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ORIGINAL PAPER

Mapping and functional analysis of four apple receptor-like protein kinases related to *LRPKm1* in *HcrVf2*-transgenic and wild-type apple plants

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Abstract The *Malus–Venturia inaequalis* interaction is the most studied plant–pathogen interaction involving a woody species. Besides the cloning of an apple scab resistance gene *HcrVf2*, several sequences have been recently identified that are modulated after pathogen recognition in *Vf*-resistant genotypes. Among these, there is a putative leucine-rich repeat receptor-like protein kinase from the apple scab-resistant cv. Florina, named *LRPKm1* that is induced after *V. inaequalis* inoculation and salicylic acid treatment. In this work, the isolation, characterization, and mapping of four new genes belonging to the *LRPKm* multigene family are reported. According to their cumulative expression profiles in *HcrVf2*-transgenic and wild-type apple plants treated with *V. inaequalis*, *LRPKm* genes have been divided in two groups. *LRPKm1* and *LRPKm3*, giving a response related to the presence of *HcrVf2*, are probably involved in the recognition of pathogen-derived signals. *LRPKm2* and *LRPKm4*, with an expression profile unrelated to the *HcrVf2* gene, are putatively involved in the plant basal defense. Furthermore, we have localized LRPKm proteins at the cytological level in the plasma membrane of epidermal cells in resistant genotypes following pathogen challenge, thus confirming software predictions and molecular results. The possible involvement of LRPKm proteins in apple scab resistance and in the plant basal defense makes them attractive for a better comprehension of the molecular mechanisms of the signal transduction pathways after pathogen recognition.

Keywords *HcrVf2* transgenic plants · *Malus–Venturia inaequalis* interaction · Multigene family

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Introduction

Malus × *domestica* Borkh. (apple) is widely grown worldwide, and it is susceptible to several fungal diseases. Apple scab, caused by the ascomycete *Venturia inaequalis* (Cke.) Wint., is the most economically important. Natural sources of scab resistance have been found in many wild

Malus species, from which monogenic dominant resistance genes have been introgressed in *M.* × *domestica.* Among these, there are the *Vf* gene from *Malus floribunda* 821, the *Vm* gene from *Malus micromalus* and the *Vr2* gene from *Malus pumilia*. Moreover, most of the major apple scab resistance genes have already been positioned in different linkage groups (Bus et al. 2005; Gygax et al. 2004; Hemmat et al. 2003; Maliepaard et al. 1998; Patocchi et al. 2004; Patocchi et al. 2005), and it is now evident that particular symptoms are correlated with the specific type of resistance in the host plant (Chevalier 1988). On the basis of host responses and fungal sporulation, four major phenotypic classes have been defined in apple: class 1, hypersensitive reaction; class 2, resistance; class 3, from (a) weak resistance to (b) weak susceptibility; and class 4, full susceptibility (Chevalier et al. 1991). After an initial infection phase, with a similar trend both in susceptible and resistant cultivars, the fungus can be blocked at different stages, depending on the cultivar, and resistance can be expressed with reactions ranging from classes 1 to 3 (MacHardy 1996).

Plants, being constantly attacked by a variety of microbes, have evolved strategies to recognize the pathogens and subsequently activate defense mechanisms. This activation may be a consequence of specific protein–protein interactions involving pathogen-encoded elicitors (Avr proteins) and plant host receptors encoded by resistance (R) genes. This pairwise association known as gene-for-gene resistance (Flor 1971) imply that, after pathogen recognition, a cascade of cytoplasmic and nuclear responses is induced, leading to the regulation of various defense-related genes (Van der Biezen and Jones 1998; Yang et al. 1997). Even though many R genes and their corresponding pathogen effectors have been cloned, it is not clear yet if there is always a direct binding between them. In fact, plant R proteins can function either by actually detecting the corresponding Avr proteins or by perceiving alterations in plant machineries that are targets of Avr proteins action in the promotion of pathogen virulence; this is known as the "guard hypothesis" (Dangl and Jones 2001; Chisholm et al. 2006; Dodds et al. 2006).

It is now clear that one class of plant R genes encodes for receptor-like protein kinases (RLKs; Hammond-Kosack and Jones 1997). RLKs are components of signal transduction pathways spanning the plant cells plasma membrane and may allow pathogen recognition in the extracellular environment and the following intracellular induction of a defense cascade reaction (Becraft 1998). Plant RLKs can be classified according to features of the predicted extracellular domain. In fact, many RLKs contain unique features such as the S-domain-like motif (with similarity to S-locus glycoprotein; Nasrallah et al. 1985), the epidermal growth factor-like motif (Kohorn et al. 1992), the tumor necrosis factor receptor-like motif (Becraft et al. 1996), and the lectin-binding domain (Herve et al. 1996). Most RLKs contain leucine-rich repeat (LRR) motifs implicated in protein–protein interactions (Kobe and Deisenhofer 1994; Shiu and Bleecker 2001). Some LRR-RLKs have emerged as key developmental regulators (Jinn et al. 2000), while others are involved in plant–pathogen interactions. For example, the rice gene *Xa21* encodes for a typical LRR-RLK protein with a cytoplasmic kinase domain, and it confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Mew 1987; Song et al. 1995; Wang et al. 1998). The tomato *Cf* genes confer resistance to the biotrophic leaf mold pathogen *Cladosporium fulvum* (Jones et al. 1994) and contain an extracellular LRR domain, a single transmembrane domain and a small cytoplasmic tail without any known catalytic activity (Dixon et al. 1996). Also, the apple *HcrVf* genes (homologs to *C. fulvum* resistance genes of the *Vf* region; Vinatzer et al. 2001) display a predicted protein structure similar to that encoded by the *Cf* R genes. In particular, the *HcrVf2* gene, expressed under the control of the 35S CaMV promoter, confers resistance to *V. inaequalis* when inserted in the susceptible cv. Gala (Belfanti et al. 2004). Malnoy and colleagues (2008) demonstrated that *Vfa1* and *Vfa2* genes (syn. *HcrVf1* and *HcrVf2*, respectively) confer partial resistance to *V. inaequalis*, when inserted under the control of their own promoter in the susceptible cvs. Galaxy and McIntosh.

Extensive information is available about the induction of the defense mechanisms in several herbaceous plants, but little is known regarding woody plants (Paris et al. 2009). *LRPKm1* is the first apple gene to be discovered that encodes a LRR receptor-like protein kinase. It belongs to a multigene family present in *Malus* spp. and in *Pyrus* spp. as well (Faize et al. 2007) and is induced in the apple scab resistant cv. Florina after *V. inaequalis* infection and salicylic acid treatment (Komjanc et al. 1999).

Here, the isolation and characterization of four genes belonging to the *LRPKm1* multigene family and their position on the reference apple map 'Fiesta' × 'Discovery' (Liebhard et al. 2003) are reported. The work also investigated, for the first time in apple, the localization of a plant RLK protein at the cytological and ultrastructural level using in situ immunofluorescence (IF) and immunogold (IG) techniques. Moreover, expression studies on mRNA from infected leaves of either susceptible or resistant apple genotypes were carried out in order to infer their putative role in the apple defense response to *V. inaequalis*. Transgenic apple Gala lines resistant because of the insertion of the *HcrVf2* (syn. *Vfa2*) gene were also investigated (Belfanti et al. 2004) in order to verify if the expression of *LRPKm* genes is correlated with the pathogen recognition activity carried out by a *Vf* locus gene.

Materials and methods

Plant material and DNA extraction

A subset of 44 seedlings from an apple segregating progeny population derived from 'Fiesta' × 'Discovery' (Liebhard et al. 2003) was used in this study. Genomic DNA was isolated from leaves using a modified CTAB protocol (Maguire et al. 1994).

