

# Characterization of nine Mlo family genes and analysis of their expression against pathogen infections in *Vitis flexuosa*

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**Abstract** Mildew resistance locus o (Mlo) is a specific seven-transmembrane gene family that confers resistance against several abiotic and biotic stresses in plants. In this study, we identified nine Mlo-like loci, *VfMlo-like3243* (*Vitis flexuosa* powdery-mildew resistance locus o-like3243), *VfMlo-like4098*, *VfMlo-like5057*, *VfMlo-like6833*, *VfMlo-like7881*, *VfMlo-like8120*, *VfMlo-like14439*, *VfMlo-like14557*, and *VfMlo-like18484*, which showed differential expression upon transcriptome analysis conducted using next generation sequencing (NGS) of *V. flexuosa* infected with *Elsinoe ampelina*. These genes are 1773–2548 bp long and encode deduced full-length proteins of 482–587 amino acids. The predicted amino acid sequences of all genes show typical Mlo domains containing seven highly conserved transmembrane domains, a calmodulin-binding domain and 30 important amino acid residues for Mlo function. While 3 *VfMlo*-like genes were downregulated, 5 *VfMlo*-like genes were up-regulated in grapevines infected with *Erysiphe necator*, among which four (*VfMlo-like6833*, *VfMlo-like8120*, *VfMlo-like14439* and *VfMlo-like18484*) are orthologous to the *AtMlo2*, *AtMlo6* and *AtMlo12* genes involved in powdery mildew susceptibility. All genes except *VfMlo-like17881* showed upregulated expression at

48 hpi after infection of *Rhizobium vitis*. The results revealed that the tested genes are related in responses to pathogens of grapevines and can be utilized as useful resources for investigating their roles in disease resistant responses in grapevines.

**Keywords** R-gene · *Vitis* · Mildew resistance locus o (Mlo) · *Erysiphe necator* · *Elsinoe ampelina* · *Rhizobium vitis*

## Abbreviations

NBS-LRR	Nucleotide binding site-leucine rich repeat
R-gene	Resistance gene
VfMlo	<i>Vitis flexuosa</i> powdery-mildew resistance locus o

## Introduction

Because plants are continually exposed to various attacks from pathogens, they employ active and passive defense mechanisms. Two classes of genes have been shown to contribute to resistance reactions in plants: (1) resistance genes involved in the recognition of pathogens, and (2) genes involved in the defense responses (Hammond-Kosack and Jones 1996). Failure of pathogen recognition by plants or suppression of defense response by pathogens results in the occurrence of diseases. For recognition of pathogens, the presence of pathogen avirulence gene and a corresponding plant

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resistance (R) gene are required (Dangl and Jones 2001; Hammond-Kosack and Jones 1997). The genetic interaction between an R gene and an avirulent (Avr) gene drives activation of plant disease resistance signaling cascades, resulting in the induction of defense gene expressions, hypersensitive response (HR) and the elicitation of systemic acquired resistance (Dangl and Jones 2001; Hammond-Kosack and Jones 1996; Staskawicz et al. 1995). Therefore, R genes play a fundamental and initiating role in the inhibition of diseases in plants. R genes are believed to confer specific resistance to diseases caused by fungi, bacteria, viruses, oomycetes and nematodes (Hammond-Kosack and Jones 2000; Taler et al. 2004). It has been reported that the largest class of R genes is nucleotide binding site-leucine rich repeat (NBS-LRR) class (Joshi and Nayak 2011). Generally dominant plant R genes are used in breeding plants resistant to diseases, however, the durability of R genes is limited due to the appearance of the virulent strains of pathogen that can overcome resistance in a few years (Pessina et al. 2014). An alternative to the R genes is the use of plant susceptibility genes (S-genes), whose loss of function gives potential durable resistance in plants (Pavan et al. 2010). Mildew resistance locus o (Mlo) is a plant specific gene that contains seven transmembrane domains and a unique carboxyl-terminal calmodulin-binding domain (Buschges et al. 1997; Devoto et al. 1999; Kim et al. 2002a). Mlo protein resides in the plasma membrane with extracellular N-terminus and cytosolic C-terminus (Kim et al. 2002a). The Mlo gene was first identified in barley as a modulator of the defense response against the powdery mildew pathogen, *Blumeria graminis* f.sp. *hordei* (Buschges et al. 1997). Mlo is also reported as a susceptibility factor involved in adapted powdery mildew pathogens for host cell entry. Barley mutants with lack of functional *HvMlo* showed broad-spectrum resistance to all known powdery mildew isolates of *Blumeria graminis* f.sp. *hordei* (Feechan et al. 2013). Members of the Mlo gene family are believed to participate in various developmental pathways and to show responses against foreign biotic and abiotic stresses in plants (Deshmukh et al. 2014). Although genes in the Mlo class are not as large as those of the NBS-LRR and receptor like protein kinase (RLK) class, several Mlo homologs have been identified in various plant species. The *Arabidopsis* and rice genomes have been reported to contain 15 and 12 Mlo genes, respectively (Devoto et al. 2003; Liu and Zhu 2008),

while the maize (*Zea mays* L.), European cultivated grape (*Vitis vinifera* L.), wheat (*Triticum aestivum* L.), cucumber (*Cucumis sativus* L.), soybean (*Glycine max*(L.) Merr.) and tomato (*Solanum lycopersicum* L.) genomes contain 9, 17, 7, 14, 39 and 17 Mlo genes, respectively (Chen et al. 2014; Deshmukh et al. 2014; Feechan et al. 2008; Konishi et al. 2010; Zhou et al. 2013).

Cultivated grapevines (*Vitis* spp.) are exposed to many pathogenic fungi, bacteria, and viruses (Wang et al. 2011). Grapevine powdery mildew caused by an obligate biotrophic ascomycete, *Erysiphe necator* (syn. *Uncinula necator*) is the most important fungal disease of grapes. Severe infections weaken the vigor of grapevines leading to reduced fruit quality and yield as a result of berry cracking and cluster rot in grape growing regions (Riaz et al. 2011). Grape anthracnose caused by *E. ampelina* is a major devastating disease that results in loss of yield and quality of grapes (Kono et al. 2009; Mirica 1994; Yun et al. 2007). Grape crown gall by *R.vitis* causes serious economic loss in fruit productivity via tumor formation (Eastwell et al. 2006). Because the application of commercial fungicides to control diseases is very expensive and causes various adverse effects on the environment, interest in the development of disease resistant grape cultivars is increasing. Although cultivated grape species (*V. vinifera* L.) are highly susceptible to many diseases (Wang et al. 2011), wild cultivars such as *V. riparia*, *V. rupestris*, and *V. rotundifolia* are resistant to several important grapevine diseases (Eibach et al. 1989; Kortekamp et al. 2008; Reisch et al. 2012). Several Mlo genes were reported to show different expression levels in response to inoculation with *E. ampelina* in *V. flexuosa*, which commonly infects Korean wild grape species (Ahn et al. 2014). Therefore, in this study, we identified nine Mlo-like genes in *V. flexuosa*, analyzed their structural features and homology, evaluated the phylogenetic relationship of Mlos from *V. flexuosa* and other plants, and investigated expression patterns against infection by *E. necator*, *E. ampelina* and *R. vitis*.

## Materials and methods

### Plant materials and pathogens

Pot cultures of grapevines (*V. flexuosa* VSKO001 native to Korea) were maintained in a greenhouse at

Yeungnam University, Gyeongsan for leaf production. Leaves were subjected to infection of pathogens, *E. necator* collected from naturally infected leaves in grape germplasm of Yeungnam University, Gyeongsan, *E. ampelina* (EA-1) was isolated from infected grapes by Dr. W. K. Kim of the National Academy of Agricultural Science, RDA, Korea and *R. vitis* (Cheonan 493) provided by Prof. J.S. Cha of Chungbuk National University, Korea.

