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Isolation of resistance gene analogs in pepper using modified AFLPs

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Abstract

An efficient technique for isolation of resistant gene analogs (RGAs) in pepper from silver stained denaturing polyacrylamide gel was developed using a modified amplified fragment length polymorphism (AFLP) strategy. Pepper DNA was digested, ligated and pre-amplified as in a normal AFLP method. The selective amplification was made by using combinations with oligonucleotide primers based on conserved motifs in and around nucleotide binding site (NBS) of known NBS-leucine-rich repeats resistance proteins from known resistant genes. The amplified products were separated by using denaturing polyacrylamide gels and silver staining instead of radioactive labelling. We isolated specific polymorphic AFLP bands directly from the gels with one round of polymerase chain reaction amplification, in order to confirm, after sequencing, that these bands have homologies with products of resistance genes described so far. Two bands (R2: 250 bp and R6: 150 bp) are particularly highlighted because they could be considered as RGAs related to resistance to *Phytophthora capsici* in pepper, because their sequences have a very high homology with other resistant gene analogs that have already been described. Besides, they were only detected in the resistant parent and in the bulked resistant segregants but not in the susceptible parent or susceptible F_2 segregants. We can conclude that the technique used is clean, quick and efficient for the isolation of RGAs in pepper.

Additional key words: amplified fragment length polymorphism, *Capsicum annuum*, *Phytophthora capsici*.

Introduction

Plants rely upon a vast array of cellular and molecular defences to fight against pathogen attack. Resistance genes, providing a mechanism by which the plant can recognise a pathogen and execute a defence against, initiate many defence responses (Bóka *et al.* 2002). Common defences include cell wall fortification, phytoalexin production, induction of pathogenesis-related proteins (Egea *et al.* 1996) and the hypersensitive response (HR). The HR is one of the efficient mechanisms by which plants resist pathogen attack. The genetically controlled induction of HR is triggered in plant-pathogen interactions only if the plant contains a disease-resistance gene (*R*) that recognises the corresponding avirulence gene (*avr*) from the pathogen. In the absence of a functional *R* gene or *avr* gene product, no recognition occurs and the interaction between the plant and the pathogen results in disease. *R* genes involved in race-specific interactions often provide disease resistance, what is well known from conventional breeding programs. In the last few years, many *R* genes have been cloned (Jones *et al.* 1994, Mindrinos *et al.* 1994, Grant *et al.* 1995, Ellingboe 2001).

A notable finding from the cloned disease-resistance genes is their similarity in amino acid sequences or conserved structures. Most of them encode proteins containing domains involved in signal transduction or

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Abbreviations: AFLP - amplified fragment length polymorphism; dNTP - deoxinucleoside triphosphate; DTT - dithiothreitol; LRR - leucine-rich repeats; NBS - nucleotide binding site; PCR - polymerase chain reaction; RGA - resistant gene analogs.
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protein-protein interaction (Takken and Joosten 2000). In particular, they have a nucleotide binding site (NBS) and leucine-rich repeat (LRR). If these highly conserved structures are common among many resistance genes, there exists an enormous potential for isolating new resistance genes based on sequence homology. In a number of plant species, researchers have used degenerated primers (designed on the basis of the conserved motifs in all the resistance genes) in a PCR approach, in order to identify resistance gene analogs (RGA), which are associated with important disease resistance genes (Kanazi *et al.* 1996, Leister *et al.* 1996, Yu *et al.* 1996, Collins *et al.* 1998).

Molecular markers have proved to be invaluable for the understanding of the genetic make-up of agricultural crops. Molecular markers take the advantage of the technologies that allow scientists and plant breeders to observe genetic differences between two or more individuals. AFLP is a PCR-based marker system whereby restriction-site and size polymorphisms are detected between lines. DNA is digested with specific enzymes and then oligonucleotide adapters are ligated to the restriction ends. These adapters serve as the recognition site for primers during the subsequent PCR reaction. Three selective nucleotides at the 3'-end of the primer ensure amplification of only a small subset of the digested and ligated genomic fragments. These fragments are labelled and separated on a polyacrylamide gel in order to detect polymorphic bands (Vos *et al.* 1995).

