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Defense response genes co-localize with quantitative disease resistance loci in pepper

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Abstract Functional bases of polygenically inherited disease resistance are still unknown. In recent years, molecular dissection of polygenic resistance has led to the identification and location of quantitative trait loci (QTLs) on many plant genetic linkage maps. This process is a pre-requisite for resistance QTL characterization at a molecular and functional level. Here, we report the use of a candidate gene approach based on the hypothesis that some resistance QTLs previously mapped in pepper may correspond to defense response (DR) genes. Degenerate oligonucleotide primers were designed for conserved regions of two DR gene families: pathogenesis-related proteins (PR) of class 2 (β-1,3-glucanase) and PR proteins of class 5 (antifungal activity). Cloned pepper PCR-products as well as other solanaceous DR gene families were used as RFLP probes for mapping in three intraspecific maps of the pepper genome. A total of 12 probes out of 23 were positioned and generated 16 loci. Some DR probes revealed multiple gene copies in the pepper genome (PR5, β-1,3-glucanase, chitinase and Glutathione S-transferase). Genes encoding acidic and basic β-1,3-glucanases were clustered on linkage group (LG) P1a, whereas genes encoding chitinases occurred on several LGs (P1b, P2a and P5). A class-III chitinase gene co-localized with a major-effect QTL controlling resistance to *Phytophthora capsici* on LG P5. PR4, PR2 and PR10 loci mapped within the region of resistance QTLs to *P. capsici* (LG P1b), *Potato virus Y* (LG P1a) and *Potyvirus E* (LG P3), respectively. A digenic interaction between a PR4 and a PR2 loci explained a large effect (35%) of the resistance to *Potyvirus E*.

Keywords *Capsicum annuum* · Candidate gene approach · Quantitative trait locus · β-1,3-Glucanase · PR5 · Class III chitinase · *Phytophthora capsici*

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Introduction

Improving crops with enhanced disease resistance is a major objective for breeders. Considerable efforts have been made to understand the molecular and functional bases of resistance induction during the plant-pathogen interaction. Genetically simple resistance (particularly those fitting the gene-for-gene model) has received much attention, but genetically complex resistance i.e., polygenically controlled resistance, is still poorly understood despite being widely used in plant breeding programs. Furthermore, polygenic resistance is theorized to provide a more durable protection. The advent of molecular markers provided the opportunity to determine the number, positions, and individual effects of resistance loci showing quantitative inheritance (Tanksley 1993; Young 1996). Until now, the biological functions of genes underlying individual quantitative loci involved in disease resistance remained unknown. A combination of partial resistance factors from different polygenic resistance sources has led to transgressive genotypes, i.e. an increase of the resistance level or a widening of the resistance spectrum (Parlevliet and Zadocks 1977; Vear and Tourvieille de Labrouhe 1984; Palloix et al. 1990). Several studies demonstrated that a quantitative trait locus (QTL) may control several resistance components or specifically one component (Freymark et al. 1993; Young et al. 1993; Wang et al. 1994; Lefebvre and Palloix 1996). A QTL may affect the resistance to several pathogen isolates or, specifically, to one pathogen isolate (Danesh et al. 1994; Leonards-Shippers et al. 1994; Caranta et al. 1997a). These results indicate that resistance factors probably control different mechanisms of

resistance with complementary effects. Consequently, the knowledge of biological functions that underlie partial resistance factors (QTLs) will help to construct genotypes with a more efficient and durable resistance. Disease resistance gene analogues (RGAs) mapped in the vicinity of resistance QTLs (Gentzbittel et al. 1998; Pflieger et al. 1999; Geffroy et al. 2000), and a recent comparative genetic study that showed major resistance genes occurring in trans-generic clusters with QTLs (Grube et al. 2000a), suggested sequence similarities, and probably function similarities, between major resistance genes and quantitative resistance loci. To date, numerous defense response (DR) genes have been cloned and the mapping of both QTLs and DR genes is feasible. Several map co-localizations between resistance QTLs and DR gene markers have been reported in plant species (Giese et al. 1993; Nodari et al. 1993; Leonards-Schippers et al. 1994; Ferreira et al. 1995; Faris et al. 1999; Geffroy et al. 2000). These results strengthen the hypothesis that some resistance QTLs may correspond to DR genes and suggest that molecular polymorphisms within the DR gene result in allelic diversity and may be related to the resistance level (Pflieger et al. 2001).

Plant DR is an active mechanism following pathogen attack. DRs involve various metabolic pathways such as synthesis of reactive oxygen species, antimicrobial secondary metabolites (phytoalexins), lignification of host cell walls and de novo transcription of a large number of genes including those encoding pathogenesis-related (PR) proteins (e.g. β-1,3-glucanases, chitinases, antifungal components), glutathione-S-transferases, thionins, defensins, and enzymes of secondary metabolism (e.g. phenylalanine ammonia lyase, Ortho-diphenol-O-methyltransferase, hydroxycinnamyl alcohol dehydrogenase) (Lamb et al. 1989; Hammond-Kosack and Jones 1996). A similar set of DR genes is activated in resistant and susceptible genotypes but with a different gene expression time-course (Lamb et al. 1989) suggesting a differential induction. The defensive arsenal is effective against different types of pathogens (viruses, fungi, bacteria and nematodes) and is conserved within mono- and dicotyledons. The antimicrobial activity of some defense components was shown using in vitro analyses (e.g. Sela-Buurlage et al. 1993). Moreover, constitutive expression of some DR genes in transgenic plants led to an enhanced resistance level (e.g. Liu et al. 1994; Jongedijk et al. 1995; Stark-Lorenzen et al. 1997; Leckband and Lörz 1998). All these observations suggest that DR genes might be good candidates for characterizing loci involved in complex induced resistance.