#### Inoculation of pathogens

Spore suspensions ( $10^6$  spores/ml) of *E. necator*, grape powdery mildew pathogen, were prepared from infected grape leaves by washing conidiospores using brush and distilled water (Bai et al. 2003) and were sprayed onto leaves for inoculation. Mycelia of *E. ampelina* were cultured on potato dextrose agar (PDA) medium (PDA; 4 g potato starch, 20 g dextrose and 15 g agar per litre) and several colonies of the *E. ampelina* were transferred to Fries liquid medium and incubated in a shaking incubator (140 rpm) at 28 °C for 21 days. Pellets of cultures were then harvested by centrifugation, ground with a homogenizer in sterile distilled water, poured onto V-8 juice agar medium [20 % (v/v) V-8 juice, 2 % (w/v) agar] and incubated at 28 °C under a near ultraviolet lamp for 2 days to produce spores (Yun et al. 2007). *E. ampelina* were then collected by scraping-off the plates with sterile distilled water. The concentrations of the spore suspensions were measured by hemocytometer and adjusted to  $10^6$  spores/ml. Inoculation of spore suspensions was performed by spraying onto leaves. For bacterial inoculation, *R. vitis* was grown on PDA media in the dark for 3 days, after which a single colony was transferred to YEP medium (10 g yeast extract, 5 g bacto peptone, 5 g NaCl per litre and pH 7.0) and grown at 28 °C in a shaking incubator for 16–18 h. The bacterial cell was subsequently collected by centrifugation and resuspended by sterile water at a concentration of  $OD_{600} = 1$ . Then, the leaves were injured slightly with a pencil tip and inoculated by dropping 20  $\mu$ l of *R. vitis* cell suspensions onto the wounded portion of the leaves. The leaves inoculated with pathogens were subsequently incubated in a moist box at 22–28 °C for 48 h, after which they were harvested at different hours (0, 1, 6, 12, 24, and 48) post inoculation (hpi), immediately frozen in liquid nitrogen and stored at –80 °C for further analysis.

#### RNA isolation and real time PCR analysis

Leaf samples were ground in liquid nitrogen using a mortar and pestle, after which total RNA was extracted using the modified pine tree method (Chang et al. 1993). The RNA quality was then measured based on the absorbance at 230, 260 and 280 nm using a Nano Drop spectrophotometer (ACTGene ASP-3700; ACTGene, Inc., Piscataway, Middlesex County, New Jersey, USA). Next, first-strand cDNA was synthesized from the total RNA (500 ng) using a GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) for use as a PCR template. Real-time PCR was conducted on a C1000™ Thermal Cycler (BioRad, Hercules, CA, USA) using SYBR Premix Ex Taq (TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye. Amplification was conducted by subjecting the samples to 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Transcript levels were calculated using the standard-curve method and normalized against the grapevine actin gene (AB372563) as an internal control and non-treated leaves (at time zero) as a reference. In addition, melting curves of the amplified products were recorded. For each gene, the reference sample was defined as the  $1 \times$  expression level and the results were expressed as the fold increase in mRNA level over the reference sample. Experiments were replicated three times to minimize the error. The gene specific primers shown in Table 1 were designed for real time PCR based on nucleotide sequence alignment.

#### Sequence analysis of genes

The nucleotide sequences of genes were converted to amino acid sequences using translation software (<http://web.expasy.org/translate/>), after which analysis of primary structures of the genes was performed with the ExPasyprotParam (<http://web.expasy.org/protparam/>) and the secondary structure was analyzed using SOPMA ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=npsa\\_sopma.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html)). Multiple alignment of protein sequences was performed using clustalw (<http://www.genome.jp/tools/clustalw/>). Homologue protein sequences of the genes were recovered from the NCBI database by BLAST (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches of the “nr” database. Additionally, the VfMlo-like genes were blasted against each other to check for gene duplication events. To verify the presence of the Mlo

**Table 1** Specific primers based on alignment of nine VfMlo-like genes used for real time PCR

Gene	Primers	Sequences
<i>VfMlo-like3243</i>	FP	TCTGGGGTGAATTTTCAGTGC GA
	RP	CAAGAGAGGGGAAACGGGTTGT
<i>VfMlo-like4098</i>	FP	CATCCGTTCACTTCCCATCGCT
	RP	GCGATGGGTTTTGAAGAGCGTG
<i>VfMlo-like5057</i>	FP	AGTAGGAGGAATGGCAGGGAGG
	RP	TCCCAGAAACACGCCCAATACC
<i>VfMlo-like6833</i>	FP	ACTTGAACGCTGTAGAACGAGC
	RP	GTGATTGATGGGCCGTGGAGAT
<i>VfMlo-like7881</i>	FP	AGGAACCGCAATTCAGCTTGGA
	RP	TTTCCTCAGCGGCACCTGTAAG
<i>VfMlo-like8120</i>	FP	AGAGACGGTGCCTACTAAACGG
	RP	TCACTGACAAGCGATATGGGCA
<i>VfMlo-like14439</i>	FP	GTCTTCGATCCCTTGTCCTGC
	RP	TATGGGGAAGGAGCAGGGTCTC
<i>VfMlo-like14557</i>	FP	TGATGACTTCCCACCTTTCACGG
	RP	CTCCGACTTAGACGCCGAAACT
<i>VfMlo-like18484</i>	FP	CTGGCATGGTACGGGTCAAGAT
	RP	GAGTATCGCCACCGTGAGGATA

FP forward primer, RP reverse primer

domain, the VfMlo-like protein sequences were analyzed using the web tool, SMART ([http://smart.embl-heidelberg.de/smart/set\\_mode.cgi?GENOMIC=1](http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1)) (Letunic et al. 2009). The transmembrane domain was analyzed using the consensus prediction of membrane protein topology (TOPCONS) (<http://old.topcons.net/>) (Bernselet et al. 2009). Phylogenetic neighbor-joining analyses of gene sequences were performed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Saitou and Nei 1987; Tamura et al. 2013). The tree branches were evaluated using the bootstrap method (Felsenstein 1985). The conserved motifs of the predicted proteins of VfMlo-like genes were analyzed using the Multiple Expectation Maximization for Motif Elicitation (MEME) online program (<http://meme.sdsc.edu/meme/intro.html>). The optimized parameters of MEME were employed as follows: maximum number of motifs 10; minimum motif width 10; and maximum motif width 50.

## Results and discussion

### Identification and sequence analysis of Mlo family genes in grapevines

Nine of 16 transcripts with different transcription levels at 24 h in the transcriptome of *V. flexuosa*

VISKO001 infected with *E. ampelina* by NGS (Ahn et al. 2014) were selected and verified as Mlo-like genes by detecting the Mlo domain using the Simple Modular Architecture Research Tool (SMART) ([http://smart.embl-heidelberg.de/smart/set\\_mode.cgi?GENOMIC=1](http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1)). A protein homology study of nine transcripts conducted using the Basic Local Alignment Search Tool of the NCBI database showed a high degree of homology with *Vitis vinifera* Mlo genes. In this study, these genes were characterized and denoted *VfMlo-like3243* (*Vitis flexuosa* powdery-mildew resistance locus *q*-like 3243), *VfMlo-like4098*, *VfMlo-like5057*, *VfMlo-like6833*, *VfMlo-like7881*, *VfMlo-like8120*, *VfMlo-like14439*, *VfMlo-like14557*, and *VfMlo-like18484* and deposited in the National Agricultural Biotechnology Information Center (NABIC), Rural Development Administration, Korea database under accession numbers NS-0713- NS-0721, and in the GenBank under accession numbers KX462171- KX462179, respectively. The VfMlo-like genes were then compared by blasting them (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to each other to check gene duplication events. Less than 70 % similarity in deduced amino acid sequences was observed among them (Table 2). Kong et al. (2013) reported that gene duplication has occurred

when the coverage of aligned regions is more than 80 % and the homology of the aligned region is greater than 80 %. According to Kong et al. (2013), these findings indicated that there were no duplication events among the nine VfMlo-like genes tested in this study. The primary structure and characteristics of these genes of *V. flexuosa* were analyzed using bioinformatic tools (Table 3). The results showed that the size of the nine loci of the VfMlo-like genes varied from 1773 to 2548 bp, while the open reading frame (ORF) ranged from 1449 to 1764, encoding 482 to 587 amino acids (55.20 to 67.30 kDa). The predicted isoelectric points of these proteins showed basic characters, which are common features in Mlo proteins (Chen et al. 2014).

