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Plant Defense Genes Associated with Quantitative Resistance to Potato Late Blight in *Solanum phureja* × Dihaploid *S. tuberosum* Hybrids

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Markers corresponding to 27 plant defense genes were tested for linkage disequilibrium with quantitative resistance to late blight in a diploid potato population that had been used for mapping quantitative trait loci (QTLs) for late blight resistance. Markers were detected by using (i) hybridization probes for plant defense genes, (ii) primer pairs amplifying conserved domains of resistance (R) genes, (iii) primers for defense genes and genes encoding transcriptional regulatory factors, and (iv) primers allowing amplification of sequences flanking plant defense genes by the ligation-mediated polymerase chain reaction. Markers were initially screened by using the most resistant and susceptible individuals of the population, and those markers showing different allele frequencies between the two groups were mapped. Among the 308 segregating bands detected, 24 loci (8%) corresponding to six defense gene families were associated with resistance at $\chi^2 \geq 13$, the threshold established using the permutation test at $P = 0.05$. Loci corresponding to genes related to the phenylpropanoid pathway (phenylalanine ammonium lyase [PAL], chalcone isomerase [CHI], and chalcone synthase [CHS]), loci related to WRKY regulatory genes, and other defense genes (osmotin and a *Phytophthora infestans*-induced cytochrome P450) were significantly associated with quantitative disease resistance. A subset of markers was tested on the mapping population of 94 individuals. Ten defense-related markers were clustered at a QTL on chromosome III, and three defense-related markers were located at a broad QTL on chromosome XII. The association of candidate genes with QTLs is a step toward understanding the molecular basis of quantitative resistance to an important plant disease.

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While many chromosomal regions have been associated with quantitative resistance to plant disease through mapping studies based on anonymous markers, the information has had limited utility because the estimated location of a quantitative trait locus (QTL) can span several centimorgans. Similarly, studies of plant–microbe interactions (most recently involving the powerful tools of genomics) have generated a substantial body of information regarding the genes involved in plants' response to pathogen attack, but it has often been difficult to demonstrate conclusively that these pathogen-induced genes have roles in plant defense, what the roles may be, and which genes could be important targets for selection in plant-breeding programs. The use of gene-based markers in genetic studies can take advantage of progress in both genetics and genomics, while resolving some of the issues left open by both. Systematic analysis of plant defense genes in linkage disequilibrium with resistance, associated with QTLs, or both, may allow the more precise identification and localization of genes conditioning quantitative resistance and may identify suitable targets for marker-assisted selection, cloning, or both.

The increasing availability of cloned genes has made it possible to seek associations among QTLs and genes potentially contributing to trait expression. Sequences of these genes are rapidly accumulating and are available in publicly accessible gene banks. These resources make it possible to test associations among genes involved in selected biochemical pathways and QTLs involved in a trait of interest. This "candidate-gene" approach has been successfully applied to grain characters (Goldman et al. 1993) and corn earworm resistance (Byrne et al. 1996) and more recently to quantitative resistance in wheat and bean (Faris et al. 1999; Geffroy et al. 2000).

Because of the agricultural importance of potato late blight disease, caused by the oomycete *Phytophthora infestans*, several studies have been undertaken to locate QTLs for resistance to potato late blight on potato chromosomes using anonymous markers. Leonards-Schippers and colleagues (1994) identified a series of restriction fragment length polymorphism (RFLP) markers associated with quantitative late blight resistance, as measured by using a leaf-disk assay in a dihaploid population. Meyer and colleagues (1998) identified additional QTLs for late blight resistance by using a tetraploid mapping population and amplified fragment length polymorphism markers. Ghislain and colleagues (2001) mapped a series of QTLs for late blight resistance in a diploid population ("PD") derived from a cross between a resistant *Solanum phureja* accession and a dihaploid *S. tuberosum* clone.

The current study was undertaken with two major objectives: to evaluate the utility of known plant defense genes as

candidates to explain quantitative resistance to late blight in the PD population and to assess the efficiency of different molecular techniques for the assay of plant defense genes in a segregating plant population. Several specific hypotheses were considered in relation to the first objective: that markers derived from resistance (R) genes, R-gene analogs, or both, explain a substantial proportion of the variance in quantitative resistance in the selected population; that genes involved in transcription regulation, signal transduction, or both, and/or genes involved in the general defense response contribute significantly to the variance for resistance; and that QTL peaks could be explained by the location of plant defense genes.

The technical aims of the study were related to the detection of allelic variation, particularly for gene families. Because many plant defense genes are members of gene families in potato, available hybridization-based or polymerase chain reaction (PCR)-based techniques allow only very limited resolution of allelic variation. For instance, the phenylalanine ammonium lyase (PAL) gene has been estimated to have 50 copies per haploid genome of potato (Joos and Hahlbrock 1992). For gene families such as PAL, DNA blot hybridization often produces complex banding patterns that are difficult to score. PCR-based markers obtained through amplification and digestion (cleaved amplified polymorphic sequences [CAPS]) (Konieczny and Ausubel 1993) frequently do not allow resolution of different members of a gene family, particularly for highly conserved families like PAL. Because they are based on dot hybridization, cDNA microarray systems are not appropriate for differentiation of gene family members (Schenk et al. 2000). We tested the hypothesis that the ligation-mediated PCR method (LM-PCR) (Hornstra and Yang 1993) could be used to amplify sequences flanking loci of defense genes in order to allow efficient resolution of even large, highly conserved families. We also used this method to amplify members of gene families that share only small conserved domains.

RESULTS

In this study, trait-marker associations were sought for quantitative resistance to late blight by using genes involved in plant defense. The loci tested represented 27 genes, gene ele-

Table 1. Defense-related genes and sequences analyzed for association with resistance, grouped by pathway or category

