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EXPERIMENTS FOR DEVELOPING MARKERS FOR TOBAMOVIRUS-RESISTANCE IN PEPPER (*CAPSICUM* SPP.) USING NBS PROFILING APPROACH

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Abstract
For generating molecular markers for Tobamovirus-resistance alleles in pepper, we tested the application of NBS-profiling procedure, which specifically produces markers in or near resistance genes. The NBS-profiling method was applied to controlled crosses, in which the donor plants with L1, L3 and L4 resistance alleles were crossed with sensitive C. annuum 'Albaregia'. Individuals of F2 progenies containing the three different resistance alleles, were phenotypically characterized and used for further analysis. DNA of resistant and susceptible F2 plants was digested and ligated with adapter. In the PCR reaction one adapter primer and one degenerate NBS specific primer were used. The PCR products were analyzed on 6% polyacrylamid gels. Partially co-segregating bands with the resistant phenotype L3 were found.

1. Introduction

Pepper (*Capsicum* spp.) is grown worldwide. Its geographical dispersal exposes it to a high number of pathogens. Tobamovirus spp. cause big damage in pepper yield, so genes providing resistances against Tobamoviruses are extremely important from agricultural point of view. Tobamovirus particles are easily transmitted mechanically and with seeds. Freshly consumed pepper is produced almost exclusively in greenhouses in Hungary. Technology of greenhouse-production (pruning, tying) provides ideal conditions for transmitting Tobamoviruses. As a consequence, resistance against Tobamoviruses is not only a recommendation, but a strong requisite in Hungarian pepper breeding strategies [11]. Pathological tests are time consuming and results of phenotypical characterization are influenced both by environmental factors and by developmental stage of plant, therefore molecular markers linked to virus resistance genes represent great value in plant breeding.

Resistance to Tobamovirus spp. in pepper (*Capsicum* spp.) is provided by the L gene. At this locus 5 alleles are known, providing broadening spectra of resistance to different pathotypes of Tobamoviruses (Fig. 1.). L0 is related to susceptibility. L1 provides resistance to P0 pathotypes of TMV (tobacco mosaic virus). L2 provides resistance to P0 and P1 pathotypes of PaMMV (Paprika Mild Mottle Virus) that overcome L1 resistance. L3 provides resistance to P0, P1, and P1,2 pathotypes of PMMoV (Pepper Mild Mottle Virus) that overcome L2 resistance. L4 provides resistance to P0, P1, P1,2 and P1,2,3 pathotypes of PMMoV that overcome L3 resistance.

Besides the classical L alleles, further alleles with different temperature sensitivity (L1a, L1c and L2b) were described [12].

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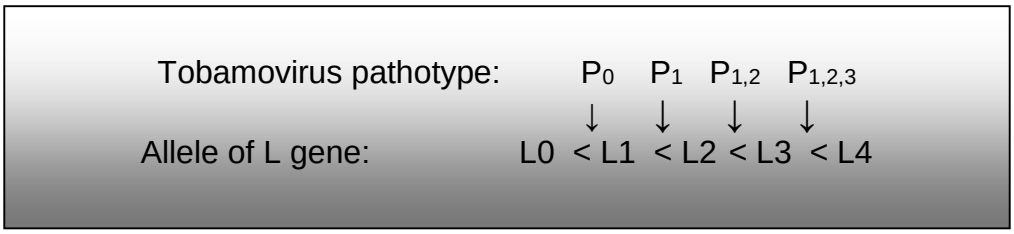

Figure 1. L alleles providing resistance to different pathotypes of Tobamovirus spp. (based on Salamon, 2006 [10,11])

For development of molecular markers closely linked to plant disease resistance genes, a wide range of techniques are available that are capable to directly target genetic loci conferring tandem arrays of resistance genes and Resistance Gene Analogues (RGAs). Most resistance genes (R genes), characterized till now, belong to the NB-LRR class of resistance genes that encode proteins having conserved NBS (Nucleotide Binding Site) and LRR (Leucin Reach Repeat) domains [5]. The NBS region contains highly conserved P-loop and kinase-2 motifs. NBS containing genes are abundant in all plant species. In potato - a close relative of pepper - Jupe et al. [6] identified 438 NB-LRR type genes by motif-based search, based on sequence data available from the potato genome sequencing project. The majority of NB-LRR containing genes have been found to be organized into 63 clusters, from which 50 clusters are homogeneous and are supposed to contain NB-LRR motifs originating from a recent common ancestor [6]. A similar situation can be expected in pepper as well. Genome sequences and extensive transcriptomic data became available in pepper recently [7, 9], providing resources for similar diversity studies.

The NBS–profiling technique is a domain-directed profiling approach, which specifically targets conserved NBS motifs of the R genes. The method was described in 2004 [13], and was applied in different plant species, like tomato, barley, lettuce [13], potato [13] [1] [14], apple [2] and durum wheat [8].