The objective of this study is to develop and test a PCR-based strategy that combines the speed, efficiency and high throughput of AFLP with the resistance gene degenerate primers approach. This technique was used in pepper (*Capsicum annuum*) with different degree of resistance to the fungus *Phytophthora capsici*, and also in bulks of the F_2 population (resistant × susceptible cultivars) to try to identified RGAs confering resistance to this fungus.

Materials and methods

Plants: *Capsicum annuum* L. cvs. Serrano Criollo de Morelos (SCM, resistant) and Americano (A, susceptible) were used. The culture conditions and the criteria used for assessing the differential sensitivity of each cultivar to the fungus *Phytophthora capsici* were described previously (Candela *et al.* 1995). The F_2 population SCM × A was used for a bulked segregant analysis. This population consists of 100 individuals. The F_2 population was tested for resistance by watering each plant with 3 cm^3 of a *Phytophthora capsici* zoospore suspension (10^6 zoospores cm^{-3}). The susceptible plants die and the resistant ones survive one month after the inoculation with the fungus.

DNA extraction: Total genomic DNA was isolated from young frozen leaves of two parental cultivars, using the procedure described by Porebski *et al.* (1997). The DNA from 20 resistant and susceptible lines of the F_2 population was pooled to form bulked-resistant (B_R) and bulked-susceptible (B_S) samples. The F_2 DNA was extracted using the same method described above.

AFLP protocol for pepper: AFLP analysis was carried out according to Vos *et al.* (1995) with the modifications described below. The DNA was digested using two restriction enzymes, *Mse*I and *Eco*RI (*New England Biolabs*, Beverly, MA, USA). Digestion was carried out in a final volume of 0.035 cm^3: 10 mM Tris-acetate, 10 mM Mg-acetate, 50 mM dithiotreitol (DTT), pH 7.5, 8 U of each enzyme, and 500 ng of genomic DNA, for 3 h at 37 °C. Two different adapters, designed to avoid the reconstruction of these restriction sites, one for the *Mse*I sticky ends and another for the *Eco*RI sticky ends, were ligated to the DNA by adding 0.005 cm^3 of a mix: 50 pmol of *Mse*I adapter, 5 pmol of *Eco*RI adapter, 8 mM ATP, 10 mM Tris-acetate, 10 mM Mg-acetate, 50 mM DTT, pH 7.5 and 1.4 U of T4 DNA ligase (*Boehringer*, Ingelheim, Germany), to the digestion. The ligation was incubated for 3 h at 37 °C and overnight at 4 °C. The *Mse*I adapter consisted in the combination of the two primers: 5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5'. The *Eco*RI adapter consisted of the combination of the two primers: 5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGTTAA-5'.

Digested-ligated DNA fragments were diluted with 5-fold to be used as templates for the first amplification reaction, the pre-amplification step, prior to the selective PCR. The pre-amplification consisted in a PCR reaction using primers which are complementary to the adapters *Mse*I and *Eco*RI with an additional selective 3'-nucleotide. The PCR reactions were carried out in a 0.02 cm^3 of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deonucleoside triphosphate (dNTP), 5 mM of each primer *Mse*I+C and *Eco*RI+A, 0.4 U of *Taq* DNA polymerase (*Applied Biosystems*, Lincoln, CA, USA) and 0.003 cm^3 of diluted fragments. The PCR amplifications were carried out by means of 28 cycles consisting of 30 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C.

The pre-amplification products were diluted to be used as starting material for the selective amplifications. The PCR was performed in a 0.02 cm^3 of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.08 mM of each dNTP, 5 mM of each primer, 0.4 U of *Taq* DNA polymerase and 0.005 cm^3 of diluted pre-amplified DNA. The selective amplification was carried out using the following cycling parameters: 1 cycle of 30 s at 94 °C,

30 s at 65 °C, 1 min at 72 °C followed by 12 cycles in which the annealing temperature decreases 0.7 °C per cycle, followed by 23 cycles of 1 min at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. The beginning at a very high annealing temperature allows optimal primer selectivity. By the gradually decrease of the annealing temperature, the efficiency of the primer binding increases (Don *et al.* 1991).