**Table 2** Per cent identities of nine predicted proteins from the VfMlo-like gene of *V. flexuosa*

	1	2	3	4	5	6	7	8	9
1		40	39	44	44	38	41	43	42
2	40		45	48	42	45	49	43	49
3	39	45		50	38	43	48	38	53
4	44	48	50		45	50	63	43	70
5	44	42	38	45		41	44	67	44
6	38	45	43	50	41		52	40	52
7	41	49	53	63	44	52		42	64
8	43	43	38	43	67	40	42		44
9	42	49	53	70	44	52	64	44	

1 VfMlo-like3243, 2 VfMlo-like4098, 3 VfMlo-like5057, 4 VfMlo-like6833, 5 VfMlo-like7881, 6 VfMlo-like8120, 7 VfMlo-like14439, 8 VfMlo-like14557 and 9 VfMlo-like18484

**Table 3** Primary structure prediction of nine Mlo like genes of *V. flexuosa*

Gene	Nucleotide length (bp)	ORF (bp)	Protein Length (aa)	MW (kDa)	pI	Instability index (II)
<i>VfMlo-like3243</i>	2242	1692	563	64.59	9.07	45.80
<i>VfMlo-like4098</i>	2548	1692	563	63.97	9.10	39.65
<i>VfMlo-like5057</i>	1986	1554	517	60.01	9.42	51.50
<i>VfMlo-like6833</i>	2092	1764	587	67.30	9.33	48.52
<i>VfMlo-like7881</i>	1773	1449	482	55.20	8.98	31.13
<i>VfMlo-like8120</i>	2015	1533	510	58.98	8.82	49.02
<i>VfMlo-like14439</i>	1810	1620	539	62.19	9.10	50.75
<i>VfMlo-like14557</i>	2166	1521	506	57.91	9.12	28.63
<i>VfMlo-like18484</i>	1931	1752	583	67.30	9.32	45.93

ORF open reading frame, MW molecular weight, pI isoelectric point

Among nine proteins, VfMlo-like14557, VfMlo-like7881 and VfMlo-like4098 showed an instability index of less than 40 (Table 3), indicating that they were stable based on the instability index (II) (Guruprasad et al. 1990). The secondary structures of the predicted proteins of the nine VfMlo-like genes were also analyzed by the Self-Optimized Prediction Method with Alignment (SOPMA) (Table 4). The results showed that folding of polypeptides deduced from VfMlo genes largely formed a helix (33.45–49.13 %) and random coil (22.61–35.09 %), which were connected with extended stands (18.57–24.19 %) and beta turns (6.96–10.47 %).

#### Structural features of nine VfMlo-like genes

Devoto et al. (1999) showed that Mlo is an integral plasma membrane-residing protein including seven transmembrane (TM) helices, an extracellular amino-terminal and a cytoplasmic carboxyl-terminal. The TMs of the nine deduced proteins of VfMlo-like genes were predicted by the TOPCONS (Bernsel et al. 2009). Barley Mlo genes and *Arabidopsis* Mlo genes encode seven-TM (7TM) proteins (Devoto et al. 1999). All of the proteins of the Mlo genes tested in this study were found to have seven TMs, which is a common characteristic of Mlo genes. To highlight the conserved amino acid residues in the seven TM domains, the TM domain logo was constructed using the online logo tool WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) (Crooks et al. 2004). The sequence logo revealed that several conserved amino

**Table 4** Predicted secondary structures of nine proteins deduced from the VfMlo-like gene

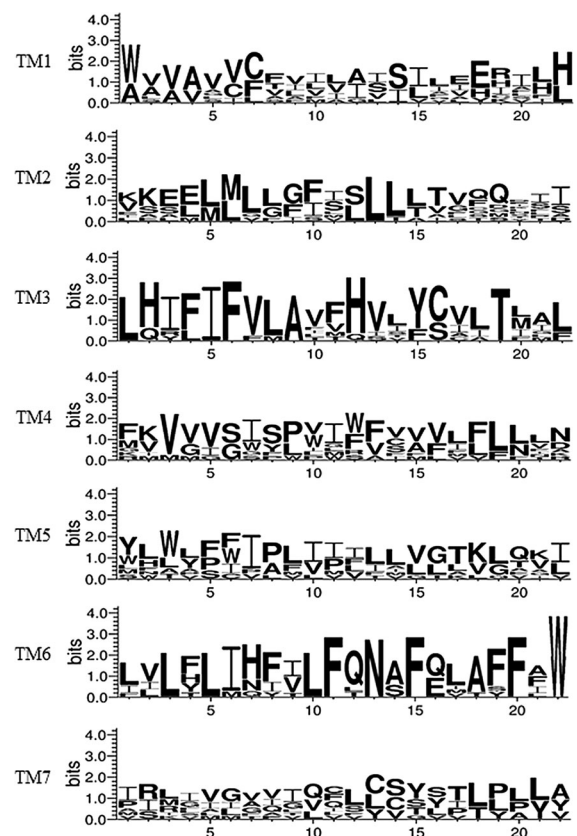
Protein	Random coil (%)	Alpha helix (%)	Beta turn (%)	Extended strand (%)
VfMlo-like3243	32.33	38.01	7.82	21.85
VfMlo-like4098	32.68	38.54	8.70	20.07
VfMlo-like5057	24.56	49.13	6.96	19.34
VfMlo-like6833	35.09	37.31	9.03	18.57
VfMlo-like7881	22.61	46.27	7.88	23.24
VfMlo-like8120	29.61	40.59	7.45	22.35
VfMlo-like14439	33.58	38.22	8.72	19.48
VfMlo-like14557	26.48	41.11	10.47	21.94
VfMlo-like18484	32.42	33.45	9.95	24.19

acids were found in all seven TM domains, such as fully conserved leucine in TM2, isoleucine, phenylalanine and threonine in TM3 and three phenylalanines, asparagines, and tryptophans in TM6 domain (Fig. 1).

#### Multiple sequence alignment and conserved motif analysis of nine VfMlo-like genes

To analyze the sequence characteristics of the predicted proteins from the nine VfMlo-like genes tested in this study, multiple sequence alignment of these candidate proteins including barley and *Arabidopsis* Mlo proteins was conducted using clustalw. The alignment results revealed that all predicted proteins from VfMlo-like genes contained seven transmembrane domains, and that the transmembrane regions, which are considered to be characteristic features of Mlo proteins (Devoto et al. 2003), were relatively well conserved (Fig. 2). The results of multiple sequence alignment also revealed the presence of 30 important amino acid residues (Fig. 2) that have previously been identified as invariable in 38 Mlo genes from various plant species (Elliot et al. 2005). Thirteen of these amino acids were located in six of the seven TM domains (Fig. 2). Further analysis of conservation of these 30 important amino acids in each VfMlo-like protein showed that four VfMlo-like proteins (VfMlo-like3243, VfMlo-like4098, VfMlo-like7881 and VfMlo-like14439) had 30 important conserved amino acids, while the other five (VfMlo-like5057, VfMlo-like6833, VfMlo-like8120, VfMlo-like18484, and VfMlo-like14557) had no conserved cysteine in position 114. Unlike barley Mlos, however, they all contained a cysteine residue in

the immediately preceding position, similar to AtMlo6. VfMlo-like5057 lost another conserved tryptophan residue at position 263 (Fig. 2). All VfMlo-like proteins commonly contained four extracellular cysteine residues that were considered



**Fig. 1** Logos of amino acid sequences of seven TMs show consensus sequences in the transmembrane domain within nine VfMlo-like genes. Horizontal lines indicate the position of amino acids and vertical lines indicate the frequency of amino acids by bit values

essential for the function of barley Mlo (Elliot et al. 2005). A calmodulin-binding (CaMBD) domain in Mlo protein has been shown to be an important domain that is necessary and sufficient for  $\text{Ca}^{2+}$ -dependent CaM complex formation. CaMBD contains three hydrophobic amino acid residues and a conserved tryptophan residue (Kim et al. 2002a, b; Bhat et al. 2005). The alignment results showed a putative CaMBD domain in all nine VfMlo proteins containing hydrophobic amino acid residues at positions 420, 427 and 433, and a highly conserved tryptophan residue at position 423 corresponding to the position of HvMlo (Fig. 2). Panstruga (2005) showed the presence of two conserved regions in the carboxyl-terminal of several Mlo proteins, which may modulate powdery mildew infection in various plants including *Arabidopsis*, cabbage, tomato, pepper, barley, maize, rice and wheat. Peptide domain I is characterized by conserved serine and threonine residues and Peptide domain II is characterized by the consensus sequence D/E–F–S/T–F (Panstruga 2005). Sequence alignment revealed that, among nine predicted proteins, VfMlo-like6833, VfMlo-like14439 and VfMlo-like18484 contained both peptide domains I and II and VfMlo-like3243 contained peptide domain I in their C-terminal regions (Fig. 2).

Subsequently, conserved motifs of VfMlo-like proteins were analyzed using the MEME online analysis tool. The results showed there were ten conserved motifs ranging from 19 to 50 amino acids in nine VfMlo-like proteins (Fig. 3; Table 5). This is consistent with the results that there were also ten conserved motifs in tomato Mlo proteins (Chen et al. 2014), and that there were ten conserved motifs in *Cucumis sativus* Mlo proteins (Zhou et al. 2014). Distribution analysis of the conserved motifs in the nine VfMlo-like proteins revealed that six contained ten motifs and three proteins had nine motifs. Six of the ten conserved motifs (motif 1, 2, 3, 4, 6 and 7) were located in seven TM domains. Motif 9 comprised the calmodulin-binding domain, while three other motifs were distributed in other regions of the predicted proteins of the nine VfMlo-like genes. The results are almost similar with previous report that seven of the ten conserved motifs were located in the seven TM domains and CaMBD domain while the remaining three motifs were located in other regions (Zhou et al. 2014).