Gene	Gene function	Source of marker used
Resistance genes (pathogen recognition)		
Pto	Tomato serine threonine kinase protein (*Pto*) conferring resistance to *Pseudomonas syringae* (Martin et al. 1993; Zhou et al. 1997)	Clone Ptc1 was provided by G. B. Martin, Purdue University, West Lafayette, IN, U.S.A.; conserved kinase and serine/threonine protein kinase domains were amplified by polymerase chain reaction (PCR) with primers corresponding to these domains in potato and tomato *Pto* genes (Chen et al. 1998; Leister et al. 1996; Martin et al. 1993; H. Leung, *personal communication*)
Mi-1	*Lycopersicon peruvianum* gene conferring resistance to root-knot nematodes and potato aphids (Vos et al. 1998)	Assayed using outward-directed ligation-mediated (LM)-PCR
NBS, LRR	Conserved domains of resistance genes: nucleotide binding site (NBS) and leucine-rich repeat (LRR)	PCR amplification was performed with AS1, AS3, S2, NBS-F1, NBS-R1, XLRRf, and XLRRr primers (Chen et al. 1998; Leister et al. 1996; Young et al. 1996)
Signal transduction and gene regulation		
Mitogen-activated protein kinase (*MAP kinase*)	Encodes mitogen-activated protein; involved in transduction of stress-related signals (Ligterink et al. 1997; Soe et al. 1995)	Primers were designed to amplified a *MAP kinase* domain and the PCR product was also used as a probe for hybridization analysis (restriction fragment length polymorphism [RFLP])
Pti4, *Pti5*, *Pti6*	Encode proteins that interact with *Pto* (Martin et al. 1993; Zhou et al. 1997)	Clones *Pti4*, *Pti5*, and *Pti6* were provided by G. B. Martin, Purdue University
WRKY1, *WRKY2*, *WRKY4*	Transcription factor family controlling the expression of different pathogenesis-related (PR) protein genes and others (Chen and Chen 2000; Eulgem et al. 2000)	Five primers were designed based on tobacco sequences for inward-directed LM-PCR
EREBP	Encodes ethylene-response element-binding protein (EREBP); transcription factor; controls the expression of *PR* genes ethylene-inducible expression (Ohme-Takagi and Shinshi 1995)	Primers were designed for inward-directed LM-PCR based on publicly available tobacco sequence
Pathogenesis-related genes		
1,3-β-glucanase gene (*glu*)	Highly expressed in potato after treatment with elicitors; the protein has been shown to restrict fungal growth in vitro (Beerhues and Kombrink 1994; Schröder et al. 1992)	Probe G46 was provided by E. Kombrink, Max-Planck-Institut für Züchtungsforschung (MPI), Köln, Germany; primers were designed for cleaved amplified polymorphic sequences (CAPS) based on publicly available sequence information
Osmotin (*osm*)	The overexpression of the gene in transgenic plants delays the development of late blight symptoms (Liu et al. 1994; Woloshuk et al. 1991; Zhu et al. 1995)	Probe pA13, cloned from *Solanum commersonii*, was provided by T. Chen, University of Oregon, Eugene, OR, U.S.A., to be used for RFLP; primers designed for CAPS and for outward-directed LM-PCR were based on sequences from *S. commersonii*
Thaumatin gene (*Tha1*)	Colocalized with quantitative trait loci (QTLs) for resistance to leaf rust in wheat (Faris et al. 1999)	Primers were designed based on publicly available sequence information for direct PCR
STH-2, *STH-21* (*PR-10*)	Strong expression was observed after infection with *Phytophthora infestans* (Constable and Brisson 1995; Matton et al. 1993)	Primers were designed based on publicly available sequence information from *S. tuberosum* for amplification of the intron region for CAPS

(*continued on next page*)

ments, or gene families, many of which are known or suspected to play a role in plant defense (Table 1). Fifteen genes involved in general metabolism were also tested (data not shown). Loci were assayed using four methods: DNA hybridization (RFLP), simple PCR, CAPS, and LM-PCR. Primers used for amplification of resistance-associated markers are listed in Table 2. Defense-gene markers were screened for possible QTL association by testing on the phenotypic extremes of PD, a diploid potato population previously used for QTL mapping. The two groups of plants differed significantly in disease reaction across three field evaluations (Table 3). A total of 308 candidate-gene loci was scored.

Using the χ^2 test for a 2 × 2 contingency table with one degree of freedom at $P < 0.01$ (significance declared at $\chi^2 \geq 6.635$), 42 loci (13.6% of those scored) corresponding to 17 defense genes were identified as significantly associated with resistance. However, when a permutation test was conducted to determine empirically the appropriate threshold for declaration of significance with the available dataset at $P = 0.05$, a threshold of $\chi^2 = 13.0$ was established. Using the limit of $\chi^2 = 13.0$, the hypothesis of independence of marker and resistance classes was rejected for 24 loci (7.8% of those scored) corresponding to six defense genes (Table 4). Different genes, groups of genes, and methods were compared for the frequency of resistance-associated bands (Table 5).

Four categories of plant defense genes were implicated as potentially contributing to quantitative late blight resistance in this population: genes of the phenylpropanoid pathway, a pathogenesis-related gene, cytochrome P450 genes, and transcriptional regulators known to play a role in the defense response. The results suggested that key genes of the phenylpropanoid pathway might contribute significantly to quantitative resistance in this population. Of the 77 bands corresponding to the phenylpropanoid pathway (PAL, 4-coumarate CoA ligase [4CL], chalcone isomerase [CHI], and chalcone synthase [CHS]), 18 (23%) were in linkage disequilibrium with resistance (Fig. 1; Tables 4 and 5). For PAL, bands representing 14 distinct loci were in significant linkage disequilibrium with resistance (Table 4). Resistance-associated bands were also detected for CHI and CHS but not for 4CL.

Table 1. (*continued from preceding page*)

Gene	Gene function	Source of marker used
Phenylpropanoid pathway (phytoalexin synthesis and lignin synthesis)		
PAL, *4CL*, *CHS*, and *CHI* genes	Phenylalanine ammonium lyase (PAL), 4-coumarate CoA ligase (4CL), chalcone isomerase (CHI), and chalcone synthase (CHS); PAL and 4CL are induced by infection with *P. infestans* (Cuypers et al. 1988; Fritzemeier et al. 1987); coordinated regulation of key genes in the phenylpropanoid pathway has been demonstrated in parsley and bean (Hahlbrock and Scheel 1989; Logemann et al. 1995; Yamada et al. 1996)	Clone pal23 (PAL) and clone pcKA5 (4CL) were provided by I. E. Somssisch, MPI, Köln, Germany; ZmCHI clone from maize was received from E. Grotewold, Ohio State University, Columbus, OH, U.S.A. (via J. Chittoor, Kansas State University, Manhattan, KS, U.S.A.); these clones were used as probes for RFLP analysis; primers for *PAL*, *CHS*, and *CHI* genes were designed for outward-directed LM-PCR
Lipid metabolism		
Phospholipase D (*PLD*) gene	Members of a superfamily encoding enzymes suspected to play in plant signaling and defense responses, particularly in hypersensitive response (Young et al. 1996)	Clones PLD6 and PLD296 were provided by D. McGee and J. E. Leach, Kansas State University, Manhattan, KS, U.S.A.
Lipoxygenase genes (*Lox1*, *Lox2*)	ROS^- (reactive oxygen species) production; play a role in signal transduction and are toxic to microbes (Rancé et al. 1998; Rosahl 1996; Royo et al. 1999)	Primers were designed based on publicly available sequence information from *S. tuberosum* to amplify the intron
Vetispiradiene synthase gene (also known as 5-epi-aristolochene synthase; *Asy1*)	Induced by *P. infestans* infection in potato (Yoshioka et al. 1999)	Primers were designed for outward-directed LM-PCR based on potato gene sequence
Others		
HMGR gene	3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase involved in phytoalexin synthesis	Primers were designed for outward-directed LM-PCR based on publicly available potato sequence
Anionic peroxidase gene	ROS^- production; involved in the accumulation of suberin and lignin in cell wall (Roberts and Kolattukudy 1989; Zeng and Hammerschmidt 1992); the gene was colocalized with QTLs for resistance to leaf rust (Faris et al. 1999)	Primers were designed for the basic peroxidase subunit (POX-UE) based on expressed sequence tag (EST) for direct PCR; primers for CAPS were also designed to amplify the intron based on tomato *TAP1* and *TAP2* genes
Metallothionein (*MTIA*)	Induced by *P. infestans* during hypersensitive response in late blight-resistant potato (Birch et al. 1999)	Primers designed from EST with homology to metallothionein isoform IA
Catalase (*Cat*)	Induced after root infection in potato by various pathogens; colocalized with QTLs for resistance to leaf rust (Faris et al. 1999)	Primers were designed for CAPS based on publicly available sequence information
Cytochrome P450	Cytochrome P450 proteins catalyze a range of oxidative reactions in both prokaryotic and eukaryotic cells; involved in the biosynthesis of alkaloids, fatty acids, flavonoids, and other compounds (Hutvágner et al. 1997); induced by *P. infestans* and wounding; member of the hydroxylase subfamily CYP71D (Schneider 2001)	Primers were designed for outward-directed LM-PCR using sequences of *S. chacoense* and *Nicotiana tabacum*
Solanidine UDP-glucose glucosyltransferase gene	Biosynthesis of γ-chaconil; wound-induced (Moehs et al. 1997)	Clone pSGT was provided by W. Belknap, U.S. Department of Agriculture, Albany, CA, U.S.A.
Glutathione S-transferase (*Gst1* = *Prp1*)	Detoxification, expression increased after infection with *P. infestans* (Hahn and Strittmatter 1994)	Primers based on the potato glutathione S-transferase gene (*gst1* = *prp1*) of potato (Taylor et al. 1990)

