Cloning and analysis of resistance gene analogs in sunflower

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Summary:

Resistance to the sunflower downy mildew, caused by *Plasmopara halstedii*, is conferred by major and dominant genes named *Pl*. Genetics studies have shown that some *Pl* genes can be clustered. Using a PCR-based methods, we have cloned some cDNA and genomic fragments from the sunflower line RHA266 containing the *Pl1* locus. Sequence comparison of these clones has showed that *Pl* genes belong probably to the large TIR-NBS-LRR class of plant resistance genes. In addition, these clones are members of a complex family in sunflower.

Résumé :

Le tournesol *Helianthus annuus* L. constitue l'une des premières sources d'huile végétale dans le monde. Cependant les rendements peuvent être affectés par de nombreuses maladies fongiques dont le mildiou causé par *Plasmopara halstedii*. Les interactions entre ce champignon et le tournesol correspondent à un système gène pour gène. Les lignées résistantes développent une réaction de type hypersensible à l'interieur de l'hypocotyle. En utilisant des amorces dégénérées nous avons amplifié et cloné un fragment génomique homologue aux gènes de résistance de la classe NBS-LRR chez les plantes (Gentzbittel & Mouzeyar *et al*., 1998). Ce fragment a été utilisé pour sélectionner des amorces et amplifier un cDNA et des clones génomiques complets chez une lignée de tournesol résistante. L'analyse et la comparaison de ces séquences montrent qu'elles appartiennent toutes à la classe des NBS-LRR, avec notamment le domaine NBS présent à l'extrêmité 5' et les répétitions riches en leucine présentes à l'extrêmité 3'. De plus une variabilité concernant aussi bien l'extrêmité 5' que l'extrêmité 3' a été mise en évidence.

Introduction

Several resistance genes involved in "gene for gene" interactions between plants and parasites have been recently cloned in different plant species. The parasites include viruses, bacteria, fungi and nematode and despite the wide diversity among them, the corresponding resistance genes share several common features and even more conserved domains (Hammond-Kosack and Jones, 1997). Structural and functional comparisons of plant resistance genes have been reviewed by Jones et al., (1999); and De Witt et al., (1999). Almost all the resistance genes cloned so far contain Leucin-Rich Repeats (LRR), for example the *Cf9* gene in tomato (Jones *et al*., 1994), *N* gene in tobacco (Whitham et al., 1995) and *L6* gene in flax (Lawrence *et al*., 1995). The prominant subclass contains resistance genes with a Nucleotid Binding Site (NBS) motif at the N terminus and a LRR region at the C terminus.

Comparison of mutants lacking resistance to flux rust provided evidence that the LRR region plays a key role in the specificity of resistance and that the number of these LRR can affect this specificity (Anderson *et al*., 1997). Massive sequencing and comparison of resistance gene sequences have showed that the NBS domains are comparatively less variable than the LRR region and that probably these genes are evolving by recombination, unequal crossing-over and gene conversion. However, Meyers *et al*., (1998) proposed that single-base changes in the predicted β-sheet portion of the LRR region may be more important than intergenic recombination and gene conversion in the rapid generation of new specifities.

The biotrophic fungus *Plasmopara halstedii* is one of the major parasites of sunflower. Although no avirulence genes have been caracterised, the interactions between *P. halstedii* and sunflower correspond probably to a gene for gene system. In the incompatible interaction, sunflower seedlings develop a hypersensitive-like reaction within the hypocotyl (Mouzeyar *et al*., 1993). In addition, transcripts of some defense genes associated with hypersensitive reactions accumulate after infection of sunflower with avirulent races (Mazeyrat *et al*. 1998).

The resistance in sunflower is conferred by major dominant genes named *Pl* (Vranceanu, 1970) and genetic studies showed that, like resistance genes in other plants, some *Pl* genes are clustered (Vear *et al*., 1997). Recently, Gentzbittel & Mouzeyar *et al* (1998) used a PCR-based strategy and cloned a genomic fragment from sunflower which shows sequence homology to resistance genes belonging to the TIR-NBS-LRR class. Subsequent RFLP localisation of homologous sequences revealed that this clone is a member of a large family and that some loci cosegregate with a major locus for resistance to sunflower downy mildew conferring resistance to all known races of P. *halstedii*. Thus, this clone shares severall features with resistance genes characterised from other plant species and constitute a candidate gene for downy mildew resistance. Before initiating positional cloning of *Pl* genes, we need further molecular characterisation of the different *Pl* loci.

