Identification and expression analysis of Fragaria vesca MLO genes involved in interaction with powdery mildew (Podosphaera aphanis)

OPEN ACCESS
Freely Available Online

JOURNAL OF ADVANCES IN PLANT BIOLOGY
ISSN NO: Coming Soon

RESEARCH

DOI : Coming Soon

Identification and expression analysis of *Fragaria vesca MLO* genes involved in interaction with powdery mildew (*Podosphaera aphanis*)

Shridhar Jambagi[1, 2 *] and Jim M. Dunwell[1, 2]

[1]School of Biological Sciences, University of Reading, Whiteknights, Reading, RG6 6AS, UK

[2]School of Agriculture, Policy and Development, Earley Gate, University of Reading, Reading, RG6 6AR, UK

Abstract

Strawberry powdery mildew, caused by *Podosphaera aphanis* is a major fungal disease that affects strawberry yield and quality. In the model plant species *Arabidopsis* and the crop plants barley, tomato and pea, the Mildew resistance locus O (MLO) proteins have been found to be required for powdery mildew susceptibility. The present study, based on the sequence of a wild plum (*Prunus americana*) MLO protein, identified 16 *MLO* genes within the genome of woodland strawberry, *Fragaria vesca* and examined their expression pattern in response to powdery mildew infection in three diploid strawberry cultivars. Phylogenetic analysis showed that the *FvMLO* genes can be classified into six clades. Four *FvMLO* genes were grouped into clade III, which comprises *MLO* genes from *Arabidopsis*, tomato and grapevine that mediate powdery mildew susceptibility. A RNA-seq analysis of two diploid strawberry cultivars, *F. vesca* ssp. *vesca* accession Hawaii 4 (HW) and *F. vesca* f. *semperflorens* line "Yellow Wonder 5AF7" (YW) at 1 d (1 DAI) and 8 d (8 DAI) after infection showed the expression of 12 out of the 16 *FvMLO* genes. The comparison of Fragments Per Kilobase of transcript per Million mapped reads (FPKM values) detected by RNA-seq and expression values of qRT-PCR for *FvMLO* genes showed substantial agreement. The *FvMLO3* gene, which was grouped in clade III and orthologous to the *Arabidopsis*, tomato and grapevine genes, was highly expressed in YW compared to other *FvMLO* genes across varieties. The results showed that *FvMLO* genes can be used as potential candidates to engineer powdery mildew resistance in strawberry based on *MLO* suppression or genome editing.

Corresponding author: Dr. Shridhar Jambagi; School of Ecology and Conservation, Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore 560 065, India; e-mail: shridharjambagi@gmail.com

Running title: Expression analysis of *MLO* genes in *Fragaria vesca*

Key words : *Fragaria vesca*, powdery mildew, *FvMLO* genes, gene expression, RNA-seq, qRT-PCR

Received: Oct 24, 2017 **Accepted**: Oct 31,2017 **Published:** Nov 22, 2017

Introduction

The *mildew resistance locus o* (*MLO*) genes encode a specific class of plant-specific proteins that possess seven transmembrane helices and a C-terminal calmodulin-binding domain [1, 2]. An *MLO* gene was first identified in barley [3] and currently, a varying number of *MLO* genes have been identified in *Arabidopsis*, *Medicago truncatula*, chickpea, rice, maize, wheat, soybean, cucumber, tomato, rose, grapevine, peach, apple, sweet orange, cultivated Solanaceae, *Brachypodium*, and Cucurbitaceae species respectively [4-18]. Although the biological functions of *MLO* genes are not yet completely known, they act as suppressors of defense responses.

The mutant *MLO* genes confer durable and broad spectrum powdery mildew resistance in monocots and dicots and the functions of these genes are highly conserved in the plant powdery mildew interactions. In barley, the presence of *HvMLO* is an absolute requirement for the powdery mildew fungi to penetrate the host cell wall successfully [19], whereas the recessive loss-of-function alleles of *MLO* gene showed durable and broad spectrum resistance to these pathogens by forming cell-wall appositions [20-23]. Recently, Acevedo Garcia et al. [24] identified simultaneous mutations in the three wheat homologues of *TaMlo*, *TaMlo-A1*, *TaMlo-B1* and *TaMlo-D1* and produced homozygous triple and double mutant lines that showed increased resistance to powdery mildew. In another study, the knock-down of grapevine *MLO* genes (*VvMLO7* in combination with *VvMLO6* and *VvMLO11*) decreased powdery mildew severity up to 77 % [25]. Like barley *mlo*-mutants, the *A. thaliana mlo2* mutants exhibited resistance to multiple powdery mildew species [26] and also the same defense response was identified in the tomato *Slmlo1* mutant [27]. Antisense down-regulation of *RhMLO1* enhances resistance to powdery mildew in *Rosa multiflora* [28], and RNAi methods have been used to knock-down expression of selected *MLO* genes and reduce susceptibility to powdery mildew in strawberry [29], apple [30], and petunia [31]. Previously, the loss-of-function mutations in *PsMLO1* showed durable and broad-spectrum resistance to powdery mildew in pea plants [32], and a transposable element insertion in the susceptibility gene *CsMLO8* results in hypocotyl resistance to powdery mildew in cucumber [33]. In recent studies, genome editing technologies, TALEN (Transcription activator-like effector nucleases) and CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease), were used in hexaploid bread wheat to introduce targeted mutations in three *TaMLO* homoeoalleles which resulted in heritable resistance to powdery mildew [34]. The *MLO* genes therefore serve as good candidate genes for genetic engineering strategies to impart resistance against powdery mildew pathogens.

Podosphaera aphanis (syn. *Sphaerotheca macularis* f. sp. *fragariae*) infection of the cultivated strawberry is a serious problem in strawberry production worldwide, and affects yield and quality. About 50% of strawberries are grown under protected conditions in most parts of the Europe; this leads to persistent powdery mildew problems, since the humid conditions in green/glasshouse is greatly favourable to the pathogen[35]. The application of systemic fungicides to control powdery mildew is expensive and is limited by legal restrictions in most parts of the world. For this reason, genetic strategies offer an alternative for controlling disease by the identification of desired genes, and their introgression and pyramiding in new genotypes. The genetic source of powdery mildew resistance in strawberry is not well studied and the introduction of new resistant cultivars has been limited [36]. These authors used SSR markers for screening of resistant and susceptible seedlings, but none of the markers co-segregated with powdery mildew resistance. The results underlined the difficulty to investigate the allelic linkage for powdery mildew resistance and suggested that efforts should be made to determine linkages in the diploid genome and then further adapt to the octoploid genome.

The available genome sequence of the diploid strawberry, *Fragaria vesca* ssp. *vesca* accession Hawaii 4 [37] allows a comprehensive overview of the strawberry *MLO* gene family. A few studies have previously reported the identification of *FvMLO* genes in the *F. vesca* genome sequence [15, 38]; however, the level of expression of these genes in response to disease pressure has not been studied. In the present study, we identified *FvMLO* genes in the *F. vesca* genome based on the sequence information of the *Prunus americana* MLO protein and analyzed their expression levels in response to powdery mildew pathogen in three diploid strawberry cultivars, *F. vesca* f. *vesca* (Fv), *F. vesca* ssp. *vesca* accession Hawaii 4 (HW), and a highly inbred line, "Yellow Wonder 5AF7 (YW5AF7)" *F. vesca* f. *semper-florens* (YW), and Eluica,

a susceptible variety of the octoploid cultivated octoploid strawberry *Fragaria* × *ananassa*. The resistance level of Fv and HW to powdery mildew is not known, whereas YW is reported to be susceptible to powdery mildew [39]. This paper describes the identification and phylogenetic relationship of *FvMLO* genes, and transcript analysis of these genes in response to *P. aphanis* infection using RNA-seq data and qRT-PCR. In future studies, *FvMLO* genes will serve as potential candidates to produce strawberry varieties resistant to powdery mildew.

