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Derick Jiwan, Eric H. Roalson, Dorrie Main & Amit Dhingra

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Antisense expression of peach mildew resistance locus O (*PpMlo1*) gene confers cross-species resistance to powdery mildew in *Fragaria x ananassa*

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Abstract Powdery mildew (PM) is one of the major plant pathogens. The conventional method of PM control includes frequent use of sulfur-based fungicides adding to production costs and potential harm to the environment. PM remains a major scourge for Rosaceae crops where breeding approaches mainly resort to gene-for-gene resistance. We have tested an alternate source of PM resistance in Rosaceae. Mildew resistance locus O (MLO) has been well studied in barley due to its role in imparting broad spectrum resistance to PM. We identified *PpMlo1* (*Prunus persica Mlo*) in peach and characterized it further to test if a similar mechanism of resistance is conserved in Rosaceae. Due to its recalcitrance in tissue culture, reverse genetic studies involving *PpMloI* were not feasible in peach. Therefore, *Fragaria x ananassa* LF9 line, a taxonomic surrogate, was used for functional analysis of *PpMlo1*. *Agrobacterium*-mediated transformation yielded transgenic strawberry plants expressing *PpMlo1* in sense and antisense orientation. Antisense expression of *PpMlo1* in transgenic strawberry plants conferred resistance to *Fragaria*-specific powdery mildew, *Podosphaera macularis*. Phylogenetic analysis of 208 putative *Mlo* gene copies from 35 plant species suggests a large number of duplications of this gene family prior to the divergence of monocots and eudicots, early in eudicot diversification. Our results indicate that the *Mlo*-based resistance mechanism is functional in Rosaceae, and that *Fragaria* can be used as a host to test mechanistic function of genes derived from related tree species. To the best of our knowledge, this work is one of the first attempts at testing the potential of using a *Mlo*-based resistance strategy to combat powdery mildew in Rosaceae.

Keywords Rosaceae · Powdery mildew · Peach · *Fragaria* · Mildew locus O

Introduction

Powdery mildews represent one of the major plant pathogen that infects all tissue types in over 10,000 plant species (Takamatsu et al. 2010; Glawe 2008). Frequent and repeated application of sulfur-based fungicides is used to combat powdery mildew, increasing the cost of crop production with potentially harmful impact on the environment. Most strategies to develop powdery mildew resistant crops have relied on

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D. Jiwan · D. Main · A. Dhingra (✉)
Department of Horticulture, Washington State University, PO Box 646414, Pullman, WA 99164, USA
e-mail: adhingra@wsu.edu

D. Main
e-mail: dorrie@wsu.edu

E. H. Roalson
School of Biological Sciences, Washington State University, PO Box 644236, Pullman, WA 99164, USA

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the gene-for-gene resistance approach which is dependent on the identification of a resistance gene (R) in the host specific to a corresponding avirulence gene (Avr) in the pathogen (Flor 1971). Major classes of R genes contain an N-terminal nucleotide binding site and C-terminal leucine-rich repeat (LRR) of various lengths. Another class is comprised of genes containing a kinase and/or a LRR domain (Tor et al. 2009). In almost all cases, R gene-mediated resistance leads to localized cell death in the host, which has a critical role in containment of the pathogen (Staskawicz et al. 1995; Zhou et al. 1995). Structural similarities in R gene-derived proteins in different plant species indicate the existence of a common downstream biochemical defense mechanism. Although these mechanisms remain unclear, localized cell death at the site of infection, known as the hypersensitive response (HR), accompanies many incompatible race-specific interactions (Staskawicz et al. 1995). This type of resistance is genetically dominant in nature and can be rapidly introgressed into new crops via molecular marker-assisted breeding.

Besides the aforementioned approach, plants are known to combat disease through other novel mechanisms that have been identified in mutants spread across several plant species. These mutants either exhibit resistance to single or multiple classes of pathogens. Some of the well documented examples include a loss of function mutation in the Mildew locus O (*Mlo*) gene in barley conferring broad spectrum resistance to powdery mildew (*Blumeria graminis* f. sp. Hordei) as well as the *sl* mutant (Sekiguchi lesion) in rice conferring resistance to the rice blast fungus, *Magnaporthe grisea* (Jorgensen 1992; Yin et al. 2000). The powdery mildew resistant (pmr) 1–4 mutants in *Arabidopsis* abolish the growth of *Erysiphe cichoracearum* (Vogel and Somerville 2000). Additionally, in Arabidopsis, *edr1* and *lsd1* mutations confer resistance to bacterial and fungal pathogens respectively (Rusterucci et al. 2001). Several such gene targets have been reviewed recently (Pavan et al. 2010). It has been proposed that such a mechanism of resistance should be integrated into developing durable and broad-spectrum resistance strategies.

In plants, spontaneous mutations that alter gene function are either lethal or remain recessive unless paired with a recessive allele in a heterozygous situation or in a genetically homozygous recessive state. Thus, such mutations are difficult to detect. The mutation within *Mlo* in barley is one such example where natural barley populations were found to possess resistance to multiple races of *Blumeria graminis* f. sp. Hordei (Jorgensen 1992). *Mlo* codes for a novel plant-specific membrane-integrated protein with one or more hitherto unknown biochemical functions (Devoto et al. 2003; 1999). However, it is known that in nature it acts as a negative regulator of a plant's defense response. In barley and Arabidopsis, homozygous recessive mutant *mlo* alleles confer broad spectrum resistance to the biotrophic powdery mildew fungus, *Blumeria graminis* f. sp. *Hordei* and *Golovinomyces orontii* respectively (Buschges et al. 1997; Consonni et al. 2006). In this genetic background, the fungus is unable to penetrate the epidermal cell wall, thus rendering the host resistant to the pathogen. The interaction, or the lack thereof, is mediated by cell wall remodeling and oxidative cross-linking processes fortifying the cell wall at the site of entry (Thordal Christensen et al. 1997; Huckelhoven et al. 1999). Although cell wall reinforcement is likely to contribute to the resistant phenotype, knowledge of all molecular events leading to cessation of the fungal attack remains elusive. In *A. thaliana*, it was shown that the process operates independent of ethylene, jasmonic acid, and salicylic acid but requires syntaxin, glycosyl hydrolase, and an ABC transporter (Consonni et al. 2006). It is thought that isoforms of the MLO protein modulate vesicle-associated defense responses at the cell periphery and that the powdery mildew pathogen possibly exploits these proteins for successful host cell entry (Panstruga 2005).