Positive evidence was obtained for one of the four pathogenesis-related genes tested (osmotin) (Tables 1 and 4). No evidence was obtained to support the hypothesis that R genes explained a substantial proportion of the quantitative resistance in this population. Among the 76 segregating markers tested, corresponding to two R genes and two conserved domains (Table 1), no bands were significantly associated with resistance.

Strong evidence was obtained to suggest that the WRKY family of transcription factors might play a role in quantitative late blight resistance (Fig. 2; Table 4). Bands amplified by using WRKY-derived primers showed the strongest "allele effects." Across the three phenotypic datasets, the groups of plants with and without these WRKY markers were significantly different in disease level (the average P value for the z tests was 0.0006). The WRKY bands were associated with reductions in disease of up to 29% of the area under the disease progress curve (Table 6). One of the WRKY bands with a high allele effect cosegregated with bands corresponding to PAL and CHI. A *P. infestans*-induced cytochrome P450 was also associated with resistance.

Map locations were estimated for many of the markers identified in this study. When map locations had been previously established, our findings corroborated the published results. For instance, PAL loci were known to occur on chromosome III (C. Gebhardt, *personal communication*). A band hybridizing to the PAL probe was mapped to chromosome X, as previously found by Gebhardt and colleagues (1991). To our knowledge, the chromosomal locations of WRKY-related loci have not previously been reported.

Of the 24 resistance-associated markers identified, 18 were located on the PD QTL map. Fifteen of these were colocalized with two of the five QTL peaks identified by Ghislain and colleagues (2001) for the *S. tuberosum* parent. A QTL on chromosome III (designated Q-D-III) harbored 10 loci defined by candidate-gene markers corresponding to WRKY, PAL, CHI, CHS, and cytochrome P450. These loci were located within a chromosomal segment spanning 8 cM defined by three anonymous markers and explaining up to 13% of the phenotypic variance in the PD population (Ghislain et al. 2001) (Fig. 3). Most of the candidate-gene loci were defined by a single band and were separated from their closest neighbor in the cluster by a single recombinant. One locus, however, consisted of cosegregating CHI, PAL, and WRKY bands. This locus was associated with the strongest effect on disease ($z = 4$; $P = 0.00005$) (Fig. 4), which was more significant than the effects associated with the anonymous markers in the cluster ($0.01 < P < 0.0005$). Three resistance-associated bands were mapped but did not colocalize with a QTL identified by Ghislain and colleagues (2001).

Table 2. Oligonucleotides (primers and linkers) used for amplifying resistance-associated markers by the LM-PCR[a]

Name	Sequence (5′ to 3′)[b]	Source and details[c]
PAL2	5′-ATTTTGTGCAATTGATGGTGC-3′	1,082 to 1,062 base pairs (bp) of the 5′ flanking conserved coding region of the potato phenylalanine ammonium lyase (*PAL*) gene (accession no. X63104); reverse primer used with Bamlinker2 as a forward linker primer
PAL6	5′-AATGTAGCAACAATTCTGGAAG-3′	3,605 to 3,626 bp of the 3′ flanking conserved region of the potato *PAL-1* gene (accession no. X63103); forward primer used with Bamlinker2 as a reverse linker primer
CHI-f	5′-AGGTCCMTTTGAGAAATT-3′	1,688 to 1,705 bp of the 3′ flanking conserved coding region of the petunia chalcone isomerase genes *CHI-A* and *CHI-B* (accession nos. X14589 and X14590, respectively); forward primer used with Bamlinker2 as a reverse linker primer
CHS-f	5′-TTTTGGATTTGGGCCTGGG-3′	1,176 to 1,194 bp of the 3′ flanking conserved region of the *Solanum tuberosum* chalcone synthase 1a gene (*Chs1a*; accession no. U47739); also homologous with the potato *Chs1b* gene, the potato *Chs2* gene, the tomato *Chs1* and *Chs2* genes, and the petunia *Chs-A* gene; forward primer used with E00 as a reverse linker primer
WRKY1-R	5′-CCTAGGATTTGGATTGCC-3′	1,095 to 1,078 bp at the conserved amino acid sequence GNPNPR at the 3′ end of the tobacco *NtWRKY1* gene (accession no. AB022693); reverse primer used with EtLM-F as a forward linker primer
WRKY2-R	5′-GCYCKTTCCACATGTTTYC-3′	1,181 to 1,163 bp at the conserved amino acid sequence RKHVERA at the 3′ end of the tobacco *NtWRKY1/2/3/4* gene (accession no. AB020590); reverse primer used with EtLM-F as a forward linker primer
WRKY4-F	5′-GTCCRGTCAAGAARAAGG-3′	1,029 to 1,046 bp at 5′ end of the tobacco *NtWRKY4* gene sequence (accession no. AB026890), with homology to the parsley *WRKY1* gene; forward primer used with MiKpn-r as a reverse linker primer
Osm 1-2	5′-AGGACCACATGGACCTTGAG-3′	570 to 551 bp of the *S. commersonii* osmotin-like protein gene (accession no. X67244); reverse primer used with E00 linker primer for LM-PCR assay
P450f	5′-CTTCATCAACAACAACTG-3′	925 to 942 bp of the 3′ flanking conserved coding region of the *S. chacoense* P450 genes (accession no. U48434); forward primer used with Bamlinker2 as a reverse linker primer
LinkBamH.1 LinkerBamHI2 Link-BamH1 LinK-BamH2 Bamlinker2 EtLM-F	5′-ATGCTCGACTGCGTACC-3′ 5′-GGATCGGTACGCAGTC-3′ 5′-CTCGTACTCAGGTCAGGTCGG-3′ 5′-GGATCCCGACCTGACCTGAGT-3′ 5′-GACTGCGTACCGATCC-3′ 5′-TCAGGTCAGGTCGGGATC-3′	For inward- and outward-directed LM-PCR, oligonucleotides LinkBamH.1 and LinkerBamHI2 are complementary and used together as a *Bam*HI linker in conjunction with gene-specific primers having $T_m < 52°C$ (with T_m = melting temperature), this linker was used with the linker primer Bamlinker2; Link-BamH1 and LinK-BamH2 form a *Bam*HI linker used at 53°C – 58°C and using EtLM-F oligonucleotide as a linker primer
EcoA1 EcoA2 E00	5′-CTCGTAGACTGCGTACC-3′ 5′-GAATTGGTACGCAGTC-3′ 5′-GACTGCGTACCAATTC-3′	Oligonucleotides EcoA1 and EcoA2 are complementary and used together as an *Eco*RI linker; used with E00 linker primer
Link-kpn1.1 LinKpn1.2 MiKpn-r	5′-GGATGCGTCAG-3′ 5′-ATGCTCCTGACGCATCCGTAC-3′ 5′-CTGACGCATCCGTAC-3′	Oligonucleotides Link-kpn1.1 and LinKpn1.2 are complementary and used together as a *Kpn*I linker; used with MiKpn-r linker primer