The aim of the present study was to clone and map DR genes in the pepper genome and to look for map co-localizations with resistance loci. Pepper is an interesting case-study since numerous loci involved in resistance against several pathogens (fungi, viruses, nematodes and bacteria) were located on pepper maps: dominant and recessive major genes controlling resistance to viruses (*Tobacco mosaic virus*: Lefebvre et al. 1995; *Potato virus Y*: Caranta et al. 1996; Murphy et al. 1998; Grube et al. 2000b; *Tomato spotted wilt virus*: Jahn et al. 2000; Moury et al. 2000), nematodes (*Meloidogyne* spp.: Djian-Caporalino et al. 2001) and bacteria (*Xanthomonas campestris*: Pierre et al. 2000), as well as QTLs for resistance to *Phytophthora capsici* (Lefebvre and Palloix 1996), Potyviruses (Caranta et al. 1997a) and *Cucumber mosaic virus* (Caranta et al. 1997b). Recently, the genetic positions of disease resistance loci were compared within the Solanaceae (Grube et al. 2000a) allowing molecular synteny studies between pepper, tomato and tobacco.

In this paper, we report the cloning of members of two pepper DR gene families (β-1,3-glucanase and PR5) using a PCR approach. We chose these families since they were shown to have an antifungal activity (Woloshuk et al. 1991; Vigers et al. 1992; Sela-Buurlage et al. 1993). Then, pepper β-1,3-glucanase and PR5, as well as other solanaceous DR gene clones, were located on pepper genetic maps and co-localizations with resistance QTLs to different pathogens were noted.

Materials and methods

Isolation of pepper DR gene clones

Degenerate oligonucleotide primers were designed using conserved amino-acid motifs of two DR gene families: β-1,3-glucanase (PR2) and PR5 genes. These motifs were deduced from the alignment of 18 β-1,3-glucanases (accession numbers X54430, X54431, M59443, M60460, M60464, M80604, X07280, M23120, U01901, U01902, M80608, M20618, M20620, X74905, X54456, X74906, Z28697 and X69794) and ten PR5 (accession numbers X67244, M64081, X61679, S44889, M29279, X70787, X66416, X65700, X67121 and X72926) nucleotide sequences from Solanaceae. Alignments were performed using the pileup program of the GCG software (Genetics Computer Group, University of Wisconsin, Madison; version 9.1). PR2-S (5-RYIGGWGTWTGYTAYGG-3) and PR2-AS (5-CADCCRCTYTCIGAYAC-3) primers corresponded to the conserved amino-acid motifs LGVCYG and VSESGW, respectively. PR5-S (5-AACAAYTGYCCRTACACCGT-3) and PR5-AS (5-GGATCATCTTGWGGRTARCTATA-3) primers corresponded to the conserved amino-acid motifs PYTVWAA and CPDAYSYP, respectively (Y=T or C, R=A or G, W=A or T). Primers were synthesized by Eurogentec (Belgium).

PCR reactions were performed on genomic DNA of two pepper inbred lines: Perennial (an Indian hot pepper line) and Yolo Wonder (an American bell pepper line). Both primer combinations were tested on the DNA of other plant species: tomato, tobacco, potato, eggplant (kindly provided by colleagues of INRA Montfavet, France) and *Arabidopsis thaliana* (provided by M. Fourmann, INRA Versailles, France). β-1,3-glucanase amplifications were performed using 20 ng of genomic DNA, 0.85 U of *Taq* DNA polymerase (Promega), 2 mM $MgCl_2$ (Promega), 0.1 mM dNTPs (Boehringer), 16.5 pmol of each primer and 1×enzyme buffer (Promega) in 25 µl. A 5-min denaturation step at 94°C was followed by 35 cycles: 30 s at 94°C, 1 min at 50°C and 2 min at 72°C, and a 10-min final extension cycle at 72°C. PR5 amplifications were performed using 50 ng of genomic DNA, 0.75 U of *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$ (Promega), 0.1 mM dNTPs (Boehringer), 20 pmol of each primer and 1× enzyme buffer (Promega) in 25 µl. PCR conditions were identical to those described for β-1,3-glucanase amplifications, except that 30 cycles were performed at an annealing temperature of 55°C during 30 s. PCR products were visualized in a 1% agarose gel stained with ethidium bromide, and cloned into the PCR2.1 vector from the T-A cloning kit (Invitrogen, Netherlands) according to the manu-

Table 1 DR gene clones mapped on the pepper genome. Abbreviations: GC: known-function gene; PR: *Pathogenesis-Related*