In the present study NBS-profiling approach was applied in pepper populations segregating for 3 different L alleles (L1, L3, L4) of Tobamovirus-resistance, with the aim of generating markers closely linked to the resistance genes.

2. Materials and methods

Plant material has been obtained from Pál Salamon (Agricultural Biotechnology Center, Gödöllő). After lyophilization of leafs of the plants, DNA was isolated as described by Fulton et al. [3]. 400 ng of DNA was digested with frequently cutting restriction enzymes, in appropriate buffer provided by producer (Life Technologies B.V.). The method - summarized on Fig. 2. -, was described earlier in details in van der Linden et al. in 2004 [13].

After digestion, adapter with the following sequences has been ligated to the cut sites:

Adapter long arm: 5′-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3,

Adapter short arm: 5′-TGGGATCTATACTT-3′ (with 3′ aminogroup).

The NBS-specific primers were labeled in a T4 Polynucleotide Kinase reaction using γ 33P ATP. Then PCR products were labeled in PCR reaction using the γ 33P labeled NBS-specific primers. PCR Conditions (Perkin Elmer Gene AmpTM PCR system 9600) were as follows:

15 min 95°C/ 10 cycles of 30 sec 95°C, 1 min 40 sec 50-65°C, 2 min 72°C/ 20 min 72°C.

Amplification of NBS-specific fragments has been conducted in a two steps PCR reaction: First an asymmetric PCR reaction with a limited amount of NBS-specific primer using 1-2 μl ligation mix as template, then both NBS-specific and adapter primers were added to the linear product and regular PCR reaction was carried out. PCR reactions were performed using HS Taq DNA polymerase kit (Qiagen, Germany) in a PTC-200 Thermocycler (MJ research). PCR conditions in both PCR reactions were: 15 min 95°C/ 30 cycles of 30 sec 95°C, 1 min 40 sec 50-65°C, 2 min 72°C/ 20 min 72°C.

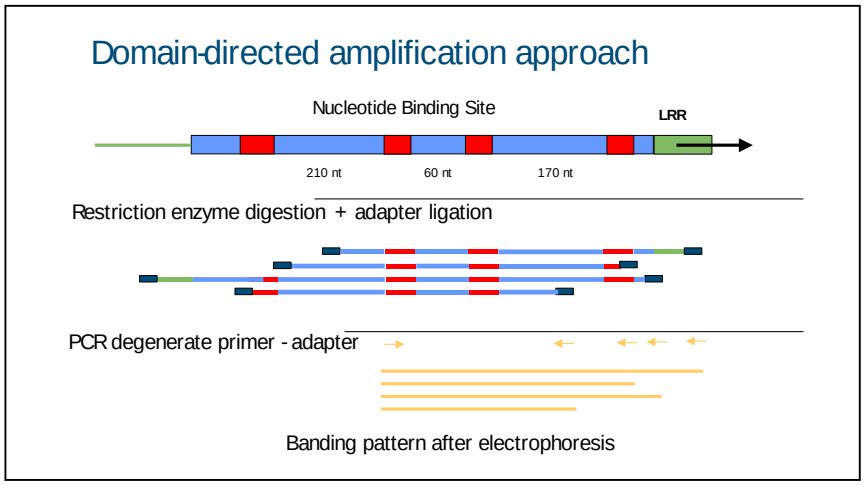

Figure 2. Short summary of NBS-profiling approach

Adapter primer: 5′-ACTCGATTCTCAACCCGAAAG-3′
NBS-specific primers (For location and orientation of primers see Fig. 3.):
NBS1: 5′-GCIARWGTWGTCTTICCYRAICC -3′;
NBS2: 5′-GTWGTYTTICCYRAICCISSCAT-3′
NBS5: 5′-YYTKRTHGTMITKGATGATGTITGG-3′;
NBS6: 5′-YYTKRTHGTMITKGATGATATITGG-3′

PCR products were separated on 6% Poliacrylamide gel (SequaGel-6, Ready-To-Use 6% Sequencing Gel Solution, National Diagnostics). Labeled samples were diluted with equal volume of loading buffer (98% formamide, 10mM EDTA pH 8.0, Bromophenol blue and Xylene cyanol). After 3 min on 95°C, samples were kept on ice. Gel electrophoresis took 2-3 hours depending on the size of fragments (Electrophoresis unit: Bio Rad sequi-Gen II 38×50cm). Gels were fixed on Wattman 3MM and dried at 80 °C. PCR products were visualized by autoradiography on Kodak X-OMAT AR, 35×43 cm film.