[a] Oligonucleotides were used to generate the resistance-associated gene markers using ligation-mediated polymerase chain reaction (LM-PCR).
[b] Y = C/T; M = A/C; K = G/T; and R = A/G.
[c] All accession numbers are from the EMBL/GenBank database.

Another group of markers was associated with a broad QTL peak on chromosome XII (Q-D-XII) that explained up to 43% of the variance for late blight resistance in field tests (Ghislain et al. 2001). Three markers, corresponding to PAL, CHI, and osmotin, formed a tight cluster located at or near the QTL peak (Fig. 3). Four additional markers showing significant associations with resistance were also located on chromosome XII of the *S. tuberosum* parent, corresponding to WRKY, PAL, cytochrome P450, and CHI.

Not all genes were assayed using all methods, so rigorous comparison of the efficiencies of different methods was not attempted. However, inspection of the available data suggests that simple PCR, CAPS, RFLP, and LM-PCR were not equally efficient, since the frequencies of "hits" per assay and hits per band were quite different (Table 5). Using direct PCR and CAPS, a total of 127 segregating bands was detected. None of these were found to be significantly associated with resistance. Using RFLP, 27% of assays yielded significant associations, and 9% of segregating bands were found to be associated with resistance. Using LM-PCR, 65% of assays resulted in the detection of significant associations, and 18% of segregating bands were associated with resistance.

The different methods used were complementary in terms of detecting loci associated with resistance. For PAL, 26 segregating loci were identified using RFLP, nine of which were significantly associated with resistance. Using LM-PCR, an additional 20 segregating loci were identified for PAL, five of these were associated with resistance. Only one locus was identified both by RFLP and LM-PCR, although groups of linked markers were defined by the two techniques (data not shown). For CHI, nine segregating loci were found using RFLP, none of which were associated with resistance. Using LM-PCR, 13 segregating loci were identified, three of which were associated with resistance. For osmotin, the single locus associated with resistance was identified using LM-PCR, although CAPS and RFLP assays were also conducted for this gene family.

DISCUSSION

The use of plant defense genes as markers allowed identification of candidate genes potentially explaining two QTLs for resistance to potato late blight. By systematically screening the phenotypic extremes of the segregating PD population, we identified several markers differing in allele frequency between the resistant and susceptible groups. Several of the markers associated with late blight resistance in this study were mapped in clusters that colocalized with QTL peaks. Groups of candidate-gene markers were tightly associated with resistance and potentially explained the QTLs on chromosomes III and XII. The clustering of defense-response genes has been noted in other plant genomes, such as bean, rice, and wheat (Faris et al. 1999; Li et al. 1999; Wang et al. 2001).

Not all the QTLs identified in the study of Ghislain and colleagues (2001) were associated with candidate-gene markers in this study. Thus, it appears that the set of genes tested in this study represented a subset of the genes contributing to quantitative resistance. This is to be expected, since we have tested only a subset of the genes known to be induced in response to infection of potato with *P. infestans* (Birch et al. 1999). We further recognize that the techniques utilized did not enable us to visualize all alleles of the gene families assayed.

The defense genes assayed in this study can be considered to act at three different levels of a regulatory hierarchy. R genes are considered to play a role in specific pathogen recognition. Transcriptional regulators are induced either by R-gene products or by other stimuli and control the expression of the general defense-response genes. General defense-response genes affect pathogen fitness, either directly or indirectly. Resistance-associated markers were obtained for transcriptional regulatory genes and general defense genes in this study.

It has been suggested that R genes or R-gene analogs are likely to account for a significant proportion of quantitative variation for disease resistance (Michelmore 1995). This hypothesis is supported by the observations that QTLs have been mapped to the same chromosomal regions as major genes (Leister et al. 1996; Shen et al. 1998) and that defeated R genes have residual effects (Ordoñez et al. 1998). Using conserved R-gene sequences in sunflower, Gentzbittel and colleagues (1998) found markers associated both with monogenic resistance to downy mildew and with quantitative resistance to *Sclerotinia sclerotiorum*. Our results, however, do not support the hypothesis that quantitative resistance to late blight can be explained largely by known R-gene analogs. None of the 76 segregating bands amplified using R-gene-derived primer sequences were found to be significantly associated with resistance.

In contrast, support was obtained for the hypothesis that regulatory genes can explain QTLs for disease resistance. Three markers corresponding to the WRKY family of transcription factors were associated with strong contributions to resistance in the PD population. The WRKY proteins comprise a family of plant-specific zinc-finger-type factors implicated in the regulation of genes associated with pathogen defense. These proteins bind specifically to W-box promoter elements within the pathogenesis-related genes (Eulgem et al. 2000; Hara et al. 2000; Maleck et al. 2000).

Evidence was also obtained to support the hypothesis that the genes of the general defense response are associated with disease resistance. For three of the four genes encoding key enzymes of the phenylpropanoid pathway (PAL, CHS, and CHI), bands strongly associated with resistance were detected. The phenylpropanoid pathway is central in the plant defense response, and PAL has been colocalized with QTLs for resistance to other diseases (Faris et al. 1999; Geffroy et al. 2000; Wang et al. 2001). One cluster of phenylpropanoid pathway genes was found on chromosome III at a chromosome segment that has been associated with quantitative late blight resistance in other studies (Collins et al. 1999; Ewing et al. 2000; Oberhagemann et al. 1999). Osmotin, a pathogenesis-related gene (PR-5), was associated with significant effects on disease. The osmotin band was mapped to a QTL on chromosome XII,

Table 3. Phenotypic values for four field experiments for the 34 most resistant individuals (R) and the 33 most susceptible individuals (S) among the 246 diploid hybrids of the *Solanum phureja* × *S. tuberosum* population "PD"[a]

Field experiments[b]	R[c]	S[c]	Phenotypic difference[d]	*z* value (*P*), AUDPC
Huancayo, Peru; 1997	1,323	2,787	1,464 (53%)	–21.61 (>0.00)
Quito, Ecuador; 1997	483	1,682	1,199 (71%)	–32.98 (>0.00)
Comas, Peru; 1999	69	89	20 (25%)	–14.4 (>0.00)

[a] The *z* test was used to compare the two groups.
[b] Sites at which field experiments were conducted; year of experiment.
[c] Area under the disease progress curve (AUDPC) for the resistant and susceptible groups of plants.
[d] Difference between average AUDPCs of the resistant and susceptible groups (% = difference/susceptible value).

where genes of the phenylpropanoid pathway had larger allele effects than the osmotin marker.

