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Research Article

A homolog of the *RPS2* disease resistance gene is constitutively expressed in *Brassica oleracea*

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Abstract

In this study, we identified disease resistance gene homologs in *Brassica oleracea* and assessed their expression in lines resistant and susceptible to *Xanthomonas campestris* pv. campestris (Xcc). Two DNA fragments of approximately 2.5 kb (BI-16/RPS2 and Lc201/RPS2) were amplified by PCR from two *Brassica* lines using primers based on an *RPS2* homologous sequence previously described in the *Brassica oleracea* ecotype B117. The sequences of these fragments shared high similarity (95-98%) with *RPS2* homologs from various *Brassica* species. The digestion of these fragments with restriction enzymes revealed polymorphisms at the *Xba* I restriction sites. The length polymorphisms were used as a co-dominant marker in an F_2 population developed to segregate for resistance to Xcc, the causal agent of black rot. Linkage analysis showed no significant association between the marker and quantitative trait loci for black rot. RT-PCR with specific primers yielded an expected 453 bp fragment that corresponded to the RPS2 homologs in both resistant and susceptible lines inoculated with the pathogen, as well as in non-inoculated control plants. These results suggest that these homologs are constitutively expressed in *B. oleracea*.

Key words: Xanthomonas campestris pv. *campestris*, disease resistance gene homolog, black rot.

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Introduction

In recent years, numerous resistance genes (R) have been cloned and their sequences have revealed common motifs (Martin, 1999), including leucine rich repeats (LRRs) and nucleotide binding sites (NBS) (Jones, 2001; Staskawicz, 2001). NBS-LRRs resistance proteins serve to recognize avirulence (*Avr*) gene products from extracellular pathogens, thereby initiating a cascade defense response (Schaal and Olsen, 2000; Dangl and Jones, 2001; Bergelson *et al*., 2001). An interaction between R and *Avr* genes was demonstrated when a mutation within the C-terminal portion of an *Avr* gene led to the loss of recognition (Axtel *et al*., 2001), indicating a role for LRR domains in this phenomenon (Banerjee *et al*., 2001).

The nucleotide sequences of these conserved domains have been used to generate primers and to amplify homologous disease resistance genes in correlate and distant species. In several cases, these homologs are linked to resistance genes (Leister *et al*., 1998; Collins *et al*., 1998; Shen *et al*., 1998; Speulman *et al*., 1998; Aarts *et al*., 1998; Melotto and Kelly, 2001).

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The *RPS2* gene of *Arabidopsis thaliana* confers resistance to *Pseudomonas syringae* pv. *tomato* (Bent *et al*., 1994; Mindrinos *et al*., 1994). This gene, which is located on chromosome 4, encodes a protein with 909 amino acids and contains NBS and LRRs domains, as well as a leucine zipper (LZ). Recently, Banerjee *et al*. (2001) showed that more than one gene in *Arabidopsis* is associated with the defense response to *P. syringae*. A high level of nucleotide polymorphism at the 3' end of the *RPS2* gene has also been demonstrated (Caicedo *et al*., 1999).

A homolog of the *RPS2* gene in *Brassica oleracea* (Wroblewski *et al*., 2000) shares 83% sequence identity with the nucleotide sequence of the *A. thaliana RPS2* gene and 80 % similarity to its predicted aminoacid sequence. Southern blot analysis revealed a single copy and no introns, as in *A. thaliana*. Nucleotide polymorphisms of this homolog found in members of the tribe Brassiceae have been compared in an attempt to determine their evolutionary history. In this study, we examined the expression of a homolog of the *RPS2* gene in two *B. oleracea* lines and assessed the linkage between this homolog and quantitative trai loci (QTL) controlling resistance to *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot. Two fragments (BI-16/RPS2 and Lc201/RPS2) homologous to the *RPS2* gene were amplified by PCR and were

found to be constitutively expressed. There was no linkage to resistance QTLs.

Material and Methods

Plant material and inoculation procedure

The cabbage inbred line Badger Inbred-16 (BI-16), resistant to Xcc (Camargo *et al*., 1995) and the susceptible `fast cycling' inbred line Lc201 (Williams and Hill, 1986) were used in this study. An F_2 population derived from the cross BI-16 x Lc201 previously screened for black rot resistance (Malvas *et al*., 1999) was used in a segregation analysis to study the linkage relationships between disease resistance homologs and quantitative trait loci (QTL) controlling resistance to *X. campestris* pv.*campestris*.