#### Phylogenetic relationship of Mlo-like genes in *V. flexuosa* with other plants

A phylogenetic tree of 40 Mlo members from dicots and monocots including nine VfMlo-like proteins was constructed by the neighbor joining method using MEGA6 to illustrate the genetic relationships of VfMlo-like with other plant proteins (Fig. 4). The members were divided into six major clades (Clade I–VI) with strong bootstrap support for the monophyly of each clade. There was also evident bootstrap support for a sister clade relationship between subfamily III and IV. The designation of these clades was based on the classification by Devoto et al. (2003). Phylogenetic analysis showed that the VfMlo-like proteins were distributed in five of the six clades. VfMlo-like3243, VfMlo-like4098 and VfMlo-like5057 were clustered with clade I, III and VI, respectively. Clade II contained two VfMlo-like proteins, VfMlo-like7881 and VfMlo-like14557, while clade V included four VfMlo-like proteins, VfMlo-like6833, VfMlo-like8120, VfMlo-like14439 and VfMlo-like18484. *HvMlo*, the first identified Mlo gene responsive to biotic stress, was clustered with clade IV, which was specific for monocots (Buschges et al. 1997; Deshmukh et al. 2014). Devoto et al. (2003) also found this clade to be a monocot specific group. Members of clade IV, V and VI were previously identified as biotic stress responsive genes (Chen et al. 2006; Elliot et al. 2002; Consonni et al. 2006). Although plant Mlo members involved in biotic stress response were clustered into clade IV, V, and VI, nine VfMlo proteins were distributed in all five clades except monocot-specific clade IV in the tree. In each group, VfMlos are clustered closely with clones originating from *V. vinifera* (Fig. 5).

It was reported that tomato *SiMlo1*, pepper *CaMlo2*, eggplant *SmMlo1*, potato *StMlo1* and tobacco *NtMlo1* were characterized to be causally associated with PM susceptibility, and they were clustered into clade V in the molecular level (Panstruga 2005; Appiano et al. 2015a). Appiano et al. (2015a, b) reported that all the monocot and dicot Mlo homologs shown to be required for powdery mildew susceptibility were also included in clade V in the tests with transgenic plants. In this study, VfMlos were further analyzed with Mlos from other plants by multiple alignment with respect to conserved amino acid residues. The results showed that VfMlo-like8120,

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Vf4 1 ----MAGATGGRLEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOS-LIISFC
Vf9 1 ----MAGSKDRLEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-TIAGFC
Vf7 1 ----MADEEERLEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-LIIRKFC
At 1 ----MAQVQKELTEPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-LIIRKFC
Vf6 1 MAGDEETTTTTTALLETPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-SIAKIC
Vf3 1 ----MATGNSVRELEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-PSKIC
Hv 1 ----MSDKGVFARELEPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-LIIRKFC
Vf2 1 ----MAGDSSGTSASRELEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-FIVKIC
Vf5 1 ----MAGGAEETLEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-LIIRKFC
Vf8 1 ----MTVAGETLEQPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-TIAGFC
Vf1 1 ----MAENEKETRELEPTWAVAVVCFVYVLIISIIIEYIHLGKWLKKEKALAELEKVKSELMMLLGPISLLLTVCOSG-MISFC

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TM1

TM2

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Vf4 84 LSKSIAAIFHPCSPSEKSTITEESDTES---DNRRKLLSISGFGGSRVLAEEEDKSAKGVFVSDVHQLHIFIFVLAHFV
Vf9 84 LSEKTAGIHPCCGKCKIKYVSNEED---YGKRRLEISDSGNSNRVLAAGDDKCEGKVPVSDVHQLHIFIFVLAHFV
Vf7 84 LSESISGHPCCKIKYVSNEED---ETKDFKNCSKGVPLVSVSGHQLHIFIFVLAHFV
At 84 LKKNIASMHPCSSASBEARKYKGDVPEKEEENLRKLLQLVDSLIPRRSLATKGYDKAEKGVAVFVSDVHQLHIFIFVLAHFV
Vf6 90 LERSVGETLPCRTLPS-----DSEETKEEQGKNSISROGVEELQYLFVLAHFV
Vf3 85 LEPYVAIKMLPCRRLLVDDQ-----SANTTDPKSKGVPLVSVSGHQLHIFIFVLAHFV
Hv 87 LSEDAIVMMCKRKGKGRKPS-----KYVDYCPKGVPLVSVSGHQLHIFIFVLAHFV
Vf2 89 LIEAAATMLPCPYNGEKDSSSETESRRRL-----LWHNHRLAAATYSSSCKEYDPLISVNGHQLHIFIFVLAHFV
Vf5 86 LKHHVSHLLPCLPBE---KHATQSLSHWALGRHLLS-----SAPSSCNEKVPPLISLALHQLHIFIFVLAHFV
Vf8 83 LKHYSDLLCKKKEKDSSESSEESTSHFQTYHFTSG---GARRLAEHSASTSYAKRGGDPLISLALHQLHIFIFVLAHFV
Vf1 85 LRSFYDSPAPCSADVEEGNERKSSQERKLLMGSVLP-----HSFRVNLVLSQNSCKEHEBIVSYEQLHIFIFVLAHFV

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TM3

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Vf4 171 LVCETLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf9 166 LYCVATLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf7 135 LYCVATLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
At 173 LYCVATLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf6 145 LYCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf3 141 LYCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Hv 143 LSVLITLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf2 164 LCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf5 155 LCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf8 166 LCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI
Vf1 167 LCVLTLALGRKMRMWRANEKETITVEYOSHDPER---FRFARFSGRRHLS---FWKTPFLIMVCFRQFVRSVKVDYFLRHGFI

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Vf4 258 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf9 253 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf7 222 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
At 260 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf6 232 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf3 228 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Hv 229 MAHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf2 251 TVHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf5 244 MHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf8 253 MHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV
Vf1 256 MHLAPSHAKFDFOKYINRSLEEDFKVVVGISSPWFVFAVFLNTHGWHSYLWLPFIPLVILVGTGKLOIITKMLGRICERGEVV

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TM4

TM5

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Vf4 348 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf9 343 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf7 312 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
At 350 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf6 320 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf3 316 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Hv 317 KGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf2 339 QGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf5 332 EGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf8 341 EGVVPLVGLDGLFWFNRELRVLIHLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST
Vf1 343 AKAQKBRNDLFWFKKHELHFLHFLQNAFQLAFFANWYEFGLKSCFYAHTEDVIRISGVIITOLCSYITLPLVALVTQMGST

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TM6

TM7

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Vf4 438 MKPPIFENRVAKALNWHIAKKNKHNKHSG---LATPMS-----RPTPSHGTSFAYLLRYRDMDSLQASPRRNLMEHW
Vf9 433 MKPPIFENRVAKALNWHIAKKNKHNKHSG---AVTPVSSRAGTPFSSRPGTPLHGMSPLLRRHRELDVSQTSPRMSNFHEGL
Vf7 402 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPAFPSYGMSPHLLQGYHNHTPMSPRRNLNMYGE
At 440 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPTPSHGTSFAYLLRYRDMDSLQASPRRNLMEHW
Vf6 410 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPAFPSYGMSPHLLQGYHNHTPMSPRRNLNMYGE
Vf3 406 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPTPSHGTSFAYLLRYRDMDSLQASPRRNLMEHW
Hv 407 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPAFPSYGMSPHLLQGYHNHTPMSPRRNLNMYGE
Vf2 429 MKPPIFENRVAKALNWHIAKKNKHNKHSG---RPAFPSYGMSPHLLQGYHNHTPMSPRRNLNMYGE
Vf5 422 FKKAFTDVOAGLVGAQAARKTITGNANR-----DAPSRGSPPSRGSSPHLLHKGMRSDDPQASPT
Vf8 431 FKKAFTDVOAGLVGAQAARKTITGNANR-----DAPSRGSPPSRGSSPHLLHKGMRSDDPQASPT
Vf1 432 YKAAIIPHRETITGKAAARREKELMFDSDS-----IHTDTEHMSVEEDNIRLFDPTVTHRGCMTIEPI