3. Results and discussion

Our preliminary experiments with genomic Southern hybridization showed that there is no high degree sequence homology between the tobacco N gene and the different L alleles. In this study controlled crosses - in which L1, L3 and L4 TMV-resistance profiles were phenotypically characterized by Pál Salamon (Agricultural Biotechnology Center, Gödöllő, Hungary) - were used. Donor plants were crossed with susceptible *C. annuum* 'Albaregia' (Table 1.). Individuals of F2 progenies containing three different L resistance alleles were used for further analysis.

Table 1. Production of F2 populations containing the different L alleles

Parents		*F2 population*		
Parent 1.	*Parent 2.*	*Resistance*	*Number of plants*	
Susceptible	*Resistant*		*Resistant*	*Susceptible*
'Albaregia'	Yolo Wonder L1 / L1 (homozygous)	L1	16	13
'Albaregia'	*C. chinense* PI 159 234 L3/ L3	L3	21	11
'Albaregia'	(*C. annuum* Jav. cecei x C. chacoense) L4 / L4	L4	23	8

NBS-profiling - applied in our experiments -, is a tool for studying resistance against pathogens on a more direct way compared to other molecular marker techniques. The method is based on

selective amplification of fragments containing NBS as domain (Fig. 2., Fig.3). DNA of resistant and susceptible F2 plants was digested and ligated with adapter. In the PCR reaction one adapter primer and one degenerate domain specific primer were used [13]. The degenerate primer directs the PCR reaction to NBS-containing resistance genes and gene analogues, providing bands highly enriched in RGA sequences. Compared to the similar method of Hayes et al. [4] - where about 20-25% of the PCR products were RGAs -, the NBS-profiles of different crops provides 40-90% RGAs in general [13].

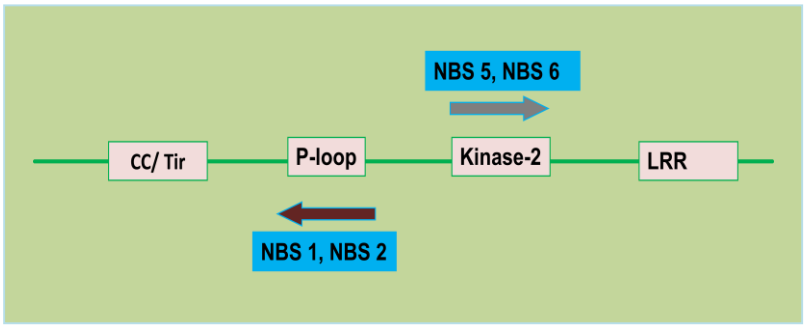

Figure 3. Schematic representation of location and orientation of the domain specific primers applied in our experiments

In our experiments four different enzymes (Rsa I, Hae III, Mse I and Alu I), and four different specific primers (NBS1, 2, 5 and 6) were used. These primers were designed based on the conserved sequences of NBS region (Fig. 4.) as described in van der Linden et al. in 2004 [13].

