

Cloning and Characterization of Nbs-Lrr Class Resistance-Gene Analogs Sequences in Sunflower

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Abstract: A number of disease resistance gene-like DNA sequences were cloned from wild species of sunflower (*Helianthus argophilus*), using a PCR approach with degenerate primers designed from conserved NBS (nucleotide-binding site) motifs found in a number of plant resistance genes. After sequencing of cloned fragment, the similarity to the known resistance genes was found (NBS2, NBS5 – NBS-LRR class resistance-gene analogs in sunflower (*Helianthus annuus*), RLG5, c27 – resistance-gene homologues in soybean (*Glycine max*), FRGA-A5 – NBS-LRR resistance-gene analog in apple-tree (*Malus domestica*). These results indicate that resistance-gene analogs sequences amplified with the NBS-derived degenerate primers are valuable sources for developing markers in disease resistance-gene tagging, mapping and cloning

Key words: Disease resistance genes, sunflower, *Helianthus argophilus*, resistance-gene analogs, molecular marker.

Introduction

Sunflower is a one of the most important oil culture in the world. Cultivated sunflower varieties are susceptible to many diverse pathogens including viruses, fungi and bacteria. This susceptibility causes huge losses to the food-oil industry. Breeding for resistance to important diseases has been one of the top priorities in sunflower cultivar improvement programs. However, improvements have been severely hindered by the lack of effective and efficient selection procedures for disease resistance.

Molecular-marker technologies have developed very rapidly in the last decade, and they have allowed genetists and breeders to locate and map resistance

genes. The availability of molecular markers allowing speeding up breeding process and making it more cheaply.

A number of disease resistance (R) genes have been cloned from several model plant species. Many of these cloned resistance genes appear to encode components of signal transduction pathways, and their protein products share some common structural domains (Baker et al. 1997; Hammond-Kosack and Jones 1997). One of the common domains is the nucleotide-binding site (NBS). Motifs of this domain are well conserved in several R-genes, including *Arabidopsis RPS2* (Bent et al. 1994; Mindrinos et al. 1994), tobacco *N* (Whitham et al. 1994) and flax *L6* (Lawrence et al. 1995). Degenerate primers designed from the conserved amino acids in this domain have allowed successful PCR-amplification of multiple DNA sequences from a number of plant species that share striking similarity to the NBS-LRR (leucine-rich repeat) class of resistance genes (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Seah et al. 1998; Shen et al. 1998; Speulman et al. 1998). These sequences have been called resistance-gene analogs (RGAs) (Kanazin et al. 1996) or resistance-gene candidate (RGC) sequences (Shen et al. 1998). Genetic analyses have associated a number of these sequences to known gene loci that confer resistance to viruses, bacteria, fungi or nematodes (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Aarts et al. 1998; Seah et al. 1998; Shen et al. 1998; Speulman et al. 1998). Some of these sequences appear to be part of the resistance genes themselves. These studies seem to indicate that PCR approaches using degenerate primers based on the conserved NBS domains of cloned R-genes can provide an attractive strategy to amplify multiple resistance-gene analogs sequences can be developed into molecular markers for use in marker-assisted selection (MAS) or even lead to molecular cloning of new disease resistance genes.

In this study, we cloned a number and characterized one NBS-LRR class resistance-gene analogs sequences from wild species of sunflower *Helianthus argophilus*. This species of sunflower shown high level of resistance against broad spectrum of pathogens in studies wich was performed by the Veidelevskii Institute of Sunflower Ltd. (Russia).

Materials and Methods

For degenerate primers were used in three combinations to amplify RGAs sequences by PCR (Table 1.).

Tab. 1. Degenerate primers and vector used in the cloning of sunflower RGAs sequences

Primer combination	Degenerate primers		Vector
	Forward	Reverse	
I	F11	R11	pUC19
II	F11	R16	pUC19
III	F11	R18	pUC19

Primer F11 was one of the two oligonucleotides designed in the sense direction corresponding to the amino-acid sequence GVGKTT found in the P-loop of *N*, *L6*, *RPS2*; primers R11, R16, R18 were three of the eight oligonucleotides

based on the sequence GLPLAL in the anti-sense direction (which is part of proposed weak hydrophobic region in *N, L6, RPS2*). Their sequences were: F11, 5' – GG(A/G/T) GT(A/G/T) GGN AA(A/G) AC(A/T) AC; R11, 5' – AGI GC(A/C/T) AGN GGN AGN CC; R16, 5' – AGN GC(A/C/T) AGN GG(C/T) AAN CC; R18, 5' – AAN GC(A/C/T) AGN GG(C/T) AAN CC (Z. Deng *et al.* 2000). The PCR template used was genomic DNA of *H. argofillus*, which was isolated from seeds. Amplifications were performed on a "Tercik-MC2" thermal cycler ("DNA-technology", Moscow) in a 25- μ l reaction volume; each reaction contained 50 mM Tris-HCl pH 8.3, 2 mM MgCl₂, 800 μ M dNTPs, 25 μ M forward and reverse degenerate primers, 150 ng of genomic DNA and 1 unit of *Taq* polymerase. The initial denaturation was 93°C for 2 min, followed by 42 cycles of 1 min at 92°C, 1 min at 50°C and 2 min at 72°C. PCR products were separated on agarose gels; desired bands were excised, re-amplified and purified before cloning. Vector pUC19 (SibEnzyme, Novosibirsk) was used in the course of cloning. We prepared T-vector before cloning.