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CaMBD

I

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Vf4 516 LIDGSPSPSHPHGDGSSHHNQLHGQTSLEHRRDISAPSSQVPLDOPTPHQHIDVVRKDFSFDRRERT----
Vf9 519 LIDGSPSPSHPHGDGSSHHNQLHGQTSLEHRRDISAPSSQVPLDOPTPHQHIDVVRKDFSFDRRERT----
Vf7 480 GAGSPGKKDDDEHEKEKESREQQG---IEDS-----SSTQLPLGRPIRTOHINITLSDFSFAKR----
At 518 LIDGSPSPSHPHGDGSSHHNQLHGQTSLEHRRDISAPSSQVPLDOPTPHQHIDVVRKDFSFDRRERT----
Vf6 473 LAGYLTVESDEETVAKR-----EPKQLKCFGFGDLRRTS----
Vf3 469 GSPTSKEIIVEVEEVEVRNEAASR-----FRSAMPILQAVKRDGRWKERE----
Hv 484 PRTQOEARMYVUVVAVPHVRLNPNDRRRSASSALADIPSAADFSFQSG----
Vf2 506 LVSDEAEPESPTSTNLIVRVDDSD-----EPETEPPVSHPEHDTSGEIEFSVKKPAPQKGP----
Vf5 476 GAAEENK-----
Vf8 485 LSAEEKNVGEIHPATSGGSK-----
Vf1 502 PASHITLDPSPVANETSFRAGTPLL-----PSASVSASVALHLHBTGLRSSNTLRSSMPSISRE----

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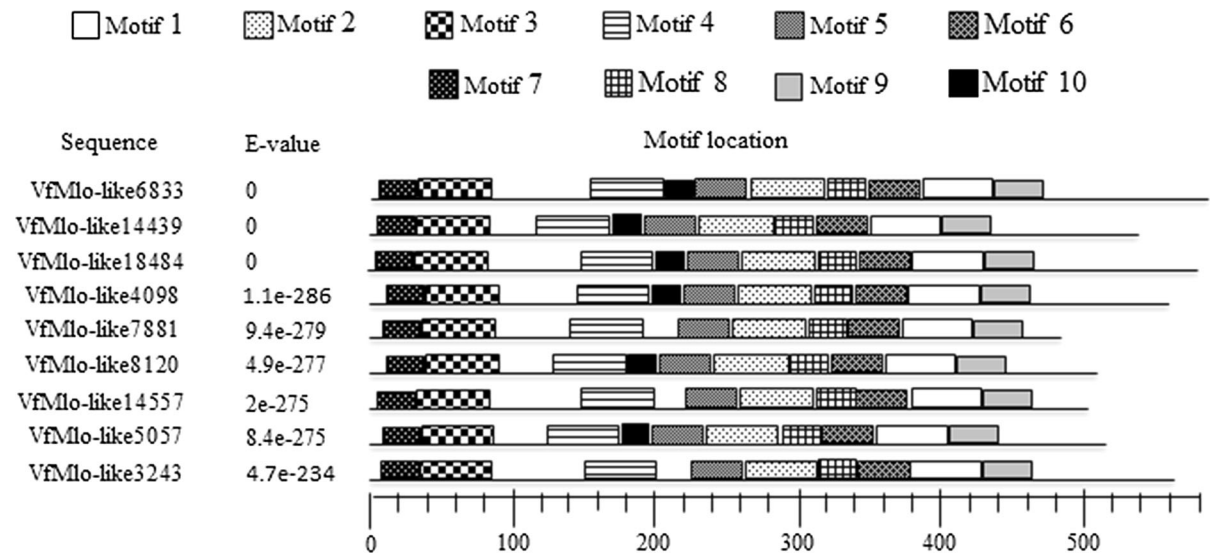
I



**Fig. 2** Multiple alignment of nine predicted proteins from the VfMlo-like gene and two other Mlo proteins. The bars indicate the positions of the seven TM domains, CaMBD and two conserved domains inferred from HvMlo, Devoto et al. (1999, Kim et al. (2002a, b) and Panstruga (2005), respectively. Hydrophobic amino acid residues and a highly conserved tryptophan residue under the CaMBD domain were indicated by an open box and a filled circle, respectively. Black dots under the aligned sequences indicate the position in barley Mlo of the 30 conserved residues (Elliott et al. 2005). Vf1 VfMlo-like3243, Vf2 VfMlo-like4098, Vf3 VfMlo-like5057, Vf4 VfMlo-like6833, Vf5 VfMlo-like7881, Vf6 VfMlo-like8120, Vf7 VfMlo-like14439, Vf8 VfMlo-like14557, Vf9 VfMlo-like18484, At AtMlo6, Hv HvMlo

VfMlo-like6833, and VfMlo-like18484 were clustered into clade V including AtMlo2, AtMlo6, and AtMlo12, which are considered as PM susceptibility factors. It suggests that they can be the most functionally important factors in causing the susceptibility to PM in *V. flexuosa* plants.

A BLAST search of the NCBI database also showed that the deduced amino acid sequences of the nine VfMlo-like genes had high similarity to Mlo protein sequences originating from *V. vinifera* with homology and query coverage values greater than



**Fig. 3** Schematic representation of motif compositions in the predicted proteins of the VfMlo-like gene. Different motifs (1–10) are displayed in different boxes. The names of all

members are displayed on the left-hand side and the bottom scale indicates the length of the motifs

**Table 5** Ten best possible matched sequences of motifs presented in Fig. 3

Motif no.	Best possible matched sequence	Length (aa)
1	FAWTWYEFGFDSCFHNKTEDIIIRISMGVIVQVLC SYVTLPLYALVTQMG	50
2	FDFQKYMKRSLEEDFKV VVGISPYIWFV VVFLLLNVHGWH SYFWIPFIP	50
3	LHLIGKWLKKKNKRALYEALEKIKEELMLLGFISLLLTVCQDMI AKICIP	50
4	HQLHIFIFVLA VFHVLYCVLTMALGRAKMRQWKAWEDETR TDEYQFSNDP	50
5	PVLLWVVCFFRQFYRSVTKVDYLT LTRHGFIMAHCAP	36
6	GTPVVQPSDDYFWFNRPRLVLYLIHFILFQNAFQLA	36
7	RSLEQTPTWAVAVVCFVMVAISIIE	26
8	ILLVGTKLQHITKMALEIAERHEVV	26
9	SNMKPTIFNERVAKALRNWHHAARKNTKHGNNNSN	34
10	RFRFARDTSFGRRHLSFWS	19

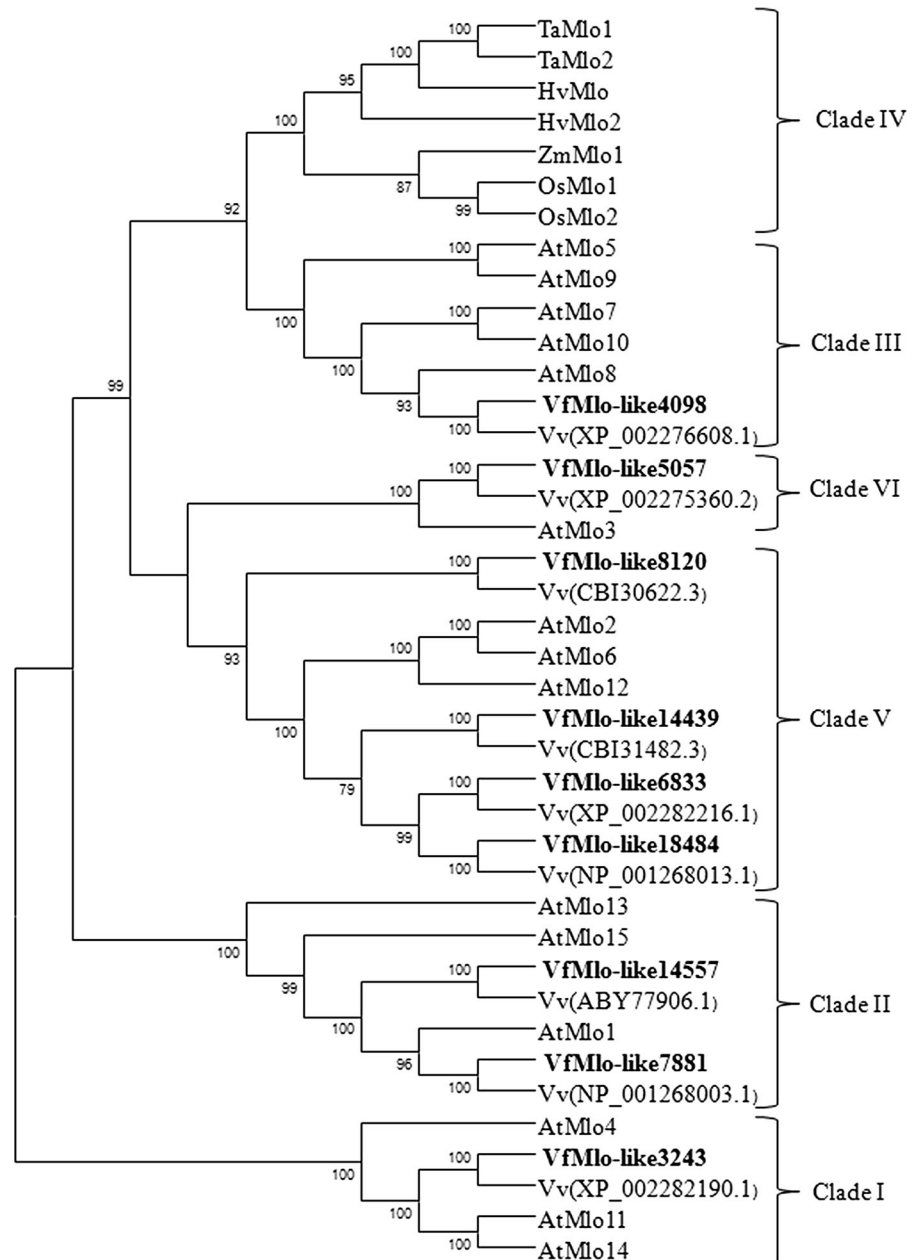
98 % and E values of 0.0, indicating their relatively conserved evolutionary relationship at the protein level (Table 6).