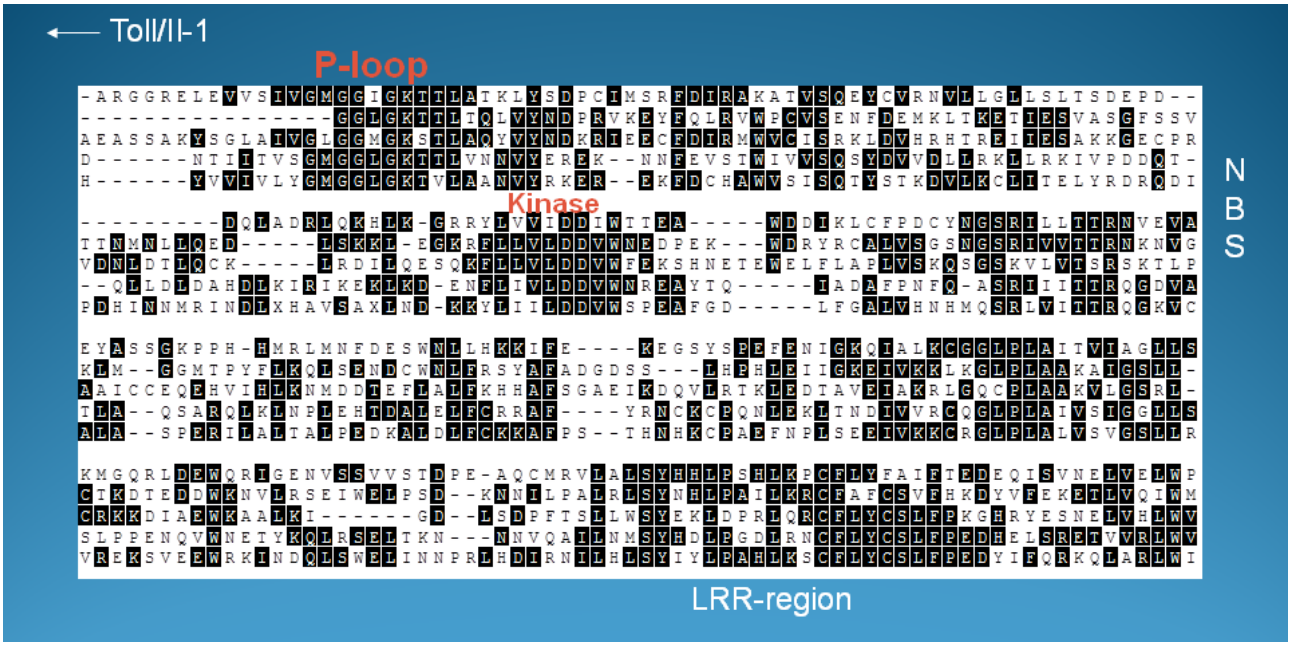

Figure 4. Protein sequences of NBS regions used for primer design

NBS1 and 2 is based on a part of the conserved P-loop motif and amplifies DNA towards 5' end of the targeted gene outside the NBS region, while NBS5 and NBS6 were designed based on the kinase-2 motif (Fig. 3.).

In the first experiments all the enzymes and specific primers were tested on DNA of three or four resistant and three or four susceptible plants of all different resistance profiles (L1, L3, L4) (data not shown). In case of L1 resistance using the Hae III enzyme, co-segregation of bands with the

resistant phenotype were found, using the NBS1, NBS2 and NBS5 primers (Fig. 5A.). Then the experiments that resulted in co-segregating bands were repeated with an extended set of DNA samples (L1: 12 resistant-12 sensitive plants; L3: 9 resistant-9 sensitive plants (Fig. 5B.); L4: 12 resistant-6 sensitive plants).

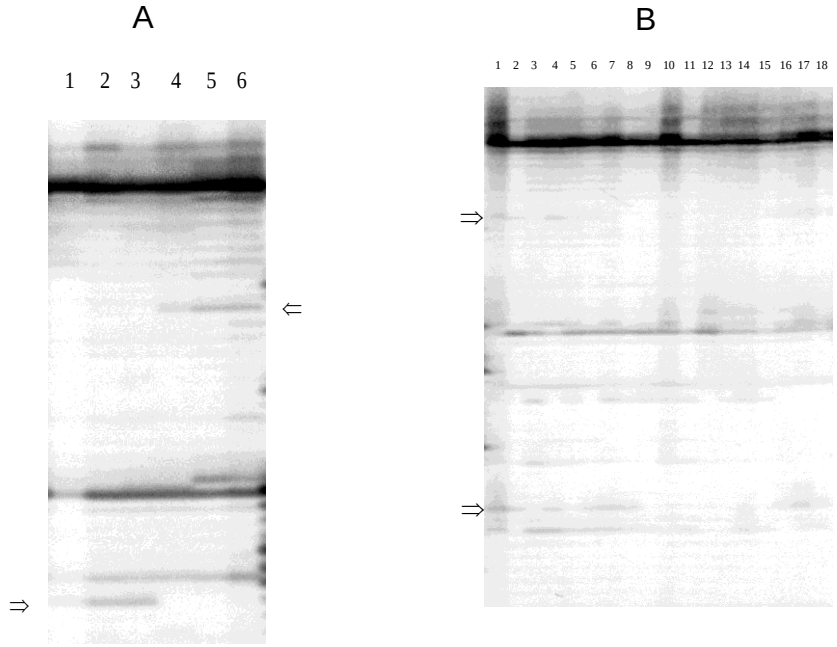

Figure 5. NBS-profile of L3 resistant and susceptible pepper ('Albaregia' x C. chinense PI 159 234 L3 / L3) DNA of F2 plants was digested with Hae III. In the PCR reaction NBS 5 was used as domain specific primer. A: Complete co-segregation of bands (indicated by arrows) with L3 profile was found using DNA of three resistant and three sensitive plants. 1-3: resistant 4-6: susceptible B: Partial co-segregation of bands with the L3 resistance profile were found (indicated by arrows) using the full set of DNA samples 1-9: resistant, 10-18: susceptible

In this study, we demonstrated that NBS-profiling can successfully be applied for generating molecular markers associated to disease resistance traits in *Capsicum* spp.

Even though no bands showing complete co-segregation with the resistant phenotype could be found in our preliminary experiments, our results demonstrated the power, flexibility and reproducibility of the NBS-profiling technique. For the development of molecular markers closely linked to the targeted resistance genes, the application of a higher number of primer/enzyme combinations and larger segregating populations might be necessary. In combination with *in silico* profiling approaches, the "wet" NBS-profiling technique represent a powerful tool for isolation and characterization of agronomically important resistance genes, as well as a versatile tool for development of molecular markers for resistance breeding.

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