In this paper, we report the cloning of resistance gene analogs (RGA) in sunflower and the analysis of sequence variability of these genes using PCR-based methods.

Materials and Methods

Cloning of complete cDNA.:

The RGA genomic fragment obtained by Gentzbittel & Mouzeyar *et al*., 1998 was used to design specific primers and to clone by RACE-PCR the corresponding complete cDNA.

mRNA were extracted from 15-days-old RHA266 seedlings according to the method described by Mazeyrat *et al*. (1998). RACE-PCR analyses were carried out using "Marathon cDNA amplification kit" from Clontech's (Ozyme, France). First and second cDNA strands were obtained according to the manufacturer's instructions. Five prime and 3' ends were amplified using adaptor primers (AP1) and 5' or 3' gene specific primers, designed from the partial genomic fragment (Gentzbittel *et al*., 1998). PCR products were cloned using pGemT-easy vector from Promega (France).

Cloning of genomic RGA:

One forward specific primer containng the ATG codon and one reverse primer containing the stop codon TGA were used to amplify genomic RGA from the sunflower line RHA266 (*Pl1*).

Sequences analysis:

Selected clones were sequenced by Génome Express (Grenoble, France) on both strands. Homology searches and sequence analyses were carried out using the NCBI tools such as BLAST (Altshul *et al*., 1990). Alignments were performed using the Clustal programme (Higgins *et al*., 1992).

Results and discussions

RACE-PCR Cloning of RGA homologs:

RACE-PCR yielded two fragments corresponding to the 5' and 3' ends of Resistance gene analogs (RGA) in sunflower. Comparison with data banks showed that these two clones are homologous to resistance genes belonging to the TIR-NBS-LRR class, the highest homology scores were found with the N gene from tobacco (Whitham *et al*., 1994) and the *L6* gene from flax (Lawrence *et al*., 1995)

As depicted in the figure 1, sunflower contains genes having all the features of resistance genes in other plant species. These features include one region homologous to the Toll and Interleukin Receptor genes (TIR) at the N-terminus, a central region containing a Nucleotide Binding Site (NBS) and a long stretch containing Leucin Rich Repeats (LRR). Although we have no experimental evidence that the *Pl* genes belong to the TIR-NBS-LRR class of resistance genes, cosegregation of these sequences and resistance to *P. halstedii* indicates that *Pl* genes are probably TIR-NBS-LRR. To verify this hypothesis, we initiated an antisense experiment as was demonstrated by Ori *et al*., 1998 in tomato.

Cloning of Genomic RGA:

We used specific primers to amplify and clone several genomic sequences wich are highly homologous to plant resistance genes. Five different clones were further selected on the basis of their sizes (Figure 2) and sequenced.

MAVEFMERLRENTSANTGLSPNTLFTLFATGVTVYYMLSVFFRGSSDHHQQHSPTSSEED
RSAASSSSASHSISASTSQSWNHDVFLSFRGEDTRNSFVDHLYAALVQQGIQTYKDDQTL
PRGERIGPALLKAIQESRIAVVVFSQNYADSSWCLDELAHIMECMDTRGQIVIPIFYFVDP
SDVRKQKGKYGKAFRKHKRENKQKVESWRKALEKAGNLSGWVINENSHEAKCIKEIVAT
(1)
ISSRLPTLSTNVNKDLIGIETRLQDLKSKLKMESGDVRIIGIW**GVGGGGKT**TLASAAYAE
ISHRFEAHCLLQNIREESNKHGLEKLQEKILSLVLKTKDVVVGSEIEGRSMIERRLRNKS
(2)
V***LVVLDDV***DDLKQLEALAGSHAWFGKGSRIIITTRDEHLLTRHADMIYEVSLLSDDEAME
(3)
LFNKHAYREDELIEDYGMLSKDVVSYAS***GLPL***ALEILGSFLYDKNKDDWKSALAKLKCIP
NVEVTERLKISYDGLEPEHQKLFLDIACFWRRRDMDEAMMVLDACNLHPRIGVKVLIQKS
LIKVSDVRFSKQKVFDMHDLVEEMAHYIVRGAHPNHPEKHSRIWKMEDIAYLCDMGEDAV
PMETEALAFRCYIDDPGLSNAVGVSDVVANMKKLPWIRFDEYPASSFPSNFHPTELGCLE
LERSRQKELWHGYKLLPNLKILDLAMSSNLITTPNFDGLPCLERLDLEGCESLEEIHPSI
GYHKSLVYVDMRRCSTLKRFSPIIQMQMLETLILSECRELQQFPDIQSNK