#### Expression analysis of VfMlo-like genes

To investigate the expression patterns of VfMlo-like genes against pathogen infections, expression analysis was performed by quantitative real-time PCR using

gene specific primers based on nucleotide sequence alignment. The expression levels of tested genes in grapevine leaves infected with pathogens were examined as relative expression compared to uninoculated control samples. The expression levels of nine VfMlo-like genes against infection of powdery mildew pathogen (*E. necator*) in grapevines are presented in Fig. 6a. *VfMlo-like6833* and *VfMlo-like14439* showed up regulated expression whereas *VfMlo-like3243*,

**Fig. 4** Phylogenetic tree of predicted proteins from the VfMlo-like genes with other plant Mlo proteins including *Arabidopsis* (AtMlo), *Vitis vinifera* (VvMlo), rice (OsMlo), barley (HvMlo), wheat (TaMlo), and maize (ZmMlo). The unrooted tree was generated using the MEGA 6.0 software and the neighbor-joining method. Bootstrap values (above 70 %) from 1000 replicates are indicated at each node



**Table 6** Homology analysis of nine predicted proteins from the Mlo-like gene of *V. flexuosa*

Protein	Top matched clones	Top homologous species	Identity (%)	Query cover (%)	<i>E</i> value
VfMlo-like3243	XP_002282190.1	<i>Vitis vinifera</i>	99	100	0.00
VfMlo-like4098	XP_002276608.1	<i>Vitis vinifera</i>	99	100	0.00
VfMlo-like5057	XP_002275360.2	<i>Vitis vinifera</i>	99	100	0.00
VfMlo-like6833	XP_002282216.1	<i>Vitis vinifera</i>	98	100	0.00
VfMlo-like7881	NP_001268003.1	<i>Vitis vinifera</i>	98	100	0.00
VfMlo-like8120	CBI30622.3	<i>Vitis vinifera</i>	98	100	0.00
VfMlo-like14439	CBI31482.3	<i>Vitis vinifera</i>	99	100	0.00
VfMlo-like14557	ABY77906.1	<i>Vitis vinifera</i>	98	100	0.00
VfMlo-like18484	NP_001268013.1	<i>Vitis vinifera</i>	99	100	0.00

*VfMlo-like4098*, *VfMlo-like5057* and *VfMlo-like14557* showed down regulated expression at all time points after infection of *E. necator*. *VfMlo-like7881* and *VfMlo-like8120* were up regulated at 1 hpi and then their expressions decreased at later time points. *VfMlo-like18484* showed up regulated expression at 24 hpi.

The response of VfMlo-like genes against *E. ampelina* infection are presented in Fig. 6b. *VfMlo-like14439*, *VfMlo-like14557* and *VfMlo-like18484* were shown to be upregulated at all time points, whereas *VfMlo-like3243*, *VfMlo-like4098*, *VfMlo-like7881* and *VfMlo-like8120* were downregulated at all time points under pathogen infection relative to the control. *VfMlo-like5057* and *VfMlo-like6833* did not show any unique pattern of expression in plants infected with *E. ampelina*. Among the nine VfMlo-like genes, there were considerable changes in expression levels in *VfMlo-like7881* and *VfMlo-like14557* in response to pathogen challenge. While *VfMlo-like14557* was highly upregulated, *VfMlo-like7881* was highly downregulated at all time points after pathogen inoculation. The highest expression was observed in *VfMlo-like14557* at 1 hpi, which was upregulated 4-fold relative to the control.

All transcripts showed active responses against *R. vitis* inoculation (Fig. 6c). All genes except *VfMlo-like8120* and *VfMlo-like14557* showed upregulated expression at 1 hpi, whereas *VfMlo-like8120* and *VfMlo-like14557* showed upregulated expression at later time points after *R. vitis* infection. The expression of *VfMlo-like5057*, *VfMlo-like14439* and *VfMlo-like18484* was up-regulated at all time points. The

highest expression was observed at 1hpi in *VfMlo-like18484* by *R. vitis* inoculation, which was 3 fold increased induction compared to control.

The Mlo genes are plant specific seven transmembrane gene family considered to be induced in response to biotic and abiotic stress and expressed in various developmental pathways (Chen et al. 2006; Kim et al. 2002b; Pessina et al. 2014; Piffanelli et al. 2002). The Mlo protein acts as a modulator of defense and cell death in response to biotic and abiotic stress stimuli (Piffanelli et al. 2002). We attempted to investigate the change of Mlo transcripts against infection with pathogenic fungi and bacteria in grapevines. Chen et al. (2006) found that expression of AtMlo genes from multiple clades was affected by abiotic stress. Konishi et al. (2010) also found that the expression patterns of seven wheat Mlo members against abiotic stress were not related to their phylogenetic classification. Although some apple genes were clustered into a clade including biotic stress responsive genes such as *AtMlo3* and *HvMlo*, they showed no response against the apple powdery mildew pathogen, *Podosphaera leucotricha* (Pessina et al. 2014).

Among nine genes tested in this study, five showed upregulated expression and three showed down regulated expression against powdery mildew pathogen (*E. necator*) infection. Among upregulated genes, four (*VfMlo-like6833*, *VfMlo-like8120*, *VfMlo-like14439* and *VfMlo-like18484*) are orthologous to the *AtMlo2*, *AtMlo6* and *AtMlo12* genes. Feechan et al. (2008) also reported that four grape Mlo family genes (*VvMlo3*, *VvMlo4*, *VvMlo9* and *VvMlo17*), which are also orthologous to the *AtMlo2*, *AtMlo6* and *AtMlo12* genes,