Materials and methods

In silico identification of MLO predicted proteins in strawberry and primer design

MLO protein sequences in *F. vesca* were identified by using a MLO sequence from the related Rosaceous species *Prunus americana* by using the Basic Local Alignment Search Tool (BLAST) of the strawberry genome tools (https://www.rosaceae.org/blast). Multiple sequence alignment was carried out with ClustalW to search for conserved regions and primers were designed using primer3 software. Primer sequences are listed in Table S1.

Phylogenetic analysis

For phylogenetic analysis of MLO proteins, a selection of known monocot and dicot MLO protein sequences downloaded from NCBI GenBank were aligned with the FvMLO protein sequences using ClustalW with default settings [40]. The aligned sequences were used to generate a phylogenetic tree using Neighbour-Joining [41] method in MEGA4 [42]. All positions containing gaps and missing data were eliminated (Complete deletion option). The bootstrap value was calculated with 500 replicates to support the branching arrangements.

Plant material and powdery mildew infection

Three diploid strawberry cultivars, Fv, HW (originally provided by East Malling Research, UK), and YW (seed provided by Dr Janet Slovin, Beltsville, USA), and two octoploid varieties, Emily and Eluica (plants provided by East Malling Research, UK) were used in the present study. The diploid plants were grown in hydroponic medium as previously described by Bindschedler, et al. [43] and maintained in a Fisons growth chamber at a temperature of 20°C with 65% humidity, and a 10 h photoperiod with a photosynthetic photon flux density of 250-260 µmol/m^2/s. The octoploid plants were maintained in a glasshouse under natural day length conditions in pots containing potting compost. Leaf material from an Eluica cultivar infected with powdery mildew were used to infect four week old *F. vesca* plants with *P. aphanis* by gently tapping conidia from infected leaves above the experimental plants. The leaves (100 mg) from five randomly selected plants were harvested from fully grown healthy (control) plants and infected plants at 1 d and 8 d after infection (DAI), frozen in liquid nitrogen, and stored at -80°C until use.

RNA sequencing and data analysis

We used RNA-seq data that was generated previously in our laboratory for two diploid cultivars, HW and YW at control, 1 d and 8 d after powdery mildew infection [44]. We manually searched for the expression of *FvMLO* genes in each library and absolute FPKM values were used to analyze their pattern of expression.

Semiquantitative and quantitative real-time PCR analysis

To examine the expression of *FvMLO* genes in different strawberry cultivars, total RNA was isolated from the control and 8 DAI frozen leaf samples using an RNAqueous® Kit (Ambion, UK) and then subjected to DNAse treatment with Turbo DNA-free kit (Ambion, UK). First-strand cDNA was synthesized from total RNA using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen, UK). For qRT-PCR analysis, the first experiment was conducted using a semiquantitative RT-PCR evaluating the expression of all the identified *FvMLO* genes at 8 DAI in all strawberry cultivars. The qRT-PCR was performed on a Rotor-Gene 6000 (Corbet Life Science, UK) by using the sensiMix™ SYBR No-ROX Kit (Bioline, UK) and gene specific primers. The strawberry gene *FaGAPDH2* (AF421145) was used as a reference gene, the primer,
forward: 5′-CAGACTTGAGAAGAAGGCCACCTA-3′ reverse: 5′-GATACCCTTCATCTTTCCCTCAGA-3′. PCR mixtures (20 µL total volume) contained 10 µL 2x SensiMix™ SYBR® No-ROX, 0.5 µL each forward and reverse primer (10 µM), 1 µL cDNA template and 8 µL Molecular Biology Grade water (Accugene, UK). A negative control reaction without cDNA template was included to monitor the non-specific binding. The thermal cycling parameters were one cycle at 95°C for 10 min, 39 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. A standard curve was generated for each run using a cDNA template dilution series and output data were analyzed using the software provided. A Two standard curve method was employed

for the analysis of real-time quantitative PCR results. The melting curve analysis was carried out from 50 to 90 °C with a hold of 1 min for every 1 °C. The expression level of *FvMLO* genes in control leaves was used as a control to compare the relative expressions of the genes in 8 d infected leaves. The relative expression levels of the *FvMLO* genes were calculated using the 2^{-CT} method [45].

Results

In silico identification of *F. vesca MLO* genes

Homology search of the published genome sequence of the strawberry against the known MLO sequence from *P. americana* was performed to identify the number of *MLO* genes in the *F. vesca* genome. Initially, seventeen genes were identified as putative members of the *MLO* gene family; these were designated as *FvMLO* genes (*FvMLO1* to *FvMLO17*). However, based on the chromosomal location of *FvMLO* genes, *FvMLO4* was found to be an isoform of the *FvMLO3* gene and therefore, the former was excluded in the phylogenetic analysis (see next paragraph). The information of *FvMLOs*, including accession numbers, and their chromosomal locations are listed in Table 1.

Phylogenetic analysis of the *F. vesca MLO* genes

A phylogenetic analysis was performed with the amino acid sequences encoded by the 16 *FvMLO* genes together with a set of 17 *VvMLO* protein sequences from grapevine [9, 46] and selected MLO proteins from barley, rice, wheat, maize, tomato, *A. thaliana*, *P. americana* and *M. toringoides*. The analysis identified six distinct clades and also showed that *F. vesca* MLO family members were distributed between all clades (Fig. 1). Clade V comprises the monocot MLO proteins previously characterized to be involved in powdery mildew susceptibility [2, 20, 47]. The FvMLO8 protein was found to be closely related to VvMLO14 protein and clustered within clade V; this finding is noticeably different from the general conclusion that this clade contains only monocot homologues [8]. The barley HvMLO protein involved in powdery mildew susceptibility is orthologous to wheat TaMLO-1 and rice OsMLO2 proteins, induce powdery mildew resistance in barley mlo-mutants [47]. Of the 16 FvMLO proteins analyzed, four (FvMLO3, FvMLO5, FvMLO11 and FvMLO14) clustered with the dicot MLO proteins in clade III. This clade is important in terms of putative function as this group contains the MLO proteins from *A. thaliana* (AtMLO2, AtMLO6, and AtMLO12), tomato (SlMLO1) and grapevine (VvMLO3, VvMLO4 and VvMLO17) that have been previously reported to be induced during powdery mildew infection [9, 26, 27]. This allowed for the analysis of the *F. vesca* MLO proteins for domains that are conserved between all proteins responsible for mildew susceptibility. Fig. 2 clearly demonstrates that the protein sequences (1 to 100 and 501 to 540) are highly conserved among all members of clade III at the position of the predicted transmembrane domains and the calmodulin-binding sites, which are important features of this protein family.

