common mosaic virus* (BCMV; Potyviridae) pathosystem is of critical importance to bean geneticists, breeders and pathologists because of the worldwide distribution of both the virus and germplasm containing this resistance gene. In order to learn more about the molecular responses characteristic of this resistance gene, a cDNA-AFLP screen was conducted on homozygous NILs of *P. vulgaris* variety 'Black Turtle Soup' (BT), containing either the *I* locus allele for resistance (BT_{II}) or susceptibility (BT_{ii}) to BCMV. Eight conditions were compared in a factorial analysis: BT_{II} versus BT_{ii}; mock inoculated versus BCMV inoculated; 26 versus 34°C. Transcripts induced in response to viral infection and that were further responsive to temperature, genotype or both were isolated and cloned. Sequence analysis of the resultant clones revealed several classes of putative genes, including transcription-related and signal transduction-related genes. Review of disease resistance literature suggests further avenues of research involving the candidates isolated in this screen.

Introduction

Disease resistance research is currently at the forefront in the field of plant biology and much effort has gone into the elucidation of what occurs between the recognition of a pathogen and the onset of defense. While resistance is the endpoint of known pathogen recognition signal transduction pathways, and genetic and biochemical evidence confirms roles for several genes in defense responses, the exact connections and intersections between the chemical, biochemical and physical reactions producing recognition-based or basal resistance are as yet unresolved (Rathjen and Moffett 2003; Belkhadir et al. 2004). Pathogenesis related (PR) genes are often expressed as a result of signal transduction (Maleck et al. 2000; Shah et al. 2001) and specific transcription factors have been identified whose interaction with PR gene promoters is required for their salicylic acid-dependent induction (Zhang et al. 1999, 2003; Zhou et al. 2000). Yet most PR genes have neither antimicrobial activity nor any direct effect on pathogen attack at all. What are the molecules that literally and immediately confer resistance? What stops the pathogen attack? Possible candidate molecules for the resistance endpoint are those, such as ubiquitin and protease inhibitors, that in some way modify or inhibit invading pathogen molecules (Heath et al. 1997; Jones and Takemoto 2004). However, these defense genes are hard to discover due to the likely scenario that the ultimate inhibition or death of the pathogen may be multifaceted and genetically redundant.

Efforts to dissect the resistance response employ both classical genetics and genomics—ranging from mutant screens to microarray studies. The use of transformation technology to confirm gene function in model species such as *Arabidopsis* and *Nicotiana* has resulted in large databases of phenotypic data. However, mutant screens can be confounded by epistasis and dominance, and microarrays can be too costly to examine all the relevant genotypes, environments, and pathogens and the microarrays themselves may not even include all the relevant genes. A great deal of data has been generated using these methods, but the complete picture still eludes us. Further, work in nonmodel systems can significantly limit a researcher's technology arsenal. An alternative approach—a differential dis-

Communicated by F. J. Muehlbauer

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play-type method—can be sensitive enough to detect flux through a pathway while being cost-effective enough to screen multiple genotypes under multiple conditions. In particular, cDNA-AFLP can be performed under conditions of much higher stringency than other differential display methods, thus eliminating some potential for false positives (Bachem et al. 1996). Further, small variations at multiple points along a pathway as well as differences in timing or tissue-specificity of expression can be revealed (Bachem et al. 1996). As a result, differential screens often identify housekeeping or seemingly nonsensical genes; however, this is not entirely unreasonable especially in light of the Guard hypothesis (Dangl and Jones 2001; van der Biezen and Jones 1998). That is, if a cell is under attack by a pathogen it is feasible that normal metabolism or functioning may be perturbed and, in fact, this may well be what an R gene monitors (Dangl and Jones 2001; van der Biezen and Jones 1998).