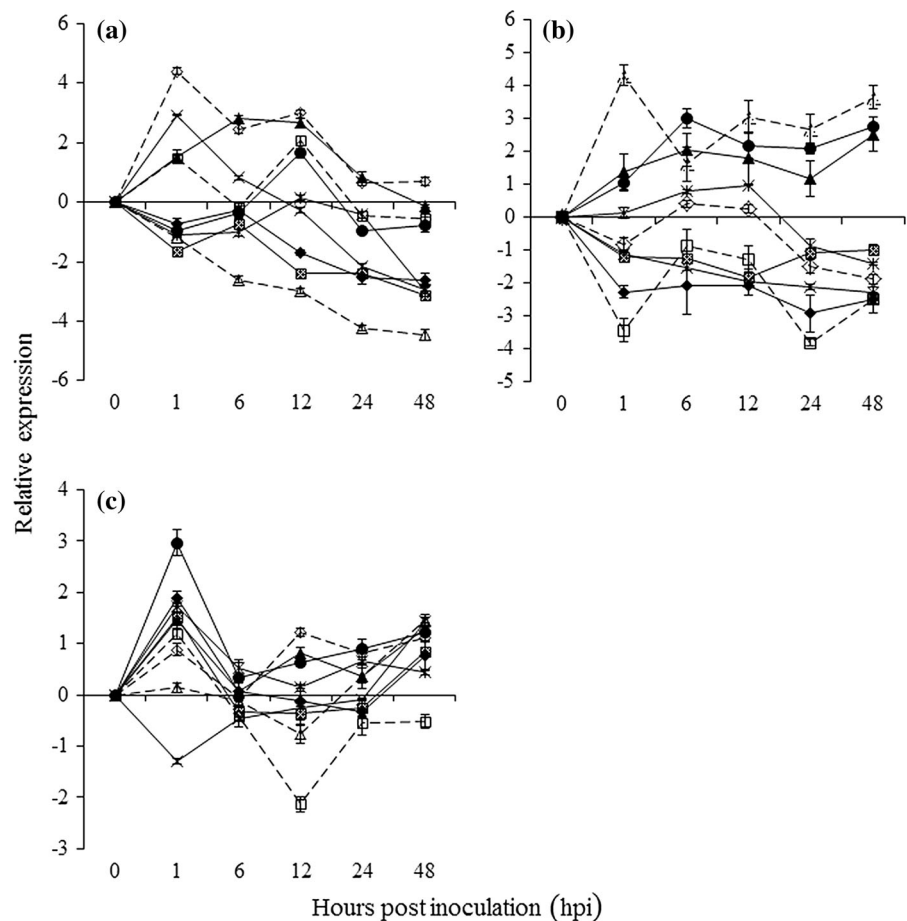
VfMlo-like6833	1	-----MAGATGGRSLEQTPPTWAVAVVCFVLLVLSIIIEYIHLIGKWLKKKHKKALYEALEKVKSELM
VfMlo-like18484	1	-----MAKGSKDRSLEQTPPTWAVAVVCFVLLVLSIIIEHLLHLIGKWLTKRNRKALYEALEKVKSELM
VfMlo-like14439	1	-----MADELEERSLEETPTWAVAVVCFVLLVLSIIIEHLLHLLIGKWLKGRHRRALYEALEKVKAEML
AtMlo2	1	-----MADQVKERTLEETSSTWAVAVVCFVLLVLSIIVLEHSHKIGTWFKKHKQALFEALEKVKAEML
AtMlo6	1	-----MADQVKERTLEETSSTWAVAVVCFVLLVLSIIVIEKLIHKIGSWFKKRNKALYEALEKVKAEML
AtMlo12	1	-----MAIKERSLEETPTWAVAVVCFVLLVLSIIMIEYIHLIGHWFKKHKKALSEALEKVKAEML
VfMlo-like8120	1	MAGDEBETTTTTTATLETTSTWAVAVVCFVLLVLSIIIEHALHLAKYFNKRRRSIIHALNNVKSELM
		<b>TM1</b>
VfMlo-like6833	65	LGFI SLLLT VGGSLISSICISKVAATWHHCCKSEERKSTTTESDTE-----SDNRKLLSISGFGGGS
VfMlo-like18484	65	LGFI SLLLT VGGGTIAGICISEKIAGTWHPCGKKQEIKYVSNED-----YGRKRLLEISDSGDSN
VfMlo-like14439	65	LGFI SLLLT LLDYISKICISEVGETWHPCCKETKDFKNTCSKG-----
AtMlo2	65	LGFI SLLLT LGQTFISNICISQKVASSTHPCSAAEAKKYGKAGKDDGDDGDKPGRLLLELAE SYH
AtMlo6	65	MGFI SLLLT LGQYISNICIFPNIAASMHPCSAASEARKYGGKVPKED-----EENLRRLQLVLDLIP
AtMlo12	63	LGFI SLLLT VLTQPVSEICIPENIAATWHPCSNHQEIAKYGDYID-----DGRKILEDSDSNDFYSP
VfMlo-like8120	71	LGFI SLLLT VCGKSIACICIPESVETLPCCKLTESDSEE-----
		<b>TM2</b>
VfMlo-like6833	130	RRVLAARFADKCSAKGQVFFVSSDAHQHLLHIFVLAIFHVLYCILTALGTAKMRRWKAWEKETRTVEY
VfMlo-like18484	126	RRVLAAGDDKCGEG-KVFFVSNYGIHQHLLHIFVLAIFVAVFHVLYCILTALGTAKMRRWKAWEKETRTVEY
VfMlo-like14439	110	-----KVPLVSSYGIHQHLLHIFVLAIFHVLYCILTALGTAKMRRWKAWEKETRTVEY
AtMlo2	135	RRSLATKGYDKCAEKGVAFVSAIGHQLHIFVLAIFVAVVHVLYCIVTVAEGIKMRTKWSWEPETKTIEY
AtMlo6	132	RRSLATKGYDKCAEKGVAFVSAIGHQLHIFVLAIFVAVVHVLYCIVTVAEGIKMRRWKAWEKETRTVEY
AtMlo12	126	RRNLATKGYDKCAEKGVAFVSAIGHQLHIFVLAIFVAVFHVLYCILTALGTAKMRRWKAWEKETRTVEY
VfMlo-like8120	112	-----ETKCEEGKMSLISROGWEELQYLIFVLAIFHVLYCIVTVAEGIKMRRWKAWEKETRTVEY
		<b>TM3</b>
VfMlo-like6833	200	QESHDPERFRFARETSFGRRHLSFWKSTHTLWIVCFRQFFVRSVVKVDYFLTRHGFI MAHLAPGSHAKF
VfMlo-like18484	195	REANDPERFRFARDTSFGRRHLSFWKSTHTLWIVCFRQFFVRSVVKVDYFLTRHGFI MAHLAPGSHAKF
VfMlo-like14439	164	QYSHDPERFRFARDTSFGRRHLSFWKSTHTLWIVCFRQFFRVSNNVDYFLTRHGFI MAHLSPGSAKF
AtMlo2	205	QYSDPERFRFARDTSFGRRHLSFWKSTHTLWIVCFRQFFSVTKVDYFLTRHGFI MAHLAPGNSRF
AtMlo6	202	QYSHDPERFRFARDTSFGRRHLSFWKSTHTLWIVCFRQFFRVSNTVDYFLTRHGFI MAHLAPGNSRF
AtMlo12	196	QYANDPERFRFARDTSFGRRHLSFWKSTHTLWITCFRQFFSVTKVDYFLTRHGFI MAHLAPGSAKF
VfMlo-like8120	174	QETNDRFRRLHQTSFGKQHLRYSRHRRLRWPACRIRQFVRSVVKVDYFLTRHGFI MAHLAPGNSR--Y
		<b>TM4</b>
VfMlo-like6833	270	DFQKYINRSLEEDFKVVVVGISEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
VfMlo-like18484	265	DFQKYIKRSLEEDFKVVVVGISEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
VfMlo-like14439	234	DFRNYIKRSLEEDFKVVVVGISEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
AtMlo2	275	DRKYIQRSLEKDFKTVEI SEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
AtMlo6	272	DRKYIQRSLEEDFKTVEI SEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
AtMlo12	266	DFQKYIKRSLEEDFKVVVVGISEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
VfMlo-like8120	242	DFQKYIKRALEKDFGVVVGISEPIWFAVFLFLLNTHGWYSYLWLPFIPLIIVLIVGTQLQVITKMLGR
		<b>TM5</b>
VfMlo-like6833	340	IQERGEVVKGVPPVQLGDLLEWENRPLVLYLHIFVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
VfMlo-like18484	335	IQERGEVVKGTPVVEPGDDLFWFNQPLRLLYLNFLVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
VfMlo-like14439	304	IQERGDVVKGT PVVEPNDLFWFNRPRLFLFLINFLVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
AtMlo2	345	IQEKGDVVRCAPVVPQGGDLFWFGPRFLFLIHLVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
AtMlo6	342	IQEKGDVVKGTPVVPQGGDLFWFGPRFLFLIHLVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
AtMlo12	336	IQEKGDVVKGAPVVEPGDDLFWFGPRFLFLIHLVLEFQNAFQAFFAWVYEFGLKSCFHEI EDVVIR
VfMlo-like8120	312	SHDKALVVRGTLVVRPESDHFVWFGRELLFLHLLHFLLEQNSFQAFFAWVYEFGLKSCFHEI EDVVIR
		<b>TM6</b>
VfMlo-like6833	410	ISMGVLIQLCSYVTLPLLYALVTQMGSTMKPTIHERVATALRNWHHTAKKNKHNKHSG----LATPMS
VfMlo-like18484	405	ISMGVTVQLCSYVTLPLLYALVTQMGSTMKPTIFNDRVAKALRNWHHTAKKNKHNKHSG----AVTPVS
VfMlo-like14439	374	ISMGVTLQLCSYVTLPLLYALVTQMGSTMKPTIFNDRVATALRSHWQAKRHTKHKGRHNSGVSESS---
AtMlo2	415	IVVGAIVQLCSYVTLPLLYALVTQMGSKMPTVFNDRVATALKKWHHTAKNETKHKGRHNSGNTPEFSS---
AtMlo6	412	ISIGLIVQLCSYVTLPLLYALVTQMGSKMPTVFNDRVATALKSWHHTAKKNKHNKHSGNTPEFSS---
AtMlo12	406	ITMGVLIQLCSYVTLPLLYALVTQMGSTMKPTIFNDRVANALKKWHHTAKKQTKHG--HSGNTEHSS---
VfMlo-like8120	382	IVVGVVQLLCGYVTLPLLYALVTQMGSTMTIVTEGVEGLNRWRRRRAKKNHARRNHSARPSLDAS---
		<b>TM7</b>
VfMlo-like6833	476	S-----RPTTPSHGTSAPYLLRYRSDMDSLQASPRRNLNDEMHWETDGSFSPSPHHPHGDGSSHHN
VfMlo-like18484	471	SRAGTPFSSRPGTFLHGMSPVHLLRHRSELDSVQTSFRMSNFDEGLETDEYR-----HREDISWSEHH
VfMlo-like14439	441	-----RPATPSYGMSPVHLLRHRSELDSVQTSFRMSNFDEGLETDEYR-----PGKDDDEH
AtMlo2	482	-----RPTTPHGSPIHLLNHNNSVENYFSSPSPRYSGHGHHPHQFWDPESECHQEAETSTHH
AtMlo6	479	-----RPTTPHGSPIHLLNHNNSVENYFSSPSPRYSGHGHHPHQFWDPESECHQEAETSNSH
AtMlo12	472	-----RPTTPHGMSPVHLLNHNNSLDQTSFTASPPFRFSDYSQGGHGH--CHFFDPEQNH
VfMlo-like8120	450	-----LDNSESFNTLDTSFVLDLQPSDAGYLVEISDEETVATKRPEPRQKLCGCFEGF
		<b>CaMBD</b>
VfMlo-like6833	538	QLHQGTSLEHDRDISAFSSQVPLPQPTPHQHEIDVVRKDFSEDRRERT
VfMlo-like18484	536	RNFG--PEEGRDTHNRITRTPAPQADNAQHEIDVQPMDFSEDKRART
VfMlo-like14439	493	EKEKFEFSRQGGIEDSSSTQLPLGPRPRTQHEINILSDFSEAKR---
AtMlo2	542	SLAHESSEP-----VLASVELPIR-----TSKSLRDFSEKK---
AtMlo6	538	HRSRFGEBESEKKFVSSVELPPGPGQIRTQHEISTSLRDFSEKR---
AtMlo12	531	SYQREITDSEFSNSHHFQVDMASPVREEKEIVEHVKVDLSSEFTEKK---
VfMlo-like8120	505	DLRETS-----