BAC library screening with *LRPKm1* gene

In order to screen the apple bacterial artificial chromosome (BAC) library obtained from the Vf scab resistant cv. Florina (Vinatzer et al. 1998), the nylon filters containing BAC clones were hybridized with a DNA probe specific for the LRR region (68–607 amino acid) of the *LRPKm1* gene. *LRPKm1*-specific primers were designed on the published sequence AF053127 (Komjanc et al. 1999), and a 1,617-bp DNA fragment was generated by polymerase chain reaction (PCR) from 'Florina' genomic DNA. PCR reactions were performed in a Perkin Elmer thermal cycler 9600 according to Komjanc et al. (1999). Probe labeling, prehybridization, hybridization, and posthybridization washes were carried out according to the Gene Images AlkPhos Direct Labeling and Detection System with CDP-star™ detection reagent (Amersham, Amersham, UK). The autoradiography technique was used to detect positive clones.

BAC extraction, Shotgun technique, and spotting

DNA was extracted from positive BAC clones following the protocol published on www.chori.org/bacpac/bacpacmini.htm, mechanically fragmented to the size of 2–3 Kb employing a nebulizer and subcloned in pCR® 4Blunt-TOPO® vector using the TOPO® Shotgun Subcloning Kit (Invitrogen, Carlsbad, CA, USA). Colonies were propagated in liquid culture plates, and plasmid DNA was extracted according to Sambrook (2001) protocol and placed into MicroAmp®Optical 96-Well Reaction Plates (Applied Biosystems, Foster City, CA, USA). Hybond™-N^+ nitrocellulose filters (Amersham, Amersham, UK) were placed onto a layer of filter paper 3MM (Whatman) and soaked with denaturing solution (0.5 N NaOH, 1.5 M NaCl). A multiblot replicator (V&P Scientific, Inc.) was used to spot the DNA of the BAC clones onto the filters. Each sample was spotted in two adjacent positions as control. Filters were then placed onto a layer of filter paper (3MM) soaked with neutralizing solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl, 1 mM EDTA) for 15 min, dried by blotting them on filter paper (3MM), and left to air dry overnight, then baked for 2 h at 80°C, and exposed to the UV (30 s each side) to improve the fixing of the DNA to the membranes. The filters were screened in order to isolate single sequence repeats (SSRs) using a biotinylated microsatellite probe (TC_{15}). Probe labeling, prehybridization, hybridization, and posthybridization washes were carried out as describe above.

Sequencing, sequence analysis, and algorithms

Clones giving positive signal were sequenced using an ABI PRISM 3100 Genetic Analyzer and BigDye Terminator Cycle Sequencing Kit 3.1 (Applied Biosystems, Foster City, CA, USA). Sequences were then assembled using AutoAssembler (Applied Biosystems, Foster City, CA, USA) software package. Sequence analyses were performed using ENTREZ/BLAST (http://www.ncbi.nlm.nih.gov/; Altschul et al. 1990), ExPASy (http://www.expasy.ch/; Gasteiger et al. 2003), CLUSTALW servers (http://www.ebi.ac.uk/clustalw/; Thompson et al. 1994), and gene identification softwares such as GeneScanW (http://genes.mit.edu/; Burge and Karlin 1998).

SSR primers design, PCR amplification, and electrophoresis

Primers to amplify microsatellite regions found in the positive BAC clones (Table 1) were designed using Primer3 software (www.primer3.org). PCR amplifications were performed according to Gianfranceschi et al. (1998) using reverse primers labeled with a fluorescent chemical (6-FAM). PCR products were separated by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyzer. The size of the amplified bands was calculated using an internal DNA size standard (GeneScan-500 ROX, Applied Biosystems, Foster City, CA, USA), and raw data were analyzed with GeneScan and Genotyper softwares (Applied Biosystems, Foster City, CA, USA).

SSR mapping

Segregation analysis was performed on a subset (44 individuals) of the 'Fiesta' × 'Discovery' progeny apple plants (Liebhard et al. 2003). All linkage analysis and map calculations were performed with JoinMap 3.0 (Van Ooijen and Voorrips 2001). A LOD score of 5.0 was applied to determine markers belonging to the same linkage group (LG). The thresholds for the following steps were set so that all data were included (LOD=1.0, REC=0.4), and Kosambi (1944) mapping function was applied.

Plant material for inoculation with *V. inaequalis* and RNA extraction

One-year-old plants of 'Florina' (*Vfvf*), 'Golden Delicious', and 'Gala' (*vfvf*), grafted on the rootstock M9 and grown in greenhouse, were inoculated with a suspension of

Table 1 Primers used in this study

Primer name	Direction	Primer sequence (5′ to 3′)
LRPKm1 specific[a]	Sense	CCAGTTGGGATCCAATCCTG
LRPKm1 specific[a]	Antisense	CCCCTTTCTGATCCAATGT
Elongation Factor-1α[b]	Sense	GCTCCTGGACATCGTGACTT
Elongation Factor-1α[b]	Antisense[d]	tatgaaCTTGGAAATACCGGCTTCA5A
28S[b]	Sense	GGGTTTGCTCTTGGGTTAG
28S[b]	Antisense[d]	gactgaaACGCAGGTTCTTCATCTTCAG5C
LRRall[b]	Sense[d]	gaaacgAACCTCTCCTACAACCCGT5TC
LRRall[b]	Antisense	ACTAGGTTGCACTCGGTGAGC
LRR2–4[b]	Sense[d]	caaacaCCCACACTCATTGCTTTGT5TG
LRR2–4[b]	Antisense	GATGCCTCGGATCATGGAAG
LRR2–3–4[b]	Sense[d]	ccaccATACACAATGAGTTTTCCGG5GG
LRR2–3–4[b]	Antisense	GGTCAAGCTCTGGCACTCAC
52M12 SSR[c]	Sense	GCATGCATGTTTGTTCT
52M12 SSR[c]	Antisense[e]	TTTTGATTTAACTTCCTCCT
71M6 SSR[c]	Sense	GTTGCTCACGTGGGATGTC
71M6 SSR[c]	Antisense[e]	CCCACCCCTCTGAAATCTCT
18K21 SSR[c]	Sense	CCAGTGGGATCGTTTCTGAT
18K21 SSR[c]	Antisense[e]	CATCCACAGCAACCTGTGAC
45P16 SSR[c]	Sense	CTTTGAAAATTTCGGCCA
45P16 SSR[c]	Antisense[e]	GCTACAAATGTTAGTAAATTCGCGT

[a] Primer specific for the LRR region (68–607 amino acid) of the LRPKml gene

[b] Primer used for real-time RT-PCR

[c] Primer used for BAC-specific SSR mapping

[d] Primer LUX (Invitrogen)

[e] Primer FAM

V. inaequalis conidia (at least 2×10^5 conidia/ml). Two independent scab resistant Gala lines, expressing the R-gene *HcrVf2* and named Ga2-2 and Ga2-21, were rooted and acclimated in greenhouse and then inoculated with the same inoculum, prepared using scabbed leaves of cvs. Gala, Red Chief, and Golden Delicious grown in an untreated orchard, as described in Belfanti et al. (2004). Control plants were inoculated with distilled water, and the growth chamber conditions were set at 19±5°C and 100% relative humidity for at least 2 days after inoculation. Young expanded leaves for RNA extraction were collected 0, 24, 48, 72, and 96 h postinoculation (pi) for cvs. Florina and Golden Delicious and 0, 24, 48, and 72 h pi for 'Gala' and the transgenic genotypes. The harvested leaf material was stored at −80°C until the RNA was extracted. RNA extractions were carried out according to Komjanc et al. (1999).

Real-time RT-PCR

Because of the high identity between *LRPKm* genes, it was quite difficult to design specific primers to discriminate each gene from the others by PCR. Initially, a first primer pair ("LRRall") was designed that recognized all the members of the family in order to evaluate their collective expression at different times after scab inoculation. Afterward, as the cDNA sequences of *LRPKm2* and *LRPKm4* were nearly identical, a second pair of primers ("LRR2–4") was designed showing the collective expression of those two genes. Finally, a third primers pair ("LRR2–3–4") was designed that showed the expression of *LRPKm2–3–4* all together. Each reverse primer, labeled with a fluorescent chemical (light upon extension (LUX); Invitrogen, Carlsbad, CA, USA), and their unlabeled counterparts (Table 1) were designed using the LUX™ Primer Design software (available at www.invitrogen.com/lux).