Clone	Function	Species	Reference	Number of loci revealed in each mapping population *(concerned linkage groups)*		
				HV	PY	YC
CHI_GC015	Acidic class III chitinase-lysozyme (PR8)	Tobacco	Stintzi et al. 1993	1 *(P5)*		
CHI_GC016	Basic class III chitinase-lysozyme (PR8)	Tobacco	Stintzi et al. 1993		1 *(P1b)*	
OMT_GC017	Class II Ortho-diphenol-*O*-methyltransferase	Tobacco	Pellegrini et al. 1993		1 *(Indigo=P6)*	
PI_GC018	Proteinase inhibitor (class I antisubtilisin)	Tobacco	Heitz et al. 1993	1 *(P10)*		
CHI_GC021[a]	Acidic class II chitinase (PR3)	Petunia	Linthorst et al. 1990a	–	–	–
GLU_GC023[b]	Acidic class II β-1,3-glucanase (PR2)	Tobacco	Linthorst et al. 1990b	–	–	–
CHI_GC024	Basic class I chitinase (PR3)	Potato	Beerhues and Kombrink, 1994		1 *(P1b)*	
GLU_GC025[c]	Basic class I β-1,3-glucanase (PR2)	Potato	Beerhues and Kombrink 1994	–	–	–
CHI_GC027	Acidic class II chitinase (PR3)	Potato	E. Kombrink, Max Planck Institut, Koln, Germany		1 *(Jaune=P2a)*	
PR10_GC028	PR10	Potato	Matton and Brisson 1989	1 *(P3)*		
GST_GC030	Glutathione S-transferase	Potato	E. Kombrink, Max Planck Institut, Koln, Germany		3 *(P9)*	
GLU_GC034	Acidic β-1,3-glucanase (PR2)	Tobacco	K.A. Lawton, Novartis, North Carolina, USA	1 *(P1a)*	2 *(P1a)*	
PR4_GC036	PR4	Tobacco	K.A. Lawton, Novartis, North Carolina, USA		1 *(P1b)*	1 *(P1b)*
GLU_GC169	Basic β-1,3-glucanase analogue (PR2)	Pepper	this work			1 *(P1a)*
		Total number of loci mapped		4	10	2

[a] CHI_GC021 raised the same RFLP pattern as CHI_GC027
[b] GLU_GC023 raised the same RFLP pattern as GLU_GC034
[c] GLU_GC025 raised the same RFLP pattern as GLU_GC169

Table 2 Pepper mapping populations and corresponding linkage maps used for DR gene markers mapping. Abbreviations: DH: doubled haploid lines; *P. capsici*: *Phytophthora capsici*; CMV: *Cucumber mosaic virus*

Code	Cross	Size and nature of the population	Number of markers	Map size (cM Kosambi)	Bibliographic reference	Segregation of polygenically inherited disease resistance		
						P. capsici	CMV	Potyviruses
PY	Perennial×Yolo Wonder	94 DH	377	2414 cM	Pflieger 1998; Lefebvre et al. 1997; Lefebvre et al. 1995	Yes	Yes	Yes
HV	H3×Vania	98 DH	300	417 cM	Lefebvre et al. 1997	Yes	Yes	No
YC	Yolo Wonder×CM334	151 F_2	255	520 cM	Unpublished data	Yes	No	No

facturer's instructions. Sequencing of plasmid inserts was performed by Genome Express (Grenoble, France). Sequences were analyzed for homology with databases using the blastx and blastn algorithms (Altschul et al. 1990). Percentages of identity were calculated using the gap program of the GCG software (Genetics Computer Group, University of Wisconsin, Madison; version 9.1). Multiple alignments were performed using the ClustalW software (version 1.81) at the Infobiogen Web site (http://www.infobiogen.fr).

DR genes from other solanaceous species

DR genes cloned from other solanaceous species were used as RFLP probes. They included genes encoding PR proteins [PR1 (antifungal activity), PR2 (β-1,3-glucanase), PR3 and PR8 (chitinase), PR4 (unknown function), PR5 (antifungal activity), and PR10 (ribonuclease)], enzymes of the phenylpropanoid and lignin pathway (ortho-diphenol-O-methyltransferase, phenylalanine ammonia lyase, hydroxycinnamate-CoA ligase), proteinase inhibitor (PI), Glutathione-S-transferase and a ribonuclease-homolog. Genes mapped in our pepper populations are mentioned in Table 1.

Genetic mapping of DR gene clones

A total of 23 clones were used as probes in Southern blotting (as described by Lefebvre et al. 1993) using five restriction enzymes to look for RFLPs between parental lines (Perennial, Yolo Wonder, CM334, H3 and Vania) of three pepper linkage maps (Table 2). The annealing temperature used in molecular hybridization was 65°C and 55°C for homologous and heterologous probes, respectively. When several RFLPs were detected with a single enzyme, the different RFLP markers were identified with a

number following the name of the probe. Some probes were tested on digested DNA of 50 *Lycopersicon pennelli* introgression lines (kindly supplied by D. Zamir; Eshed et al. 1992) when no polymorphism could be detected in the three pepper crosses.

The Mapmaker/EXP software (version 3.0b) (Lander et al. 1987) was used to map new DR gene markers on the maps where polymorphism could be detected. Markers were added using the "try" command (minimum LOD score of 4.0 for the PY cross and 3.0 for the other crosses; maximum recombinant fraction 0.30). DR gene markers mapped on the *L. pennelli* introgression lines map were putatively located on pepper maps thanks to common RFLP markers between pepper and tomato reference maps (Tanksley et al. 1988; Prince et al. 1993, Lefebvre et al. 1995, 1997; Livingstone et al. 1999).

QTL detection at DR gene markers

QTL analyses were performed in the three pepper crosses using the phenotypic data for resistance to *P. capsici* (three components: root index, receptivity, and stability) (Lefebvre and Palloix 1996, V. Lefebvre, unpublished data), to *Potyvirus E*, to different pathotypes of *Potato virus Y* (PVY) (Caranta et al. 1997a) and to *Cucumber mosaic virus* (Caranta et al. 1997b) (Table 2).