**Fig. 5** Multiple alignment of seven members of phylogenetic tree clade V (Four VfMlos and three AtMlos protein). The bars indicate the positions of the seven TM domains, and CaMBD domains inferred from AtMlo (Kim et al. 2002a, b; Panstruga 2005)

were found to be induced at the transcriptional level during infection by grapevine powdery mildew pathogen. It has been reported that three apple genes, *MdMlo11*, *MdMlo18*, and *MdMlo19*, were upregulated after inoculation with the powdery mildew pathogen, *Podosphaera leucotricha*. *MdMlo19* was also reportedly down regulated by the same pathogen in the ‘Gala’ variety of apple (Pessina et al. 2014). Piffanelli et al. (2002) showed that Mlo mRNA of barley leaf increased transiently after spore inoculation with powdery mildew pathogen. Chen et al. (2006) reported that *AtMlo2*, *AtMlo3*, *AtMlo6* and *AtMlo12* were upregulated by the biotrophic fungal pathogen *Erysiphe*

*cichoracearum*, that *AtMlo3* and *AtMlo12* were induced by the related fungal pathogen *Golovinomyces*. In the case of *E. ampelina* infection three showed upregulation and four were downregulated in this study. Chen et al. (2006) also reported that *AtMlo6* and *AtMlo12* were induced by the hemibiotrophic oomycete, *Phytophthora infestans*, and the necrotrophic fungus, *Botrytis cinerea*. Expression of the rice Mlo gene was also reported to be strongly induced by fungal pathogens and plant defense signaling molecules (Kim et al. 2002b). In the case of *R. vitis* inoculation, all transcripts of VfMlo-like gen except *VfMlo-like8120* and *VfMlo-like14557* transiently increased rapidly at 1 hpi, then decreased (Fig. 6b). A similar expression pattern was observed in rice Mlo after infection with a blast fungus in which the transcript level of *OsMlo* transiently increased at 1 hpi, then declined to basal levels by 12 hpi (Kim et al. 2002b). The apple Mlo genes *MdMlo11* and *MdMlo18* also showed a similar

**Fig. 6** Expression pattern of nine Mlo-like genes of *Vitis flexuosa* against *E. necator* (a), *E. ampelina* (b) and *R. vitis* (c). *VfMlo-like3243* (—■—), *VfMlo-like4098* (—●—), *VfMlo-like5057* (—\*—), *VfMlo-like6833* (—◇—), *VfMlo-like7881* (—□—), *VfMlo-like8120* (—×—), *VfMlo-like14439* (—▲—), *VfMlo-like14557* (—△—), *VfMlo-like18484* (—◆—). The error bars represent the standard error of the means of three independent replicates



pattern of expression with increased levels of expression at early time points and decreased levels at later time points (Pessina et al. 2014).

Expression of genes related with disease resistance or defense responses against plant pathogen attacks were investigated in pathogen-inoculated plants compared to healthy plants or mock-inoculated plants. In this study, expressions of MLO genes in pathogen-inoculated vines were compared healthy vines instead of mock inoculated plants according to procedures in previous reports, which presented the expression pattern of apple Mlo and tomato Mlo genes using non treated control samples (Pessina et al. 2014; Zheng et al. 2016). Although it was observed that the expressions of VfMlo-like genes by pathogen inoculation were similar to Mlo's expression patterns reported in apple trees and tomato plants, it should be considered that there would be variations between healthy plants and mock inoculated plants used as controls to compare gene expression by pathogen infections in the further studies.

Although it was reported that MLO proteins were related with developmental functions (Osakabe et al. 2005), they were involved in downstream defense-regulated pathways by biotic and abiotic stimuli (Chen et al. 2006). In expression of transcripts, VfMlo-like genes showing different expression patterns by different pathogens suggests that VfMlo-like genes function in diverse response processes against various pathogens. Transcriptional accumulation indicates that Mlo susceptibility genes are upregulated by challenging with mildew fungi (Bai et al. 2008). We identified five upregulated genes i.e. *VfMlo-like6833*, *VfMlo-like7881*, *VfMlo-like8120*, *VfMlo-like14439* and *VfMlo-like18484* by the infection of grapevine powdery mildew pathogen (*E. necator*) in grapevines. In phylogenetic tree, *VfMlo-like6833*, *VfMlo-like8120*, *VfMlo-like14439* and *VfMlo-like18484* belong to clade V, which is specific for powdery mildew susceptibility. Therefore, based on transcriptional and phylogenetic analysis these four VfMlo-like genes may have function in susceptibility of grapevine powdery mildew. Barley mlo mutants have been employed to control powdery mildew (Feechan et al. 2013). Therefore, it suggested that it will be possible to develop resistant cultivar against powdery mildew by mutation of VfMlo-like genes which were related to powdery mildew susceptibility of grapevines.

## Conclusions

Mlo proteins are known to be plant specific proteins containing seven transmembrane domains and a C-terminal calmodulin-binding domain, which are involved in modulation of infection by the powdery mildew pathogen in barley, as well as several other pathogens of other crops (Jorgensen 1992; Wolter et al. 1993). In this study, we identified nine non-duplicated Mlo-like genes selected from transcriptome analysis by NGS of *V. flexuosa* inoculated with *E. ampelina*. Structural analysis and a comparison study confirmed that the tested genes encoded typical Mlo proteins that contained seven transmembranes and a C-terminal calmodulin-binding domain and showed common structural features and cellular localization, which were consistent with other typical Mlo proteins. Additionally, all of the predicted proteins showed a high degree of homology with other Mlo proteins. We also investigated the expression pattern of these VfMlo-like genes in response to fungal and bacterial pathogen infection through real time PCR using gene specific primers. All tested genes showed expression in response to the inoculated pathogens, including upregulated expression of five VfMlo-like genes against mildew pathogen indicating that these genes may respond against other pathogens as well as powdery mildew pathogen in grapevines. This findings might provide valuable information and useful molecular genetic resources in elucidating the complex molecular mechanisms of resistant responses to diseases in grapevines.

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