In order to discover some of the transcriptional variation that occurs as either resistance or susceptibility develops, we evaluated a genetically simplified, yet agronomically relevant pathosystem: *Bean common mosaic virus* (BCMV) infection in *I* gene-containing *Phaseolus vulgaris*. BCMV is a member of the *Potyviridae* and, as such, encodes only eight proteins, whose functions have been characterized in BCMV and other closely related Potyviruses (Bos 1971; Urcuqui-Inchima et al. 2001). No single virulence factor or movement protein for this virus is known at this time, although several genes have been implicated as being involved with the latter (Urcuqui-Inchima et al. 2001). The *P. vulgaris* host genotypes used here are inbred, nearly isogenic lines (NIL) varying most significantly at the *I* locus, so any variation detected in a differential expression study should be attributable to this locus. Resistance to BCMV conferred by the incompletely dominant *I* allele is considered to be extreme at 26°C (Cadle-Davidson and Jahn 2005; Collmer et al. 2000; Drijfhout 1991). The "extreme" descriptor refers to the lack of any visible symptoms and the inability to recover virus from inoculated plants. However, upon inoculation at higher temperatures (above 30°C), resistant plants develop necrotic lesions, systemic venal necrosis and, ultimately, apical death (Drijfhout 1991). Susceptible plants show chlorotic (sometimes necrotic) lesions and systemic mosaic at either temperature, although at high temperature symptoms develop faster and often lead to premature death (Drijfhout 1991).

In the present study a factorial cDNA-AFLP screen was conducted to compare expression between resistant and susceptible NILs of cv. 'Black Turtle Soup' (BT), BT_{II} and BT_{ii}, at 26 and 34°C. Bands of interest were cloned and sequenced and their sequences submitted for BLAST searches against the nonredundant and EST databases in Genbank. The results of these sequence analyses were compared with known genes involved in plant disease resistance responses.

Materials and methods

Germplasm

Near isogenic lines of *P. vulgaris* variety BT homozygous for either the dominant (BT_{II}) or recessive allele (BT_{ii}) were used in these experiments (Cadle-Davidson 2005; Provvidenti 1983). The NILs were originally resistant and susceptible selections from a seed lot of BT (Provvidenti 1983). Since this selection the lines have been backcrossed for five generations in order to homogenize the genetic background (Cadle-Davidson 2005). In theory, this level of backcrossing retains approximately 1.5% of the donor genome, however the actual remnant in these lines is likely much less due to the fact that the initial genotypes were already very closely related. All experimental plant material was maintained in a growth chamber at 26°C days/22°C nights or 34°C days/31°C nights with a 16 h photoperiod.

Viral isolate maintenance and inoculation

Bean common mosaic virus NY15 68/95 was obtained from R. Provvidenti (Geneva, NY, USA) and maintained on the *P. vulgaris* variety 'California Light Red Kidney' (CLRK), a mosaic-producing host. Rub inoculations were performed using sap extracted from fresh, highly symptomatic CLRK leaves by grinding in a chilled mortar with carborundum (400 mesh) and 0.05 M KH_2PO_4.

RNA isolation and cDNA-AFLP

Samples for RNA extraction and cDNA-AFLP analysis were collected at 4 days post inoculation, immediately frozen in liquid nitrogen and stored at −80°C. This material consisted of: BT_{II} mock-inoculated (buffer only), 26°C; BT_{II} BCMV-inoculated, 26°C; BT_{II} mock-inoculated, 34°C; BT_{II} BCMV-inoculated, 34°C; BT_{ii} mock-inoculated (buffer only), 26°C; BT_{ii} BCMV-inoculated, 26°C; BT_{ii} mock-inoculated, 34°C; BT_{ii} BCMV-inoculated, 34°C. A single primary (inoculated or mock-inoculated) leaf from each sample was used as starting material for the RNA preparation. RNA isolation and cDNA-AFLP procedures and primer sequences were as in Bachem et al. (1996). All 256 primer combinations described by Bachem et al. (1996) were used. RNA extraction buffer volumes were scaled down to one fourth of the original protocol to account for the reduced amount of tissue used here.