One technical aim of our study was to demonstrate a general method for QTL discovery in segregating populations that could be applied to populations developed for mapping, germ plasm characterization, and breeding programs. Our results indicate that screening the phenotypic extremes of a segregating population with candidate genes for a quantitative trait can be an efficient approach to QTL discovery. Markers differing significantly in frequency between the two extreme subpopulations can be inferred to be linked to a QTL controlling the character (Lander and Botstein 1989; Lebowitz et al. 1987; Tanksley 1993). The results obtained with this method are encouraging and set the stage for a broader application of the approach. We are now embedding this strategy into an ongoing breeding program at the tetraploid level and into an effort to characterize germ plasm, with positive results (Manosalva et al. 2001). As in the current study, alleles showing linkage disequilibrium in the phenotypic extremes can be subsequently located on the potato chromosomes by using available mapping populations.

Our second technical aim was to develop a simple and reliable method for analyzing alleles of genes belonging to complex gene families. Many genes affecting key traits belong to families. Differential expression patterns have been found among members of defense-related gene families such as PAL and peroxidase (Chittoor et al. 1997; Sarma et al. 1998; Shufflebottom et al. 1993). This suggests that the different family members have different efficiencies, functions, or both, and thus merit differentiation and analysis on an individual basis. In an effort to overcome the limitations encountered using the RFLP and CAPS techniques to analyze multigene families such as PAL and WRKY, we adapted and applied the LM-PCR

Table 4. Defense-related gene markers found to be associated with resistance to late blight in the diploid potato population ("PD")[a]

Target gene or marker	Marker type[b]	Locus[c]	Band derived from P or D[d]	Number of observed (tested)		χ^2	*P*
				R[e]	S[e]		
WRKY-1	LM-PCR	WRKY1r-*B*-0.33, -0.51	D	25 (34)	4 (33)	23.3	<0.00001
WRKY-2	LM-PCR	WRKY2r-*B*-0.9	D	25 (34)	5 (33)	20.8	<0.00001
WRKY-4	LM-PCR	WRKY4-f-*K*-0.9	D	5 (34)	23 (33)	18.6	0.000016
Osmotin (PR-5)	LM-PCR	Osm1-2-*E*I-0.29	D	26 (30)	5 (26)	23.0	<0.00001
Phenylalanine ammonium lyase (PAL)	RFLP	PAL-*E*I-2.8	D	25 (34)	3 (31)	24.4	<0.00001
	RFLP	PAL-*E*I-3	P	31 (34)	10 (33)	23.6	<0.00001
	RFLP	PAL-*Hin*-2.8, -2.7	D	9 (34)	22 (27)	16.1	0.00006
	RFLP	PAL-*N*-3.6	D	26 (34)	8 (33)	16.3	0.00006
	RFLP	PAL-*N*-4	D	9 (34)	26 (33)	16.3	0.00005
	RFLP	PAL-*N*-4.4	D	9 (34)	27 (33)	18.5	0.00002
	RFLP	PAL-*N*-5.9	P	22 (34)	6 (33)	13.1	0.0003
	RFLP	PAL-*N*-6	D	26 (34)	1 (33)	34.6	<0.00001
	RFLP	PAL-*N*-6.8, -6.9	D	26 (34)	5 (33)	22.9	<0.00001
	LM-PCR	PAL2-*B*-0.075	D	9 (34)	26 (32)	17.7	0.00003
	LM-PCR	PAL2-*B*-0.41	D	27 (34)	6 (32)	21.9	<0.00001
	LM-PCR	PAL6-*B*-0.420	D	23 (30)	4 (27)	19.4	0.00001
	LM-PCR	PAL6-*B*-0.5	D	9 (30)	22 (27)	13.2	0.00028
	LM-PCR	PAL6-*B*-0.5a	D	4 (30)	24 (29)	25.8	<0.00001
Chalcone isomerase (CHI)	LM-PCR	CHI-f-*B*-0.320, -0.315	D	27 (34)	5 (32)	24.4	<0.00001
	LM-PCR	CHI-f-*B*-0.53	D	8 (34)	24 (32)	15.5	0.00008
	LM-PCR	CHI-f-*B*-0.75a	D	4 (24)	22 (27)	18.8	0.00001
Chalcone synthase (CHS)	LM-PCR	CHS-f-*E*I-0.159	D	28 (33)	5 (31)	27.5	<0.00001
Cytochrome P450 (CYP71D)	LM-PCR	P450f-*B*-0.18	D	26 (34)	5 (32)	22.1	<0.00001
	LM-PCR	P450f-*B*-0.298	D	8 (34)	24 (32)	15.5	0.00008

[a] Loci in linkage disequilibrium with quantitative resistance. Cosegregating bands obtained with the same probe or primer combination were considered as a single locus. Cosegregating bands obtained with different probes or primer combinations were considered as two independent loci.
[b] LM-PCR = ligation-mediated polymerase chain reaction, RFLP = restriction fragment length polymorphism.
[c] Loci defined for RFLP markers, loci are indicated by gene code - restriction enzyme - size in kilobases; for LM-PCR markers, loci are indicated by primer code - restriction enzyme - size in kilobases. *E*I = *Eco*RI; *B* = *Bam*HI; *K* = *Kpn*I; *Hin* = *Hin*dIII; and *N*= *Nde*I.
[d] P = *Solanum phureja*; D = *S. tuberosum* dihaploid.
[e] R = resistant; S = susceptible.

Table 5. Summary of results obtained for the diploid potato population "PD" using different gene classes, genes or gene families, and analytical methods

Class; gene or gene family; or method	No. assays	No. segregating loci	No. resistance-associated loci ("hits")	Hits per assay (%)	Hits per segregating locus (%)
Overall	106	308	24	23	8
Gene class					
Resistance (R)-gene analogs	13	76	0	0	0
Signal transduction and gene regulation	22	41	3	14	7
Phenylpropanoid pathway	13	77	18	139	23
Pathogenesis-related (PR) genes	16	37	1	6	3
Lipid metabolism	12	13	0	0	0
Other genes	30	64	2	7	3
Method[a]					
PCR (direct)	30	106	0	0	0
CAPS	19	21	0	0	0
RFLP	34	99	9	27	9
LM-PCR	23	82	15	65	18

[a] CAPS = cleaved amplified polymorphic sequences, LM-PCR = ligation-mediated polymerase chain reaction, PCR = polymerase chain reaction, and RFLP = restriction fragment length polymorphism.

technique. This technique, which had been used for DNA fingerprinting of pathogen strains, footprinting assays, sequencing, and cloning (Hornstra and Yang 1993; Palittapongarnpim et al. 1993), was found to be efficient for detecting polymorphism for complex gene families. While many loci were identified using LM-PCR, not all loci identified using other methods were also detected using LM-PCR. It seems that a range of techniques should be applied and that the risk remains that not all alleles will be effectively sampled.