The exact biochemical mechanism by which MLO operates remains to be determined. However, recent data has shown that MLO-mediated defense suppression in barley functions independently of heterotrimeric G-proteins and that calmodulin interacts with MLO to dampen defense reactions against the powdery mildew fungus (Kim et al. 2002). In addition, a small GTP-binding protein of the barley RAC family (RACB) may play a role in MLO-mediated powdery mildew compatibility (Schultheiss et al. 2002).

Barley *mlo* mutants have been known for more than 60 years and have been successfully employed in European barley agriculture for more than 25 years (Lyngkjaer and Carver 2000), emphasizing the principal durability of *mlo*-mediated disease resistance under agricultural conditions. This makes *Mlo* an important target gene to characterize for its use in economically important crops.

Results from previous phylogenetic studies support an early evolutionary diversification of the *Mlo* subgroups well before the diversion of monocots and dicots (Devoto et al. 2003). MLO homologs from *Hordeum vulgare*, *Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Arabidopsis thaliana*, *Brassica rapa*, *Citrullus lanatus*, *Glycine max*, *Gossypium hirsutum*, *Linum usatissimum*, *Lotus japonicus*, *Solanum esculentum*, *Medicago truncatula*, *Solanum tuberosum*, and *Sorghum bicolor* were identified and placed in five clades (Devoto et al. 2003). Clade IV contained only monocot homologs, while clade V represented only dicot members. Highly divergent homologs from Arabidopsis and *Zea mays* comprise the remaining phylogenetic clades. Maintenance of these clades when other plant MLOs were added to the analysis, suggests preservation of an early functional diversification. Further phylogenetic analysis using 17 members of the *V. vinefera Mlo* gene family (*VvMlo*) and members from rice, barley, tomato, maize, wheat and Arabidopsis, resulted in the formation of six clades with clade VI containing AtMLO3, which in previous analyses was identified as a single divergent lineage (Feechan et al. 2008).

In this study, we have functionally tested a *Mlo* gene from Peach (*PpMlo1*) as a first step towards understanding its function in Rosaceae. We demonstrate that antisense expression of *PpMlo1* in transgenic strawberry plants, which mimics the loss of function mutation reported in other plants, confers resistance to *Fragaria*-specific powdery mildew, *Podosphaera macularis*. Our results indicate that silencing of *Mlo* is potentially a viable strategy in imparting resistance to powdery mildew within Rosaceae, and that *Fragaria* can be used as a host to test function of genes derived from related tree species. Further, we have reconstructed the phylogenetic tree to gain an insight into the evolution of Rosaceae MLO genes and their relationship with previously reported members of this family in other plant species.

Experimental procedures

Identification and sequencing of the *Mlo* gene

Based on the region in linkage group 2 of the TxE linkage map where a QTL for powdery mildew resistance has been previously mapped (Lalli et al. 2005), we identified clone 058P54 from the peach rootstock Nemared BAC library (Georgi et al. 2002) containing the QTL of interest. Sequence for this BAC was obtained using the ABI3730xl DNA Analyzer at the Clemson University Genomic Institute (CUGI). Evaluation of sequence data, assembly of contigs and prediction of open reading frames was done using custom scripts, PHRED and PHRAP with manual editing using CONSED (Ewing et al. 1998; Gordon et al. 1998). Homology searches of the BAC sequences were carried out against the non-redundant GenBank protein database, using the BLASTX program at the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov). Sequences were manually annotated using gene, protein and motif prediction programs including FGENESH (Salamov and Solovyev 2000), GeneMark.hmm (Lukashin and Borodovsky 1998), GENSCAN (version 1.0) (Burge and Karlin 1997), SMART (Schultz et al. 1998) and InterProScan (Quevillon et al. 2005).

Cloning of *PpMlo1* cDNA and construction of plant transformation vectors

All recombinant DNA manipulations were performed in *Escherichia coli* strain XL-1 Blue MRF'. The DNA plasmid constructs described in this work were confirmed by restriction digestion analysis and subsequent DNA sequencing. Sequence of all oligonucleotides used in the study is listed in Table 1. Based on homology with barley and *Arabidopsis Mlo* genes, a partial coding region of *PpMlo1* gene was identified from the sequence of Peach BAC clone number 058P54. To obtain the upstream 5′ region of *PpMlo1* cDNA, 5′-RACE (rapid amplification of cDNA ends) was carried out using a cDNA amplification kit (CLONTECH) according to the manufacturer's instructions. The 5′ region of *PpMlo1* was amplified from cDNA synthesized from mRNA of peach fruit using a *PpMlo1* specific oligonucleotide DAJP1 (Table 1) and an adaptor primer from CLONTECH. The amplified product was sub-cloned into the *EcoRI* site of pBluescript II SK (Invitrogen) and sequenced. Full-length *Mlo* cDNA was PCR amplified from peach fruit cDNA using gene specific primers DAJP2 and DAJP3 (Table 1) and *Pfu* Turbo proofreading DNA polymerase (Stratagene, CA). The cDNA was cloned into pCR 2.1-TOPO plasmid vector as per the manufacturer's instructions and confirmed by sequencing.