The following different primer combinations were used in the second round of the amplification: *Mse*I-AAA, *Mse*I-AAG, *Mse*I-AAT, *Mse*I-CCT, *Mse*I-ATA, *Mse*I-AGT, *Eco*RI-GAA, *Eco*RI-TAA, *Eco*RI-CGA, *Eco*RI-AAC, *Eco*RI-TAT, with degenerated primers corresponding to four amino acid motifs conserved among the known resistance proteins (P-loop, kinase-2, GLP and MHD) and two primers set also influenced by similarities between resistance proteins and the products of RGAs isolated by Collins *et al.* (1998) in maize. The sequence of the resistant primers used is shown in Table 1.

Denaturing polyacrylamide gel analysis: Before running PAGE, 0.01 cm^3 of PCR products were checked on a 1 % agarose gel. The reaction products were then analysed on 6 % denaturing polyacrylamide gels. The denaturing polyacrylamide gel was prepared with 6 % acrylamide solution, 7 M urea, and 1 X TBE buffer. The gel was pre-run at 60 W until the temperature reached 50 °C. The AFLP samples were denatured by adding an equal volume of formamide-buffer (98 % formamide, 10 mM EDTA, pH 8.0, 0.05 % bromophenol blue and 0.05 % xylene cyanol) and heated for 2 min at 94 °C, followed immediately by chilling on ice. From 0.002 to 0.003 cm^3 of each sample were loaded into wells. To prevent the gel from cooling, gel-loading time did not exceed 15 min. The gel was run at a constant 50 W for 4 h.

Detection, isolation and amplification of AFLP bands: After electrophoresis, gels were fixed to the glass plate, stained with silver nitrate according to the manufacturer's instructions (*Promega Cat: TMD005*, Southampton, UK) and photographed with a 10-s film exposure.

Specific bands were excised directly from the gel on the glass plate using a razor blade. The bands were used directly as template in a total PCR reaction volume of 0.05 cm^3, using the same primers that generated the polymorphic product under the same or less-stringent conditions. The PCR products were checked in a 1 % agarose gel to be sure that we cut only one band and that they have the proper-size.

Sequence analysis: PCR products were sequenced using an *ABI Model 373A* DNA sequencer (*Applied Biosystems*, Warrington, UK). Nucleic acid sequences were analysed for the homology using the NCBI Blast server available on the Internet (http://www.ncbi.nlm.nih.gov/blast).

Table 1. Oligonucleotide primers used to amplify resistance gene analogs in pepper for AFLP modified technique.

Conserved amino acid motifs	Primer name	Primer sequence (5' to 3')
GVGKTT (P-loop)	RGA 1	AAGAATTCGGNGTNGGNAAAACAAC
	RGA 2	AAGAATTCGGNGTNGGNAAAACTAC
L(I/V/L)VLDDV (kinase-2)	RGA 3	CTACTGNTNCTNGACGACGT
	RGA 4	CTACTGNTNCTNGACGATGT
GLPLAL	RGA 5	AACTCGAGAGNGCNAGNGGNAGCCC
KQCFAFCSI	RGA 6	ATAGAA/GCAA/GT/AAIGCG/AAAACA
WMAxG(F/I)V	RGA 7	AT/CA/GAANCCNTNGGCCATCCA
MHD	RGA 8	CGACAGTCNGTCATGCAT
	RGA 9	CGACAGTCNGTCGTGCAT

Results

Oligonucleotide primers based on conserved motifs in and around the NBS of known NBS, LRR resistance proteins and other described in resistance genes were used to amplify sequences from amplified fragment length polymorphism in pepper. All the 45 possible *Eco*RI+3/RGA primer combinations and the 54 possible *Mse*I+3/RGA primer combinations were tested with the bulk and parental DNA samples. A total of 36 polymorphisms were observed among the approximately 1650 visualised bands. In most of the tested cases, polymorphism was very low or even non-existent but we can value an average of 20 loci per AFLP primer combinations.