QTLs with major additive effects were detected by the interval mapping method using the Mapmaker/QTL software (Lander and Botstein 1989) (significance level: LOD≥3) and by one-way analyses of variance (ANOVA) ($P \leq 5\times10^{-3}$) using the GLM procedure of the Statistical Analysis System software (SAS Institute Inc., 1989, version 6.11). To detect QTLs with minor additive effects, the effect of the interval or the individual marker linked to the major-effect QTL was "fixed" using both the interval mapping method and two-way ANOVA ($P \leq 5\times10^{-3}$). Finally, two-way ANOVAs with an interaction component between all pairs of markers were performed to detect digenic interactions ($P \leq 4\times10^{-5}$). Interactions between loci were considered only when the four genotypic classes showed an equilibrated number of individuals (according to a χ^2 test, $P>5\%$). QTL effects were estimated by the coefficient of determination (R^2 value). A more precise position of the major-effect QTL on LG P5 was estimated with the composite interval mapping (CIM) detection method (Zeng 1994) using model 6 of QTL Cartographer software (Basten et al. 1997). A Forward-Backward stepwise regression was performed to choose cofactors before performing QTL detection by CIM. Five cofactors, with the highest *F* values, were taken into account. A window size of 10 cM around the tested interval, where the cofactors were not considered, was chosen.

Results

Isolation and identification of β-1,3-glucanase and PR5 clones in pepper

Two PCR primer combinations were used to amplify pepper, other solanaceous species and *A. thaliana* genomic DNA. PR2 primers generated two major bands of approximately 750 and 1,100 bp in pepper. A common 750-bp fragment and other fragments were amplified in other species. PR5 primers generated a single band of approximately 550 bp in all the tested species.

Only pepper PCR products of expected sizes (deduced from the theoretical distance between primer annealing regions) were cloned (750 bp for β-1,3-glucanase and 550 bp for PR5 genes). One β-1,3-glucanase pepper clone (GLU_GC169) and three PR5 pepper clones (PR5_GC166, PR5_GC167, PR5_GC168) were sequenced. Significant homologies were found between GLU_GC169 and β-1,3-glucanase sequences of potato (96% and 97% nucleotide identity with accessions U01900 and U01901, respectively) and tomato (93% nucleotide identity with the accession M80608) (Fig. 1A). PR5_GC166 was homologous with a tomato PR5 sequence (90% nucleotide identity with the accession M21346). PR5_GC167 and PR5_GC168 were homologous with a potato PR5 sequence (91% nucleotide identi-

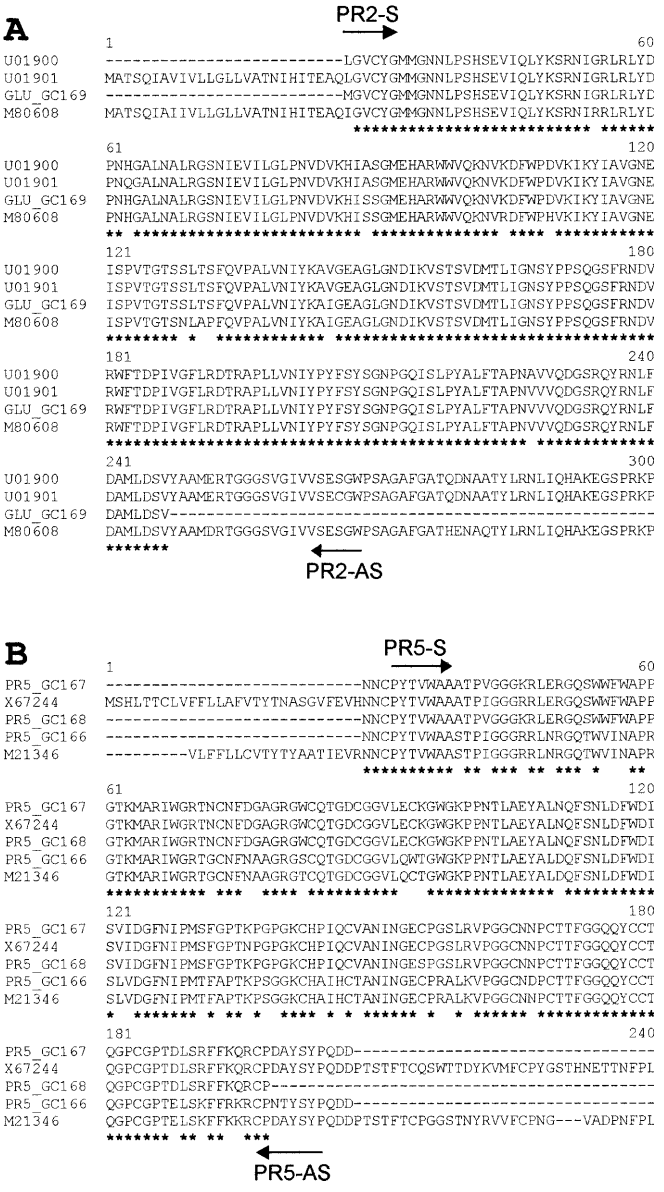

Fig. 1A, B Comparison of the deduced amino-acid sequences of the PR2 (**A**) and PR5 (**B**) pepper sequences with the best homologies found with Blastx. The *asterisks* indicate sequence identity. The position and orientation of PCR primers used to amplify pepper PR2 and PR5 sequences are shown with *arrows*. Multiple alignments were performed using ClustalW (1.81). Nucleotide accessions: U01900 and U01901, endo-1,3-β-glucanases, *S. tuberosum*; M80608, β-1,3-glucanase, *L. esculentum*; X67244, osmotin-like protein, *Solanum commersonii*; M21346, PR5 protein, *L. esculentum*