Table 1 List of Oligonucleotides used in this study

Primer	Sequence	Experiment
DAJP1	GCCCTAAACCCATGGTGATGACGC	Gene specific primer for 5′ RACE
DAJP2	GGTGAATTCATGGCAGCATCAGAG	Forward primer—full length PpMlo1 amplification
DAJP3	ATGATATCATCATGTTTCTTTATCCGG	Reverse primer—full length PpMlo1 amplification
DAJP4	GTGTGGCCATGGCAGCATCAGAGAG	Forward primer with *Nco*I site
DAJP5	ATCTGTTCTCTAGATTTCTTTATCCGGGG	Reverse primer with *Xba*I site
DAJP6	ACAGTCGAGGGATCTAGACCG	Forward primer with *Xba*I site
DAJP7	TTTGCCATGGGTCTTGCGG	Reverse primer with *Nco*I site
DAJP8	ACAGCCGGAACACGGCGGCATCAGAG	Forward primer: anneals to *npt*II in sense and antisense construct
DAJP9	TCTCCAGAGCTTCAAACAGAGCTCTC	Reverse primer: anneals to PpMlo1 in sense construct
DAJP10	AAAGTTGGAACGTTCCTCACGGATAGGC	Reverse primer: anneals to PpMlo1 in antisense construct
DAJP11	TTTGCACGCGAATGCGCTCCATCCG	Forward primer: *virB1* gene
DAJP12	TAATGCTCGTCGTCAGCCGTGTTGCCG	Reverse primer: *virB1* gene
DAJP13	AAAGTTGGAACGTTCCTCACGGATAGGC	Forward primer: *PpMlo1* expression
DAJP14	AAAATGAAGAACCAACTGAGGCCG	Reverse primer: *PpMlo1* expression

For sense orientation, the 5′ end of the *Mlo* gene was modified by changing the sequence immediately 5′ to the translation initiator ATG codon to an *NcoI* restriction site and the stop codon was changed to include an *XbaI* restriction site using DAJP4 and DAJP5 oligonucleotides (Table 1). PCR was performed using *Pfu* Turbo proofreading DNA polymerase. For antisense orientation, specific primers were used to introduce an *NcoI* site at the 3′end of the gene and an *Xba*I site at the 5′end of the gene using primers DAJP6 and DAJP7 (Table 1) respectively. The sense and antisense *PpMlo* amplicons were digested with *Nco*I/*Xba*I and used to replace smGFP in pAD 120 derived from pAVA121 containing a double CaMV35S promoter, Tobacco Etch virus translational enhancer and Nopaline synthase (Nos) terminator (von Arnim et al. 1998) to obtain plasmid pDAJ2. The entire expression cassette including the 35S promoter-Enhancer-*Mlo* (sense/antisense)-TNos was released from pDAJ2 using *HindIII* and cloned in *HindIII* digested pCAMBIA 2300 to obtain pDAJ3 and pDAJ4 binary transformation vectors.

Fragaria plant growth and propagation

Axenic cultures of *Fragaria x ananassa* LF9 used in this study were kindly provided by Dr. Kevin Folta (University of Florida, USA). Plants were propagated in MS media (Murashige and Skoog 1962) as previously described (Folta et al. 2006) and maintained at 24 °C with a 16/8 h light/dark photoperiod.

Agrobacterium-mediated transformation and regeneration of transgenic plants

Agrobacterium strain AGL0 carrying pDAJ3 or pDAJ4 binary plant transformation vectors was used for co-cultivation with leaf explants derived from LF9 plants. In this study, pre-conditioned leaf explants were used for *Agrobacterium* co-cultivation. Preconditioning involved incubation of explant material on shoot regeneration medium (SRM) for 2 weeks in dark prior to co-cultivation. This pre-treatment of leaf explants resulted in formation of meristematic primordia that are presumed to readily receive foreign DNA. Explants were co-cultivated with bacterial suspension (OD $600 = 0.8$) which also includes acetosyringone (50 μM) and incubated in the dark for 2 h and occasionally swirled. Explants were placed on SRM plates and incubated for 48 h at 24 °C in the dark as described earlier (Folta et al. 2006). Explants were rinsed with sterile water and subsequently washed in 2 MS + 500 mg/L Timentin once. Explants were incubated in antibiotic solution for

10–20 min, blotted on sterile paper to remove excess liquid and then placed on SRM supplemented with 2.5 μg/ml Kanamycin. These plates were incubated at 22 °C with a 16/8 h light/dark photoperiod under low light (~25 μmol/m^2/s) for 10 days. Transgenic plants were sub-cultured and subsequently selected on an increasing concentration of antibiotic. The following concentrations were used for selection: 5.0 μg/ml (one round), 7.5 μg/ml (four rounds) and 10 μg/ml (four rounds). Each round constituted 10 days after which the explants were sub-cultured onto fresh medium. After molecular analysis, transgenic plants were moved into soil and maintained in the greenhouse at 24 °C with a 16/8 h light/dark photoperiod.

Molecular analysis of transgenic plants

Total cellular DNA or RNA was isolated from leaf tissue using Qiagen DNeasy or RNeasy kits respectively following manufacturer's protocols. For reverse transcription PCR (RT-PCR), cDNA was synthesized directly from RNA using SuperScriptII reverse transcriptase (Invitrogen, CA). PCR was used to check for the presence of sense and antisense expression cassettes in the strawberry genome using primers annealing to *nptII* and *PpMlo1* genes. Primers used for this test were: DAJP8 and DAJP9 in the plants transformed with pDAJ3 and, DAJP8 and DAJP10 in plants transformed with pDAJ4. PCR was performed with annealing at 69 °C to ensure stringent PCR conditions. Reverse transcription PCR was performed to check for the expression of the transgene using *PpMlo*-specific primers DAJP12 and DAJP13 (Table 1; Fig. 3).

Pathogen challenge and analysis

Fragaria-virulent powdery mildew, *Podosphaera macularis,* conidia were obtained from infected strawberry plants provided by Dr. Patrick Moore (Washington State University). Infected plants were maintained in a separate greenhouse at 22 °C 16/8 h photoperiod under ambient humidity. Conidia were brushed onto three transgenic plants and one wild type control plant grown under disease-free conditions as described previously (Attanayake et al. 2010). Plants were incubated in an incubator at 22 °C 16/8 h photoperiod under low light (25 μmol/m^2/s). Leaf samples were observed 3 dpi (days post inoculation) and 7 dpi. Inoculated leaves were analyzed under bright field microscope and photographed. The pathogen challenge experiment was repeated three times with similar results. Quantification of infected leaf area was performed once on three different leaves from each of the three sense and antisense lines. Infected area of the leaf was quantified using ImageJ (Collins 2007), an image processing program developed by NIH, as spread of the pathogen is assumed to be directly related to the susceptibility of the plant.