Seven dominant markers R1 (420 bp), R2 (250 bp), R3 (380 bp), R4 (550 bp), R5 (400 bp), R6 (150 bp) and R7 (300 bp), were identified which seem to be linked to bands amplified by this procedure and detected by silver staining. These bands were detected only in the resistant parental and in the resistant bulk. Two examples using two different primer combinations are shown in Fig. 1. The *arrows* indicate polymorphism bands only detected in the resistant parental and the resistant bulk from F_2.

Some more bands were only detected in the susceptible parental but we did not use them for later analysis.

Sequence analysis was done only in the bands that are present in the resistant parental and absent in the susceptible parental. The analysis of the polymorphic bands indicated that all contain the sequence corresponding to the degenerated primer used to amplify them. Only two of them: R2 and R6, however, contains an additional sequence that shows homology to product from other RGA sequence in *Arabidopsis thaliana* (accession number: NM_112307) and *Oryza sativa* (accession number: AF146275) respectively (Fig. 2). We considered this two bands are putatively RGAs in pepper since their sequences had high homology to other RGAs and also they were detected only in the resistant bulk segregation and resistant parental. The rest of the bands show sequences that show homology to transposon and protein kinases in others plants but no homology with resistance genes.

Discussion

An efficient cloning technique is indispensable for harnessing the potential AFLP or other multilocus scanning techniques to produce usable polymorphic markers. The conversion of markers developed with random amplification techniques into diagnostic PCR-based markers, and the further characterisation of interesting polymorphisms, rely on cloning and the sequencing of purified DNA fragments. In this study we used a modified AFLP approach in pepper in order to get markers related with the resistance.

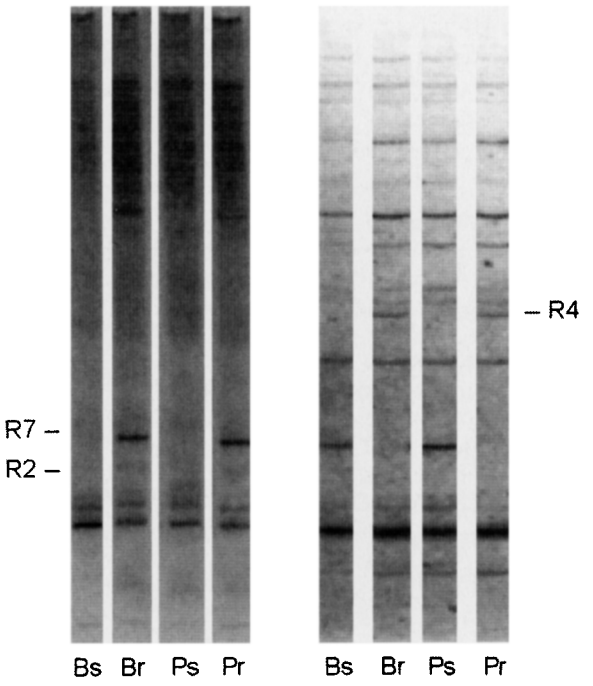

Fig. 1. Homology-based AFLP gel of parents and bulks of the population SCM × A displaying linked polymorphisms with two primer combinations. *Lanes* are as follows: Pr - SCM (resistant parental), Ps - A (susceptible parental), Br - bulk resistant and Bs - bulk susceptible. Primer combinations are as follows: *Eco*RI+AAC/ RGA 1 (*left*) and *Mse*I+CCT/ RGA 9 (*right*). The first primer combination amplified linked markers R2 and R7, and the second one amplified linked marker R4.