Expression analysis of *FvMLO* transcripts using RNA-seq data

In our previous study [44], RNA-seq data was generated for two diploid strawberry cultivars, HW and YW, in response to powdery mildew infection. Global transcriptome analysis of two *F. vesca* cultivars of healthy (control) and infected leaves (1 DAI and 8 DAI) allowed us to identify the transcriptional changes during *P. aphanis* infection. In the present study, RNA-seq data were used to analyze the level of expression of all 16 identified *FvMLO* genes in HW and YW between control and 1 DAI, and control and 8 DAI. Based on the FPKM values, only 12 *FvMLO* genes that are grouped into different putative clades in the phylogenetic tree were expressed at all stages; these included gene13023 (*FvMLO1*), gene09653 (*FvMLO3*), gene23198 (*FvMLO5*), gene10558 (*FvMLO7*), gene28541 (*FvMLO9*), gene09651 (*FvMLO10*), gene29285 (*FvMLO12*), gene10166 (*FvMLO13*), gene29770 (*FvMLO14*), gene10346 (*FvMLO15*), gene31264 (*FvMLO16*) and gene14592 (*FvMLO17*). By using the RNA-seq based absolute fold change values, the transcript levels of all 12 *FvMLO* genes showed a steady increase in response to *P. aphanis* infection between control and 1 DAI in both HW and YW with fold change values ranging from 0.5 to 3.6 (Fig. 3A). Three genes, *FvMLO3*, *FvMLO10* and *FvMLO15* showed much higher levels of expression with fold change values of about 30, 4.5 to 7.3, and 13 to 20 between control and 8 DAI in both HW and YW (Fig. 3B). The time course between control and 1 DAI, and control and 8 DAI clearly showed an increase in expression of *FvMLO* genes.

Expression analysis of *FvMLO* genes by qRT-PCR

The level of expression of *FvMLO* genes was analyzed in three biological replicates using qRT-PCR in leaf tissue of the three diploid strawberry cultivars, Fv, HW, and YW, and one octoploid variety, Eluica, between

Table 1 The *FvMLO* genes as predicted in *Fragaria vesca* genome sequence

Gene	Chromosome location	Transcript name
FvMLO1	LG1: 7017413 - 7022793	mrna13023.1-v1.0-hybrid
FvMLO2	LG5: 20075140 - 20093212	mrna31488.1-v1.0-hybrid
FvMLO3	LG6: 8563501 - 8567082	mrna09653.1-v1.0-hybrid
FvMLO4	LG6: 8563518 - 8566426	mrna09653.1-v1.0-hybrid
FvMLO5	LG7: 14517490 - 14520807	mrna23198.1-v1.0-hybrid
FvMLO6	LG3: 10707557 - 10713141	mrna03210.1-v1.0-hybrid
FvMLO7	LG2: 15724387 - 15725931	mrna10558.1-v1.0-hybrid
FvMLO8	LG5: 20171268 - 20174872	mrna31498.1-v1.0-hybrid
FvMLO9	LG3: 17105660 - 17112982	mrna28541.1-v1.0-hybrid
FvMLO10	LG6: 8541201 - 8546252	mrna09651.1-v1.0-hybrid
FvMLO11	LG7: 16370664 - 16374969	mrna26428.1-v1.0-hybrid
FvMLO12	LG5: 19055228 - 19057796	mrna29285.1-v1.0-hybrid
FvMLO13	LG1: 697457 - 707892	mrna10166.1-v1.0-hybrid
FvMLO14	LG3: 4660882 - 4663962	mrna29770.1-v1.0-hybrid
FvMLO15	LG3: 25834627 - 25838931	mrna10346.1-v1.0-hybrid
FvMLO16	LG3: 9515163 - 9524099	mrna31264.1-v1.0-hybrid
FvMLO17	LG1: 7820289-7827918	mrna14592.1-v1.0-hybrid

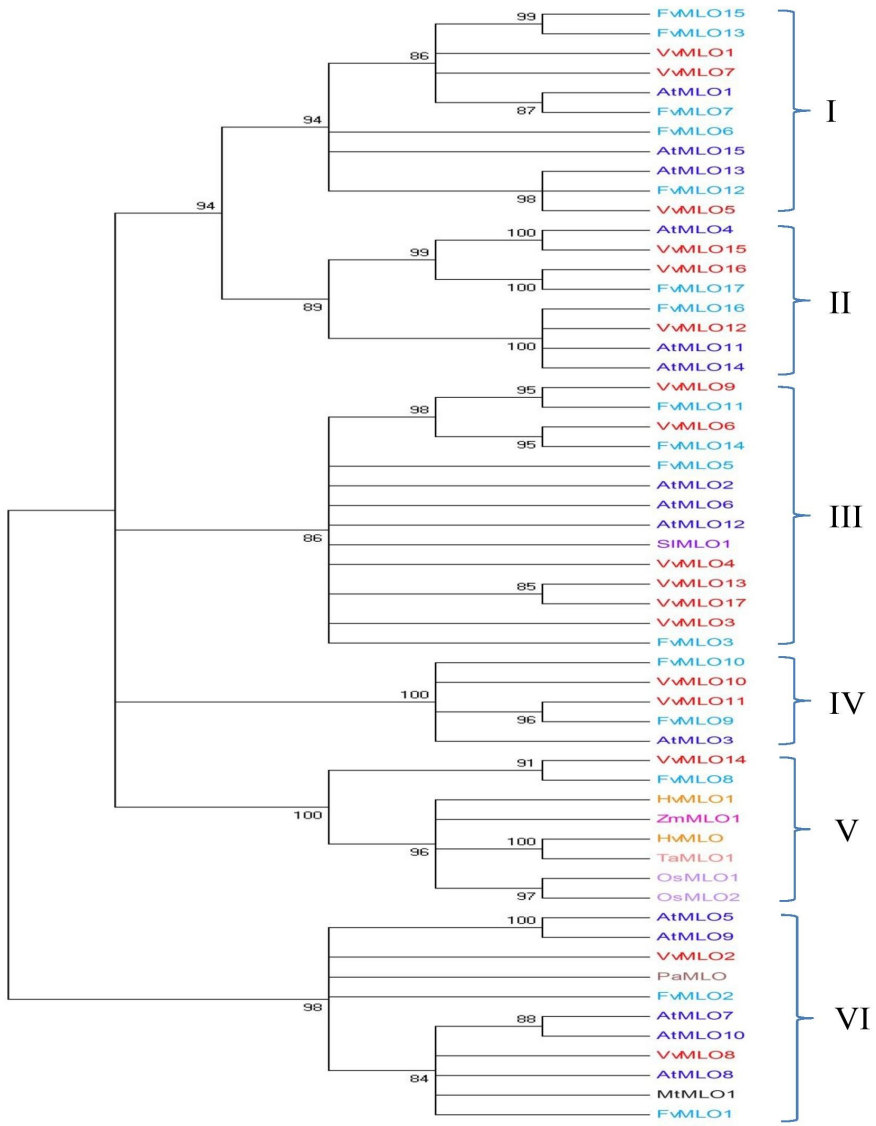

Figure 1 Phylogenetic relationship of MLO proteins inferred using Neighbour-Joining method

Phylogenetic tree of the amino acid sequences of MLO family members of *F. vesca*, *V. vinifera*, *A. thaliana* and selected MLO orthologs from barley, rice, wheat, maize, tomato, *P. americana* and *M. toringoides* is shown. Numbers at the branch nodes indicate bootstrap values from 500 replications. Branch lengths are proportional to sequence distance. The species containing different MLO sequences are colour coded. FvMLO sequences are colour coded in sky blue.