Phylogenetic analysis

As many *Mlo* or putitive *Mlo* sequences from land plants were included as possible, as assessed by BLASTing known *Mlo* sequences against GenBank. Accessions included in these analyses are listed in Supplementary material Section 1. Outgroups were chosen from putative *Mlo* sequences from the algae *Chlamydomonas reinhardii* and *Ostreococcus lucimarinus*. Sequences were aligned using MUSCLE (Edgar 2004). Aligned sequences were analyzed using MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Ten million generations were run with four chains (Markov Chain Monte Carlo), the heating parameter set at 0.05, and a tree was saved every 1,000 generations. Priors for all analyses included the mixed amino acid model implementing a covarion model, as applied in MrBayes. The covarion model allows for rates to change across the topology (Galtier 2001; Huelsenbeck et al. 2002; Tuffley and Steel 1998). In order to test for the occurrence of stationarity, convergence, and mixing within 10 million generations, multiple analyses were started from different random locations in tree space. The posterior probability distributions from these separate replicates were compared for convergence to the same posterior probabilities across branches. Majority rule consensus trees of those sampled in Bayesian inference analyses yielded probabilities that the clades are monophyletic (Lewis 2001). Discarding the trees generated within the first 2,500,000 generations (those sampled during the ''burnin" of the chain (Huelsenbeck and Ronquist 2001), to only include trees after stationarity was established. Posterior probability values (pp) are presented on a consensus tree averaged from the post-stationarity distribution of Bayesian trees in order to demonstrate average branch lengths across the distribution of trees.

Comparison of dN/dS Calculations of the ratio of synonymous to nonsynonymous substitutions were performed using the SNAP program (Korber 2000) (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html), which calculates synonymous and non-synonymous substitution rates based on a set of aligned codons. For this analysis, we used the external and internal loops I, II and III including the region which has the conserved cysteine residues. These were used to assess if components of these loops were directly responsible for the formation of the new clade in the analysis performed in this study. For comparison of dN/dS ratios, we used the sequences of *PpMlo*, *HvMlo*, *AtMlo2*, *AtMlo6*, *AtMlo12*, *OsMlo1*, *ZmMlo1*, *VvMlo3* and *VvMlo6* (Feechan et al. 2008; Buschges et al. 1997; Consonni et al. 2006). These were selected due to the availability of complete sequence and functional evidence of their involvement in the disease resistance pathway. *AtMlo5*, *AtMlo7*, *AtMlo8*, *AtMlo9*, *AtMlo10*, *VvMlo2* and *VvMlo8* were also used for analysis as they belong to the same clade as *PpMlo1* and the members of this clade have not been well characterized. The nucleotide sequences corresponding to the amino acid residues of domains mentioned above were aligned using ClustalW (Thompson et al. 1994). Thereafter, the SNAP program was used to calculate dN/dS ratios for each comparison in a multiple alignment and an average dN/dS ratio for each region was used for comparisons.

Results and discussion

Characterization of *MloI* from peach and construction of transformation vectors

PpMloI exons were identified from the sequence of peach root BAC 058P54 by sequence similarity searches with barley and Arabidopsis homologs. Amino acid sequence comparison of *PpMloI* with *AtMlo2*, previously reported to be involved in powdery mildew resistance (Consonni et al. 2006), demonstrated a high degree of similarity between the two genes at the amino acid level thereby indicating a possible involvement of *PpMloI* in powdery mildew resistance. At the nucleotide sequence level the two genes were highly dissimilar obviating the possibility of using *AtMLO2* as a control in this study. Full-length *PpMloI* was obtained via a sequential series of RT-PCR and 5′ RACE PCR steps and cloned in sense and antisense orientation into a binary vector to obtain the plant transformation vectors pDAJ3 and pDAJ4 (Fig. 1a, b).

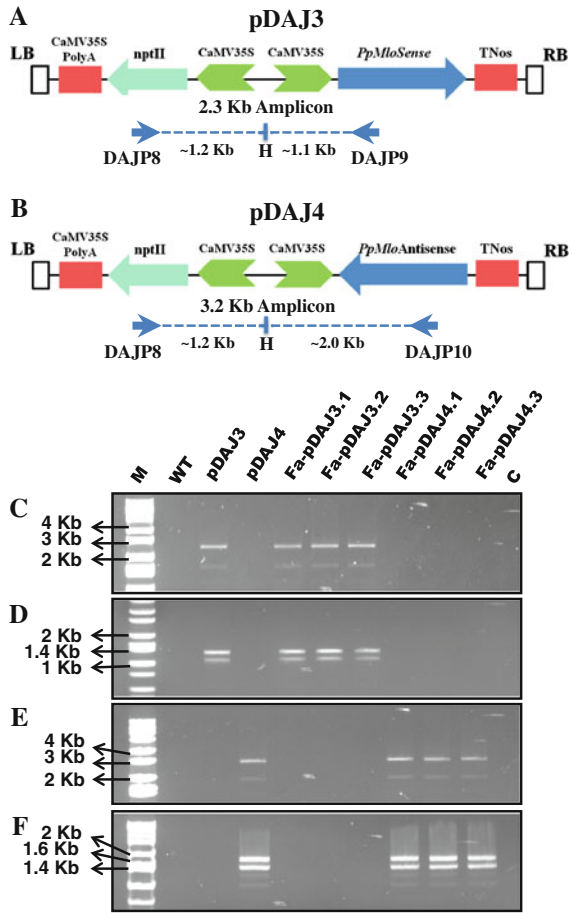

Fig. 1 Strawberry transformation for sense and antisense expression of *PpMlo1*. **a** and **b** Schematic representation of plant transformation cassettes expressing *PpMlo* in sense or antisense orientation. Primers used for molecular analysis are shown along with expected amplicon sizes. DAJP8, DAJP9 and DAJP10 anneal to *npt*II, PpMloSense (pDAJ3) and PpMloAntisense (pDAJ4) regions respectively. **c** An expected 2.313 kb amplicon is visible in pDAJ3 and corresponding transgenic lines Fa-pDAJ3.1–3.3. **d** Restriction digestion of the 2.313 kb amplicon with *Hin*dIII generates expected fragments of 1.227 and 1.086 kb. **e** An expected amplicon of 3.236 kb is visible in pDAJ4 and corresponding transgenic lines Fa-pDAJ4.1–4.3. **f** Restriction digestion of the 3.236 kb amplicon with *Hin*dIII generates expected fragments of 1.227 and 2.009 kb. *Lane* marked as *M* represents DNA ladder