Parental lines were inoculated with the isolate PHW 1205 of *X. c.* pv. *campestris* by clipping the leaf tip and rubbing a cotton swab saturated with bacterial suspension over the wounded site as previously described (Camargo *et al*., 1995). Control plants were inoculated with water. Inoculated plants were analyzed for their phenotypic reaction to the pathogen seven days after inoculation.

Cloning and sequencing of the *RPS2* homologs BI-16/RPS2 and Lc201/RPS2

A pair of primers was designed based on the partial sequence of a published *B. oleracea* genomic fragment (GenBank accession number AF180355) homologous to the *RPS2* gene (Wroblewski *et al*., 2000) and used to amplify homologous fragments from our lines by PCR.

The PCR mixtures contained 150 ng of genomic DNA, 0.2 mM dNTPs (Life Technologies, Rockeville, MD), 0.25 µM of each primer, 4.0 mM $MgCl_2$, 1x reaction buffer and 5 U of high fidelity *Taq* DNA polymerase (Life Technologies, Rockeville, MD) in a final volume of 50 µL. The reactions were run in a PTC100 thermal cycler (MJ Research) with the following conditions: one cycle of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 65 °C and 5 min at 72 °C, and a final extension of 15 min at 72 °C. The PCR products were resolved on 1% agarose gels in 0.5x TBE buffer.

The PCR fragments were sonicated for 30 s at 750 W-s in an ultrasonic homogenizer (Cole Parmer), and fragments 500 to 750 bp in size were excised from the agarose gel and purified with a Sephaglas Band prep kit (Amersham Pharmacia Biotech, Piscataway, NJ). Sonicated fragments were cloned into pUC18 using a Sure Clone kit (Amersham Pharmacia Biotech). Plasmid DNA was purified according to Sambrook *et al*. (1989) and used as the template in sequencing reactions with a Ready Reaction Dyedeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA). The DNA sequences were obtained using an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) and evaluated using internet-based facilities such as BLAST (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997) and the computer program Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI).

Analysis of restriction fragment length polymorphisms between BI-16/RPS2 and Lc201/RPS2

DNA sequences were analyzed for their restriction patterns by electronic digestion with the software Sequencher 3.0. The restriction enzymes *Pst* I, *Eco*R I, *Eco*R V, and *Xba* I were used to digest the DNA fragments amplified from the parental lines BI-16 and Lc201 as well as from an F_1 individual in order to confirm the length polymorphisms. Enzymes that revealed polymorphisms were then used to digest the RPS2 homologous fragment amplified from the F_2 population. The digestion reaction contained 1x buffer, 10 U of restriction enzyme and 8 µL of PCR mix which was incubated for 6 h at 37 °C. The restriction fragments were visualized in 0.8% agarose gels.

RT-PCR

Twenty-four hours after inoculation, 100 milligrams of leaf tissue were harvested, frozen with liquid nitrogen and stored at -80 °C until mRNA isolation. mRNA was isolated with an Oligotex Direct mRNA midi kit (Qiagen GmbH, Hilden, Germany) and quantified spectrophotometrically. cDNA was synthesized from approximately 3 µg of mRNA using the Superscript One Step RT-PCR kit with Platinum Taq (Life Technologies Inc. Galthersburg) and amplified with specific primers designed between nucleotides 580 and 1100 of the BI-16/RPS2 homolog in order to produce a fragment of approximately 500 bp. DNA contamination of RNA samples was monitored with a negative control by using mRNA as template in the reactions. The assays were run using one cycle of 2 min at 93 °C, followed by 35 cycles of 45 s at 93 °C, 45 s at 51 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C, after which the PCR products were separated in 1% agarose gels with 0.5x TBE buffer.

Results

A set of primers based on the *RPS2* homologous sequence previously described in *B. oleracea* ecotype B117 (Wroblewski *et al*., 2000) was used to amplify a fragment of approximately 2.5 kb from our parental lines. The forward and reverse primer sequences were 5'-GCAGGACATTAAGACTGATCTTAAGC-3' and 5'-GCTGGCAAGTTTGGCTGGACCCTTTCC-3', respectively. PCR using these primers amplified a single 2.5 kb DNA fragment in B1-16 and Lc201 (not shown). Sequencing of the clone amplified fragments revealed 95% sequence identity between BI-16/RPS2 and Lc201/RPS2 was 95%. Comparison of the sequences identified 112 base

substitutions (4.3% of bases) and 18 indels (insertions/deletions; 0.7% of bases).