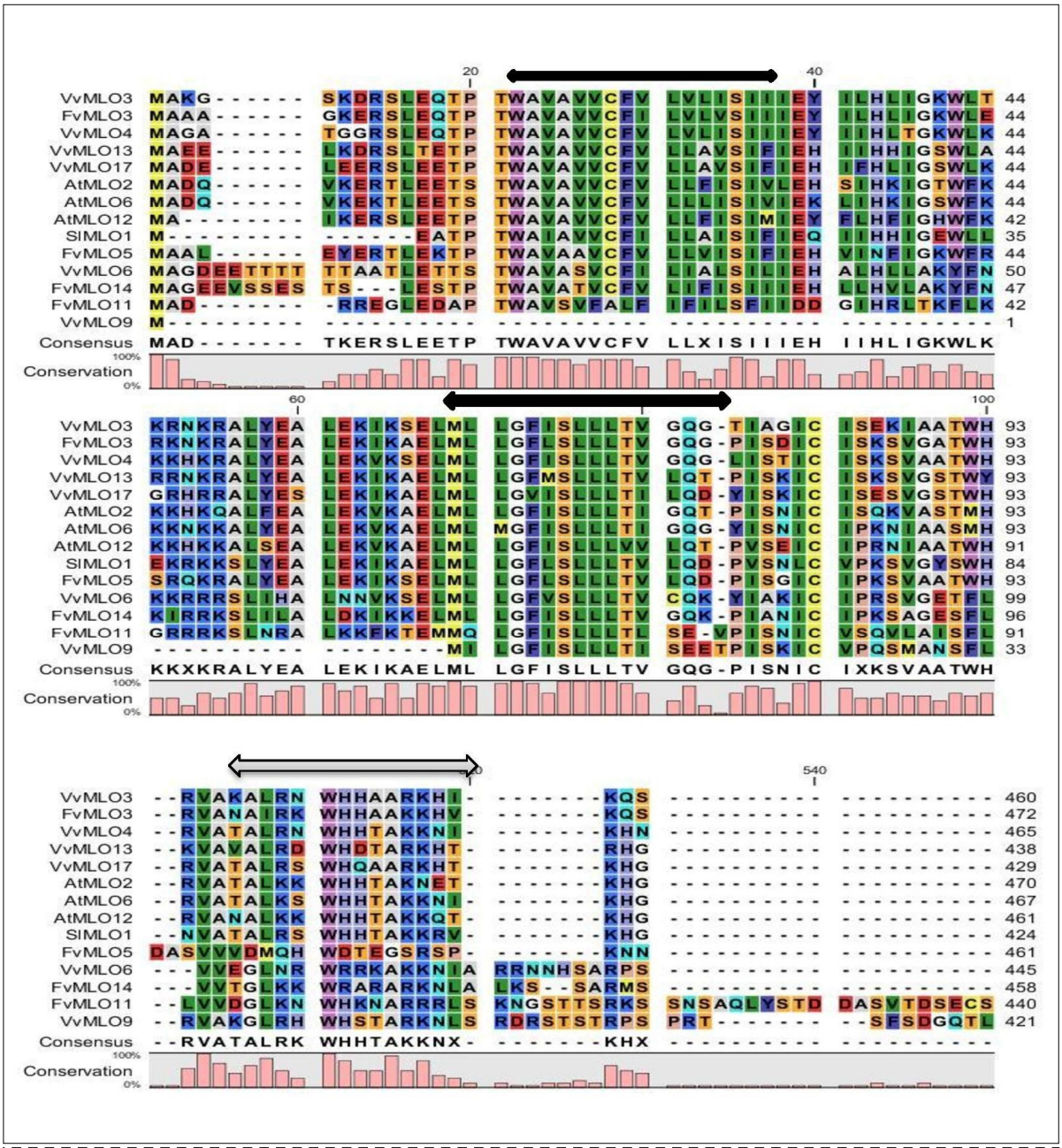

Fig. 2. Multiple sequence alignment of FvMLO proteins

Multiple sequence alignment of FvMLO proteins in clade III with selected MLO proteins that have been shown to be involved in powdery mildew susceptibility in *Arabidopsis* (AtMLO2, AtMLO6 and AtMLO12; Consonni et al., 2006; Consonni et al., 2010), grape (VvMLO3, VvMLO4, VvMLO6, VvMLO9, VvMLO13 and VvMLO17; Feechan et al., 2009) and tomato (SlMLO1; Bai et al., 2008). The alignment was generated by CLC Sequence Viewer 6.6.2. The positions of the seven transmembrane domains (Devoto et al., 1999) are indicated by black arrows and the position of the calmodulin-binding site (Panstruga, 2005) is indicated by a grey bar above the sequences.

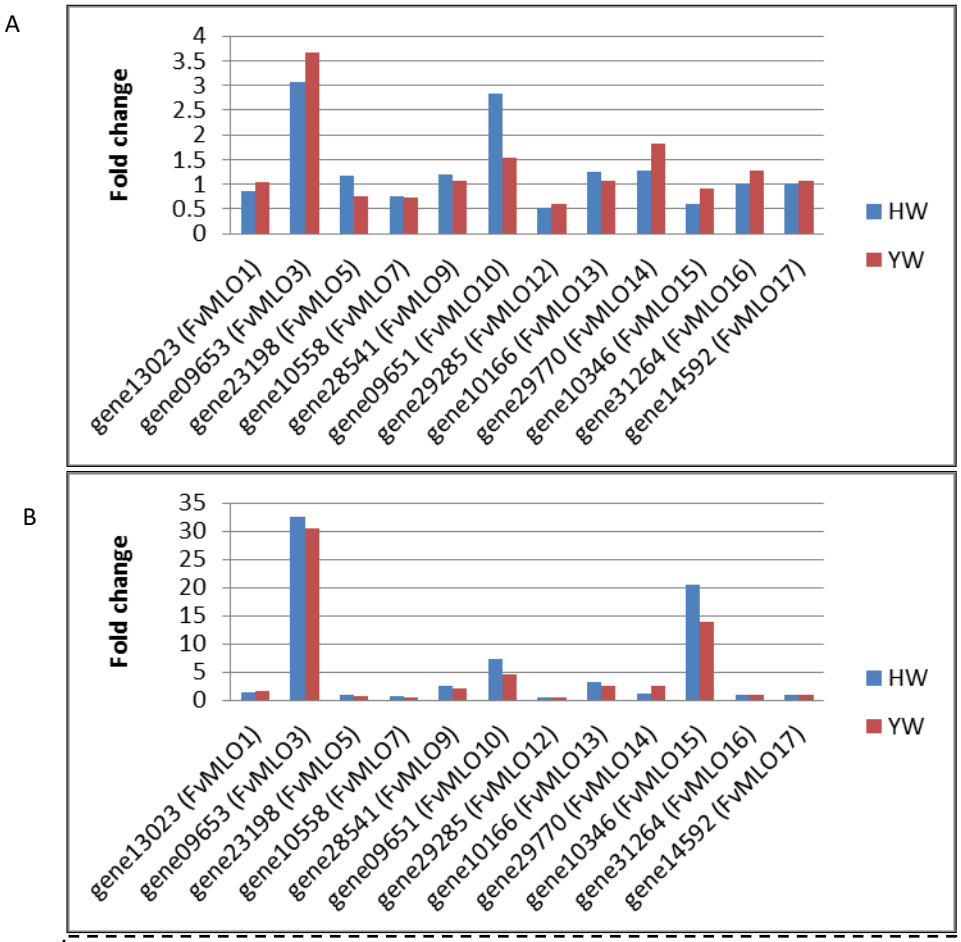

Figure 3 Graph showing the expression of 12 *FvMLO* genes based on RNA-seq absolute fold change values in HW and YW between control and 1 DAI (A), and control and 8 DAI (B).

control and 8DAI.