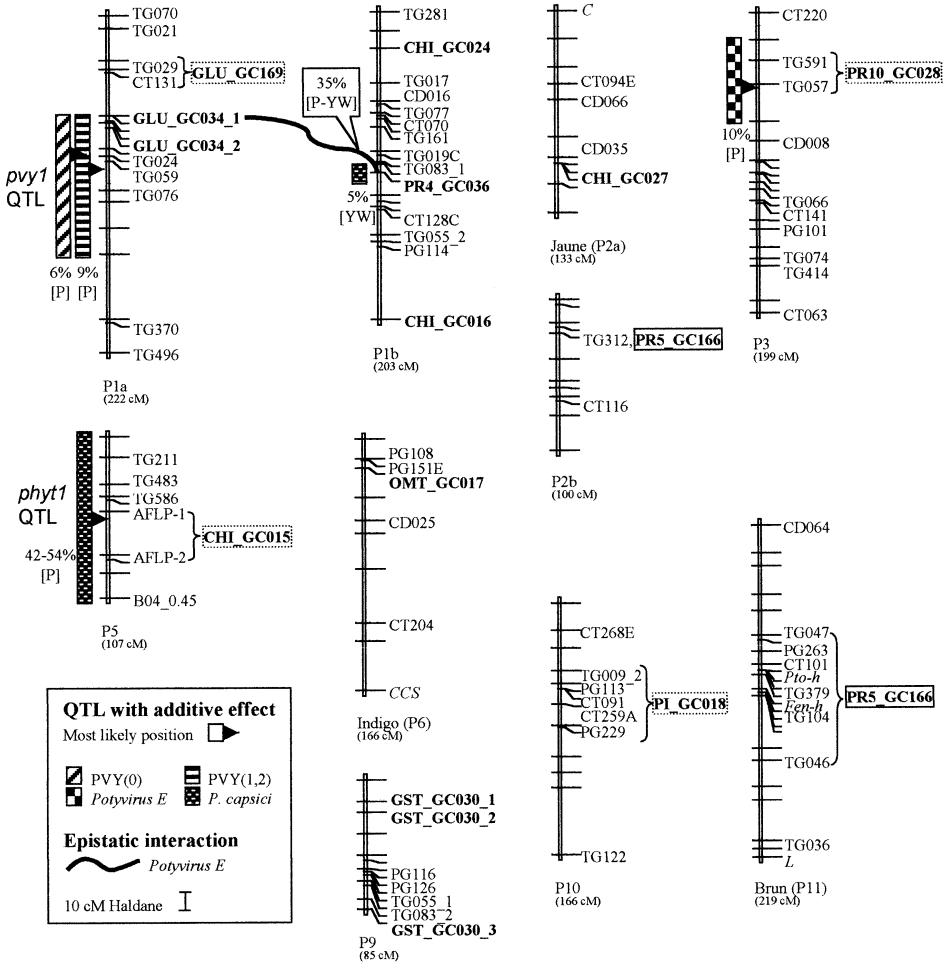

Fig. 2 Map location of DR gene loci on the PY intraspecific map of pepper. *Vertical bars* represent linkage groups (LGs). Only LGs holding DR gene loci are shown. LG numbers are given according to the nomenclature of Livingstone et al. (1999). Three LGs were assigned to chromosomes designated by *French colour names* (Pochard 1970). The *marker names* are given on the right of LGs and were described by Lefebvre et al. (1997). DR gene markers are shown in *bold*. DR gene markers localized *via* comparative mapping with other pepper maps or with the *L. pennelli* introgression lines map are *framed with a dashed line or a continuous line*, respectively. *Boxes* on the left of LGs represent QTLs associated with pathogen resistance. *Arrows* indicate the position of the maximum LOD score value. The R^2 value and the allele conferring resistance (*P*: Perennial, *YW*: Yolo Wonder) are indicated. QTL names given by Grube et al. (2000a) are noticed for the QTLs on LG P1a and LG P5. The *curved line* indicates a digenic interaction between two loci

ty with the accession X67244) (Fig. 1B). When compared to each other, the three pepper PR5 sequences shared between 80 to 99% nucleotide identity.

Mapping of solanaceous DR gene clones on the pepper genome

The 23 DR gene probes were first tested for polymorphism between five parental lines. Some probes showed identical RFLP patterns: CHI_GC027 and CHI_GC021 (acidic chitinases), GLU_GC034 and GLU_GC023 (acidic β-1,3-glucanases), GLU_GC169 and GLU_GC025 (basic β-1,3-glucanases). This result is probably due to high nucleotide homology between the DNA sequences of these probes. Only CHI_GC027, GLU_GC034 and GLU_GC169 segregation data were scored. Seven probes, mapped on the PY population, generated ten loci located on five linkage groups (LGs). Four probes, mapped on the HV population, generated four loci located on four LGs. Two probes, mapped on the YC population, generated two loci located on two LGs (Table 1, Fig. 2).

Two clones (GLU_GC034 and PR4_GC036) revealed polymorphism within two crosses (Table 1). Resulting loci displayed comparable positions between maps. The position of four loci, mapped on the HV or YC map (CHI_GC015, PI_GC018, PR10_GC028 and GLU_GC169), were reported on the PY map by a comparison of common marker positions (Fig. 2). Indeed, Lefebvre et al. 1997 showed that some RFLP and RAPD markers could be locus-specific between the intraspecific pepper maps allowing their alignment. The PY map thus counted 14 DR gene loci (10+4) generated by 11 probes and located on eight different LGs.