Clone isolation and validation

Bands that reflected gene induction relevant to the pathosystem being studied were isolated and cloned. Selection criteria consisted of: (1) Responsiveness to viral infection; (2) Responsiveness to heat; (3) Responsiveness to genotype—specifically the presence of the *I*

allele; and (4) Combinations of 1 and 3. Gel slices corresponding to the bands of interest were boiled and used as template for PCR using the primer sets from the original amplification. Following this PCR and confirmation of product by agarose gel electrophoresis, the PCR product was cloned into EcoRV-digested pBlue-Script KS- to which a T overhang had been added by incubation at 70°C with 1 U Taq DNA polymerase and 50 mM dTTP. All clones were sequenced using the M13 forward primers by the Bioresource Center, Cornell University, Ithaca, NY, USA. Validation of clones was performed by northern analysis using a panel of RNAs extracted from a second experiment using the same genotypes, inoculation, and environmental conditions as were used for the original screen.

Sequence analysis

All sequence analysis was performed using the Lasergene suite of sequence analysis programs (DNAstar) and Sequencher (Gene Codes Corporation). Redundant clones were eliminated at this point. Following annotation of sequence data, all clones were submitted to NCBI BLAST comparisons in an attempt to determine their identities. The procedure used for identifying each clone was to first BLASTn each sequence in the nonredundant (nr) database. If no hits were found in this database that met the Expect ($E \leq 10^{-5}$) criterion, the sequence was then submitted to BLASTn against the EST database. The original sequence was also used to perform a translated BLAST (BLASTx) on the protein database. If the original sequence returned a significant match only in the EST database, the resultant EST was used to BLAST the nr and protein databases.

Results

Several banding patterns were present in the materials tested here including uniform expression for the majority of bands detected. This was expected due to the fact that the starting plant materials are NILs that differ primarily at the *I* locus. Seven different banding patterns (Fig. 1) were considered interesting in the context of these experiments and only bands that fit into these categories (labeled a–g) were extracted and cloned. With the exception of (e), all bands cloned were virus-dependent. The relevant banding patterns were: (a) temperature-dependent size variant; (b) BT_{II} specific, temperature-independent; (c) genotype-dependent differential abundance; (d) heat responsive, virus-dependent; (e) heat responsive, virus-independent; (f) high temperature induction; and (g), BT_{II}-specific, high temperature only. Pattern (a) was selected without knowledge of whether the bands were derived from the same or different genes and both bands were isolated. In total, 59 bands were identified as being meaningful and were extracted from the gels.

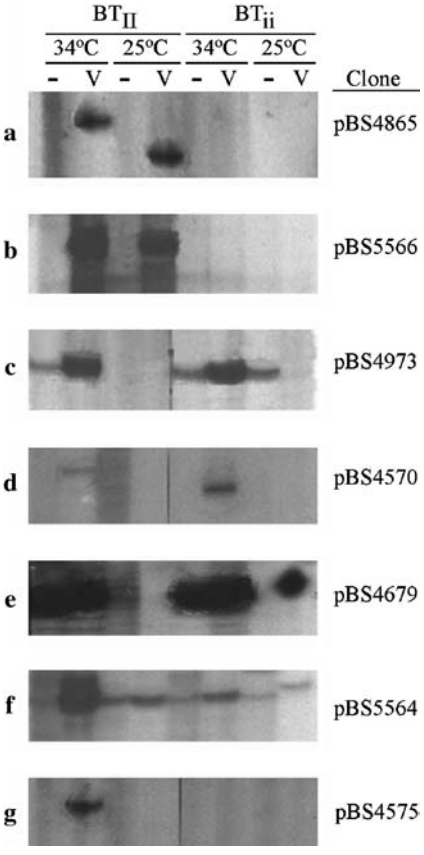

Fig. 1 Examples of expression patterns from cDNA-AFLP gels when BT_{II} and BT_{ii} were compared at 25 and 34°C. RNA for this analysis was collected 4 days post inoculation or mock inoculation. Letters a–g represent classes of banding patterns and are represented by images of single clones each. *V* BCMV-inoculated sample, - mock inoculated sample. **a** Temperature-dependent size variation, **b** BT_{II} specific, temperature-independent, **c** genotype-dependent differential abundance, **d** heat responsive, virus-dependent, **e** heat responsive, virus-independent, **f** high temperature induction, **g** BT_{II}-specific, high temperature only