Initial analysis by semiquantitative RT-PCR showed the expression of 12 out of the 17 *FvMLO* genes (*FvMLO1*, *FvMLO3*, *FvMLO4*, *FvMLO5*, *FvMLO7*, *FvMLO9*, *FvMLO10*, *FvMLO12*, *FvMLO13*, *FvMLO14*, *FvMLO16* and *FvMLO17*). Among the 12 expressed *FvMLO* genes, *FvMLO3* showed expression only in infected leaves, whereas *FvMLO14* showed expression only in control leaves of all strawberry varieties (Fig. 4). Expression of the other 10 *FvMLO* genes varied widely between all varieties in both control and infected leaves.

The expression levels of all of the 12 *FvMLO* genes were further investigated using the qRT-PCR method. The genes that did not show expression in the initial analysis of semiquantitative RT-PCR method were not included in the qRT-PCR expression analysis. In measuring the relative expression level, the PCR efficiencies and coefficients of correlation (R2 value) of reference (*FaGAPDH2*) and target (*FvMLO*) genes ranged from 0.89 to 0.97 and from 0.98 to 0.99, respectively (Fig. S1). The cDNA synthesized from total RNA extracted from Emily (resistant) octoploid plant was used as a standard. The transcript levels of individual *FvMLO* genes were normalized against the transcript levels of *FaGAPDH2* in each cDNA sample.

The absolute expressions of *FvMLO* genes in control and infected leaves of all strawberry varieties are shown in Table S2. During powdery mildew infection, two genes *FvMLO7* and FvMLO14 showed low level of expression among all varieties, whereas six genes *FvMLO1, FvMLO3, FvMLO9, FvMLO10, FvMLO13 and FvMLO16* were upregulated but the level of relative expression across varieties was significantly different (Fig. 5). The expression level of *FvMLO1, FvMLO3* and *FvMLO13* genes were higher in YW and Eluica, whereas *FvMLO9* showed higher expression in Eluica, *FvMLO10* in YW and HW, *FvMLO16* in HW and Eluica. The level of expression of four genes, *FvMLO4*, *FvMLO5*, *FvMLO12* and *FvMLO17* varied widely across varieties in response to infection. The *FvMLO4* gene, which was

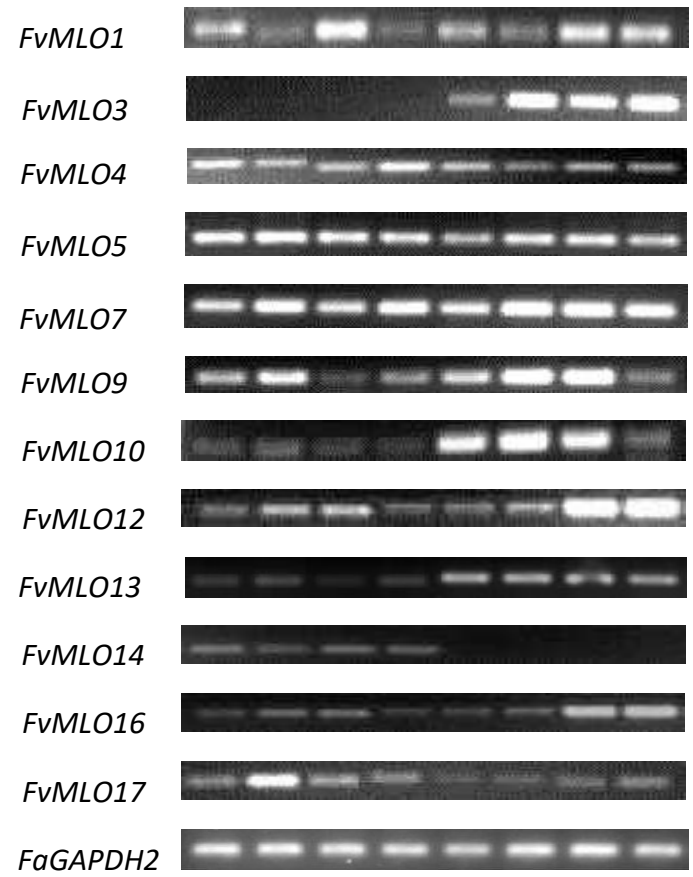

Figure 4 Semiquantitative RT-PCR products of *FvMLO* genes and reference gene (*FaGAPDH2*) in control and infected (8 DAI) leaves of four strawberry varieties. 1-4: Fv, YW, HW and Eluica (control), 5-8: Fv, YW, HW and Eluica (infected).

found to be an isoform of *FvMLO3*, was downregulated in Fv but highly expressed in YW, HW and Eluica. The expression of *FvMLO5* was lower in Fv and HW, whereas it was higher in YW and Eluica. *FvMLO12* and *FvMLO17* showed low levels of expression in Fv and YW but were highly expressed in HW and Eluica. Among all the genes expressed across varieties in response to infection, *FvMLO3* showed a very high level of expression (164 fold) in YW. As far as inter-varietal response to infection is concerned, the genotypes exhibited a differential expression pattern of *FvMLOs*. In the Fv genotype, all *FvMLO* genes except *FvMLO9* were down-regulated under infected conditions, whereas in HW (except *FvMLO5*, *FvMLO7* and *FvMLO14*), YW (except *FvMLO7*, *FvMLO12*, *FvMLO14* and *FvMLO17*) and Eluica (except *FvMLO7* and *FvMLO14*) all *FvMLOs* showed higher expression in infected plants compared to control.

Comparison of RNA-seq and qRT-PCR based FvMLO genes expression

RNA-seq based expression of *FvMLO* genes in response to P. *aphanis* infection was compared with the qRT-PCR based expression levels of *FvMLO* genes in two *F. vesca* cultivars, HW and YW. The comparison showed that the *FvMLO4* gene was expressed in semiquantitative RT-PCR but was not expressed in the RNA-seq experiment. Additionally, RNA-seq data showed the expression of *FvMLO15*, which was not expressed in semiquantitative RT-PCR. Overall, the transcripts FPKM values detected by RNA-seq and qRT -PCR analysis absolute expression values in HW and YW revealed

substantial agreement in the extent of the powdery mildew-induced variation in transcript accumulation for the *FvMLO* genes (Table 2, Fig. 3 and 5).