Nine DR gene probes (PR5_GC019, PR5_GC029, 4CL_GC032, PR5_GC037, RN_GC079, PAL_GC097, PR5_GC166, PR5_GC167 and PR5_GC168) out of 23 were monomorphic in the three pepper crosses. The PR5_GC166 probe was mapped thanks to the *L. pennelli* introgression lines available at INRA Montfavet. It generated two loci in tomato (M. Causse, personal communication). Thanks to comparative mapping between tomato and pepper genomes (Tanksley et al. 1988; Prince et al. 1993, Lefebvre et al. 1995, 1997; Livingstone et al. 1999), these two loci were positioned on the pepper LGs P2b and P11 (Fig. 2). To conclude, 16 DR gene loci were putatively mapped on ten different LGs of the pepper genome, with a total of 12 probes.

Map co-localizations of DR gene loci with resistance QTLs

Detection of QTLs by interval mapping and ANOVA gave the same results, except for the resistance QTL to *P. capsici* mapped on LG P1b. All the resistance QTLs detected at DR gene loci have already been detected in previous studies (Lefebvre and Palloix 1996; Caranta et al 1997a), except the QTL on LG P1b. Nevertheless, the addition of DR gene markers may allow us to determine the most likely position of these QTLs.

A major-effect QTL associated with three *P. capsici* resistance components (named *phyt1* by Grube et al. 2000a), defined as a generalist QTL by Lefebvre and Palloix (1996), was detected in the [AFLP-3/TG483] interval on LG P5 of the HV map (Table 3, Fig. 3). A gene encoding a class-III acidic chitinase (GC015) belonging to the PR8 group was mapped within this region (CHI_GC015 marker). Results obtained with the composite interval mapping (CIM) method showed that CHI_GC015 accounted for the highest R^2 percentage for the three resistance components: stability, root index and receptivity (Fig. 3). Thus, the class-III acidic chitinase marker is a good positional candidate gene for the major-effect QTL controlling resistance to *P. capsici* in the HV population. Interestingly, this major-effect QTL was detected in the two other resistance sources (Perennial and CM334) (Table 3, Fig. 2 and Fig. 3). In the three mapping populations, this QTL covered the entire span of LG P5 when detected by interval mapping and ANOVA. The CIM detection method showed that the most likely position of this QTL corresponded to the putative location of CHI_GC015 in the [AFLP-1/AFLP-2] interval in PY and YC (Fig. 3). Indeed, no polymorphism was detectable for the CHI_GC015 clone in the PY and YC mapping populations.

Three other DR gene markers co-localized with resistance QTLs on the PY map (Table 3, Fig. 2). A minor-effect QTL associated with one component of *P. capsici* resistance (stability) was detected at the PR4_GC036 marker on LG P1b. This QTL was only detected by ANOVA and had not been detected in previous analyses. For this QTL, the resistant allele originated from the susceptible parent, Yolo Wonder. A minor-effect QTL associated with PVY resistance (Caranta et al. 1997a; named *pvy1* by Grube et al. 2000a) was detected at the GLU_GC034 loci encoding a β-1,3-glucanase on LG P1a. The position of the *pvy1* QTL of pepper corresponds to the *Cm1.1* QTL of tomato (*Lycopersison peruvianum*) controlling resistance to *Clavibacter michiganensis* (Sandbrink et al. 1995; Grube et al. 2000a). A minor-effect QTL associated with *Potyvirus E* resistance (Caranta et al. 1997a) was detected in the vicinity of the PR10_GC028 marker on LG P3. Finally, a new digenic epistasis between PR4_GC036 (LG P1b) and GLU_GC034_1 (LG P1a) was detected with a strong effect on *Potyvirus E* resistance (R^2=35%). The most resistant combination involved one allele from Yolo Wonder and one allele from Perennial.