Representative clones of one class (F11+R11) were chosen for sequencing. Double stranded plasmid DNA was sequenced by the Eurogene Ltd. (Moscow). One of cloned sequence was characterized in the database of GenBank using the BLASTN algorithm.

Results and Discussion

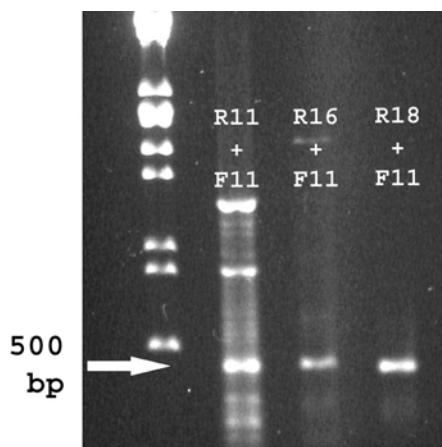


Fig.1. PCR amplification of genomic DNA with three degenerate primer combinations. In the left column – size marker.

Amplification with degenerate primers. The PCR products amplified from the genomic DNA of *H. argofillus* with three degenerate primer combinations are shown in Fig.1. All primer combination generated one major band of around 500 bp in size and a few faint bands. The approximately 500-bp band from each primer combination was close to the fragment size expected based on the sequences of the *N, L6* and *RPS2* genes; therefore, the band from F11+R11 was cloned. The presence of smaller than 500 bp bands suggested that there are homologues sequences inside the 500 bp sequence.

For increasing of quantity and quality of PCR products, we made reamplification (Fig. 2).

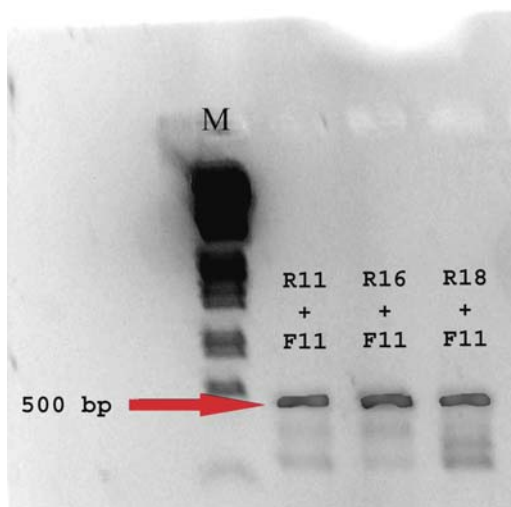


Fig. 2. PCR products after re-amplification.

Molecular cloning of RGAs sequences

After re-amplification and purification the, PCR product of F11 and R11 degenerate primers combination was ligated into the pUC19 vector (Fig. 3).

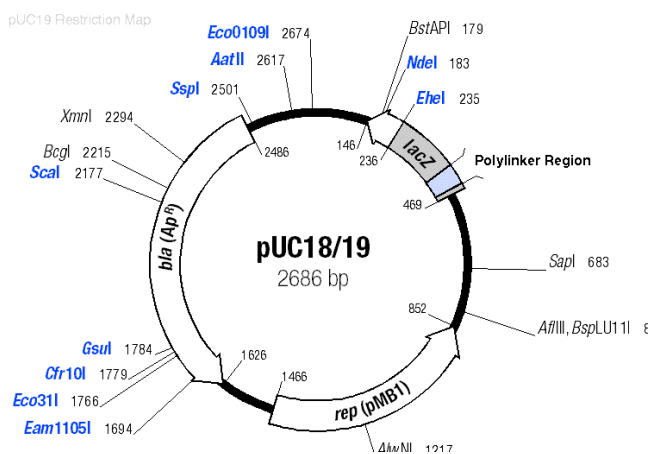


Fig. 3. Scheme of pUC19 vector

For identification of clones, which carry the 500 bp insert in vector, we used white-blue selection.

All selected clone was screened by PCR with primers on M13 fage sequence.

Sequence analysis

Searches of the GenBank database using the BLASTN algorithm revealed that the cloned sequence (Fig. 4) were highly similar to *NBS2*, *NBS5*–NBS-LRR class resistance-gene analogs in sunflower (*Helianthus annuus*), *RLG5*, *c27* – resistance-gene homologues in soybean (*Glycine max*), *FRGA-A5* – NBS-LRR resistance-gene analog in apple-tree (*Malus domestica*) and weak similarity to other known resistance genes.