Sequence analysis revealed that most of the clones were redundant and the original number of cloned bands was reduced to 19 unique clones (Table 1) with 17 of these being confirmed by northern analysis. All confirmed sequences were submitted to the Genbank database and their accession numbers are included in Table 1. Banding pattern (a) resulted in either one or two unique sequences and only the former were retained in this study. A number of reasons for the presence of two different length fragments derived from the same sequence may be cited, including alternative splicing, expression from different gene family members or alternative starts/stops. All of these are reasonable possibilities based on the fact that transcription and transcription factors are frequently influenced by environmental and biotic factors (Ferrier-Cana et al. 2005; Kaldis et al. 2003). Table 1 shows the results of the sequence analyses conducted on the cDNA-AFLP clones. The sequences identified by the cDNA-AFLP experiments presented here are comprised of genes that can be categorized as transcription-related, signal

Table 1 cDNA-AFLP clones showing differential expression between *P. vulgaris* NILs, BT_{II} and BT_{ii}, and at 25 versus 34°C

Clone (Genbank accession)	Banding pattern	BLAST results		
		GenBank accession	*E* value	Description
Transcription				
pBS4979 (DW177235)	g	NP_197184	6.00E-20	*Arabidopsis* heat shock transcription factor 3 (HSF3)
Signal transduction				
pBS4773 (DW177223)	g	AY463016.1	2.00E-11	*Musa acuminata* putative beta family G-protein
pBS4866 (DW177226)	g	AF096249	1.00E-100	*Lycopersicon esculentum* ethylene-responsive small GTP-binding
pBS5077 (DW177230)	g	BAC05575.1	4.00E-74	*Oryza sativa* protein phosphatase 2C-like protein
Defense				
pBS4575 (DW177218)	g	AY248742	4.00E-28	*L. esculentum* omega-3 fatty acid desaturase gene
pBS4771 (DW177222)	b	AJ506739.1	2.00E-05	*O. sativa* mRNA for beta 1,3-glycosyltransferase-like protein II/Avr9 elicitor response protein-like (3e-75, BAB09796.1)
Housekeeping				
pBS4571 (DW177217)	c	AJ320268	3.00E-26	*P. paniceum* malate deshydrogenase
pBS4665 (DW177219)	a	X75082	6.00E-38	*S. tuberosum* mRNA for mitochondrial citrate-synthase
pBS4679 (DW177220)	e	X14060	8.00E-41	*L. esculentum* nia gene for lnitrate reductase (EC 1.6.6.1)
pBS4764 (DW177221)	e	X05984.1	9.00E-08	Tomato rbcS3A gene for ribulose 1,5-bisphosphate carboxylase/oxygenase
pBS4973 (DW177229)	c	K00507	6.00E-50	Tobacco chloroplast atpase gene (b and e subunits)
Unknown				
pBS4777 (DW177224)	g	AAM63708.1	1.00E-71	*O. sativa* putative zinc-finger, heat shock protein
pBS4865 (DW177225)	a	AF233745	1.00E-06	*L. esculentum* chaperonin 21 precursor
pBS4872 (DW177227)		BI931463	1.00E-95	Tomato EST; predicted transmembrane domain
pBS5265 (DW177231)	g	CB483497	2.60E-02	*O. sativa* aluminium-induced EST
pBS5566 (DW177233)	b	AAC98059.1	3.00E-26	*Arabidopsis* chloroplast lumen common protein
pBS5667 (DW177234)	g	BG128668	2.00E-26	*L. esculentum* shoot/meristem EST

[a] Temperature-dependent size polymorphism
[b] BT_{II} specific
[c] Genotype-dependent differential abundance
[d] High temperature size/abundance polymorphism
[e] Heat responsive
[f] High temperature increased abundance
[g] BT_{II} specific, high temperature only

transduction-related, defense-related, "housekeeping" genes and others that do not give insight into their relevance to this study.