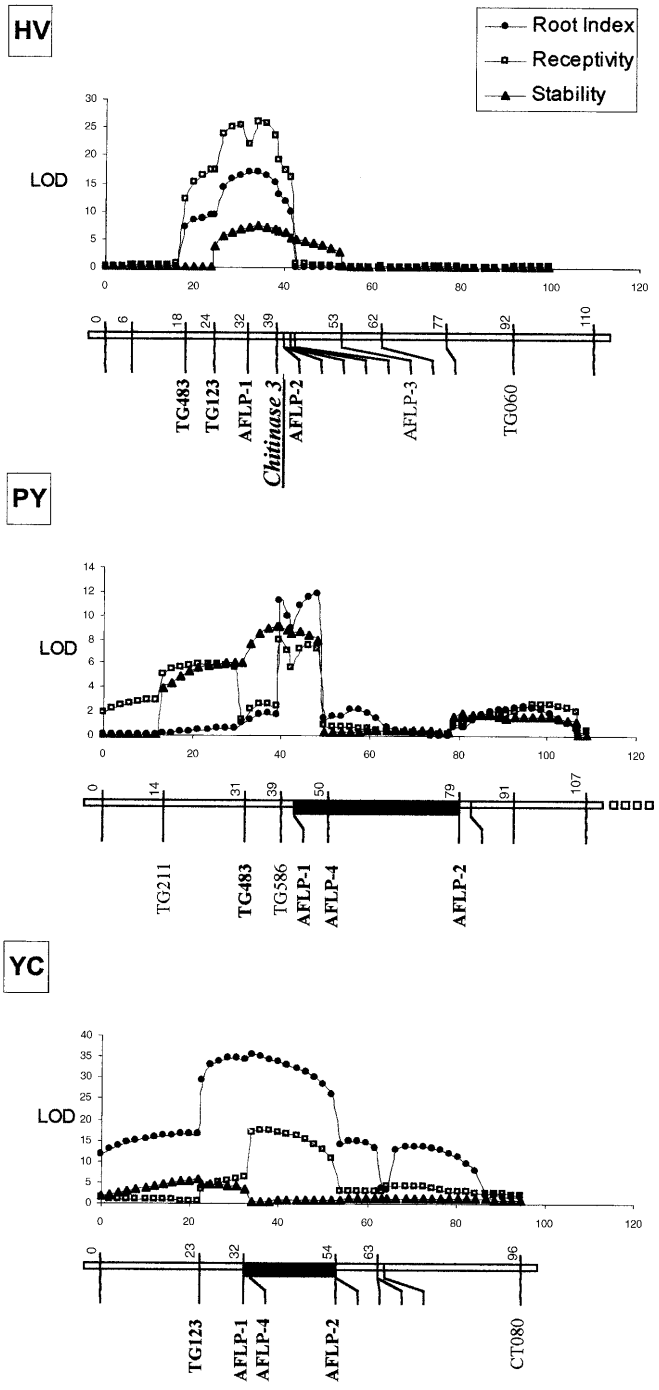

Fig. 3 Relative map position of the CHI_GC015 locus and the major-effect resistance QTL associated with the resistance to *P. capsici* (*phyt1* QTL) on LG P5 of the HV, PY and YC maps. The three graphs show the evolution of LOD score values (obtained with QTL cartographer) along the LG P5 of the HV, PY and YC maps for three *P. capsici* resistance components: root index, receptivity and stability. *Marker names and map distances* (cM Haldane) are indicated beneath and above the LG P5 representation on abscissa, respectively. *Shaded boxes* show the putative position of the CHI_GC015 marker on the PY and YC maps, which was deduced via comparative mapping; it corresponded to the [AFLP-1/AFLP-2] interval. *Identical names* for AFLP markers indicate co-migrating fragments in the three mapping populations

Table 3 DR gene regions associated with resistance to *P. capsici*, *Potato virus Y* and *Potyvirus E* in the HV, PY, and YC crosses (ANOVA results)

Pathogen resistance	LG/map	Marker	*Probability*	R^2	Resistant allele
P. capsici					
Root rot index	LG P5/HV	CHI_GC015	$<10^{-7}$	0.33	V
Receptivity	LG P5/HV	CHI_GC015	$<10^{-7}$	0.48	V
Stability	LG P5/HV	CHI_GC015	1.24×10^{-5}	0.18	V
Root rot index	LG P5[a]/PY	AFLP-4	$<10^{-7}$	0.50	P
Receptivity	LG P5[a]/PY	AFLP-4	$<10^{-7}$	0.54	P
Stability	LG P5[a]/PY	AFLP-4	$<10^{-7}$	0.42	P
Stability	LG P1b/PY	PR4_GC036 (+AFLP4) [b]	4.4×10^{-3}	0.46[c]	YW
Root rot index	LG P5[a]/YC	AFLP-4	1×10^{-7}	0.28	C
Receptivity	LG P5[a]/YC	AFLP-4	3×10^{-7}	0.27	C
Stability	LG P5[a]/YC	AFLP-4	1.6×10^{-3}	0.11	C
Potato virus Y					
Pathotype (1,2)	LG P1a/PY	GLU_GC034_1	4.8×10^{-3}	0.09	P
Pathotype (0)	LG P1a/PY	GLU_GC034_1 (+AC10_0.3)[b]	4.4×10^{-3}	0.62[c]	P
Potyvirus E	LG P3[d]/PY	TG057	1.9×10^{-3}	0.10	P
	LG P1b* LG P1a/PY	PR4_GC036* GLU_GC034_1[e]	1.8×10^{-6}	0.35	YW-P

[a] LG containing the CHI_GC015 marker on the HV map
[b] Two-way ANOVA with AFLP4 or AC10_0.3 as first components (see Materials and methods and Caranta et al. 1997a)
[c] Global R^2 with AFLP4 or AC10_0.3 as "fixed markers"
[d] LG containing the PR10_GC028 marker on the HV map
[e] These values were found with a marker narrowly linked to GLU_GC034_1

Discussion

The goal of this study was to map DR genes on the pepper genome and to look for map co-localizations with resistance QTLs. Using degenerate PCR primers, we isolated pepper clones similar to β-1,3-glucanase and PR5 genes assessing the functionality of the designed primers. We determined the map locations of 16 DR gene loci corresponding to 12 DR gene clones on the intraspecific maps of pepper. We pointed out four map co-localizations between DR gene loci and resistance QTLs, and one major-effect digenic interaction between two DR gene loci. Recently, we successfully performed such a PCR-based approach to isolate resistance gene analogues (RGA) in pepper (Pflieger et al. 1999). We showed co-localizations between RGAs and resistance QTLs different from those described in this study. Therefore, mapping both DR genes and RGAs appears as complementary approaches for characterizing quantitative resistance loci.

