Research Note

cDNA-AFLP Display for the Isolation of *Peronospora parasitica* Genes Expressed During Infection in *Arabidopsis thaliana*

Erik A. van der Biezen, Hedyani Juwana, Jane E. Parker, and Jonathan D. G. Jones

The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, U.K.

Accepted 1 May 2000.

To identify genes from the obligatory biotrophic oomycete *Peronospora parasitica* that are expressed during infection in *Arabidopsis thaliana* we employed cDNA-amplified fragment length polymorphism (AFLP) display. cDNA-AFLP fragments from infected and non-infected leaves were separated in parallel by gel electrophoresis and displayed by autoradiography. Most differential gene fragments were derived from *P. parasitica*.

Additional keywords: differential display.

The exclusively biotrophic lifestyle of certain agronomically important pathogens has hampered the identification of genes involved in pathogenicity. Several techniques have been applied to monitor alterations in gene expression that occur during compatible interactions. Although differential display reverse transcription-polymerase chain reaction (RT-PCR), differential hybridization, and subtractive hybridization have been useful techniques, they can generate a high proportion of false-positives (e.g., see Jones and Harrower 1998). We applied the cDNA-amplified fragment length polymorphism (AFLP) technique (Bachem et al. 1996) to identify *Peronospora parasitica* genes expressed during infection in *Arabidopsis thaliana*.

A. thaliana eds1 genotypes support enhanced growth of *P. parasitica* (Parker et al. 1996). To obtain high levels of *P. parasitica* infection, we therefore inoculated the *A. thaliana* ecotype Wassilewskija (Ws-0) that carries the *eds1-1* mutation with *P. parasitica* isolate Noco2 (Parker et al. 1996). Maximum levels of *P. parasitica* growth were achieved 4 days after infection of 10-day-old seedlings with a suspension of 4×10^4 conidiospores per ml at 16°C and 100% humidity. Infection levels were asserted by lactophenol trypan blue staining.

P. parasitica-infected and water-sprayed control seedlings were harvested, and mRNA was isolated. Following cDNA

synthesis, the AFLP procedure was applied (Vos et al. 1995; Bachem et al. 1996) with the *MseI* and *ApoI* restriction enzymes (Durrant et al., *in press*). The adapter-linked cDNA

Fig. 1. Example of a cDNA-amplified fragment length polymorphism (AFLP) autoradiogram. RNA was isolated from *Peronospora parasitica*-infected (+) and non-infected (–) leaves, and the cDNA was subjected to AFLP procedures. The amplified ^{32}P -radiolabeled cDNA fragments were separated in parallel by polyacrylamide gel electrophoresis and displayed by autoradiography. Gene fragments present only in samples made from infected plants (arrows) were excised from the gel, re-amplified, and sequenced.

Corresponding author: J. D. G. Jones
E-mail: jonathan.jones@bbsrc.ac.uk

Current address of Erik A. van der Biezen: Aventis CropScience N.V., Jozef Plateastraat 22, 9000 Gent, Belgium.

Nucleotide and/or amino acid sequence data are to be found at GenBank as accession nos. AW737062 to AW737077.

fragments were PCR amplified with ³³P-radiolabeled primers with two and three “selective” nucleotides, respectively (described in Durrant et al., *in press*). The cDNA fragments of infected and non-infected plants were separated in parallel by polyacrylamide gel electrophoresis and displayed by autoradiography (Fig. 1).

In total, 228 primer combinations were used, generating products ranging in size from approximately 100 to approximately 600 bp. The cDNA-AFLP procedure produced highly reproducible banding patterns with on average 70 amplification products per PCR and, hence, a total estimated number of approximately 16,000 (228 × 70) fragments. Sixty fragments (0.4%) were present only in samples made from infected plants. Twenty-three of these cDNA fragments were excised from the gel and the DNA sequence determined (Table 1). The

sequences of two cDNA fragments, CAF15 (185 bp) and CAF42 (206 bp), were extended by 3′ rapid amplification of cDNA ends (3′-RACE; Sambrook et al. 1989) giving total lengths of 506 and 326 bp, respectively.