Strawberry transformation for sense and antisense expression of *PpMlo1*

The *Fragaria x ananassa* LF9 line was transformed with pDAJ3 (*Mlo* expressed in sense orientation, Fig. 1a) and pDAJ4 (*Mlo* expressed in antisense orientation, Fig. 1b) respectively as described earlier (Folta et al. 2006). Agrobacterium co-cultivation was performed with young emerging leaves obtained from tissue culture grown plants. Four leaves were divided into 4 sections thereby generating 16 explants for each construct used. After the final round of selection on SRM supplemented with 10 μg/ml of kanamycin 8 transgenic events for pDAJ3 and 9 transgenic events for pDAJ4 were recovered. Three lines each of Fa-pDAJ3 and Fa-pDAJ4 (Fa—*Fragaria x ananassa*) were selected for subsequent molecular analyses and bioassays. We found that increasing the concentration of kanamycin progressively yielded better results than transferring explants directly to a high concentration of kanamycin in the selection medium which resulted in browning of explants and ultimate loss of the explant.

Rooted transgenic plants were transferred to soil and maintained in the greenhouse as described in "Experimental procedures". Both sense and antisense transgenic lines established well in soil but the plants expressing *PpMlo1* eventually perished due to natural powdery mildew infection. The plants expressing antisense *PpMlo1* continued to propagate vegetatively and flower.

PpMlo1 was used to perform discontiguous Mega-BLAST against predicted genes from *Fragaria* (Shulaev et al. 2011). One of the homologs (Fv_gene12974), which we have named as *FaMlo1* demonstrated 89 % nucleotide identity and an e-value of 0. A varying degree of percentage identities and e-values were observed with other *Mlo* homologs (Table 2). One future strategy could be to use 5′ or 3′ UTRs (untranslated regions) of *Mlo* homologs to specifically silence a given gene. The strategy used in this work was limited to first testing the concept of suppressing *Mlo* in a heterologous host and its impact on powdery mildew resistance. Further, we opted for an antisense, rather than RNAi approach in this study. The rationale for this was to avoid any other pleiotropic issues that may result from complete silencing of any given *Mlo* transcript. Since this gene family is conserved within the plant kingdom and some recent reports are unraveling functional information regarding the *Mlo* gene family (Chen et al. 2009), we considered it prudent to proceed with an antisense approach.

Table 2 Results of discontiguous megablast comparing *PpMlo1* with predicted genes in the Fragaria genome

Predicted genes in Fragaria	% Identity	e value
Gene12974	89	0
Gene09621	88	2.00E−15
Gene09623	72	2.00E−15
Gene28466	70	3.00E−14
Gene31395	69	6.00E−66
Gene31405	67	1.00E−19
Gene02764	64	4.00E−26

Molecular analysis of transgenic *Fragaria x ananassa* (LF9)

A PCR-based approach was used to rapidly screen for the presence of sense and antisense *PpMlo1* transgene cassettes in strawberry. Primers annealing to *npt*II gene (DAJP8) and *PpMlo1* in sense (DAJP9) or antisense (DAJP10) orientation were used to amplify respective amplicons (Fig. 1a, b). As shown in Fig. 1c, an expected amplicon of 2.313 kb is amplified in the lanes labeled as pDAJ3 (plasmid DNA as control) and Fa-pDAJ3.1 - 3.3. The amplicon is absent in WT (wild type control) and lanes labeled as pDAJ4 and Fa-pDAJ4.1 - 4.3 indicating specific amplification only in samples with the sense orientation of the *PpMlo1* cassette. A unique *Hin*dIII restriction site between the two divergent CaMV35S promoters was used for further diagnosis. Restriction digestion of the amplicon with *Hin*dIII resulted in the expected fragments of 1.227 and 1.086 kb (Fig. 1d). Similarly, an expected amplicon of 3,236 bp was amplified and can be visualized in lanes labeled as pDAJ4 (plasmid DNA as control) and Fa-pDAJ4.1 - 4.3 (Fig. 1e). The amplicon is absent in WT (wild type control) and lanes labeled as pDAJ3 and Fa-pDAJ3.1 - 3.3 indicating specific amplification only in samples with the antisense orientation of the *PpMlo1* cassette. Restriction digestion analysis of the amplicon in this case yielded the expected fragments of 1,227 and 2,009 bp (Fig. 1f). The sizes of amplicons and restriction digest fragments were calculated based on sequence analysis of the plasmid constructs. The transgenic plants were

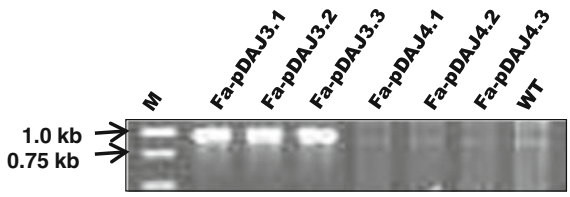

Fig. 2 Expression analysis of transgenic strawberry plants. Reverse transcription PCR with primers DAJP13 and DAJP14 show expression of *PpMlo1* in Fa-pDAJ3.1–3.3. Note the absence of a corresponding amplicon in Fa-pDAJ4.1–4.3 lines. *Lane* marked as *M* represents DNA ladder

further confirmed for lack of Agrobacterium contamination using PCR and insertion of the transgene using DNA gel blot analysis (see Supplementary material Section 2 and 3).