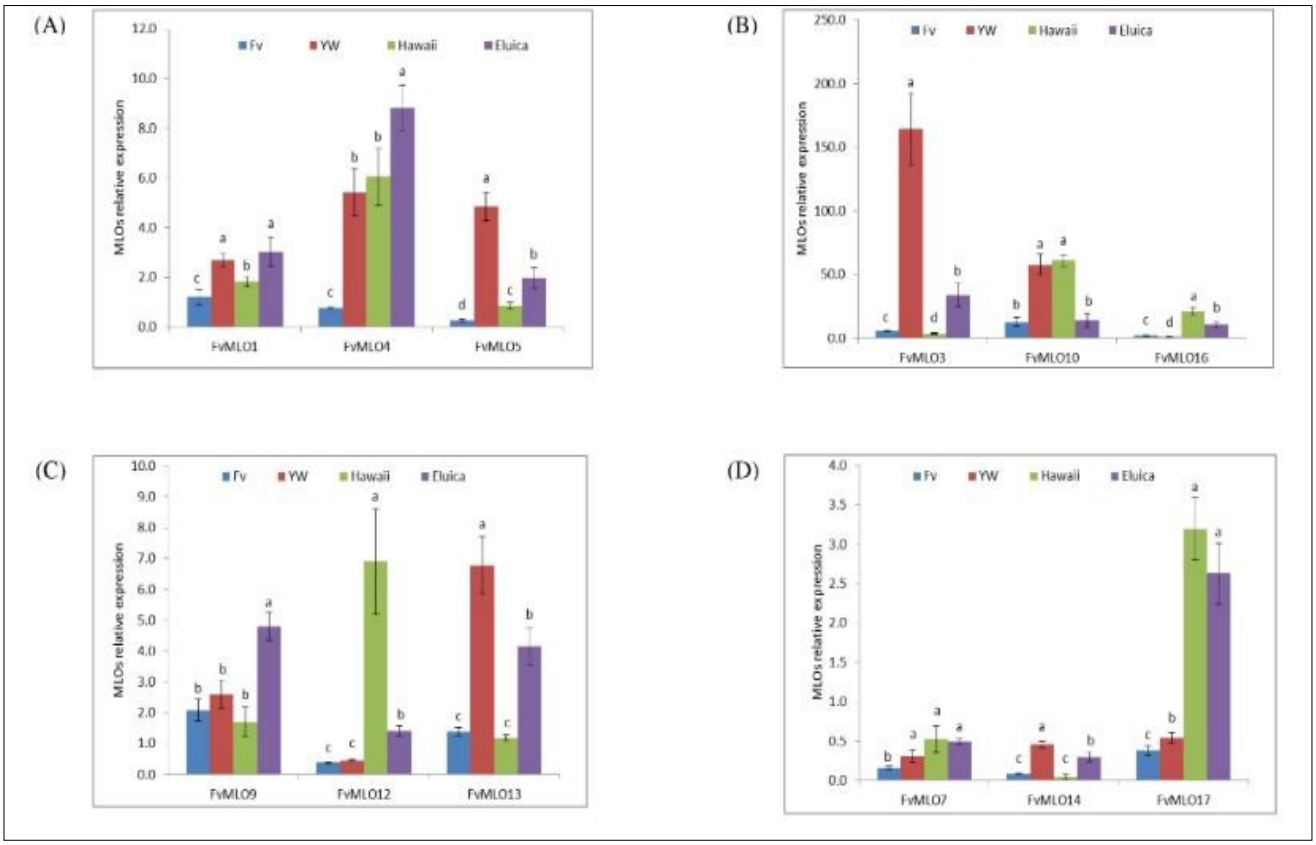

Figure 5 Bar chart showing relative expression levels of 12 *FvMLO* genes in four strawberry varieties in response to *P. aphanis* infection. Graph shows fold increase/decrease in expression under infection over control. All values are compared with control value=1. Values are shown as mean ± standard deviation (n=2). Fv: *F. vesca f. vesca*, YW: *F. vesca* f. *semperflorens* line "Yellow Wonder", HW: *F. vesca* ssp. *vesca* accession Hawaii 4 and Eluica: Susceptible octoploid strawberry variety. Different letters (a, b, c, d) denote significant difference in level of expression across varieties.

Discussion

Our study focused on identification of *MLO* genes in sequenced *Fragaria* genome by BLAST search using sequence information of a *Prunus americana* MLO protein and expression analysis of *FvMLO* genes in three diploid (Fv, HW and YW) and one octoploid (Eluica) strawberry cultivars. The *in silico* analysis allowed the identification of 16 *FvMLO* genes and the phylogenetic analysis of FvMLO proteins performed with MLO family members of grapevine, *Arabidopsis*, and selected monocot and dicot MLO proteins revealed that the proteins were grouped into six distinct clades, two of which (III and V) comprise members that are associated with powdery mildew susceptibility in dicots and monocots, respectively. In this study, it was observed that the number of *FvMLO* genes and clade numbering of the *FvMLO* gene family were in contrast to the previously categorized *FvMLO* genes. Pessina, et al. [15] identified 17 *FvMLO* genes (*FvMLO1-FvMLO17*) based on sequence information of tomato SlMLO1 amino acid and HMMER programme, whereas Miao, et al. [38] reported 20 *MLO* genes in the strawberry genome (*FvMLO01-FvMLO20*) using *Arabidopsis thaliana* and rice MLO protein sequences. Further, the phylogenetic analysis in their studies showed that *MLO*s were grouped into seven distinct clades. These results may possibly indicated that the number of *FvMLO* genes and clades varied according to the query type, target database type, and stringency used for the blast search to identify *MLO* genes, and also the specific MLO family members of selected crop species used for phylogenetic analysis.

A previous study comparing MLO sequences from grapevine with those from monocots and dicots supported our phylogenetic results [9]. Among four *FvMLOs* of clade III, the FvMLO3 protein sequence is

Table 2 Comparisons of transcripts FPKM values detected by RNA-seq and expression values (mean ± standard error) of qRT-PCR analysis for the *FvMLO* genes in HW and YW

Gene	HW					YW				
	RNA-seq (FPKM)			qRT-PCR (Mean ± SE)		RNA-seq (FPKM)			qRT-PCR (Mean ± SE)	
	Control	1 DAI	8 DAI	Control	8 DAI	Control	1 DAI	8 DAI	Control	8 DAI
gene13023 (*FvMLO1*)	7.53	6.52	10.07	0.57±0.14	1.05±0.11	6.67	7.03	11.64	0.66±0.10	1.78±0.19
gene09653 (*FvMLO3*)	0.12	0.38	3.96	15.80±3.13	55.76±7.55	0.16	0.57	4.77	0.76±0.13	123.49±21.33
gene23198 (*FvMLO5*)	14.12	16.76	13.08	12.48±4.99	10.84±1.67	23.02	17.56	17.95	3.35±1.12	16.21±1.88
gene10558 (*FvMLO7*)	63.49	47.46	45.17	0.84±0.10	0.44±0.14	78.01	57.98	45.54	1.83±0.37	0.57±0.14
gene28541 (*FvMLO9*)	12.91	15.39	32.31	1.67±0.55	2.90±0.81	11.24	12.16	22.35	0.83±0.11	2.18±0.37
gene09651 (*FvMLO10*)	1.69	4.79	12.47	2.37±0.45	144.30±10.94	1.86	2.85	8.49	1.37±0.27	78.77±11.34
gene29285 (*FvMLO12*)	87.71	46.49	41.05	0.14±0.03	0.98±0.24	86.62	52.78	42.94	0.33±0.06	0.15±0.01
gene10166 (*FvMLO13*)	10.00	12.63	31.42	17.33±4.03	20.53±1.66	10.12	10.91	26.31	2.86±0.09	19.37±2.63
gene29770 (*FvMLO14*)	0.30	0.38	0.37	1.18±0.29	0.06±0.04	0.27	0.49	0.67	0.31±0.03	0.14±0.01
gene10346 (*FvMLO15*)	0.27	0.16	5.55	-	-	0.33	0.30	4.58	-	-
gene31264 (*FvMLO16*)	3.09	3.11	2.69	0.25±0.07	5.33±0.81	2.92	3.74	3.00	1.65±0.23	1.69±0.31
gene14592 (*FvMLO17*)	21.53	21.92	21.49	0.08±0.03	0.25±0.03	21.12	22.64	19.66	0.17±0.00	0.09±0.01

highly conserved in the positions of transmembrane domains [8] and calmodulin-binding site [19] with other members in the clade and therefore this gene serves as a potential candidate gene in modulating antifungal defense response. Further, FvMLO8 was clustered with monocot MLO proteins in clade V, which includes HvMLO, TaMLO1 and OsMLO2 that have been demonstrated to be required for powdery mildew susceptibility [47]. However, the finding that FvMLO8 was not expressed in RNA-seq and semiquantitative RT-PCR analysis suggests that this protein is not likely to be involved in powdery mildew pathogenicity. The grouping of FvMLO8, a dicot MLO, into a clade that only contains monocot MLOs, raises a question about the accuracy of the published sequence for *FvMLO8* (Transcript name: mrna31498.1-v1.0-hybrid). Similar conclusions were made by Feechan, et al. [9] for the clustering of the VvMLO14 protein into the same clade. Furthermore, the *FvMLO11* grouped in clade III was not expressed in the RNA-seq data and semiquantitative RT-PCR.