Discussion

In the present study isolines for the *Phaseolus* virus resistance locus, *I*, were differentially screened using cDNA-AFLP. The rigor of the experimental design aided the discovery of candidate genes induced by the interaction of BCMV and the *I* locus due to the fact that genotype, environment and infection state were controlled for. Specifically, resistant and susceptible NILs were tested at both normal and elevated temperatures and in both the inoculated and mock-inoculated condition. The factorial design eliminates selection of many false-positive banding patterns and allows for the identification of bands that are present only in the presence of viral infection as well as in association with resistance. This combination of genetics and differential expression screen is very powerful: because there are very few genetic differences between the BT NILS, any differences in gene expression are likely due to the influence of the *I* locus. Further, the conditions tested here included virus-infected samples at "permissive" (34°C) and "restrictive" (26°C) temperatures. It is well known that viral infection leads to alteration of gene expression and that this, in part, is due to viral recruitment of the cellular transcription machinery for its own use (Hull 2002). Therefore, the differential expression seen in this study should be viewed as having

two sources: (1) defensive regulation controlled by host cells in order to combat infection and heat shock and (2) viral manipulation of cellular transcription for the purpose of replication and movement.

In a similar study, Vallejos et al. (2000) compared bulked samples homozygous for either *I* or *i* from a population of recombinant inbred lines (76 lines in the complete population). This study did much to account for the genetic background and identified RNA-level differences between resistance and susceptibility. A further result of their research was the discovery sequences genetically linked the *I* locus that may prove beneficial in the positional cloning of this resistance locus (Vallejos et al. 2000). However, they did not address the complete interaction of the *I* locus with pathogen or environment either by inoculation with BCMV or by exposure to the permissive and restrictive temperatures (Vallejos et al. 2000). Further, the clones isolated in the Vallejos et al. (2000) study were not sequenced and as a result, no additional comparisons between their study and the present one may be made.

Clones pBS4773, 4866 and 5077 show significant sequence similarity to components of an ethylene-responsive G-protein signaling system (4773, 4866) and an ABA sensitive protein phosphatase 2C (5077) (Table 1) (Roehl et al. 1995; Zegzouti et al. 1999). It is reassuring that we identified three potential components of the same signal transduction pathway in our screen and that they share the same banding pattern. At this time, stress responsive ABA signaling is the only pathway in plants known to function via G-protein signaling (Leung et al. 1997; Jones 2002). The possibility that a stress-related signaling pathway is induced in a resistant-genotype-dependent manner in response to viral infection is inviting in that it suggests that general, not specialized, pathways are used by a plant host in order to combat infection and is consistent with the growing evidence for crosstalk between signaling pathways (Kachroo et al. 2003).

By reviewing the molecular plant pathology literature, some of these genes' functions can be speculated upon; however, this is only useful in hypothesis generation. In this regard, the clones generated in these experiments are candidates for further hypothesis-driven assays into the molecular interactions of the *I* locus-BCMV pathosystem and, perhaps, virus resistance, in general.

Acknowledgements We thank L. Cadle-Davidson, G. Rairdan, S. Restrepo, P. Griffiths, S. McCouch, and T. Zitter for critical review of this manuscript and insightful discussions during the course of this work and G. Moriarty for technical assistance. M.M. Cadle-Davidson was supported in part by the Interdisciplinary Research Training Group in Molecular Mechanisms of Plant Processes (NSF Grant DBI-9420687).

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