Analysis of *PpMlo1* expression in transgenic lines

After confirming integration of the transgenic cassette, reverse transcription PCR (RT-PCR) was used to test for the expression of *PpMlo1* in the transgenic lines. RT-PCR performed with primers DAJP13 and DAJP14 yielded an expected fragment of 816 bp in Fa-pDAJ3.1 - 3.3 lines expressing *PpMlo1* in sense orientation. The corresponding fragment was missing in wild type control and Fa-pDAJ4.1 - 4.3 lines expressing *PpMlo1* in antisense orientation (Fig. 2).

Pathogen challenge and molecular analysis

The aim of this study was to test if silencing of the *Mlo* gene can be used as a potential strategy to combat the scourge of powdery mildew within Rosaceae. The litmus test for this approach would be to obtain resistance or tolerance to a strawberry-specific pathogen in plants silenced for a corresponding *Mlo* expression using the *PpMlo1* gene. It is important to note that MLO-based resistance is known to be a pre-invasive resistance that terminates fungal colonization attempts before host cell penetration (Humphry et al. 2011). Therefore, if MLO-based resistance is present, phenotyping for disease or lack thereof would be straightforward.

Conidia of *Podosphaera macularis* (*Fragaria*-specific powdery mildew formerly called *Sphaeotheca* macularis) were collected using a thin paintbrush sterilized in 90 % alcohol and brushed on to transgenic lines expressing *PpMlo1* in sense and antisense orientation under disease-free conditions. The conidia of *Podosphaera macularis* burst when suspended in water therefore necessitating this mode of inoculation (Peries 1962; Attanayake et al. 2010). Inoculated transgenic plants were screened for infection 3 days post inoculation, which is sufficient time for spore germination and mycelia growth. A second set of observations to measure mycelia growth were recorded after 7 days post inoculation. Transgenic lines Fa-pDAJ3.1–3.3 expressing *PpMlo1* in sense orientation demonstrated a larger degree of infection compared to the wild type. Over 60 % of the leaf area was covered with fungal mycelia (Fig. 3a, b) as determined by ImageJ (Collins 2007). A similar phenotype is most likely expected if a closely related homolog of *PpMLO1* from *Fragaria* (*Fragaria vesca* predicted gene: Fv_p 12974—see Fig. 4b) is overexpressed in *Fragaria*. In the wild type plant, approximately 25 % of the leaf area was infected with the mycelia. Transgenic plants Fa-pDAJ4.1–4.3 expressing *PpMlo1* gene in an antisense orientation showed less than 1 % of leaf area being infected with the mycelia (Fig. 3b). These lines demonstrated the classical pre-invasive resistance that is a characteristic feature of MLO-based resistance to powdery mildew. The wild type plant used in this study is LF9 which was derived from Strawberry Festival, a commercial strawberry cultivar that is moderately resistant to powdery mildew and was especially bred for efficient performance in reverse genetics studies focused on Rosaceae crops (Folta et al. 2006).

Leaf tissue collected from control and pathogen challenged plants at 3 dpi was processed to obtain RNA and reverse transcription PCR was performed with DAJP 13 and DAJP 14 primer set to evaluate expression of *PpMloI*. As in Fig. 2, no *PpMloI* expression was observed in the antisense lines. However, it was noteworthy to observe amplification of an expected sized fragment in WT sample challenged with powdery mildew which is most likely a *Fragaria Mlo* homolog (WT-PM; Fig. 3c) as supported by sequence identity analysis of the primer set with all the *Fragaria vesca* MLO homologs (Supplementary material Section 4). Enhanced expression of *Mlo* in the presence of a powdery mildew pathogen is consistent with the *Mlo*-based plant pathogen interaction model (Consonni et al. 2006). There was no

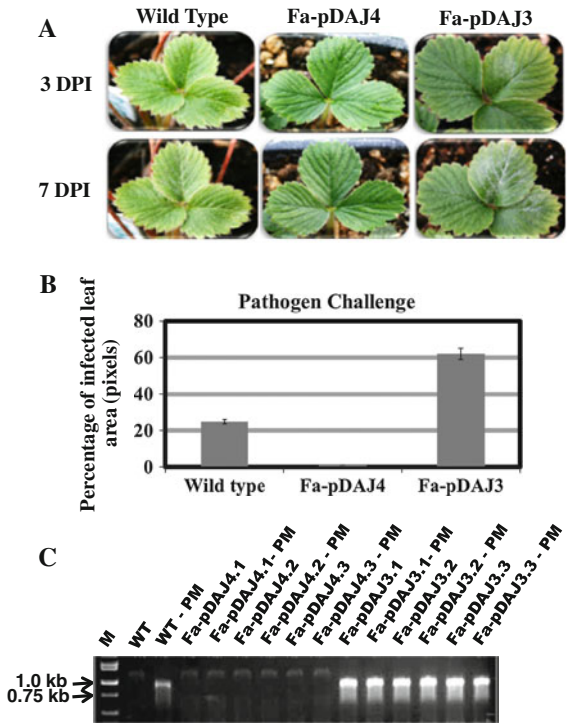

Fig. 3 Pathogen challenge. **a** Pathogen Challenge: Bioassay for pathogenesis on wild type, Fa-pDAJ3 and Fa-pDAJ4 lines. Note extensive mycelia proliferation on Fa-pDAJ3 leaf. **b** The *graph* shows area of infected leaf. Nearly 60 % of the Fa-pDAJ3.1–3.3 leaves had mycelia spread as measured using Image J. **c** Expression analysis of *PpMlo1* gene in transgenic and wild type plants upon pathogen challenge. *Lane* marked as *M* represents DNA ladder

detectable expression of *PpMlo1* or any *Fragaria* MLO homolog in Fa-pDAJ4.1-4.3 lines upon powdery mildew challenge. However, Fa-pDAJ3.1-3.3 lines expressed PpMlo1 constitutively with or without powdery mildew challenge (Fig. 3c). Thus, the gene expression results correspond to the resistant phenotype observed in response to pathogen challenge. These observations imply that an MLO-based resistance mechanism is conserved in *Fragaria*.