In this study, plant defense genes were found in clusters corresponding to previously identified QTLs in the PD population. Several members of multigene families were found together in some cases; for instance, six PAL loci and two WRKY loci were found at one QTL on chromosome III. This is consistent with previous reports of clustering of gene family members (Faris et al. 1999; Geffroy et al. 2000; Leonards-Schippers et al. 1994; Wang et al. 2001). The clustering of defense genes at QTLs confounds inferences regarding the molecular basis of quantitative resistance. It is unclear whether the association with resistance is due to effects of groups of genes, to a single member of the group, or to linked gene or genes that have yet to be identified. Even without unraveling the cause-and-effect relationships of the markers and phenotype, the information can be useful for marker-assisted selection. The gene-based markers fell in QTL peaks that were identified by interpolation between anonymous markers and thus should be useful as markers for the regions of interest. Mapping of QTL-related candidate genes could allow selection of potentially complementary chromosome segments from different sources, to be combined with the aid of markers.

Functional assays will be required to demonstrate a causal relationship between specific genes and resistance. Functional tests will include mutation analysis, transient expression assays, and transformation. If a reduction in disease can be attributed to a particular allele, direct gene transfer could become an efficient way to utilize the natural variation present in germ plasm collections and breeding programs.

MATERIALS AND METHODS

Plant material.

The diploid population "PD" consists of 246 diploid ($2n = 2x = 24$) progenies produced from a cross between the native cultivated potato *S. phureja* (INIAP accession no. CHS625 [Instituto Nacional Autónomo de Investigaciones Agropecuarias {INIAP}, Santa Catalina, Quito, Ecuador], with quantitative resistant to late blight) and the *S. tuberosum* dihaploid PS-3 (susceptible). This population was evaluated in three field experiments conducted in Peru and Ecuador in 1997 to 1999. The population showed a near-normal distribution for late blight resistance and the phenotypic datasets were highly correlated (Trognitz et al. 2001). We used available phenotypic data to select the most resistant ($n = 34$) and the most susceptible ($n = 33$) genotypes. Each tail represented approximately 14% of the

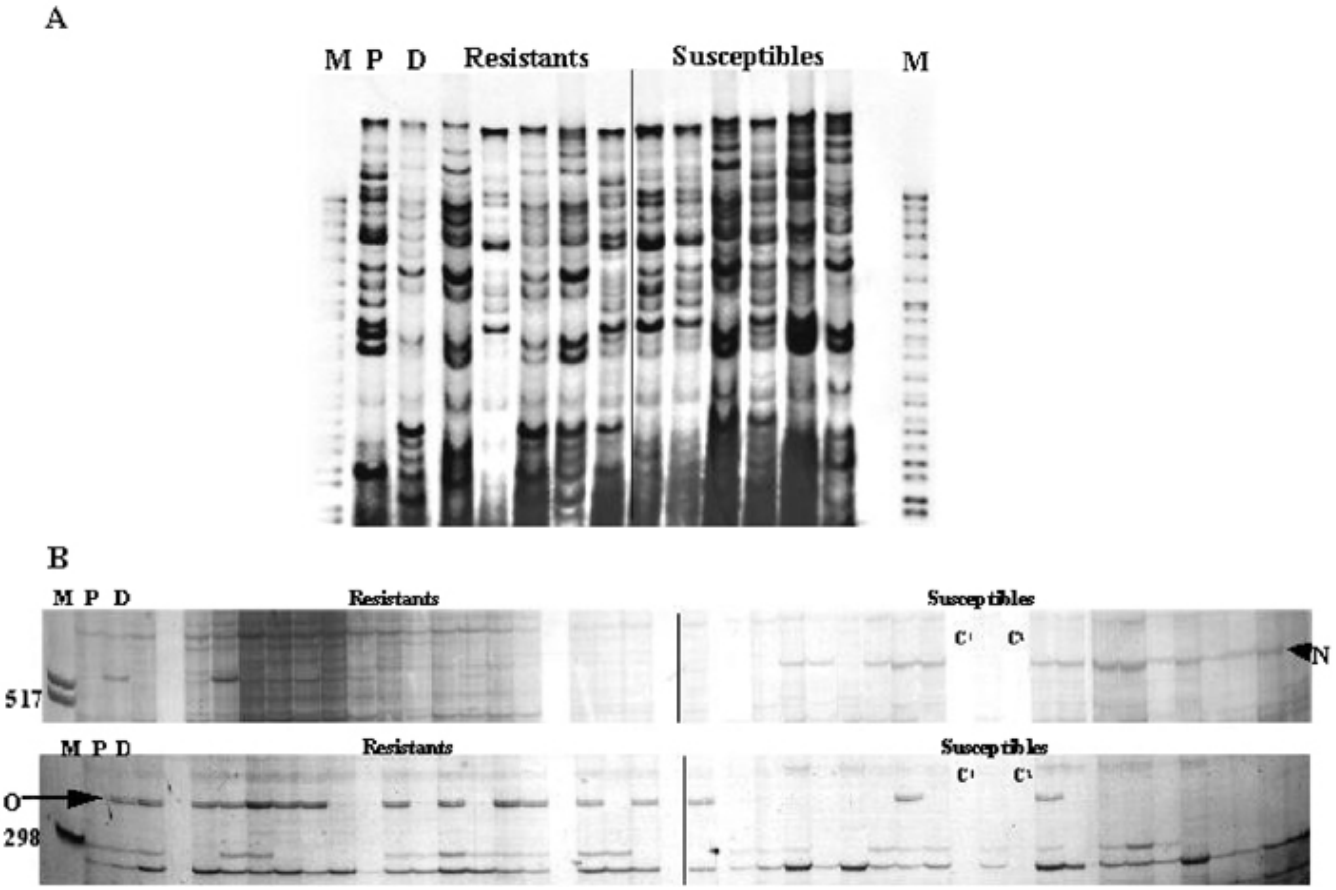


Fig. 1. Screening of loci corresponding to the chalcone isomerase gene using the phenotypic extremes of the diploid potato population "PD". Lane 1, Molecular weight marker (M); lane 2, *Solanum phureja* parent (P); and lane 3, *S. tuberosum* parent (D). **A,** Complex hybridization patterns for DNA extracts of genotypes of the PD population digested with *Eco*RI and hybridized with the probe ZmCHI. No chalcone isomerase (CHI) bands associated with resistance were detected using this approach. **B,** Ligation-mediated polymerase chain reaction (LM-PCR) with primers CHI-f and Bamlinker2. C_1, water control; and C_2, linker control (PCR mix only with Bamlinker2 primer and DNA template). Two bands nonrandomly associated with disease phenotype were detected using this technique. The band marked "N" corresponds to CHI-f-*B*-0.53 and "O" corresponds to the cosegregating doublet bands CHI-f-*B*-0.32 and CHI-f-*B*-0315.