The cDNA fragments present only in samples made from infected plants could originate from *P. parasitica* genes expressed during infection, or from *A. thaliana* genes induced by *P. parasitica* infection. An advantage of *A. thaliana* as the host species is that its determined genome sequence (>85%, as of April 2000) and extensive expressed sequence tag (EST) data base can be used to rapidly assess the origin of the cDNA fragments by data base analysis. Seven cDNA fragments were identical to genomic *A. thaliana* sequences (Table 1), indicating that these fragments were derived from *A. thaliana* genes, possibly transcriptionally induced upon *P. parasitica* infec-

Table 1. Nucleotide identity of cDNA-amplified fragment length polymorphism (AFLP) fragments (CAFs) present only in *Peronospora parasitica*-infected *Arabidopsis thaliana* seedlings with *A. thaliana* sequences and oomycete expressed sequences tags (ESTs)

CAF ^a	Origin ^b	<i>A. thaliana</i> sequence ^a	Oomycete EST ^a	P ^{c,d}
CAF01 [AW737062]	<i>P. parasitica</i>	None	PGI:S:4368	1 ⁻³
CAF02	<i>A. thaliana</i>	Genome [B23722]; ESTs [T76650, H36949, N97152, H36811, T76561]	None	
CAF05	<i>A. thaliana</i>	Genome [AC002376]	None	
CAF10	<i>A. thaliana</i>	Genome [AB023038]	None	
CAF11	<i>A. thaliana</i>	Genome [AC007133]	None	
CAF12	<i>A. thaliana</i>	Genome [AC004261]	None	
CAF13 [AW737063]	<i>P. parasitica</i>	EST [AI994318]	PGI:S:292	5 ⁻⁶
CAF14 [AW737064]	ND	None	None	
CAF15 [AW737065] ^e	<i>P. parasitica</i>	None	PGI:S:3665	1 ⁻⁸⁷
CAF16 [AW737066]	ND	None	None	
CAF23 [AW737067]	ND	None	PGI:S:565	1 ⁻²⁸
CAF33	<i>A. thaliana</i>	genome [AC004133]	None	
CAF36 [AW737068]	ND	None	PGI:S:2910	3 ⁻⁶
CAF40 [AW737069]	<i>P. parasitica</i>	None	None	
CAF41	<i>A. thaliana</i>	Genome [AB013392]	None	
CAF42 [AW737070] ^e	<i>P. parasitica</i>	None	PGI:S:2713	5 ⁻⁴⁶
CAF43 [AW737071]	<i>P. parasitica</i>	None	None	
CAF44 [AW737072]	<i>P. parasitica</i>	None	None	
CAF49 [AW737073]	<i>P. parasitica</i>	None	None	
CAF50 [AW737074]	ND	None	None	
CAF51 [AW737075]	<i>P. parasitica</i>	None	None	
CAF52 [AW737076]	<i>P. parasitica</i>	None	PGI:S:2452	2 ⁻¹⁹
CAF58 [AW737077]	ND	None	PGI:S:4308	3 ⁻⁵⁷

^a With [accession number].

^b The *A. thaliana* and *P. parasitica* origins of the cDNA fragments were deduced from the nucleotide identity with *A. thaliana* genome sequences, and determined by polymerase chain reaction and Southern hybridization with genomic DNA. ND = not determined.

^c Blast-N (Altschul et al. 1997).

^d P = number of distinct alignments with equivalent or superior score occurring purely by chance; depends upon alignment score, and lengths of both the query sequence and the data base searched.

^e cDNA-AFLP sequences extended by 3′ rapid amplification of cDNA ends (3′-RACE).

Table 2. Sequences of polymerase chain reaction primers used to amplify *Peronospora parasitica* gene fragments from genomic DNA and in 3′ rapid amplification of cDNA ends (3′-RACE) experiments.