Phylogenetic analysis

Multiple homologs of the *Mlo* gene have been reported in all land plants that have been assessed. The actual number of copies found in each species varies, but as most species have not been exhaustively sampled, it is not entirely clear whether the assumed variation in *Mlo* copy number is best attributed to duplications, deletions, or not yet finding all of the copies of the *Mlo* gene family within some genomes. In total, 208 putative *Mlo* gene copies were analyzed for 35 species (including one hybrid). The consensus Bayesian phylogeny is presented in Fig. 4a, b. Previously identified clades I–VI (Feechan et al. 2008) are also found here and are noted on Fig. 4a, and b. Also noted on the figures are clades that are eudicot specific (green stars) and monocot specific (orange stars). In most cases, the previously designated clades define gene lineages that predate the divergence of monocots and eudicots, with the exception of clade V which is dicot specific, but includes at least two and possibly three duplication events early in eudicot diversification, and clade VI which is eudicot specific and includes only one eudicot copy, although some species within the eudicots apparently have had additional duplications. It is clear that the previously identified clades do not correspond to a particular time interval.

While Rosaceae genomes have not been exhaustively searched for *Mlo* gene copies, it is clear that of the eight eudicot-specific gene lineages only six have been found to include Rosaceae *Mlo* genes (Fig. 4a, b). That being said, however, there is strong support for at least 11 or 12 paralogs in the Rosaceae, some of which have been found in multiple species and others that are represented by only one species. It should also be noted that two previously published sequences of *Mlo* genes from *Prunus americana* (*PaMlo1*, *PaMlo2*) seem quite different from other *Mlo* genes, as can be noted by the very long branches leading to these gene copies (Fig. 4b). This is particularly true of *PaMlo1*, which does not group with any other *Mlo* clade and is weakly placed as the sister group to clade III. In all, the phylogenetic hypothesis presented here suggests a large number of duplications of this gene family prior to the divergence of monocots and eudicots, early in eudicot diversification, and at more shallow phylogenetic depths (Fig. 4a, b).

We have also here included for the first time samples from the moss *Physcomitrella patens* subsp. *patens* and the lycophyte *Selaginella moellendorffii*.

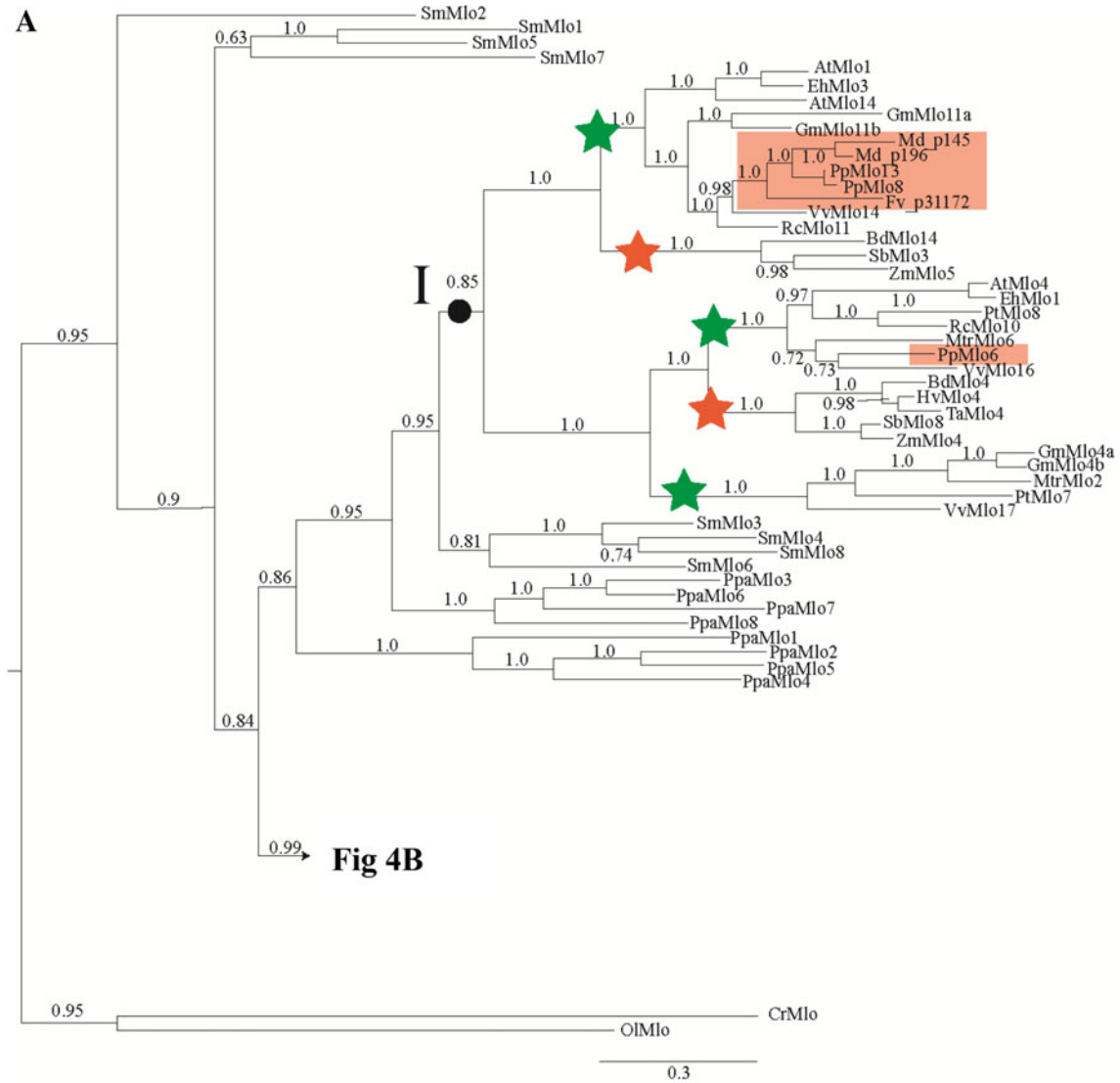

Fig. 4 Phylogenetic relationship of MLO isoforms. Bayesian phylogenetic hypothesis for the *Mlo* gene family. The figure represents a consensus tree averaged from the post-stationarity distribution of Bayesian trees in order to demonstrate average branch lengths across the distribution of trees. Posterior probabilities are noted on branches where greater than 0.5. *Black dots* and *Roman numerals* represent previously described clades (Feechan et al. 2008). *Green stars* note eudicot clades and *orange stars* note monocot clades. *Pink boxes* mark Rosaceae *Mlo* clades. Species abbreviations follow Supplementary material Section 1. **a** Outgroups and early branching events of the *Mlo* gene family. **b** The rest of the *Mlo* phylogeny. (Color figure online)