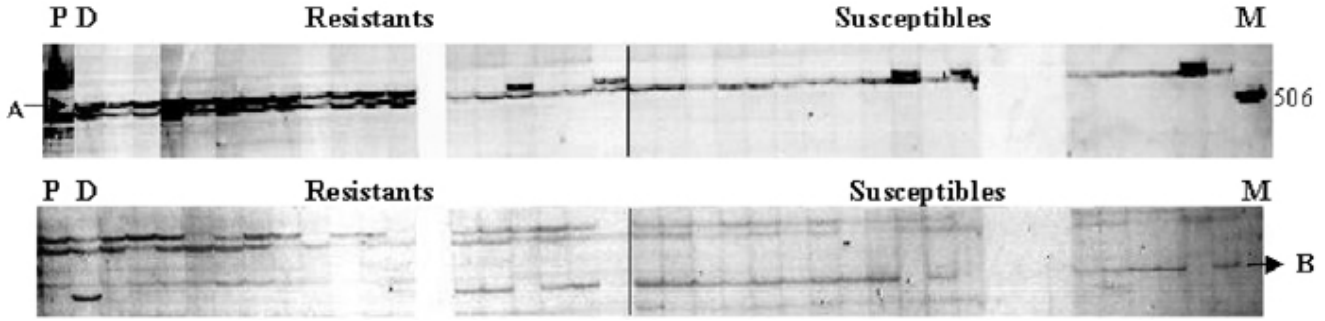


Fig. 2. Polyacrylamide gel showing the segregation of the allelic ligation-mediated polymerase chain reaction (LM-PCR) markers WRKY1r-*B*-0.33 and WRKY1r-*B*-0.51 in the phenotypic extremes of the diploid potato population "PD". **A,** The 0.51-kilobase (kb) band that is more frequent in resistant individuals. **B,** The 0.33-kb band that is present more frequently in the susceptible individuals. Both are present on *Solanum tuberosum* (D) and absent in *S. phureja* (P).

Table 6. Quantitative trail loci (QTLs), associated candidate genes mapped and allele effects for the diploid potato mapping population "PD"[a]

QTL-parent-chromosome[b] (R^2)	Locus[c]	Huancayo[d]			Quito[d]			Comas[d]		
		z	*P*	AE (%)	*z*	*P*	AE (%)	*z*	*P*	AE (%)
Q-D-III (13 %)	WRKY1r-*B*-0.33, -0.51	4	0.00006	429 (20)	4	0.00005	332 (29)	3.4	0.0006	6 (7)
Q-D-III	PAL6-*B*-0.5	–4	0.00006	429 (20)	–4	0.00005	332 (29)	–3.4	0.0006	6 (7)
Q-D-III	CHI-f-*B*-0.320, -0.315	4	0.00006	429 (20)	4	0.00005	332 (29)	3.4	0.0006	6 (7)
Q-D-III	WRKY2r-*B*-0.9	4	0.00008	427 (20)	4.1	0.00005	331 (29)	3.2	0.001	5 (6.6)
Q-D-III	PAL6-*B*-0.420	3.7	0.0003	398 (19)	3.5	0.0004	297 (26)	3.0	0.003	5 (6.5)
Q-D-III	P450-f-*B*-0.18	3	0.002	356 (17)	3.3	0.0009	291 (26)	2.7	0.007	5 (6)
Q-D-III	CHS-f-*E*I-0.159	2.1	0.04	245 (12)	2.3	0.02	200 (18)	2.3	0.02	4 (5)
Q-D-XII (43%)	PAL6-*B*-0.5a	–3.1	0.002	408 (18)	–3.7	0.0003	345 (28)	–2.8	0.006	4.9 (6)
Q-D-XII	CHI-f-*B*-0.75a	–3.1	0.002	407 (18)	–3.7	0.0002	344 (28)	–3	0.003	5 (6)
Q-D-XII	Osm 1-2-*E*I-0.29	2.8	0.005	356 (16)	3.6	0.0004	329 (28)	2.9	0.004	5 (6)
Q-D-XII	PAL2-*B*-0.075	–2.5	0.01	308 (14)	–2.5	0.01	290 (25)	–2.8	0.004	5 (6)
Q-D-XII	P450-f-*B*-0.298	–2.9	0.004	362 (17)	–2.7	0.008	249 (22)	–2.4	0.02	4 (5)
Q-D-XII	CHI-f-*B*-0.53	–1.7	n.s.	291 (13)	–2.5	0.01	298 (25)	–1.8	n.s.	4 (5)
Q-D-XII	WRKY4-f-*K*-0.9	–2.5	0.01	302 (14)	–4	0.00008	337 (29)	–4	0.00007	6 (8)
None	PAL2-*B*-0.410	1.8	n.s.	215 (10)	1.4	n.s.	128 (12)	1.7	n.s.	3 (4)

[a] Means of the different marker allele classes were compared using the *z* test at $P < 0.05$.
[b] QTLs previously detected by Ghislain et al. (2001) in the PD population. (R^2) express the phenotypic variance explained by each QTL in the PD population.
[c] Loci defined by ID code - restriction enzyme - molecular weight in kilobases. *E*I = *Eco*RI; *B* = *Bam*HI; and *K* = *Kpn*I.
[d] For each location (Huancayo, Peru; Quito, Ecuador; and Comas, Peru), the significance and magnitude of the effect of each allele on disease phenotype (area under the disease progress curve [AUDPC]) for the PD population was tested using the *z* test (*z*: the *z* statistic; *P*: the probability that the marker classes have the same phenotypic values according to the *z* test). Allele effect (AE) was calculated for each locus as the difference of the phenotypic means for the two marker classes. The effects are presented as difference in mean AUDPC of the group of individuals with and without the band of interest. The difference, expressed as the percentage of the susceptible value, is given in parentheses. The analysis was based on the 92 individuals used for the QTL mapping study (Ghislain et al. 2001). n.s. = Not significant ($P \geq 0.05$).

population of 246 plants. The two groups were significantly different in susceptibility to *P. infestans* (Table 3). A random subset of 92 individuals from this population was previously used for QTL mapping (Ghislain et al. 2001) and utilized for locating markers in the current study.

Candidate-gene classes.

Table 1 shows the genes used for this study. For PCR-based assays, gene sequences or expressed sequence tags were obtained from the MENDEL database or the National Center for Biotechnology Information (NCBI) database. Corresponding primers were designed using the program Primer 0.5 (unpublished software by S. Lincoln, M. Daly, and E. Lander; available from the Whitehead Institute/MIT, Center for Genome Research, Cambridge, MA, U.S.A.) and synthesized by GENSET (La Jolla, CA, U.S.A.). Table 2 shows the oligonucleotides used for amplification of resistance-associated markers; a complete list of oligonucleotides is available from the authors upon request.

DNA preparation and analysis by RFLP, direct PCR,and CAPS.

Plant DNA was extracted by using the procedure of Doyle and Doyle (1987), with the addition to the extraction buffer of 100 mg of polyvinylpyrrolidone (molecular weight 40,000) per gram of leaf tissue (Porebski et al. 1997). Southern hybridization was carried out according to standard protocols (Sambrook et al. 1989) by using 8 µg of digested DNA per lane. The restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I, *Nde*I, and *Dra*I (5 U/µg) were used. DNA detection was performed using the enhanced chemiluminescence protocol (Amersham, Buckinghamshire, U.K.). For several genes and markers, polymorphism was detected based on amplification with or without subsequent restriction digestion of PCR products. Most of the PCR markers were resolved by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.).

LM-PCR.