The BI-16/RPS2 and Lc201/RPS2 sequences were compared with other sequences deposited in the GenBank database using the BLASTN search software (Altschul *et al*., 1997). The alignment obtained at the nucleotide level revealed a high similarity to *RPS2* homologs previously described in *Brassica* species such as *B. montana* (AF180358; 98% identity, 0% gap), *B. oleracea* ecotype B117 (AF180357; 98% identity, 0% gap) and *B. rapa* (AF180359; 95% identity, 0% gap). The 5' end of the DNA sequence corresponding to the predicted LRR domain showed 100% similarity to the *RPS2* homolog from ecotype B117.

The BI16/RPS2 fragment amplified from the resistant line showed an open reading frame (ORF) of 2,506 nucleotides with one frame shift at the 5' end. The Lc201/RPS2 fragment amplified from the susceptible line showed stop codons, although three ORFs of 875 bp, 791 bp, and 1,452 bp were identified. In both sequences, three domains were identified and arranged in the following order leucine zipper (LZ), nucleotide binding site (NBS), and leucine rich repeat (LRR).

Alignment of the derived amino acid sequences of the two cloned homologs showed high similarity (E value of 0.0) to the RPS2 protein described in *Arabidopsis* and to the *RPS2* homolog in *Brassica* species (Figure 1), as well as to other resistance gene sequences such as *RPR1* from *Oryza sativa* (gi|4519936) and *RGC2J* from garden lettuce (gi|7489352).

To detect the expression of *RPS2* homologs in response to inoculation with Xcc, mRNA extracted from BI-16 and Lc201 leaves 24 h after inoculation was transcribed and cDNA was obtained with primers designed to amplify a 453-bp segment internal to the BI-16/RPS2 fragment. PCR analysis confirmed the presence of the fragment in both the resistant and susceptible lines (Figure 2). This result suggests that these homologs are constitutively expressed in *B. oleracea*. The sequenced cDNA fragment from the resistant line aligned with the genomic sequence between nucleotides 479 and 931 (NBS domain) and showed 14 nucleotide substitutions and two indels (97% identity).

Alignment of the BI-16/RPS2 cDNA sequence with the *Arabidopsis* genome showed 87% identity with the BAC clone F20B18 (emb|AL049483.1) located on the chromosome 4 of *Arabidopsis* that harbors the *RPS2* locus. In addition, a cluster of *Arabidopsis* EST clones has been mapped to the same location, indicating that this region is expressed as mRNA. The BI-16/RPS2 cDNA also partially aligned with one of the *Arabidopsis* EST clones (gi|1217460; 90% identity).

Of the four restriction enzymes tested, only *Xba* I detected polymorphism between BI-16 and Lc201. There were three *Xba* I restriction sites in the BI-16/RPS2 fragment and two in Lc201/RPS2 (Figure 3). This RFLP was used as a co-dominant marker in an F_2 population (91 individuals) and segregated to resistance to Xcc. A linkage analysis of the resistance phenotypes of F_2 plants and their marker genotypes indicated no significant association ($F = 0.21$, $p < 0.01$) between the marker and resistance QTLs previously mapped in this population (Malvas *et al*., 1999; Camargo *et al*., 1995).

Discussion

In this study, we identified two disease resistance gene homologs, BI-16/RPS2 and Lc201/RPS2, and demonstrated their constitutive expression in *B. oleracea* lines. PCR amplification using specific primers amplified a single 2.5 kb fragment in *Brassica* lines and the DNA sequences of the fragments showed similarity to NBS-LRR domains present in some previously cloned resistance genes, including the *RPS2* gene of *Arabidopsis*. The 5% sequence divergence between BI16/RPS2 and Lc201/RPS2 indicates that these fragments could possibly represent different alleles.

The lack of co-segregation between these fragments and QTLs for resistance to Xcc does not preclude them from being part of a resistance locus or part of a gene that confers resistance to another pathogen. The RFLP that distinguished the two fragments may be used as a marker to detect resistance genes in other segregating populations. Resistance gene homologs are not always closely associated with resistance phenotype and may be only loosely linked to a known resistance locus (Melotto and Kelly, 2001). Four other disease resistance gene homologs have been reported not to be linked to any resistance QTLs previously mapped in *B. oleracea*, probably because of the small number of resistance genes identified in this species (Vicente and King, 2001). Similarly, in maize, the *ht2*, *htn1*, *hm2* and *msv1* disease resistance gene homologs were found unlinked to any known resistance gene (Collins *et al.*, 1998). However, even in these cases, it is possible that linkage does exist, but between the homologs and another as yet undescribed R gene.

RT-PCR detected BI-16/RPS2 and Lc201/RPS2 cDNAs in non-inoculated tissue. This may indicate that these homologs are involved in resistance mechanisms since the vast majority of R genes are also constitutively expressed. The presence of the LRR and NBS domains further indicates that these homologs may have a recognition function similar to that of R genes.

