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


It is advisable to refer to the publisher’s version if you intend to cite from the work. See Guidance on citing.

Publisher: Open Access Pub

www.reading.ac.uk/centaur
CentAUR
Central Archive at the University of Reading
Reading's research outputs online
Identification and expression analysis of *Fragaria vesca* MLO genes involved in interaction with powdery mildew (*Podosphaera aphanis*)

Shridhar Jambagi1,2 * and Jim M. Dunwell1,2

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

2School 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 *Simlo1* 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 Eulica,
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/m²/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.

**Semi quantitative 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’-CAGACTTGAGAAGGCCACCTA-3’ reverse: 5’-GATACCTTATCCTTCCCTCAGA-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 (Accugenec, 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−ΔCT 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 (SIMLO1) 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

DOI: Coming Soon
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome location</th>
<th>Transcript name</th>
</tr>
</thead>
<tbody>
<tr>
<td>FvMLO1</td>
<td>LG1: 7017413 - 7022793</td>
<td>mrna13023.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO2</td>
<td>LG5: 20075140 - 20093212</td>
<td>mrna31488.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO3</td>
<td>LG6: 8563501 - 8567082</td>
<td>mrna09653.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO4</td>
<td>LG6: 8563518 - 8566426</td>
<td>mrna09653.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO5</td>
<td>LG7: 14517490 - 14520807</td>
<td>mrna23198.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO6</td>
<td>LG3: 10707557 - 10713141</td>
<td>mrna03210.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO7</td>
<td>LG2: 15724387 - 15725931</td>
<td>mrna10558.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO8</td>
<td>LG5: 20171268 - 20174872</td>
<td>mrna31498.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO9</td>
<td>LG3: 17105660 - 17112982</td>
<td>mrna28541.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO10</td>
<td>LG6: 8541201 - 8546252</td>
<td>mrna09651.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO11</td>
<td>LG7: 16370664 - 16374969</td>
<td>mrna26428.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO12</td>
<td>LG5: 19055228 - 19057796</td>
<td>mrna29285.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO13</td>
<td>LG1: 697457 - 707892</td>
<td>mrna10166.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO14</td>
<td>LG3: 4660882 - 4663962</td>
<td>mrna29770.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO15</td>
<td>LG3: 25834627 - 25838931</td>
<td>mrna10346.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO16</td>
<td>LG3: 9515163 - 9524099</td>
<td>mrna31264.1-v1.0-hybrid</td>
</tr>
<tr>
<td>FvMLO17</td>
<td>LG1: 7820289-7827918</td>
<td>mrna14592.1-v1.0-hybrid</td>
</tr>
</tbody>
</table>
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 (SimmLO1; 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).

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 downregulated 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 Fvesca 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).

**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 *MLOs* 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>
<thead>
<tr>
<th>Gene</th>
<th>HW</th>
<th>YW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mRNA-seq (FPKM)</td>
<td>qRT-PCR (Mean ± SE)</td>
</tr>
<tr>
<td>gene13023 (FvMLO1)</td>
<td>7.53</td>
<td>6.52</td>
</tr>
<tr>
<td>gene09653 (FvMLO3)</td>
<td>0.12</td>
<td>0.38</td>
</tr>
<tr>
<td>gene23198 (FvMLO5)</td>
<td>14.12</td>
<td>16.76</td>
</tr>
<tr>
<td>gene10558 (FvMLO7)</td>
<td>6.39</td>
<td>46.46</td>
</tr>
<tr>
<td>gene25541 (FvMLO9)</td>
<td>2.91</td>
<td>19.39</td>
</tr>
<tr>
<td>gene09651 (FvMLO10)</td>
<td>1.69</td>
<td>4.79</td>
</tr>
<tr>
<td>gene29285 (FvMLO12)</td>
<td>87.71</td>
<td>46.49</td>
</tr>
<tr>
<td>gene10166 (FvMLO13)</td>
<td>10.00</td>
<td>12.63</td>
</tr>
<tr>
<td>gene25770 (FvMLO14)</td>
<td>0.30</td>
<td>0.38</td>
</tr>
<tr>
<td>gene13046 (FvMLO15)</td>
<td>0.27</td>
<td>0.16</td>
</tr>
<tr>
<td>gene31264 (FvMLO16)</td>
<td>3.09</td>
<td>3.11</td>
</tr>
<tr>
<td>gene14592 (FvMLO17)</td>
<td>21.53</td>
<td>21.92</td>
</tr>
</tbody>
</table>
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, SIML01, 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 \textit{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 \textit{FvMLO} genes in control and infected (8 DAI) conditions across four strawberry varieties. Values are shown as mean ± standard error (n=2).

**References**


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