The LM-PCR method was adapted from protocols developed for DNA fingerprinting studies, genomic footprinting, and other purposes (Hornstra and Yang 1993; Palittapongarnpim et al. 1993). Although the LM-PCR technique has been used for a range of purposes, this is, to our knowledge, the first application of this method to resolve members of complex gene families. This LM-PCR method involves the generation of cohesive ends by restriction digestion of genomic DNA, the ligation of a

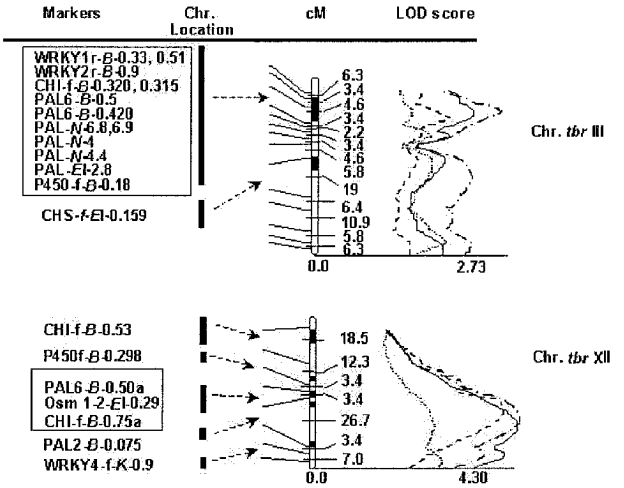
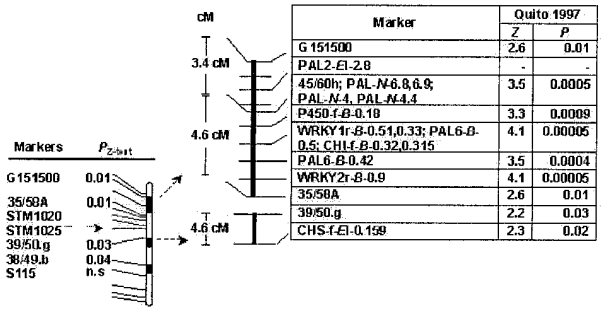


Fig. 3. Loci associated with resistance or susceptibility and colocalized with quantitative trait loci (QTLs) from *Solanum tuberosum* (tbr). Distances between anonymous markers are indicated in centimorgans (cM) (Ghislain et al. 2001). Log of the likelihood ratio (LOD) scores (Log_{10} of the odds ratio) in relation to four field data sets: – – Huancayo 1997; – · – Quito 1997; · · · Comas 1998; and — Comas 1999. The QTL on chromosome III was detected in two trials (Quito 1997 and Comas 1999) and the QTL on chromosome XII was detected in all trials except for Comas 1998.



Marker	Quito 1997	
	Z	*P*
G 151500	2.6	0.01
PAL2-El-2.8	-	-
45/60h; PAL-N-6.8,6.9; PAL-N-4, PAL-N-4.4	3.5	0.0005
P450-f-B-0.18	3.3	0.0009
WRKY1r-B-0.51,0.33; PAL6-B-0.5; CHI-f-B-0.32,0.315	4.1	0.00005
PAL6-B-0.42	3.5	0.0004
WRKY2r-B-0.9	4.1	0.00005
35/58A	2.6	0.01
39/50.g	2.2	0.03
CHS-f-El-0.159	2.3	0.02

Fig. 4. Marker loci clustering at quantitative trait loci (QTLs) on *Solanum tuberosum* dihaploid chromosome III. STM1020 and STM1025 are microsatellites markers corresponding to gene sequences (Milbourne et al. 1998). Probabilities of association with quantitative resistance to late blight were calculated using the *z* test based on data from a field trial conducted in Quito, Ecuador, in 1997.

nonphosphorylated synthetic linker to each restriction fragment, and amplification of the target sequence by using one primer complementary to the linker sequence and one primer complementary to the target gene sequence. This permits amplification of sequences flanking the gene. Theoretically, the number of fragments generated by this method should reflect the number of copies of the gene in the genome.

Specific gene primers were designed by using publicly available sequences, with preference given to potato sequences and those from other solanaceae such as tomato, petunia, and tobacco. For gene families with highly conserved sequences, such as *Mi-1*, 3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase (HMGR), and PAL, we designed primers corresponding to conserved sequences in the 5′ ends, 3′ ends, or both, of the genes for outward-directed LM-PCR. For gene families with diverse members sharing only small conserved regions (e.g., the *WRKY* and *EREBP* families of transcription regulators), primers were designed to complement the conserved regions and to amplify the introns or coding regions for inward-directed LM-PCR. To identify conserved sequences, three or more gene sequences were aligned by using the Bioedit Sequence Alignment Editor Version 4.8.4 software (T. Hall, North Caroline State University, Raleigh, NC).

The complementary oligonucleotides for a given linker were mixed in equimolar amounts (40 pM/µl) in PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% [wt/vol] gelatin), heated to 80°C, and slowly cooled to 4°C over 1 h to allow the two oligonucleotides to anneal. Depending on the DNA sequence of interest, genomic DNA was digested with *Bam*HI, *Eco*RI, or *Kpn*I (enzymes were selected based on restriction maps, to avoid cutting within the gene). Genomic DNA (1 µg) was digested for 4 h with 20 units of restriction enzyme in a 25-µl reaction. A ligation mixture (40 µl) containing 400 ng of digested DNA, 2 µl of the equimolar linker mixture (40 pM), 5× ligation buffer (GIBCO, Rockville, MD, U.S.A.), and 2 units of T4 DNA ligase (GIBCO) was incubated at 16°C overnight. After ligation, the T4 DNA ligase was inactivated by incubation at 65°C for 10 min.

Amplification was performed in a total volume of 25 µl containing 2 or 4 µl of the ligated DNA mixture, 0.5 µM linker primer, 1 µM specific primer, 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase in a 1× PCR buffer (10 mM Tris-HCl, pH 9.0; 1.25 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100; 0.01% [wt/vol] gelatin). The reaction mix was initially denatured for 10 min at 94°C and then subjected to 35 cycles of PCR (1 min at 94°C, 1 min at T_m – 5°C [with T_m = melting temperature], and 2 min at 72°C) and a final extension for 10 min at 72°C with a DNA thermal cycler (MJ Research, Watertown, MA, U.S.A.). Amplification products were resolved by PAGE and visualized by silver staining according to the manufacturer's manual (Promega).

Data analysis.

The *z* test was used to compare the resistant and susceptible groups and to assess the effect of allelic constitution at each locus ("allele effect"). Independence of marker distribution (presence or absence) and phenotypic groups (resistant and susceptible) was tested by the χ^2 test with one degree of freedom. The permutation test was performed as described by Churchill and Doerge (1994) to establish an appropriate experiment-wise threshold of significance for trait-marker associations. The marker and trait values were randomized and QTL analysis was performed using MAPMAKER/QTL (Lincoln et al. 1992). This process was repeated 1,000 times, and the resulting sets of log of the likelihood ratio (LOD) scores at 2-cM intervals were used to determine the LOD value at which (spurious) associations were detected at a frequency of 0.05. To transform from LOD scores to χ^2 values, the following equation was utilized: $\chi^2 = 2\ln_{10}\text{LOD}$. Values above this level ($\chi^2 = 13$) were considered significant. The MAPMAKER/EXP v3.0b software (Lander and Botstein 1989) was used to locate markers on the PD map, and QTL Cartographer for Windows, Version 1.01 (Basten et al. 1994), was used for QTL mapping with the expanded marker dataset.

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