Real-time RT-PCR experiments were performed in a 96-well spectrofluorometric thermal cycler ABI PRISM 7000 Sequence Detection System. Reactions were set up according to SuperScript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit. The real-time PCR amplification efficiency of the LUX primers was first tested to quantify our targets. Reactions were incubated at 45°C for 45 min, 95°C for 18 min, and then cycled (40) using 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min. The cycle thresholds (C_T) were between 15 and 33 cycles for all PCRs involving all primer sets and template dilutions. We used the comparative Ct method ($\Delta\Delta C_T$), as described in Applied Biosystems, User Bulletin No. 2 (P/N 4303859), because the amplification efficiencies of the targets (*LRPKm* genes) and the reference (*EF* gene-elongation factor-1α) were not far from 100%. The criterion used to define the validity of a primer pairs requires that the plot of the log of the input amount of template versus the ΔC_T (C_T target gene-C_T *EF*) has a slope of less than 0.1. The *EF* gene was used as a standard to validate our procedure because in

other research studies, it proved to be the most stable among all the housekeeping genes tested (Nicot et al. 2005). In order to confirm that *EF* was a good reference gene candidate, also in our biological system, it was checked versus another housekeeping gene certified to be used in real-time PCR experiments, *28S*. For each genotype, the calibrator used in the $\Delta\Delta C_T$ calculation is the time point zero of 'Gala'. All samples were run in triplicates per experiment, and the analyses were repeated twice, starting from two independent total RNA extraction. The averages of the biological replicates and standard deviations were calculated.

MAbs production

Based on LRPKm proteins hydropathy, a synthetic peptide of 25 amino acids from a highly hydrophilic region (from 31–56 aa; see Fig. 4) was constructed by the Custom Peptide Synthesis Service of the company Genosys, UK. The synthetic peptide was linked to a Keyhole Limpet Hemocyanin carrier (Sigma-Aldrich, Darmstadt, Germany) and used as antigen to immunize a Balb/c mouse to produced monoclonal antibodies. Hybridomas were produced after fusion of mouse Balb/c immunized lymphocytes with NSO myeloma cells (Loi et al. 2002). Indirect ELIZA selected 20 antibody clones producing specific MAbs against LRPKm synthetic peptide. The stable and continuously producing MAbs were selected and tested against protein extracts from cell membranes of apple cv. Florina 48 h pi (Sicilia et al. 2005). The hybridoma 8C5 was selected because the highest values of optical density in ELISA and 8C5-MAb does not produce unspecific reactions against protein extracts from membranes isolated from noninfected 'Florina' leaves nor with protein extract of *V. inaequalis.*

IF and IG staining technique

IF was performed as reported by Loi et al. (2002) on apple leaf tissue sections of cvs. Florina and Golden Delicious 24, 48, 72, and 96 h after inoculation by spraying a water suspension of *V. inaequalis* conidia (1×10^6 conidia/ml). Not inoculated tissues from cvs. 'Florina' and 'Golden Delicious' were included as control samples. Transmission electron microscopy analyses using IG staining were performed on inoculated and not inoculated cv. Florina samples after 48 h dehydrated and fixed according to Musetti et al. (2002) and included in LR Gold resin (Electron Microscopy Science, USA). Ultrathin sections were incubated with 8C5-MAb and stained with Antimouse IgG gold 10 nm conjugate (Sigma-Aldrich, Darmstadt, Germany) and observed under a PHILIPS CM 10 transmission electron microscope (Philips Scientifics, Eindhoven, The Netherlands), operated at 80 kV.

Results

Identification of BAC clones with homology to *LRPKm1* multigene family

After the BAC library screening with the *LRPKm1* gene, seven positive clones were identified: 17I14, 18K21, 45P16, 52M12, 71M6, 72K10, and AM19-5. The AM19-5 BAC clone, mapping in a region flanking the *Vf* gene (Patocchi et al. 1999), had an open reading frame (ORF) of 176 amino acids interrupted by a STOP codon and showed a lower homology to *LRPKm1* than the ORFs deducted from the other six positive BAC clones. These six clones displayed specific restriction patterns after digestion with *Eco*RI and *Hind*III (Fig. 1a, c). Southern analysis of restricted clones, after hybridization with the *LRPKm1* probe, showed a similar pattern between the following BAC pairs: 17I14 and 72K10, 18K21 and 52M12, and 45P16 and 71M6 (Fig. 1b, d). These BAC clones pairs probably have some identical or overlapping region, and they may be clustered in the apple genome. ORFs contained in 17I14 and 72K10 BAC clones have not been further investigated because they showed a lower homology to *LRPKm1* after a partial sequencing.

In order to identify regions with higher homology to the *LRPKm1* gene from 18K21, 52M12, 45P16, and 71M6 BAC clones, bands hybridizing with *LRPKm1* probe were excised from agarose gel and subcloned for further sequence analyses.

Sequencing analysis of apple BAC clones (52M12, 71M6, 18K21, and 45P16) and characterization of the *LRPKm1* gene family products

Computational analysis revealed large open reading frames similar to *LRPKm1* coding region in four BAC clones, namely 52M12, 71M6, 18K21, and 45P16, containing 3173, 3172, 3188 and 3172 bp ORFs, respectively (Fig. 2). The deducted aa sequences were respectively designated as LRPKm1 (999 aa), LRPKm2 (998 aa), LRPKm3 (1001 aa), and LRPKm4 (998 aa). The ORF contained in the 52M12 clone was named LRPKm1 because it showed 99.9% sequence identity to *LRPKm1* (cDNA accession number AF053127) also in the 5′ (accession number AF053126) and 3′ noncoding region. The pairwise sequence identities between *LRPKm1*, *LRPKm2*, *LRPKm3*, and *LRPKm4* ranged from 80% to 95% in the coding region. Each ORF was interrupted by one putative intron (Fig. 2). Intron alignment showed identities ranging from 74% (between 52M12 and 71M6) to 99% (between 18K21 and 45P16). The presence of variability across the introns and in noncoding regions located 5′ and 3′ of *LRPKm* genes suggests that these four BAC clones contain different genes. All *LRPKm* promoters contained putative TCA