In recent years many different disease-resistance genes have been cloned from plants. These genes confer resistance to fungal, viral, bacterial, insect and nematodes pathogen and come from a diverse assortment of species that includes monocots and dicots. The genes contain conserved domains that can account for many of the predicted functions of *R*-genes. Many *R*-genes contain a leucine-rich repeat (LRR) region. Some of the *LRR*-genes contain domains possibly involved in signal transduction (Young 2000) such as nucleotide binding domains (*NBD*), leucine zipper, or toll-interleukin-1 cytoplasmic receptor (*TIR*) domains. Still others contain protein kinase domains and conserved domains of unknown function (Hammond-Kosack and Jones 1997, Takken and Joosten 2000).

Using degenerated primers designed from the conserved domains of cloned *R*-genes has been possible to amplify analogs sequences from other plant species (Kanazi *et al.* 1996, Leister *et al.* 1996, Yu *et al.* 1996). In the same way, we have used these degenerated primers in the selective amplification of the AFLP technique, with the purpose of amplifying bands related with resistance or at least with general defence. We show that specific AFLP bands can be amplified directly from silver-stained polyacrylamide gels without contamination by alternate fragments (Fig. 1). These bands can be isolated after a single round of PCR amplification rather than requiring various amplification steps to obtain a pure product (Weaver *et al.* 1994).

The combinations of primers used in this study showed a very low polymorphism between the resistant cultivar (SCM) and the susceptible cultivar (A). However, a few of those combinations proved satisfactory although after band isolation and further amplification and sequencing, a significant percentage of these amplified sequences contained a resistant primers sequence but not additional sequences characteristic of disease resistance genes (unpublished data). Our results are similar, in this case, to the result obtained by Hayes and Maroof (2000) in soybean using a similar technique.

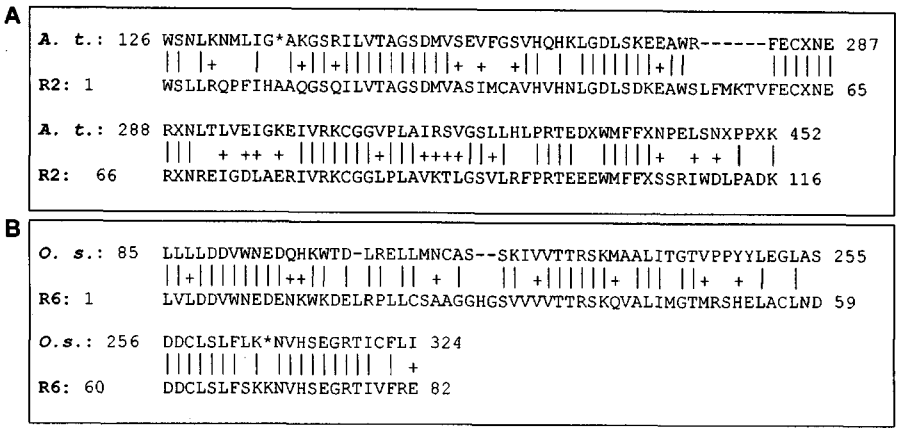

Fig. 2. Comparison of two predicted aminoacid sequence (single letter codes) for the pepper resistant gene analogs (bottom) with that from: (1) *Arabidopsis thaliana* (*A. t.*) (accession number NM_112307), (2) *Oryza sativa* (*O. s.*) RGA PIC27 (accession number AF146275). Line: identical match, +: similar properties, space: mismatch.

The results also showed a few bands detected only in the susceptible parental and susceptible bulked segregant (Fig. 1), we think that they would be related with other features such as fruit shape, capsaicin absence, *etc.*, instead of being related with the resistance because they were not detected in the resistant plants.

Of the seven bands evaluated in this study, all of which were detected in SCM and resistant bulk, only two, R2 and R6, contain motifs identical to others RGAs in *Arabidopsis thaliana* and *Oryza sativa* (Fig. 2). We suggest that they would be considered as putatively RGAs in pepper, because they were detected in the resistant plants and they were absented in the susceptible plants.