Samples from both of these species form multiple clades at the base of the tree (Fig. 4a). Interestingly, successive clades of the moss and lycophyte are sister to clade I, suggesting that these might be the orthologous copies of the angiosperm clade I copies. It should be noted, however, that each of the moss and lycophyte clades include four putative paralogs each and the angiosperm clade includes three eudicot and two monocot clades, so any inference of functional similarity has to be tentative. As more species from critical clades of land plants are added (e.g. monocots other than grasses), a better understanding of the patterns of gene duplication and loss will be possible.

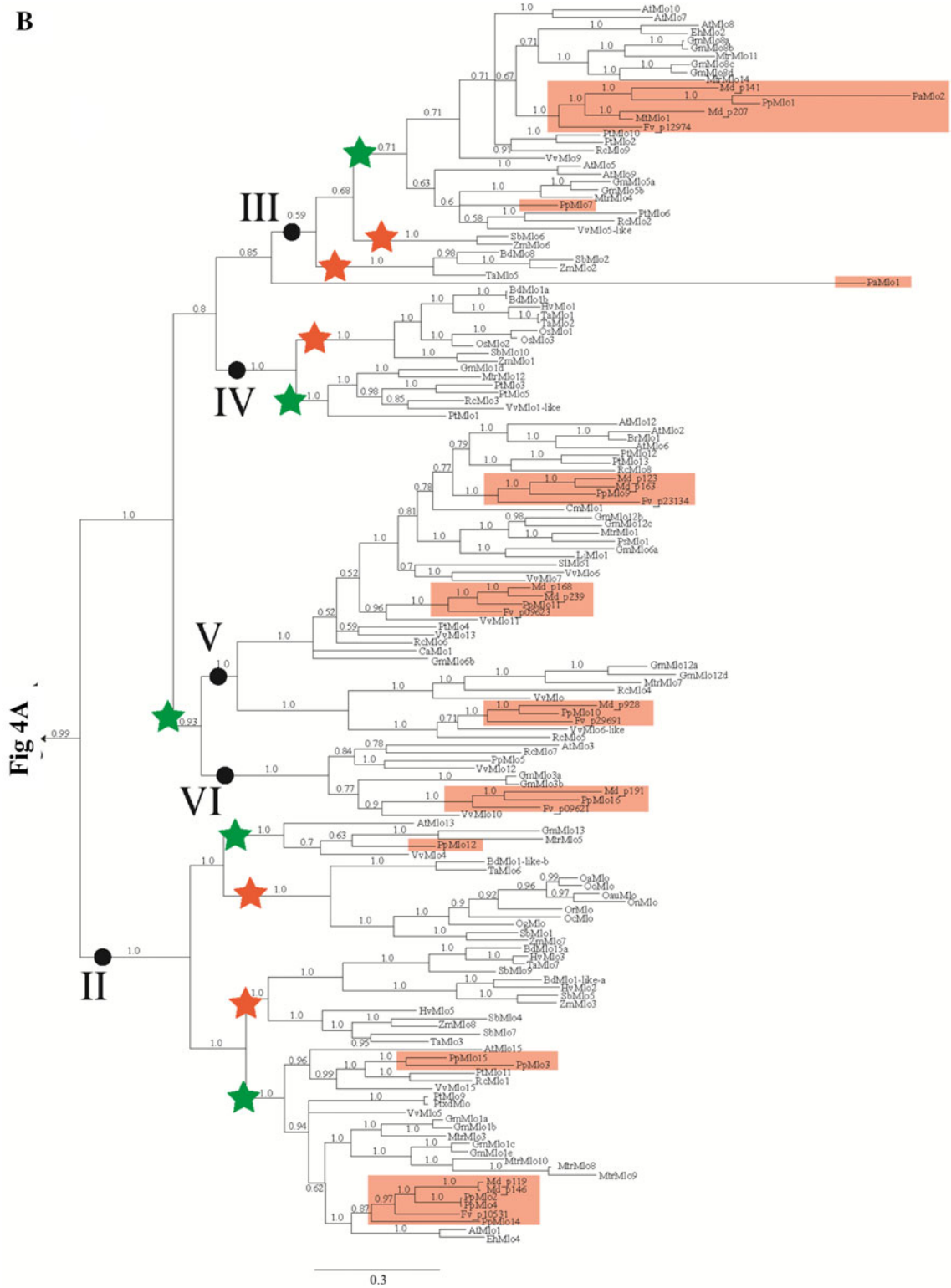

Fig. 4 continued

Conclusions

In conclusion, results obtained from the pathogen challenge experiment lead us to infer that antisense repression of *Fragaria Mlo* homolog with *PpMlo1* gene results in conferring resistance to *Fragaria*-specific powdery mildew pathogen. This further indicates that the strategy of rendering the *Mlo* homolog partially non-functional via a transgenic approach or mutagenesis will potentially be a viable strategy to counter powdery mildew within Rosaceae. To the best of our knowledge, this work is one of the first attempts at testing the potential of using a *Mlo*-based resistance strategy to combat powdery mildew in Rosaceae. Also, in cases where there are mechanistic overlaps, *Fragaria* could be a worthy taxonomic surrogate to test function of genes derived from related tree species as proposed by the Rosaceae community (Shulaev et al. 2008). Identification of gene(s) involved in conferring durable resistance to powdery mildew and the development of cultivars containing these genes is expected to contribute to an environmentally sustainable solution for the tree fruit industry.

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