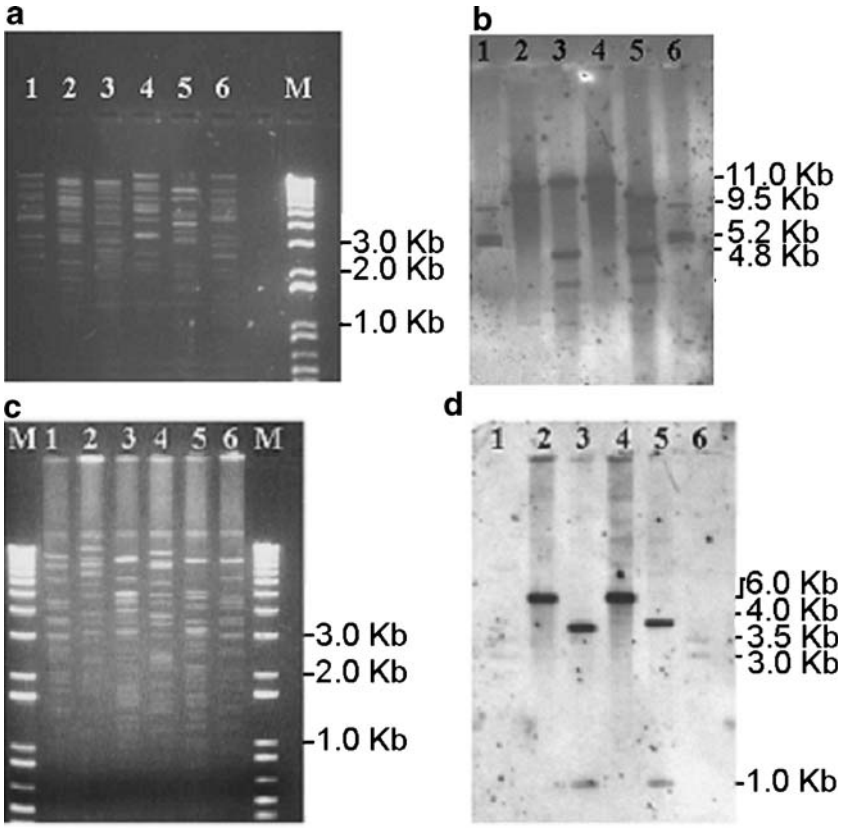


Fig. 1 Restriction pattern of six 'Florina' BAC clones with *EcoR*I (**a**) and *Hind*III (**c**). Southern blot analysis using *LRPKm1* gene as probe on BAC clones digested with *EcoR*I (**b**) and *Hind*III (**d**). *1* 17I14, *2* 18K21, *3* 45P16, *4* 52M12, *5* 71M6, *6* 72K10, *M* 1 Kb ladder

motifs (Table 2) and W-boxes, while many of them contained H-boxes/G-box, ATTTCAAA motif, and Box IV (data not shown). Interestingly, even though 5′ untranscribed regions are very different among the *LRPKm* genes, they showed the presence of DNA binding domains for WRKY proteins (W-boxes) and other consensus sequences in almost identical positions between the 450-bp upstream region and the ATG start codon in 18K21, 45P16, and 71M6 promoters (Fig. 3).

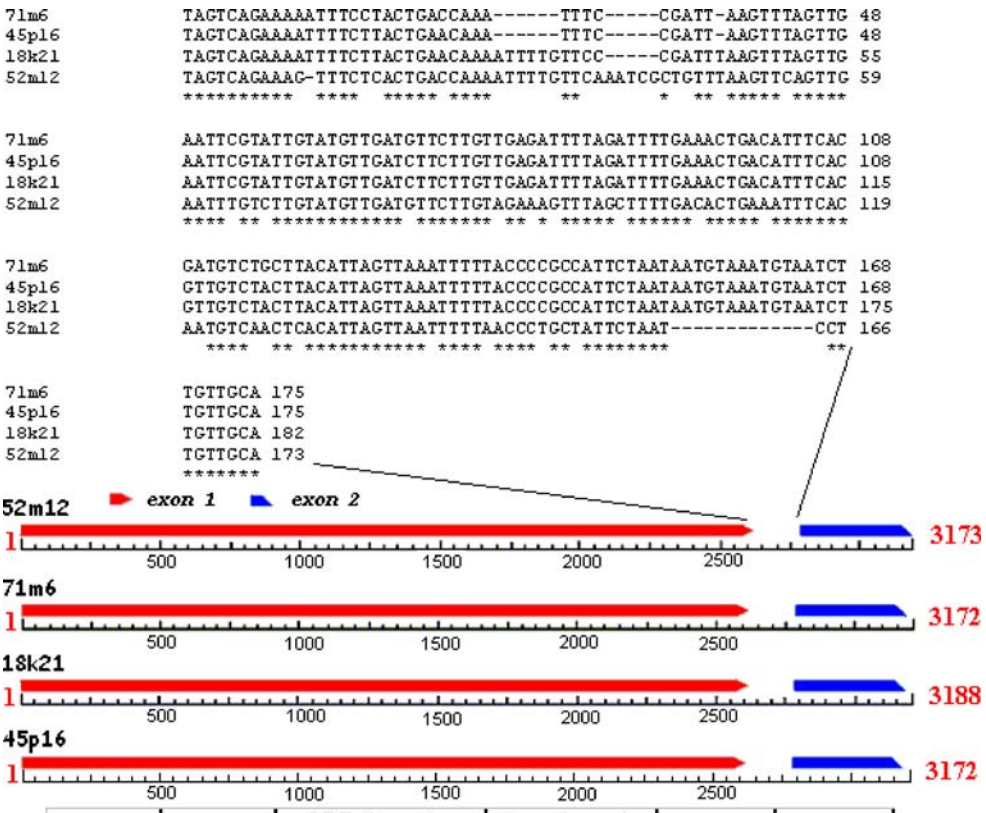
The pairwise sequence identities of deduced amino acid (aa) sequences ranged from 90% to 99%, and the kinase domains were highly conserved. LRPKm2 and LRPKm4 were almost identical (99% identity). The N-termini of all LRPKm proteins contain potential hydrophobic signal peptides that may direct the secretion of these proteins to the plasma membrane. These putative signal peptides are followed by putative extracellular regions consisting of conserved leucine zipper and LRR domains. All LRPKm proteins contain 23 complete LRRs flanked by pairs of conservatively spaced cysteine residues (Fig. 4). The predicted transmembrane regions are highly hydrophobic and are expected to form α-helices in the membranes. The putative intracellular cytoplasmic tail regions contain all conserved kinase subdomains found in serine/threonine protein kinases (Fig. 4, I–XII subdomains).

Microsatellites isolation and mapping in 'Fiesta' × 'Discovery' population

In all the BAC clones containing a *LRPKm* family gene, a microsatellite (SSR) was isolated near the coding region, and specific primers were designed. Primers were firstly checked for no cross amplification when tested on the other BAC clones, and after testing polymorphism in the population, 52M12 SSR was mapped in LG 5 of 'Fiesta' × 'Discovery' reference map (Liebhard et al. 2003), while 71M6 SSR and 45P16 SSR mapped in LG 10 of 'Fiesta' (scab susceptible) map. 71M6 SSR, 18K21 SSR, and 45P16 SSR clustered in LG 10 of 'Discovery' (scab resistant) map (Fig. 5).

Expression studies of *LRPKm* gene family

The whole *LRPKm* multigene family presented different basal level of expression (at time 0) in all the genotypes tested, and each one displayed a specific trend of induction in relation to scab infection (Fig. 6a, b, "LRRall"). More specifically, cvs. Florina and Golden Delicious had a lower basal expression level if compared to 'Gala' (scab susceptible) and to *HcrVf2* transgenic Gala lines. The results (Fig. 6a) showed a progressive increase of gene expres-



BAC name	ORF name	ORF dimension (bp)	ORF dimension (aa)	intron dimension (bp)	exon 1 (bp)	exon 2 (bp)
52M12	LRPKm1	3173	999	173	1-2610	2783-3173
71M6	LRPKm2	3172	998	175	1-2607	2782-3172
18K21	LRPKm3	3188	1001	182	1-2616	2798-3188
45P16	LRPKm4	3172	998	175	1-2607	2782-3172

Fig. 2 Schematic alignment of four genes belonging to *LRPKm* multigene family with intron nucleotidic alignment. In the table, exons, introns, and ORF dimensions are reported

sion of *LRPKm* multigene family in cv. Florina (scab resistant) and a lower and 24 h delayed induction in 'Golden Delicious' (scab susceptible), as previously demonstrated by Northern analysis (Komjanc et al. 1999). In detail, cv. Florina showed an increase in the level of expression 24 h pi, about 4.4-fold higher than the level at its time point 0, while cv. Golden Delicious displayed a similar trend of induction but starting from 48 h pi and with a significant (about 2.5-fold respect to its time point 0) but lower increase in respect to the cv. Florina.

Among the Gala-derived plants, Ga2-21 showed a higher basal level of expression of the whole *LRPKm* gene family than both 'Gala' and Ga2-2, which level was quite similar (Fig. 6b). Moreover, while 'Gala' revealed no modulation, the transgenic Gala lines showed a similar trend, with a peak in the expression of the multigene family at 24 h pi but at different extent (Fig. 6b). In fact, Ga2-21, which is a highly scab resistant line (Chevalier class 2), showed a level of induction that is 8-fold higher than 'Gala', while Ga2-2 that is reported to occasionally show some restricted fungal development (class 3b) showed a lower one.