Although, *FvMLO* genes have been identified previously, their expression levels were not examined in strawberry in response to powdery mildew infection. In our previous study, transcriptome analysis of two diploid strawberry varieties (HW and YW) upon *P. aphanis* infection provided access to a large data set and enabled new insights into the identification of genes expressed during powdery mildew interaction [44]. RNA-seq analysis of *FvMLO* genes showed the expression of 12 *FvMLO* genes in HW and YW that are distributed in different clades. Comparison of RNA-seq based *FvMLO* genes expression with qRT-PCR exhibited significant correlation between the two different techniques except for *FvMLO4* and *FvMLO15* for which more detailed analysis is needed. Two genes, *FvMLO3* and *FvMLO10*, which showed high expression levels in RNA-seq data analysis, were in considerable agreement with the qRT-PCR based expression pattern for these two genes. The *FvMLO3* gene is particularly important because it is grouped in clade III along with the *AtMLO2*, *AtMLO6*, *AtMLO12*, *SlMLO1*, *VvMLO3*, *VvMLO4* and *VvMLO17* genes that have been previously reported to be induced by powdery mildew infection [9, 26, 27]. Two other genes, *FvMLO5* and *FvMLO14* that are also grouped in clade III showed the same pattern of expression in HW and YW upon infection compared between RNA-seq data and qRT-PCR results. The *FvMLO5* expression level was lower in HW but higher in YW, whereas *FvMLO14* was found to be lowly expressed in both HW and YW in response to infection.

The level of relative expression of *FvMLOs* in all the genotypes varied significantly. In Eluica and YW, most of the *FvMLO* genes were highly expressed compared to Fv and HW (based on qRT-PCR and RNA-seq data). These results indicated that the expression of *FvMLOs* could possibly be responsible for the genotypes Eluica and YW being more susceptible to powdery mildew infection, as reported in previous studies. The results for YW in this study were in agreement with the report published by Slovin, et al. [39] that YW is susceptible to powdery mildew, thrips and aphids in a greenhouse environment. Similarly, Eluica is known to be a susceptible octoploid variety as confirmed by East Malling Research, UK. However, the resistance level of the other *Fragaria* spp. is not known [36] and further functional analysis should be conducted to test the role of these *FvMLO* genes in the susceptibility of strawberry varieties to *P. aphanis*.

Conclusion

The results of our study will allow for future strategies to generate powdery mildew resistant strawberry varieties. Previously, it has been reported that loss-of-function *mlo*-mutants powdery mildew resistance in *A. thaliana* and tomato [27, 48]. Similarly, Jiwan, et al. [29] reported that the antisense expression of peach *MLO* gene *PpMlo1* in transgenic strawberry plants reduced susceptibility to powdery mildew. Therefore, *FvMLO* genes serve as potential candidates to produce strawberry varieties resistant to powdery mildew. If resistance in strawberry can be achieved through silencing a single *FvMLO* gene, as in tomato, it would then be possible to look for naturally occurring mutant alleles within *F. vesca* germplasm collections that could be used in marker-assisted selection to produce progeny that are homozygous recessive at this locus. Conversely, if complete powdery mildew resistance in strawberry is only attained through the silencing of more than one *FvMLO* gene, then this is practically only amenable totransgenic methods using constructs designed to concurrently silence multiple *FvMLO* genes, an approach that has already been achieved in *A. thaliana*. Moreover, recent genome editing technologies, TALEN and CRISPR-Cas9, or other genome editing techniques, can be used to introduce targeted

mutations in *FvMLO* genes that result in heritable resistance to powdery mildew.

Acknowledgements

We would like to thank Felix trust for financial support, and Dr Janet Slovin, Beltsville, USA and Dr Richard Harrison, East Malling Research, UK for providing materials utilised for the study. We acknowledge Dr Ihsan Ullah, University of Reading, UK for the technical help in data analysis.

Conflict of interest

The authors declare no conflicts of interest.

Supplemental data:

Supplementary figure:

Figure S1 Standard curve graph showing PCR efficiencies and coefficients of correlation (R2 value) of reference (blue dots) and target (red dots) genes.

Supplementary table

Table S1 List of primers used in the study.

Table S2 Absolute expressions of *FvMLO* genes in control and infected (8 DAI) conditions across four strawberry varieties. Values are shown as mean ± standard error (n=2).

References

1. Devoto, A., Piffanelli, P., Nilsson, I., Wallin, E., Panstruga, R., et al. (1999) Topology, subcellular localization, and sequence diversity of the Mlo family in plants. J. Biol. Chem. 274, 34993-35004.

2. Kim, M.C., Panstruga, R., Elliott, C., Müller, J., Devoto, A., et al. (2002) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. Nature. 416, 447-451.

3. Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., et al. (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell 88, 695-705.

4. Appiano, M., Pavan, S., Catalano, D., Zheng, Z., Bracuto, V., et al. (2015) Identification of candidate *MLO* powdery mildew susceptibility genes in cultivated Solanaceae and functional characterization of tobacco *NtMLO1*. Transgenic Res. 24, 847-858.

5. Ablazov, A., and Tombuloglu, H. (2016) Genome-wide identification of the *mildew resistance locus O* (*MLO*) gene family in novel cereal model species *Brachypodium distachyon*. Eur. J. Plant Pathol. 145, 239-253.

6. Chen, Y., Wang, Y., and Zhang, H. (2014) Genome-wide analysis of the *mildew resistance locus O* (*MLO*) gene family in tomato (*Solanum lycopersicum* L.). Plant Omics 7.2, 87-93.

7. Deshmukh, R., Singh, V.K. and Singh, B.D., (2017) Mining the *Cicer arietinum* genome for the *mildew locus O* (*Mlo*) gene family and comparative evolutionary analysis of the *Mlo* genes from *Medicago truncatula* and some other plant species. J. Plant Res. 130(2), 239-253.

8. Devoto, A., Hartmann, H.A., Piffanelli, P., Elliott, C., Simmons, C., et al. (2003) Molecular phylogeny and evolution of the plant-specific seven-transmembrane MLO family. J. Mol. Evol. 56, 77-88.

9. Feechan, A., Jermakow, A.M., Torregrosa, L., Panstruga, R., and Dry, I.B. (2009) Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew. Funct. Plant Biol. 35, 1255-1266.

10. Iovieno, P., Andolfo, G., Schiavulli, A., Catalano, D., Ricciardi, L., et al. (2015) Structure, evolution and functional inference on the *Mildew Locus O* (*MLO*) gene family in three cultivated Cucurbitaceae. BMC Genomics 16: 1112.

11. Kaufmann, H., Qiu, X., Wehmeyer, J., and Debener, T. (2012) Isolation, molecular characterization, and mapping of four rose MLO orthologs. Front. Plant. Sci. 3, 244.