The differences between the cDNA and the genomic sequences of BI-16/RPS2 suggest the occurrence of two copies of this gene, which may exist in a cluster. An extra copy of the RPS2 homolog in *Brassica* has been reported by Wroblewski *et al.* (2000). It is also possible that the enzymes used to digest the fragment amplified from the genomic DNA cleaved the two copies at the same position, thereby generating fragments that co-migrated in agarose

gels. Gene duplication and the formation of clusters is common in R genes. The *RPP8* gene from Arabidopsis, for instance, is present as a single copy in the Col-0 accession but as two copies in the Ler accession (Dangl and Jones, 2001).

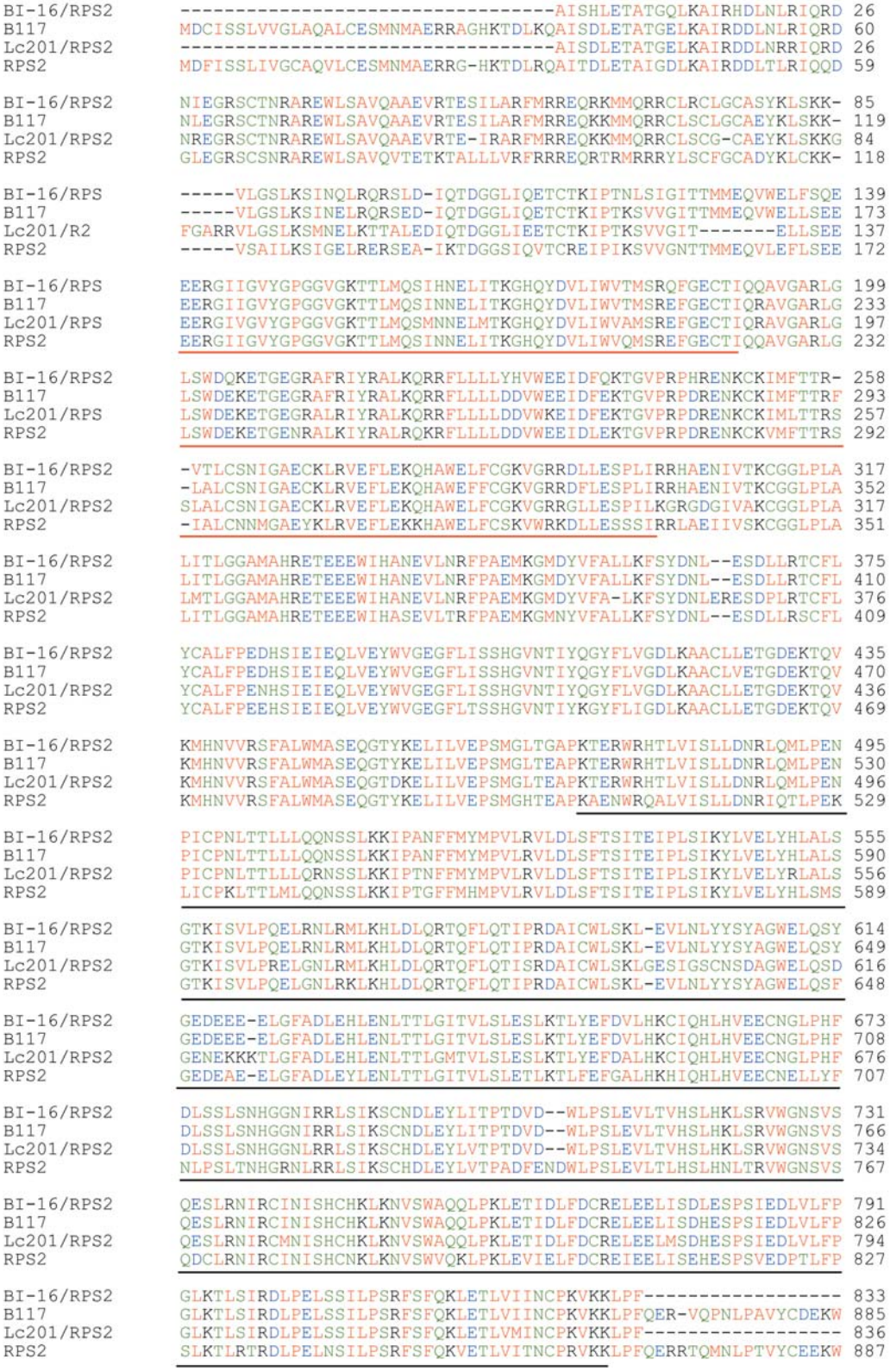

Figure 1 - Alignment of the amino acid sequences of BI-16/RPS2, Lc201/RPS2 and *RPS2* from *Arabidopsis thaliana* (ATU14158) and B117 from *Brassica oleracea* (AF180357).