From these results we can conclude that the technique used is clean (using silver staining and radioactivity is avoided), quick (bands can be directly isolated from the gel and sent for sequencing by another plain amplification) and efficient for the isolation of RGAs in pepper. Our future work is being developed in order to find more RGAs in pepper to map genomic regions containing virus and fungus resistance genes.

References

Bóka, K., Jakab, J., Király, I.: Comparison of the effect of different fungal elicitor on *Rubia tinctorum* L. suspension culture. - Biol. Plant. **45**: 281-290, 2002.

Candela, M.E., Alcazar, M.D., Espín, A., Egea, C., Almela, L.: Soluble phenolic acids in *Capsicum annuum* stems infected with *Phytophthora capsici*. - Plant Pathol. **44**: 116-123, 1995.

Collins, N.C., Webb, C.A., Seah, S., Ellis, J.G., Hulbert, S.H., Pryor, A.: The isolation and mapping of disease resistance gene analogs in maize. - Mol. Plant-Microbe Inter. **11**: 968-978, 1998.

Don, R.H., Cox, B.J., Wainwright, B.J., Mattick, J.S.: "Touchdown" PCR to circumvent spurious priming during gene amplification. - Nucleic Acids Res. **19**: 4008, 1991.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Satter, A., Innes, R.W., Dangl, J.L.: Structure of the *Arabidopsis* RPM1 gene enabling dual specificity disease resistance. - Science **269**: 843-846, 1995.

Egea, C., Alcázar, M.D., Candela, M.E.: β-1,3-glucanase and chitinase as pathogenesis-related proteins in the defense reaction of two *Capsicum annuum* cultivars infected with cucumber mosaic virus. - Biol. Plant. **38**: 437-443, 1996.

Ellingboe, A.H.: Plant-pathogen interactions: genetic and comparative analyses. - Eur. J. Plant Pathol. **107**: 79-84, 2001.

Hammond-Kosack, K., Jones, J.: Plant disease resistance genes. - Annu. Rev. Plant Physiol. Plant mol. Biol. **48**: 575-608, 1997.

Hayes, A.J, Maroof, M.A.S.: Targeted resistance gene mapping in soybean using modified AFLPs. - Theor. appl. Genet. **100**: 1279-1283, 2000.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., Jones, J.D.G.: Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon. - Science **266**: 798-793, 1994.

Kanazi, V., Marek, L., Shoemaker, R.: Resistance gene analogs are conserved and clustered in soybean. - Proc. nat. Acad. Sci. USA **93**: 11746-11750, 1996.

Leister, D., Ballvora, A., Salamini, F., Gebhardt, C.: A PCR-based approach for isolating pathogen resistance genes from potato with potential for the wide application in plants. - Nature Genet. **14**: 421-429, 1996.

Mindrinos, M., Katagiri, F., Yu, G.L., Ausubel, F.M.: The *Arabidopsis thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. - Cell **78**: 1089-1099, 1994.

Porebski, S., Bailey, L.G., Baun, B.R.: Modification of CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. - Plant mol. Biol. Rep. **15**: 8-15, 1997.

Takken, F.L.W., Joosten, M.H.A.: Plant resistance genes: their structure, function and evolution. - Eur. J. Plant Pathol. **106**: 699-713, 2000.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hones, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M.: AFLP: a new technique for DNA fingerprinting. - Nucleic Acids Res. **23**: 4407-4414, 1995.

Weaver, K.R., Caetano-Anolles, G., Gresshoff, P.M., Callahan, L.M.: Isolation and cloning of DNA amplification products from silver-stained polyacrylamide gels. - BioTechniques **16**: 226-227, 1994.

Young, N.D.: The genetic architecture of resistance. - Curr. Opinion Plant Biol. **3**: 285-290, 2000.

Yu, Y., Buss, G., Saghai Marrof, M.: Isolation of a super family of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. - Proc. nat. Acad. Sci. USA **93**: 11751-11756, 1996.