CAF	Forward primer (5′–3′)	Reverse primer (5′–3′)
CAF01	CGTACGTGAAGACGCCCATG	GCCCTGTGCGACGACGAGG
CAF13	CTTGCCAGAGCGAAGAGTAG	CGCTTCCAGCTCGACGTCGG
CAF15	GGGTGGATGTGGCCGTCACG	GCAGCATTGGCTTGCGCGTG
CAF40	CTCGGCACGTCCTCGAGTGC	GCGGTGCACGACAAACATAC
CAF42	CGAGCATTATCTAAGCAGCG	CGCAGTACGTATTTCATCTGC
CAF43	CGGTAGACCCCAAGGACTCG	GCGATCACACTTGATCAGCG
CAF44	CTGTGAGGGTATGATAGACC	CCAGGGCGACTATCATGCCG
CAF49	CCACGAGCAGTGTCTACTCG	CGTCGTATTGCCATCTGACG
CAF51	CCAGAGGGAAGCGATCTTCC	GGCGATAAAGTTGTTGTGGC
CAF52	CCAGCACCTTGCGATTCTCC	CGCCTCGCTTGACATTCCGC

Table 3. Protein similarity of cDNA-amplified fragment length polymorphism (AFLP) fragments (CAFs) present only in *Peronospora parasitica*-infected *Arabidopsis thaliana* seedlings with sequences in nonredundant data bases

CAF	Protein similarity ^{a,b}	P ^c
CAF01	3-Ketoacyl-coA thiolase, <i>Rattus norvegicus</i> [X05341]	1 ⁻²⁰
CAF02	Heat shock protein 70, <i>A. thaliana</i> [X74604]	2 ⁻³⁰
CAF05	Similar to soybean cold regulated 2, <i>A. thaliana</i> [AC002376]	7 ⁻¹⁰
CAF10	None	
CAF11	None	
CAF12	Putative alcohol dehydrogenase [AAD32792]	1 ⁻¹⁰
CAF13	None	
CAF14	None	
CAF15	60S Ribosomal protein, <i>S. pombe</i> [AF297211]	5 ⁻¹³
CAF16	None	
CAF23	40S Ribosomal protein, <i>Daucus carota</i> [AB012702]	5 ⁻¹⁵
CAF33	None	
CAF36	None	
CAF40	Small zinc finger-like protein, <i>Malus domestica</i> [AF150084]	9 ⁻⁸
CAF41	Phosphofructo 1-kinase, <i>A. thaliana</i> [AL049171]	4 ⁻⁶
CAF42	Eukaryotic initiation factor eIF3-p40, <i>H. sapiens</i> [AC007354]	2 ⁻⁵
CAF43	Uridinephosphoglucose pyrophosphorylase, <i>S. cerevisiae</i> [X69584]	1 ⁻¹²
CAF44	None	
CAF49	None	
CAF50	Anthranilate phosphoribosyl transferase, <i>Lactococcus lactis</i> [M87483]	3 ⁻⁹
CAF51	Aminotransferase, <i>Thermococcus profundus</i> [AB027131]	3 ⁻¹⁴
CAF52	Aminoacyl-tRNA synthetase, <i>Euplotes octocarinatus</i> [AJ131940]	3 ⁻¹⁸
CAF58	Actin depolymerizing factor, <i>Lilium longiflorum</i> [AF058919]	8 ⁻²²

^a With [GenBank accession number].

^b T-BLAST-X [Altschul et al. 1997].

^c P = number of distinct alignments with equivalent or superior score occurring purely by chance; depends upon alignment score, and lengths of both the query sequence and the data base searched.

tion. Eight cDNA sequences showed significant DNA identity to oomycete ESTs of the *Phytophthora* Genome Initiative at the National Center for Genome Resources (Table 1).

To determine experimentally the origin of 10 cDNA fragments present only in infected material, PCR primers were designed to amplify these gene fragments from DNA isolated from *P. parasitica* conidiospores or from *A. thaliana* Ws-0 (Table 2). *P. parasitica* asexual conidiospores were collected from heavily sporulating leaves of 12 4- to 6-week-old *A. thaliana* plants (Ws-0 *eds1-1*) that had been infected 14 days earlier. Spores were resuspended in 300 µl of extraction buffer (100 mM Tris-HCl, 50 mM EDTA; 500 mM NaCl; 5 mM β-mercaptoethanol) with 300 µL glass beads (425 to 600 microns; Sigma, Dorset, UK) and vigorously vortexed. DNA isolation was subsequently carried out according to the Nucleon Phytopure protocol (Amersham, Buckinghamshire, UK). All primer pairs amplified fragments from *P. parasitica* DNA only, showing that these 10 cDNA fragments were derived from *P. parasitica* genes that are expressed during infection in *A. thaliana*. The *P. parasitica* origin of cDNA fragment CAF42 was confirmed in an *Eco*RI-digested DNA gel blot hybridization experiment (Sambrook et al. 1989) with approximately 10 µg of *P. parasitica* DNA, and approximately 5 µg of *A. thaliana* DNA; the radiolabeled cDNA hybridized to *P. parasitica* DNA only (approximately 12 kb fragment).