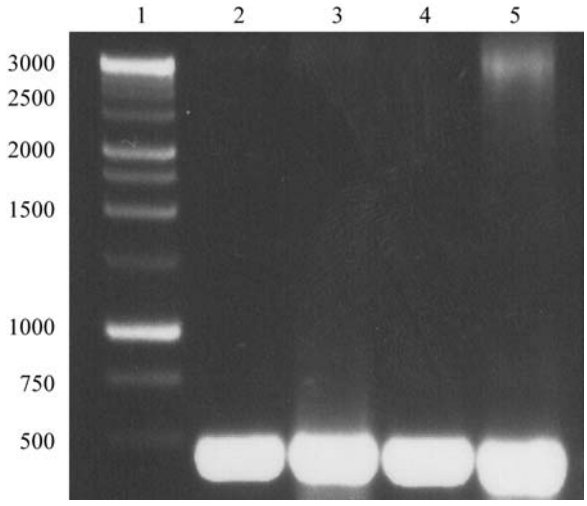

Figure 2 - RT-PCR analysis of mRNA isolated from leaves 24 h after inoculation. Lane 1 - 1kb DNA ladder (Promega), lanes 2 and 3 - positive control with genomic DNA from BI-16 and Lc201, respectively; lanes 4 and 5 - PCR with cDNA from BI-16 and Lc201, respectively.

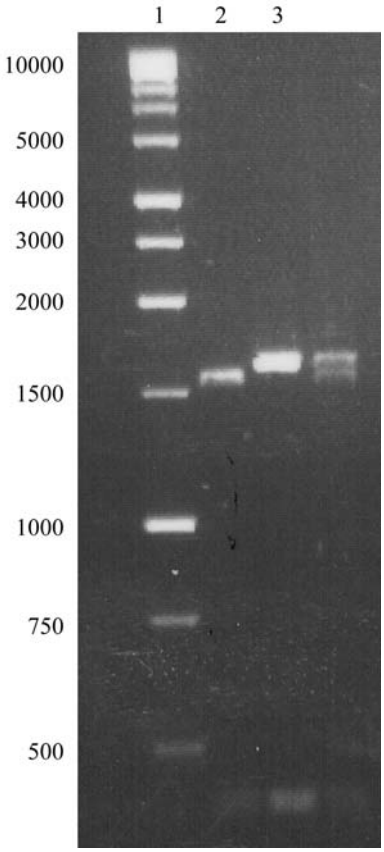

Figure 3 - Restriction patterns for BI-16, Lc201 and F1, digested with *Xba I*. Lane 1 – 1kb DNA ladder (Promega); lane 2- BI-16 ; lane 3 - Lc201.

Most of the resistance genes characterized so far belong to families of tightly linked genes. The *Rp1 locus of maize* (Sun *et al.*, 2000), *I2* of tomato (Simons *et al.*, 1998), and *Dm3* of lettuce (Shen *et al.*, 1998) span a large region of the genome. Over 24 candidate genes covering 3.5 Mb of the Dm3 locus in lettuce (Shen *et al.*, 2002) have been characterized. The occurrence of gene clusters may provide genetic variation from which new resistance genes evolve. Clusters may also confer specificity to a wide range of pathogens. Mechanisms such as duplication, unequal crossing-over, gene conversion, and transposable elements have been proposed to contribute to recombination between clustered genes (Hulbert *et al.*, 2001). The *Pto* locus has four homologs that map to a syntenic region in tomato, potato and pepper. *Fen* gene homologs isolated from two species of tomato showed 97% and 95% nucleotide identity, but only the Fen gene was effective in recognizing fenthion (Riely and Martin, 2001).

The BI-16/RPS2 homolog contained an open reading frame with a predicted frame shift. However, the partial BI-16/RPS2 cDNA sequence aligned at this very region. Frame shifts and stop codons are common in homologs (Vicente and King, 2001). Despite the presence of frame shifts and retroelement insertions in the RPP5 gene family, these genes may still be functional (Nöel *et al.*, 1999). The homologs described here may also be functional in other cultivars or species. In addition, a cluster of Arabidopsis ESTs clones with high homology to our cDNA sequences has been mapped to chomosome 4 and linked to the *RPS2* locus. Definitive proof that these sequences correspond to a resistance gene requires transgenic expression (Shen *et al.*, 1998).

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