Figure 1 : Deduced Amino Acid sequence of one RGA in the sunflower line RHA266 Description of domains is based on putative functional motifs. The first two motifs (1 and 2) are found in NBS domains. The third motif (3) is a hydrophobic conserved region in RGA. The C-terminus half of the protein is constituted of LRR.

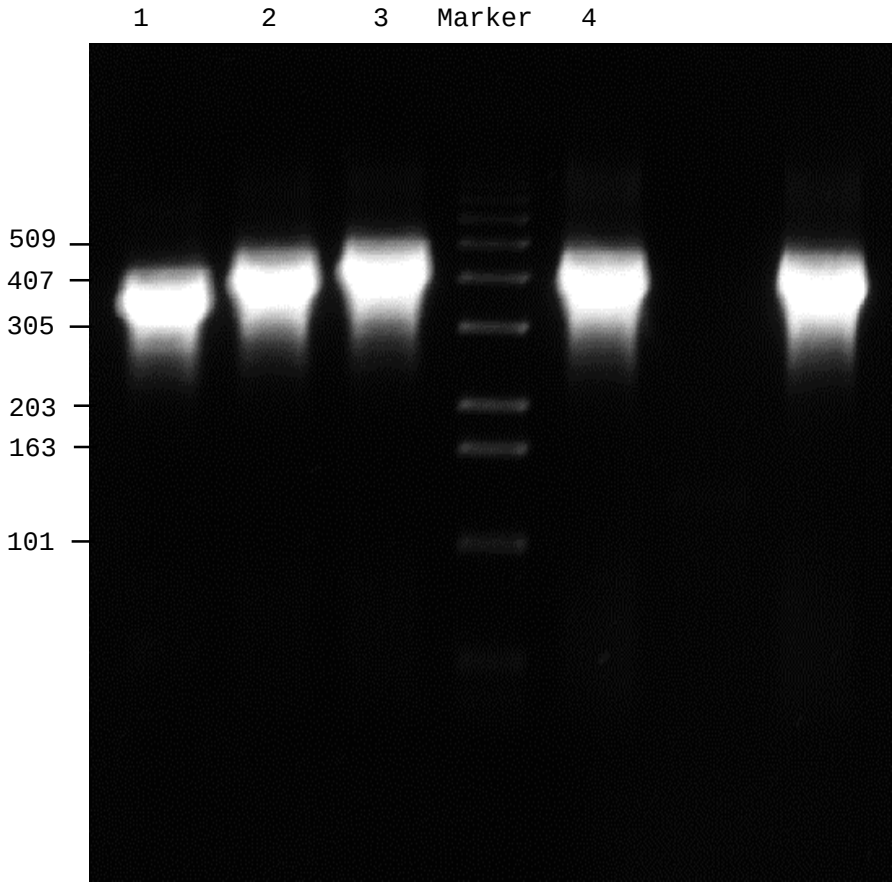

Figure 2 : Genomic RGA obtained from the sunflower line RHA266. The sizes of the fragments are between 3.5 and 4.3 kbp. The size marker is the 1 Kb ladder from Life Technologies, France.

Sequence comparisons show that these clones are highly homologous, thus they are probably members of a large resistance genes family in sunflower, as this is the case in lettuce for example (Meyers *et al*., 1998). Furthermore, nucleotide differences between these clones were found within the LRR regions but also within the TIR-NBS domains. Whether these RGA clones are functional genes and whether the differences observed represent different race or pathogen specificities have yet to be investigated.

Conclusions

We have cloned and sequenced several DNA fragments which share many features with plant resistance genes. However, we need to demonstrate that *Pl* genes are members of this class of RGA. It is expected that the first evidence will be provided by antisense experiments during the next year. Further demonstration will be the positional cloning of *Pl* genes and the transformation of susceptible sunflower lines with appropriate clones. For this purpose, a large insert Bacterial Artificial Chromosomes (BAC) library is being constructed to facilitate a chromosome walking strategy.

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