All cDNA fragments were analyzed further for similarity to protein sequences in nonredundant data bases (Altschul et al. 1997). Three of the seven *A. thaliana* predicted gene products (CAF02/05/12) showed homology to proteins involved in stress responses (Table 3). Seven of the 10 cDNA fragments shown to be derived from *P. parasitica* (CAF01/15/40/42/43/51/52) showed significant similarity to characterized proteins with "housekeeping" functions (Table 3). Other *P. parasitica*

cDNA sequences did not show significant similarity to sequences in the data bases, possibly encode novel proteins, or are too short (50 to 100 amino acid residues) to reveal significant data base similarity.

The sensitivity of cDNA-AFLP display is restricted by the maximum number of fragments generated per primer pair amplification. Rare cDNA fragments, resulting from low abundance transcripts, are probably competed out by more abundant cDNAs. Indeed, we could increase the sensitivity by using adapter primers with more "selective nucleotides." We found that these primers with higher specificity generate approximately the same (maximum) number of fragments, thus amplifying the less abundant transcripts. It is possible, therefore, that all 10 *P. parasitica* cDNA fragments, including the seven fragments predicted to encode proteins with housekeeping functions (Table 2), are relatively highly expressed. To further increase the efficiency of cDNA-AFLP display, one or more rounds of suppression subtractive hybridization could be incorporated in the procedure (Diatchenko et al. 1996). Large-scale application of the cDNA-AFLP technique establishes an EST library (Quackenbush et al. 2000) of pathogen genes expressed during infection of host tissue. EST libraries of obligate biotrophic pathogens will be very useful in combination with other "patho-genomic" approaches, such as genome sequencing, and procedures to study mutant phenotypes (Judelson 1996).

ACKNOWLEDGMENTS

Wendy Durrant (The Sainsbury Laboratory, Norwich, UK), Peter Vos and Mark Zabeau (Keygene, Wageningen, Holland), Anne Rehmany (Horticultural Research Institute, Wellesbourne, UK), and Eric Holub (Horticultural Research Institute, Wellesbourne, UK) are acknowledged for helpful advice. This project was supported in part by Zeneca, UK (E.

A. v. d. B.), and carried out in part within the UK-Indonesia Biodiversity for Biotechnology Development project funded by the UK Department for International Development (H. J.). The Sainsbury Laboratory is supported by the Gatsby foundation.

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Bachem, C. W. B., Van der Hoeven, R. S., de Bruijn, S. M., Vreugdenhil, D., Zabeau, M., and Visser, R. G. F. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant J.* 9:745-753.
- Diatchenko, L., Lau, Y.-F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93:6025-6030.
- Durrant, W. E., Rowland, O., Piedras, P., Hammond-Kosack, K. E., and Jones, J. D. G. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell.* (In press.)
- Jones, J. T., and Harrower, B. E. 1998. A comparison of the efficiency of differential display and cDNA-AFLPs as tools for the isolation of differentially expressed parasite genes. *Fundam. Appl. Nematol.* 21:81-88.
- Judelson, H. S. 1996. Recent advances in the genetics of oomycete plant pathogens. *Mol. Plant-Microbe Interact.* 9:443-449.
- Parker, J. E., Holub, E. B., Frost, L. N., Falk, A., Gunn, N. D., and Daniels, M. J. 1996. Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell* 8:2033-2046.
- Quackenbush, J., Liang, F., Holt, I., Pertea, G., and Upton, J. 2000. The TIGR indices: Reconstruction and representation of expressed gene sequences. *Nucleic Acids Res.* 28:141-145.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.