12. Konishi, S., Sasakuma, T., and Sasanuma, T. (2010) Identification of novel *Mlo* family members in wheat and their genetic characterization. Genes Genet. Syst. 85, 167-175.

13. Liu, L.P., Qu, J.W., Yi, X.Q. and Huang, H.H., (2017) Genome-wide identification, classification and expression analysis of the *Mildew Resistance Locus O* (*MLO*) gene family in sweet orange (*Citrus sinensis*). Braz. Arch. Biol. Technol. 60, e17160474.

14. Liu, Q., and Zhu, H. (2008) Molecular evolution of the *MLO* gene family in *Oryza sativa* and their functional divergence. Gene 409, 1-10.

15. Pessina, S., Pavan, S., Catalano, D., Gallotta, A., Visser, R.G., et al. (2014) Characterization of the *MLO* gene family in Rosaceae and gene expression analysis in *Malus domestica*. Bmc Genomics 15, 618.

16. Rispail, N., and Rubiales, D. (2016) Genome-wide identification and comparison of legume *MLO* gene family. Sci. Rep. 6, 32673.

17. Shen, Q., Zhao, J., Du, C., Xiang, Y., Cao, J., and Qin, X. (2012) Genome-scale identification of MLO domain-containing genes in soybean (*Glycine max* L. Merr.). Genes Genet. Syst. 87, 89-98.

18. Zhou, S., Jing, Z., and Shi, J. (2013) Genome-wide identification, characterization, and expression analysis of the *MLO* gene family in *Cucumis sativus*. Genet. Mol. Res. 12, 6565-6578.

19. Panstruga, R. (2005) Serpentine plant MLO proteins as entry portals for powdery mildew fungi. Biochem. Soc. Trans. 33, 389-392.

20. Jørgensen, J.H. (1992) Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *In* Breeding for disease resistance (Springer), pp. 141-152.

21. Wolter, M., Hollricher, K., Salamini, F., and Schulze-Lefert, P. (1993) The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Mol. Gen. Genet. 239, 122-128.

22. Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., et al. (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol. 129, 1076-1085.

23. Piffanelli, P., Ramsay, L., Waugh, R., Benabdelmouna, A., D'Hont, A., et al. (2004) A barley cultivation-associated polymorphism conveys resistance to powdery mildew. Nature 430, 887-891.

24. AcevedoGarcia, J., Spencer, D., Thieron, H., Reinstädler, A., Hammond Kosack, K., et al. (2017) *mlo*based powdery mildew resistance in hexaploid bread wheat generated by a non transgenic TILLING approach. Plant Biotechnol. J. 15, 367-378.

25. Pessina, S., Lenzi, L., Perazzolli, M., Campa, M., Dalla Costa, L., et al. (2016) Knockdown of *MLO* genes reduces susceptibility to powdery mildew in grapevine. Horticulture Res. 3, 16016.

26. Consonni, C., Bednarek, P., Humphry, M., Francocci, F., Ferrari, S., et al. (2010) Tryptophan-derived metabolites are required for antifungal defense in the Arabidopsis *mlo2* mutant. Plant Physiol. 152, 1544-1561.

27. Bai, Y., Pavan, S., Zheng, Z., Zappel, N.F., Reinstadler, A., et al. (2008) Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of *mlo* function. Mol. Plant Microbe Interact. 21, 30-39.

28. Qiu, X., Wang, Q., Zhang, H., Jian, H., Zhou, N., et al. (2015) Antisense *RhMLO1* gene transformation enhances resistance to the powdery mildew pathogen in *Rosa multiflora*. Plant Mol. Biol. Rep. 33, 1659-1665.

29. Jiwan, D., Roalson, E.H., Main, D., and Dhingra, A. (2013) Antisense expression of peach *mildew resistance locus O* (*PpMlo1*) gene confers cross-species resistance to powdery mildew in *Fragaria* x *ananassa*. Transgenic Res. 22, 1119-1131.

30. Pessina, S., Angeli, D., Martens, S., Visser, R.G., Bai, Y., et al. (2016) The knock down of the expression of *MdMLO19* reduces susceptibility to powdery mildew (*Podosphaera leucotricha*) in apple (*Malus domestica*). Plant Biotech. J. 14, 2033-2044.

31. Jiang, P., Chen, Y., and Wilde, H.D. (2016) Reduction of *MLO1* expression in petunia increases resistance to powdery mildew. Sci. Hort. 201, 225-229.

32. Humphry, M., Reinstadler, A., Ivanov, S., Bisseling, T., and Panstruga, R. (2011) Durable broadspectrum powdery mildew resistance in pea er1 plants is conferred by natural loss of function mutations in *PsMLO1*. Mol. Plant Pathol. 12, 866-878.

33. Berg, J.A., Appiano, M., Martínez, M.S., Hermans, F.W., Vriezen, W.H., et al. (2015) A transposable element insertion in the susceptibility gene *CsaMLO8*

results in hypocotyl resistance to powdery mildew in cucumber. BMC Plant Biol. 15, 243.

34. Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., et al. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nature Biotechnol. 32, 947-951.

35. Peries, O. (1962) Studies on strawberry mildew, caused by *Sphaerotheca macularis* (Wallr. ex Fries) Jaczewski. Ann. Appl. Biol. 50, 211-224.

36. Lifshitz, C., Shalit, N., Slotzky, S., Tanami, Z., Elad, Y., and Dai, N. (2008) Heritability s studies and DNA markers for powdery mildew resistance in strawberry (*Fragaria* × *ananassa* Duchesne). VI International Strawberry Symposium 842, 561-564.

37. Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., et al. (2010) The genome of woodland strawberry *(Fragaria vesca*). Nature Genet. 43, 109-116.

38. Miao, L., Jiang, M., Zhang, Y., Yang, X., Zhang, H., et al. (2016) Genomic identification, phylogeny, and expression analysis of *MLO* genes involved in susceptibility to powdery mildew in *Fragaria vesca*. Genet. Mol. Res. 15, gmr15038400.

39. Slovin, J.P., Schmitt, K., and Folta, K.M. (2009) An inbred line of the diploid strawberry *Fragaria vesca* f. *semperflorens* for genomic and molecular genetic studies in the Rosaceae. Plant Methods 5, 15.

40. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

41. Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

42. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

43. Bindschedler, L.V., Palmblad, M., and Cramer, R. (2008) Hydroponic isotope labelling of entire plants (HILEP) for quantitative plant proteomics; an oxidative stress case study. Phytochemistry 69, 1962-1972.

44. Jambagi, S., and Dunwell, J.M. (2015) Global transcriptome analysis and identification of differentially expressed genes after infection of *Fragaria vesca* with powdery mildew (*Podosphaera aphanis*). Transcriptomics: Open Access 3, 1. DOI:10.4172/2329-8936.1000106.

45. Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

46. Winterhagen, P., Howard, S.F., Qiu, W., and Kovacs, L.G. (2008) Transcriptional up-regulation of grapevine *MLO* genes in response to powdery mildew infection. Am. J. Enol. Vitic. 59, 159-168.

47. Elliott, C., Zhou, F., Spielmeyer, W., Panstruga, R., and Schulze-Lefert, P. (2002) Functional conservation of wheat and rice Mlo orthologs in defense modulation to the powdery mildew fungus. Mol. Plant Microbe Interact. 15, 1069-1077.

48. Consonni, C., Humphry, M.E., Hartmann, H.A., Livaja, M., Durner, J., et al. (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nature Genet. 38, 716-720.