After amplification with "LRR2–4" primer pairs, *LRPKm2* and *LRPKm4* genes resulted always downregulated after *V. inaequalis* inoculation in all tested genotypes (Fig. 6c). In 'Golden Delicious', the transcripts level decreased between 0 and 24 h pi, and then it was stable over time. In the other genotypes, the transcripts levels decreased markedly and progressively with the minimum detected at 96 h pi for 'Florina' or 72 h pi for Ga2-2 and Ga2-21.

With "LRR2–3–4" primers, it was possible to observe two different expression profiles (Fig. 6d). In 'Florina' and Ga2-21, a peak of expression was observed at 24 and 48 h pi, respectively, revealing the collective induction of *LRPKm2*, *LRPKm3*, and *LRPKm4*. All other genotypes proved to be downregulated, when analyzed with "LRR2–3–4" primers, showing trends similar to that displayed with "LRR2–4" primers. In synthesis, the level of expression obtained using the "LRR2–4" primers was strongly repressed (Fig. 6c), while widening the analyses to *LRPKm3* with the "LRR2–3–4" primers (Fig. 6d), an induction in the two more resistant genotypes, 'Florina' and Ga2-21, was observed at different times (24 and 48 h pi, respectively).

Table 2 Promoter regions with homology to the TCA motif in *LRPKm* genes

Gene	Sequence	Position
LRPKm1	* * * A A * * * * *	−2,269 comp.
	A A * * A * * * * *	−1,993 comp.
	* G T * G * * * * *	−1,798
	A A G * * * * * * *	−1,630
	* G * * T * * * * *	−1,519
	* * T * A * * * * G	−1,087
	* * * * * A * T * A	−742
	C T * * G * * * * *	−584 comp.
LRPKm2	G * T * A * * * * *	−2,163
	* G C * A * * * * *	−1,842 comp.
	G T T * * * * * * *	−1,220
	* T * A T * * * * *	−1,776
	* T T * T * * * * *	−332
	* G C * A * * * * *	−226 comp.
LRPKm3	* T T * T * * * * *	−332
	* T * C T * * * * *	−226 comp.
LRPKm4	* T * A T * * * * *	−776
	* T T * T * * * * *	−332
	* T * C T * * * * *	−226 comp.
	T C A T C T T C T T	consensus

Asterisks—position identical to consensus sequence

comp. the motif is located on the complementary DNA strand

In the same way, starting from these last results ("LRR2–3–4") and extending the analysis to all the members of the multigene family (with "LRRall" primers), an increase in the induction level was observed in 'Florina' 24 h pi, from 1.7- to 3.3-fold in respect to its point 0. In Ga2-21, a maximum peak of expression at 24 h pi in "LRRall" was revealed, not observed with "LRR2–3–4" primer pairs.

Localization of the LRPKm protein at cytological and ultrastructural level

In situ immunofluorescence and immunogold staining were used to localize LRPKm proteins at cytological and ultrastructural level. A specific monoclonal antibody was produced against a synthetic peptide (25 amino acids) synthesized from *LRPKm1* DNA sequence by selecting a highly hydrophilic region of the protein to guarantee peptide immunogenicity. Because this hydrophilic region is identical in all 4 *LRPKm* sequences isolated, the monoclonal antibody (MAb) very likely binds to all of them. Only three out of 638 obtained hybridomas were stable and secreted constantly specific MAbs against the synthetic peptide; among them, hybridoma 8C5 producing 8C5-MAb was used. 8C5-MAb reacts only against membrane proteins of apple cv. Florina after *V. inaequalis* inoculation and does not react with healthy and infected tissues of cv. Golden Delicious and healthy 'Florina' both in ELISA (data not shown) and IF (Fig. 7a, b) or with the fungus by itself (data not shown).

The fluorescence was detected mainly on the plasma membrane of epidermal cells under the leaf cuticle. The fluorescence appeared in cv. Florina leaves 24 h after infection, and it was enhanced to a very high level after 96 h (Fig. 7c). The IG staining revealed gold particles in the proximity epidermal cell plasmalemma of cv. Florina challenged with the pathogen, confirming the hypothesis that LRPKm proteins are localized on the membrane of epidermal cells (Fig. 8a, b).

Discussion

This work has led to the isolation in apple of four members of *LRPKm* multigene family (Komjanc et al. 1999) which

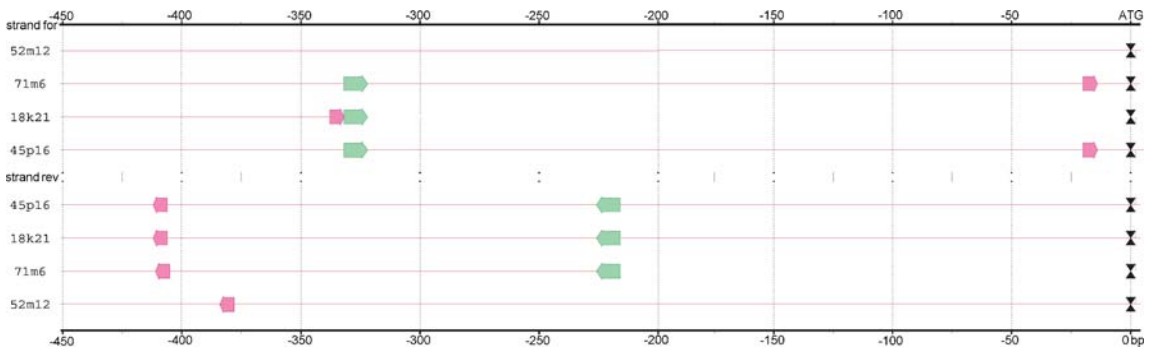


Fig. 3 5′ untranscribed regions showed the presence of DNA binding domains for WRKY proteins (W-boxes, *pink arrows*) and TCA-motifs (*green arrows*) in conserved positions between −450 bp and the ATG start codon in 18K21, 45P16, and 71M6 promoters, while they are located in different positions in 52M12 promoter. The *upper part* of the diagram shows DNA binding domains on the forward strands while the lower point out the motifs found on the reverse strands. The positions of DNA binding domains are indicated in base pairs starting from ATG codon