Mapping of DR gene probes on pepper genome

The mapping results of DR gene probes suggested the existence of multigene families. Indeed, molecular hybridization on pepper DNA showed multiple band patterns (data not shown), with some polymorphic bands mapping to several loci. These loci may be clustered on a single LG or dispersed over several LGs. For instance, markers corresponding to acidic and basic β-1,3-glucanases were clustered on LG P1a. A similar result was obtained by Jin et al. (1999) in soybean who studied the genomic distribution of β-1,3-glucanase genes. A total of 34 loci clustered on five LGs was revealed. By contrast, we showed that chitinase probes belonging to the PR3 and PR8 groups mapped to three LGs (P1b, P2a and P5). Although DR genes are not involved in pathogen recognition, their effectiveness influences disease resistance expression and pathogen spread. Bishop et al. (2000) recently showed that plant DR genes such as chitinases undergo rapid adaptive evolution as already observed for disease resistance genes (Ellis et al. 2000; Richter and Ronald 2000 for a review). Duplication of DR genes maintains the structural and functional diversity within DR gene families and provides more flexibility for spatial and temporal regulation of gene expression (Jin et al. 1999; Bishop et al. 2000).

Map co-localizations of DR gene loci with resistance QTLs

Four DR gene markers corresponding to a class-III chitinase (PR8), a β-1,3-glucanase (PR2), a PR4 and a PR10, co-localized with resistance QTLs on the pepper maps. Our results are consistent with results from other studies in which genetic linkage between DR gene markers and resistance QTLs have been reported in various pathosystems. They confirmed that certain resistance QTLs may be involved in general defense mechanisms (Giese et al. 1993; Nodari et al. 1993; Leonards-Schippers et al. 1994; Ferreira et al. 1995; Lefebvre and Chèvre 1995; Faris et al. 1999; Geffroy et al. 2000).

The genetic linkage of the class-III acidic chitinase with a QTL associated with *P. capsici* resistance is an interesting result. Although this QTL covers all the chro-

mosome when detected by ANOVA and interval mapping, its position was highly precise with the CIM method which gave a more resolutive position for this high-effect QTL. Fine-mapping experiments are currently underway in our laboratory to precisely identify the chitinase and QTL positions. This QTL had a major-effect on resistance and was detected in three resistant pepper genitors from different geographical origins. Moreover, the region containing this major-effect QTL corresponded to tomato chromosome 4. Based on marker TG483 (which revealed a single-band pattern), this region was syntenous with the *R2* (*Solanum demissum*) and *Pi1* (*Solanum tuberosum*) loci controlling resistance to *P. infestans* in potato, and with the *Hero* locus (*Lycopersicon pimpinellifolium*) controlling resistance to *Globodera rostochiensis* in tomato (Leonards-Schippers et al. 1994; Lefebvre and Chèvre 1995; Li et al. 1998; Grube et al. 2000a). It is quite surprising that a class-III chitinase may have a role in *Phytophthora* resistance. Indeed, the *Phytophthora* genus belongs to the Oomycetes class, classified as a cellulose-glucan group and characterized by the absence of chitin polymers in cell walls. However, Mort-Bontemps et al. (1997) reported the presence of chitin synthase genes in at least three Oomycetes, including *P. capsici*, suggesting the presence of chitin in cell walls. Chitinases and β-1,3-glucanases were found to be greatly induced, and accumulated in pepper leaves or stems infected by *P. capsici* (Kim and Hwang 1994). These hydrolases purified from pepper stems showed in vitro antifungal activity against *P. capsici* (Kim and Hwang 1996, 1997). Immunogold labelling experiments in pepper stems showed specific labelling of chitinase on the cell wall of *P. capsici* hyphae, 24 h after inoculation (Lee et al. 2000). Degradation of the oomycete hyphae was observed at the hyphal tip and dense gold particles were visible in this area. Class-III chitinases (named PR8) are structurally different from other classes of chitinases (I, II, IV, V, VI) (Meins et al. 1994; Neuhaus et al. 1996) since they do not possess a chitin-binding domain and have a catalytic domain with no similarity to that of the other classes (Collinge et al. 1993).

The candidate-gene approach for the characterization of resistance QTLs

In plants, no resistance QTL has yet been cloned. The candidate-gene approach is particularly well adapted to QTL characterization because of the multiplicity of genes defining a complex trait, their partial effects on phenotypic variation, and their imprecise location on genetic maps (Pflieger et al. 2001). Nevertheless, co-localizations between a candidate gene (e.g., the DR gene) and a resistance QTL is not a sufficient argument to conclude that this candidate gene is responsible for the phenotypic variation (Pflieger et al. 2001). The DR gene loci mapped on the pepper maps may correspond to functional genes as well as to pseudogenes, or the putative polymorphic candidate gene may be in linkage disequilibrium with the actual polymorphism responsible for variation in the trait (Pflieger et al. 2001). Validation of the implicated candidate genes requires further analysis, currently underway in our laboratory to verify the co-localization between the class-III chitinase (PR8) and the major-effect QTL controlling resistance to *P. capsici*.

The identification of quantitative disease resistance loci will allow improved understanding of the biological mechanisms that determine polygenically inherited resistance. Our results from this and previous studies in pepper (Lefebvre and Palloix 1996; Caranta et al. 1997a; Pflieger et al. 1999), taken together with results from other plant species, suggest that quantitative polygenic resistance will not only result from polymorphisms within RGAs, but also from polymorphisms within DR genes. Thus, breeding for polygenic resistance allows breeders to exploit different resistance mechanisms and/or a broad range of genetic diversity. Knowledge about the functions controlled by resistance loci will help breeders to construct novel resistant genotypes by pyramiding complementary mechanisms to avoid the breaking of resistance and to manage durable resistance.

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