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AGG GCAAGGGGGA GGCCGGAAGC TAAGAAACCA CACGCGATGA
AAGTGTCTCA TAATTCTTCT ACAGGGTTGT CTTGATTATA CGCATGTCTT TTTAGAAGTT
GGTGAGCCCTC ATCATCTGAT AAAAAAGTGA CACGGTAGAT ATGATCTACC CTGTGGATTI
GTAGCAAATG TTTATCTCTA GATGTGACTA TTATTCGTCT CCCACTACCA AACCAATTTT
GATTTCCAGC TAACGCCTCT AGTTGGTCAA GTTGATCCAC ATCATCCAGA ACTACCAGCA
CTTTTCTAAA ACAAAACACA CGTCTCATCA TGTGTTTTCC TTCCGAAACA CTATGTACTC
TCACATCTGT TTTCAATAGA GCTGATATAA TATTCTCTTG TAGTTTATTC AAGCCATACT
TTCTTGTTTC TTCTCTAACA TTCTCCACAA CGCAGTGAAC TTCAAAATGT TGAGAGATTT
CCCTATAAAA GGAAGTTGGA AGGGTAGTCT TTCCACCCC

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Fig. 4. Nucleotide sequence of the cloned fragment. The sites of primers annealing shown by the red color.

Conclusion

Our study shows presence of NBS-LRR class resistance-gene analogs in the genome of wild species *Helianthus argophilus*, which highly similar to known resistance genes. Thus, PCR approaches using degenerate primers based on the conserved NBS domains of cloned R-genes can provide an attractive strategy to amplify multiple resistance-gene analogs sequences can be developed into molecular markers for use in marker-assisted selection (MAS) of sunflower or even lead to molecular cloning of new disease resistance genes.

References

- Aarts M.G., te Lintel Hekkert B., Holub E.B., Beynon J.L., Stiekema W.J., Pereira A. (1998): Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. *Mol Plant-Microbe Interact* 11:251-258.
- Baker B., Zambryski P., Staskawicz B., Dinesh-Kumar S.P. (1997) Signaling in plant-microbe interactions. *Science* 276:726-733.
- Bent A.F., Kundel B.N., Dahlbeck D., Brown K.L., Schmidt R., Giraudat J., Leung J., Staskawicz B.J. (1994): RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* 265:1856-1860.

- Deng Z., Huang S., Ling P., Chen C., Yu C., Weber C.A., Moore G.A., Gmitter Jr. F.G. (2000): Cloning and characterization of NBS-LRR class resistance-gene candidates sequences in citrus". *Theor Appl Genet* 101: 814-822.
- Lawrence G.J., Finnegan E.J., Ayliffe M.A., Ellis J.G. (1995): The L6 gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene RPS2 and the tobacco viral resistance gene N. *Plant Cell* 7:1195-1206.
- Leister D., Ballvora A., Salamini F., Gebhardt C. (1996): A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. *Nature Genet* 14: 421-429.
- Seah S., Sivasithamparam K., Karakousis A., Lagudah E.S. (1998): Cloning and characterization of a family of disease resistance gene analogs from wheat and barley. *Theor Appl Genet* 97: 937-945.
- Shen K.A., Meyers B.C., Islam-Faridi M.N., Chin D.B., Stelly D.M., Michelmore R.W. (1998): Resistance gene candidate identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. *Mol Plant-Microbe Interact* 11:815-823.
- Speelman E., Bouchez D., Holub E.B., Beynon J.L. (1998): Disease resistance gene homologs correlate with disease resistance loci of Arabidopsis thaliana. *Plant J* 14: 467-474.
- Whitham S., Dinesh-Kumar S.P., Choi D., Hehl R., Corr C., Baker B. (1994): The product of the tobacco mosaic virus resistance gene N: similarity to Toll and the Interleukin-1 receptor. *Cell* 78:1101-1115.
- Yu Y.G., Buss G.R., Saghai-Maroo M.A. (1996): Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. *Proc. Natl. Acad. Sci. USA*, 93:11751-11756.

KLONIRANJE I KARAKTERIZACIJA SRODNIKA SUNCOKRETA REZISTENTNIH GENA KLASA NBS-LRR

- originalni naučni rad -

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Rezime

Određeni broj DNA gen serija rezistentnih na bolesti je klonirano iz divljih vrsta suncokreta (*Helianthus argophilus*) primenom PCR pristupa izvedenih kombinacija vezanih nukleusa nekih vrsta biljaka sa rezistentnim genima. Nakon kloniranja, utvrđena je sličnost sa poznatim rezistentnim genima. (NBS2, NBS5 – NBS-LRR klasa rezistentnih gena suncokreta (*Helianthus annuus*), RLG5, c27 – homolozi soje (*Glycine max*) rezistentnih gena FRGA-A5 – NBS-LRR srodnik rezistentnih gena jabuke (*Malus domestika*). Ovi rezultati pokazuju da srodnici rezistentnih gena, kad se uvećaju sa NBS izvedenim kombinacijama, predstavljaju dragocene izvore za 'hvatanje', 'mapiranje' i kloniranje rezistentnih gena.