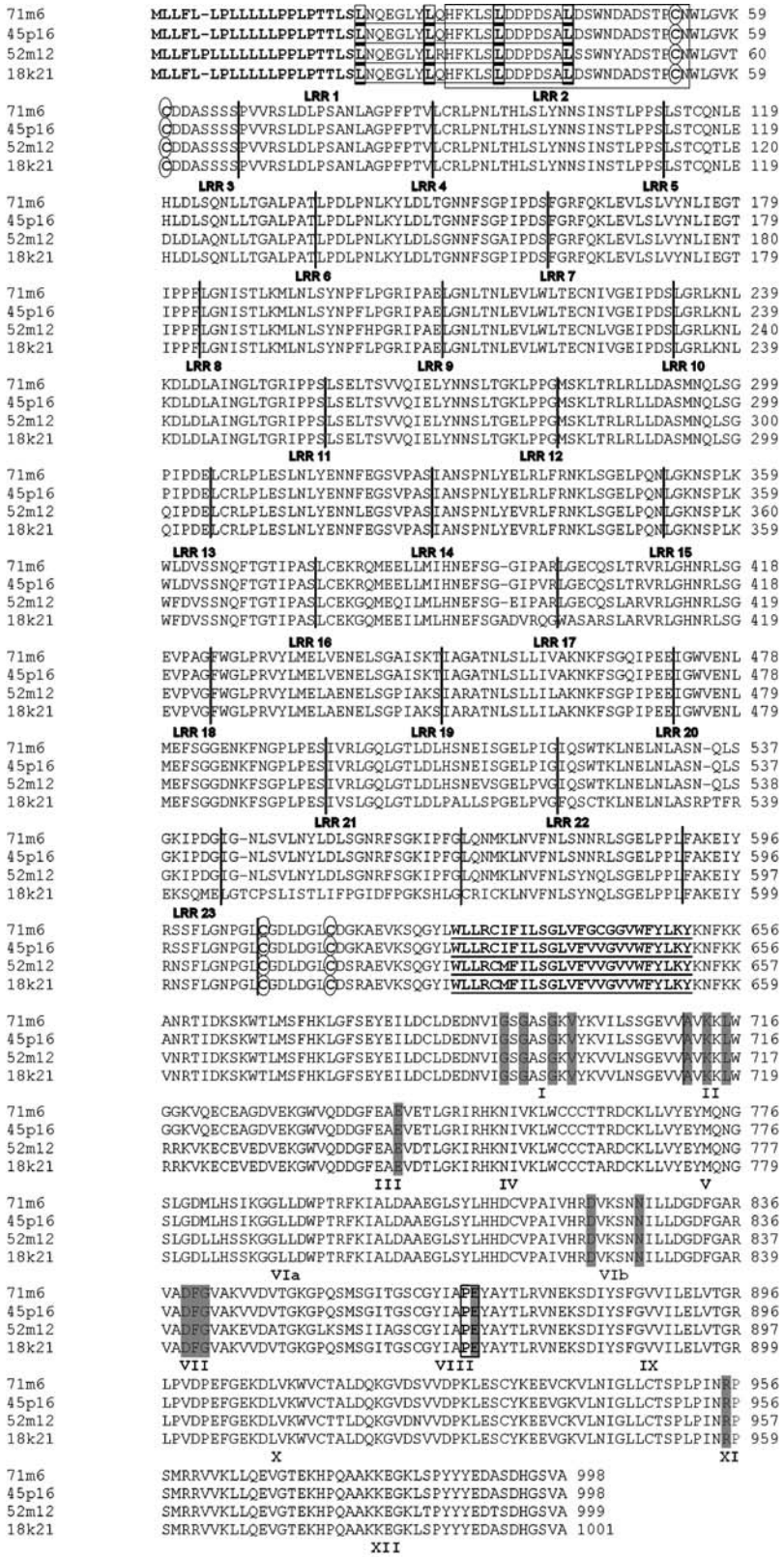


Fig. 4 Deduced amino acids sequence alignment of *LRPKm* multigene family was performed with Clustal W. In the alignment: (C), paired cysteine residues; L, putative leucine zipper; PE, junction between exons 1 and 2; *I–XII*, conserved kinase subdomains (Hanks et al. 1988). Invariant amino acid residues in all eukaryotic kinases are *shadowed*. Twenty-three LRR modules are *numbered*. The signal peptide is *bold*, and putative cleavage sites were localized using SignalP Server (Nielsen et al. 1997; Nielsen and Krogh 1998) and TMHMM Server V2.0 (Krogh et al. 2001; Sonnhammer et al. 1998). Putative transmembrane regions, *underlined* and *bold in the alignment*, are predicted to form α-helices using PSORT (Nakai and Horton 1999), SOSUI (Hirokawa et al. 1998), and Expasy servers (Gasteiger et al. 2003). The region of 25 aa where the synthetic peptide for IF was designed is included in a *rectangle*

	Putative Cleavage site (aa)	Position of LRR-domains (aa)	Transmembrane Regions (aa)
LRPKm1'	21-22	68-607	627-649
LRPKm2	20-21	67-606	627-649
LRPKm3	20-21	67-609	628-650
LRPKm4	20-21	67-606	627-649

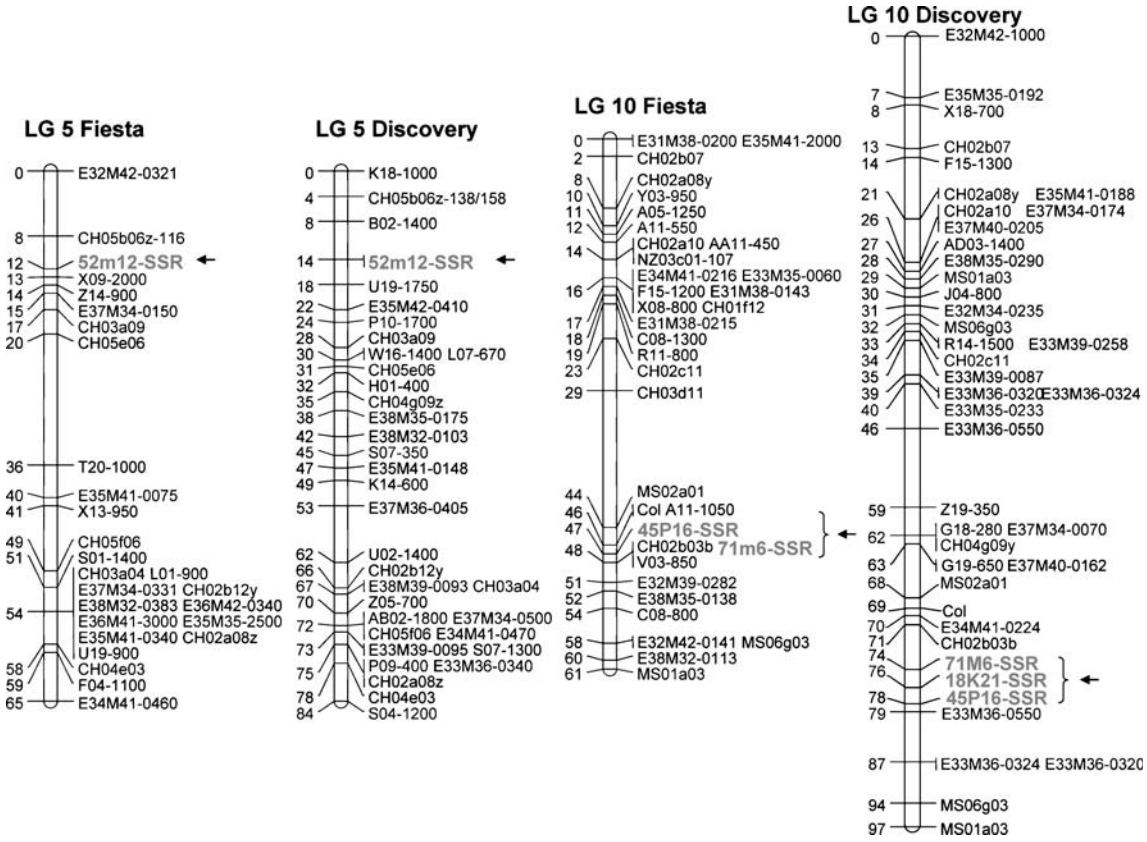


Fig. 5 Linkage groups 5 and 10 of 'Fiesta' × 'Discovery' map (Liebhard et al. 2003) with the BAC, containing *LRPKm*, associated SSRs position. *LRPKm*-SSRs are indicated by *arrows*

code for putative membrane-anchored receptor-like kinases. However, we could hypothesize the presence of more than four copies of genes encoding LRPKm-like proteins in the apple genome because the chemiluminescent methodology used for the BAC library screening is not sensitive enough and probably caused loss of information and the appearance of several faint spots that were considered as false positives. Failure in capturing other members of this gene family in apple might also be due to genome coverage of the 'Florina' BAC library (5x haploid equivalent) used in this study.

All LRPKm putative proteins contain a leucine zipper domain at the N terminus of the mature deduced proteins that may participate in the homo- or heterodimers formation (Landschulz et al. 1988), and 23 extracellular LRRs likely involved in the binding of a still unknown ligand (Kajava 1998). All *LRPKm* promoters contain putative binding sites for a number of regulatory proteins related to responses to biotic and abiotic stresses as TCA motif, W-boxes, and G/H-boxes. The TCA motif, with a TCATCTTCTT consensus sequence, is present in several plant stress-inducible genes (Goldsbrough et al. 1993). W-boxes are *cis*-elements, with a (T)TGAC(C/T) *core* sequence, known to be binding sites for the WRKY plant-specific transcription factors and are often clustered in promoters of defense-related genes (Rushton and Somssich 1998; Mahalingam et al. 2003). Moreover, a combination of H-box (CCTACC) and G-box motifs (CACGTG) has been shown to be important in gene activation after pathogen attack (Droge-Laser et al. 1997; Mahalingam et al. 2003).

For investigating the real localization of LRPKm proteins, monoclonal antibodies against a synthetic peptide obtained from a hydrophilic portion of LRPKm1 protein were prepared. This is a useful tool to localize protein expression in situ. Immunofluorescence and immunogold staining, used in apple for the first time in this work, confirmed at cytological and ultrastructural level that LRPKm accumulate in the plant cell membrane, as hypothesized using prediction tools, and especially on pathogen challenged scab resistant leaves, as postulated by molecular results. The possible involvement of these genes in the apple–*V. inaequalis* interaction was also assumed from the results of gene expression analyses that revealed a different modulation of the *LRPKm* genes.

In brief, it was not possible to discriminate between *LRPKm2* and *LRPKm4* because of their high identity nor between *LRPKm1* and *LRPKm3* because the major differences in nucleotidic sequences were located in regions where *LRPKm3* is almost identical to *LRPKm2* and

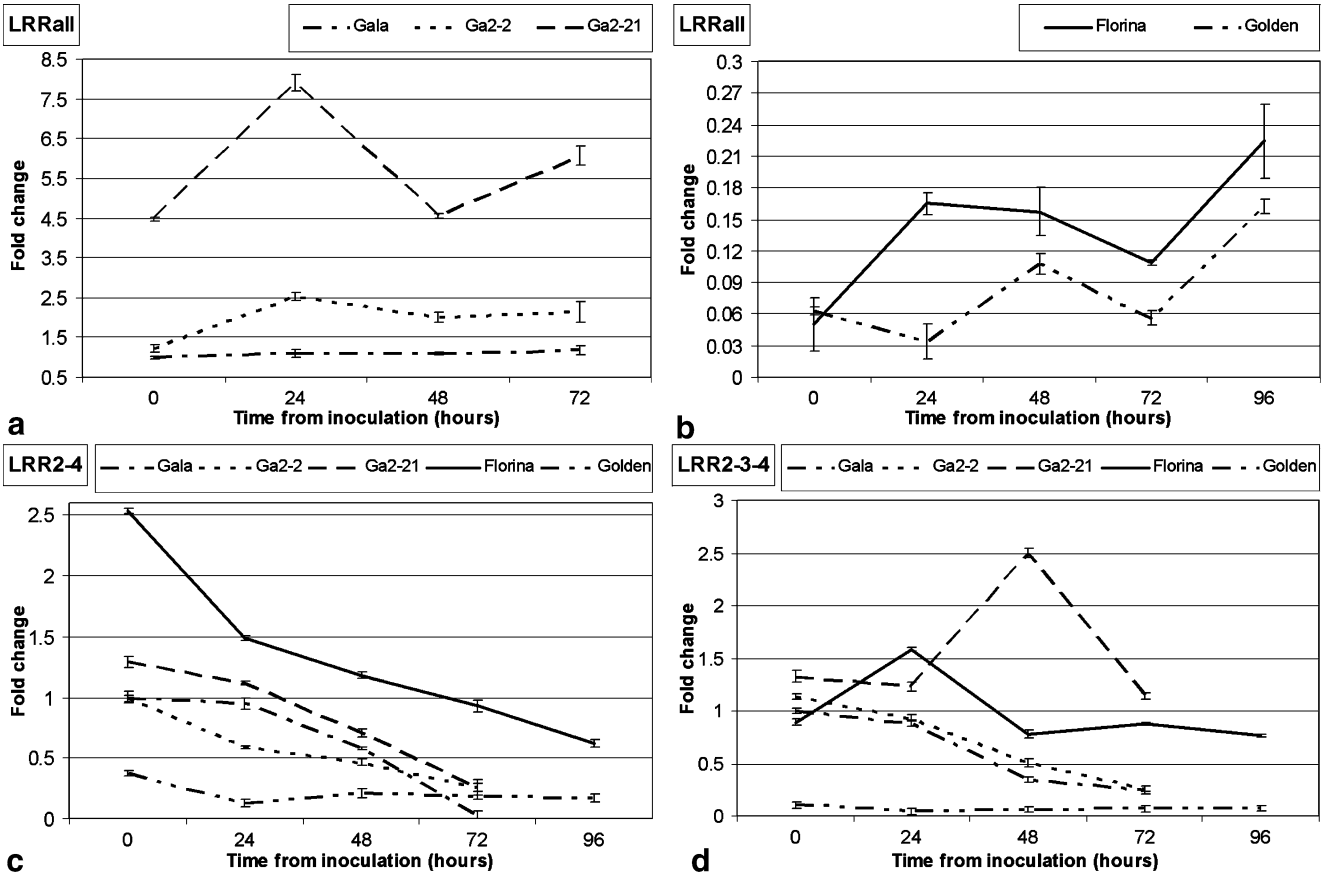


Fig. 6 Real-time RT-PCR experiments on five apple genotypes. The $\Delta\Delta C_T$ method was applied and 'Gala' time point 0 was used as "calibrator" for each primer pair. The fold change, expressed in arbitrary units, is plotted versus the time from inoculation with *V. inaequalis*, expressed in time after inoculation (hours). **a**, **b** Expression profiles of the whole *LRPKm* multigene family ("LRRall") in 'Florina' and 'Golden Delicious' (**a**) and in 'Gala', Ga2-2, and Ga2-21 (**b**). **c** Downregulation of *LRPKm2* and *4* ("LRR2–4"), reported for all genotypes. **d** Cumulative expression levels of *LRPKm2*, *LRPKm3*, and *LRPKm4* ("LRR2–3–4"). The *bars* represent the standard deviations

LRPKm4. However, comparing all the results, some conclusions can be drawn. First of all, the genes belonging to *LRPKm* multigene family seem all to react to *V. inaequalis* but in very different ways. In fact, some members of the family—i.e., *LRPKm2* and *LRPKm4*—were always downregulated after pathogen inoculation.

On the contrary, other members of the family—i.e., *LRPKm1* and *LRPKm3* genes, as can be observed comparing

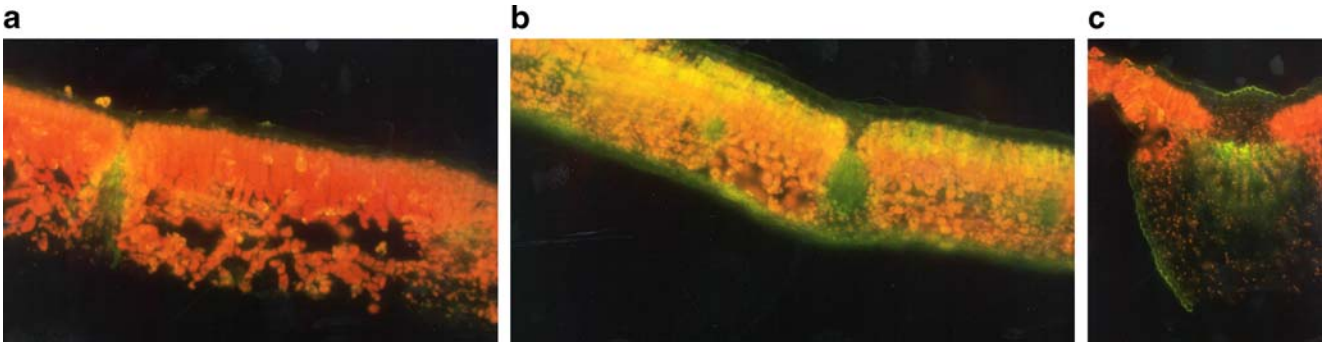


Fig. 7 **a** 'Golden Delicious' leaf tissue section 48 h after infection with *V. inaequalis*: no fluorescence is present. **b** 'Florina' leaf tissue section 48 h after inoculation with *V. inaequalis*: fluorescence is visible on the epidermal cells. Noninoculated cv. Florina leaf tissue did not display fluorescence, similarly to cv. Golden Delicious. **c** 'Florina' leaf tissue section 96 h after infection with *V. inaequalis*, when a very high level of fluorescence is present

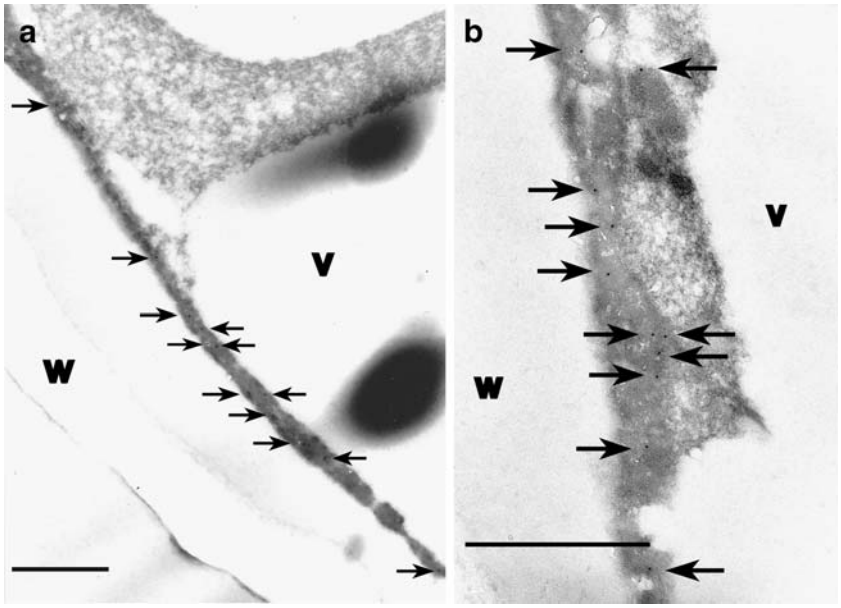


Fig. 8 **a** Micrographs of leaf tissue sections (*M.* × *domestica* cv. Florina) after 48 h from infection with *V. inaequalis*. Gold particles are localized in the proximity of the plasmalemma. **b** Enlarged detail. *W* cell wall, *V* vacuole. *Bars* correspond to 1 μm

"LRRall" with "LRR2–3–4" and "LRR2–3–4" with "LRRR2–4", respectively—were induced in resistant genotypes ('Florina' and the *HcrVf2* transgenic lines). Therefore, we suggest a division of the *LRPKm* multigene family in two subclasses: one dependent on the presence of *HcrVf2* (*LRPKm1* and *LRPKm3*) and the other independent from *HcrVf2* but nevertheless correlated to *V. inaequalis* infection (*LRPKm2* and *LRPKm4*).

More precisely, *LRPKm1* and *LRPKm3* have proved to be upregulated in 'Florina' (scab resistant-*Vfvf*) and in transgenic Gala2-21 line (scab *HcrVf2*-resistant) even if with different timing and extent. Ga2-2 indeed showed a lower modulation with a trend intermediate between 'Gala' and Ga2-21. The differences between the two transgenic lines can be due to the fact that, being derived from distinct transformation events, they may differ for the transgene integration site, which can modify transgene expression and consequently the resistance level (Kumar and Fladung 2001). In this regard, it is known that *HcrVf2*-transgenic Gala lines had different levels of resistance (Belfanti et al. 2004). In fact, Ga2-21 line had the highest resistance level (class 2) while the transgenic line Ga2-2 occasionally showed some restricted fungal development (class 3b), but it can still be considered more resistant than the *M.* × *domestica* cv. Gala (class 4; Belfanti et al. 2004).

These data support the idea that LRPKm1–3 could be involved with HcrVf2 in the incompatible apple–*V. inaequalis* interaction, maybe in the recognition of a pathogen-derived or an endogenous signal released during the infection with *V. inaequalis* downstream of the *Vf* gene product. Concerning the other *LRPKm* genes, *LRPKm2* and *LRPKm4* revealed a strong downregulation after challenge with *V. inaequalis*, and this response is independent of *HcrVf2* because it is evident in both susceptible and resistant genotypes. Moreover, as the strong downregulation was found also in 'Florina', which contains the whole cluster of *Vf* genes, we can assume that *LRPKm2* and *LRPKm4* are not even correlated to *HcrVf1* (*Vfa1*) that Malnoy et al. (2008) found out to confer partial resistance to scab. This evidence support the idea that *LRPKm2–4* expression is not influenced by the *Vf* cluster genes and that these genes are not active in the *Vf* downstream signal cascade. A similar strong downregulation of LRR-RLK genes has been previously reported as suppression of the tomato host basal defense carried out by *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 during the compatible interaction (Espinosa and Alfano 2004). Similarly in *Malus*, the downregulation of the *LRPKm2* and *4* after the pathogen recognition might be caused by the fungus *V. inaequalis* in order to decrease the host basal defense.

Noteworthy too is the genomic localization of the SSRs associated to all multigene family members. Maliepaard et al. (1998) and Liebhard et al. (2002) theorized an event of gene duplication between linkage group 5 and linkage group 10 in *Malus* for the presence of SSRs (CH02a08–CH04g09) mapping in both linkage groups. Also, *LRPKm* multigene family members map exactly in this region (LG5: 52M12-SSR; LG10: 71M6-SSR, 18K21-SSR, and 45P16-SSR) suggesting that this family could derive from a gene duplication event. Moreover, the SSRs segregation analysis pointed out that we identified four paralogs instead of different alleles because there is no independent segregation of markers sizes associated to the clones mapped in LG 10. As known, plant specificities and pathogen virulence continually adapt in response to each other; this strong selection pressure may favor gene

duplication and subsequent diversification (MacHardy et al. 2001). The clustering of R genes sequences in fact is reported in the genome of many model plants, and it represents a ready source of new variance to fight against the pathogen virulence (Bergelson et al. 2001). The evidence that three microsatellites associated to *LRPKm* genes map in cluster in a genomic region not associated to resistance QTLs may suggest that some members of this multigene family are active in response to the pathogen *V. inaequalis*, downstream of a resistance gene, and they are involved in the transduction of the signal in the defensive cascade, as already postulated.

The true mechanism involving *LRPKm* multigene family is still unknown as the RLK pathways may be further complicated by functional redundancy between receptors. In the *Arabidopsis thaliana* genome, there are 216 LRR-RLKs, but only a few have been associated with a biological function because multiple receptors may functionally overlap with each other and it is hard to find the mutant versions of the receptor (Diévart and Clark 2004). In a woody plant, like *Malus* spp., a further complication is the inability to work on mutants; for this reason, in apple, only some RLKs have been successfully studied and have been associated with a biological function.

It is therefore useful to develop transgenic plants in order to study the interaction between an endogenous gene (*LRPKm* genes) and the presence or the absence of an exogenous one (*HcrVf2* gene) or to study the silencing effect of endogenous genes involved in the resistant response.

The recent development of transgenic genotypes opens a new scene in the study of the *Malus–V. inaequalis* interaction allowing a better comprehension of the molecular bases of such interaction. A lot still remains to be discovered but assuming a transgenic approach, also with the *LRPKm* genes and through the gene silencing, we will be able to better understand the space–time positioning of these proteins in the transmission of the signals across the plasma membrane during the infection with *V. inaequalis*.

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