SHORT COMMUNICATION

IDENTIFICATION AND FUNCTIONAL INFERENCE ON THE MLO FAMILY IN VIRIDIPLANTAE

P. Iovieno[1], V. Bracuto[2], S. Pavan[2], C. Lotti[3], L. Ricciardi[2] and G. Andolfo[1]

[1]*Department of Agricultural Sciences, University of Naples "Federico II", Via Università 100, 80055 Portici, Italy*
[2]*Department of Soil, Plant and Food Science, University of Bari "Aldo Moro", Via Amendola 165/A, 70126 Bari, Italy*
[3]*Department of Agricultural, Food and Environmental Sciences, University of Foggia. Via Napoli 25, 71100 Foggia, Italy*

SUMMARY

Powdery mildew (PM) is a widespread plant disease of temperate climates caused by ascomycete fungi of the order Erysiphales. PM is an important agricultural issue since it can cause significant economic losses. Specific members of the *MLO* gene family act as susceptibility factors towards the PM disease. A step towards the stability of crop productions would be thus the characterization of *MLO* genes at the genomic level. We carried out a genome-wide characterization of the *MLO* gene family in twenty-three plant and two algal genomes providing manual curated MLO protein catalogues. In total, 180 novel proteins containing the MLO domain were identified. Evolutionary history and phylogenetic relationships were studied through maximum likelihood analysis. This highlighted eight different clades, including a new monocot-specific clade (VIII) identified for the first time. In addition, 15 and 67 putative PM susceptibility genes, clustering in clade IV and V, respectively, were identified. Results of this work may help to address further biological questions concerning MLOs involved in PM susceptibility. In follow-up studies, it could be investigated whether the silencing or loss-of-function mutations in one or more of these candidate genes may lead to PM resistance.

Keywords: MLO, Powdery mildew, Functional inference, Gene annotation.

Powdery mildew (PM) is a widespread plant disease of temperate climates caused by ascomycete fungi of the order Erysiphales (Glawe, 2008). It is an important threat for many crops and can cause significant losses in cereal crops such as wheat and barley, vegetable crops such as tomato and cucumber (Dean *et al*., 2012), tree species such as those of the family of Rosaceae (Pessina *et al*., 2014) and ornamental plants such as roses (Linde *et al*., 2006). Accordingly, the development of varieties that exhibit robust immunity to this disease is of great economic interest. The *Mildew resistance Locus O* (*MLO*) gene family encodes proteins with seven transmembrane domains and a calmodulin-binding site (Büschges *et al*., 1997; Kim *et al*., 2002). Specific homologs of the *MLO* gene family act as susceptibility factors towards PM fungi. Indeed, plants carrying recessively inherited loss-of-function mutations of these genes show durable broad-spectrum PM resistance, referred to as *mlo* resistance (Jørgensen *et al*., 1992; Buschges *et al*., 1997; Consonni *et al*., 2006). The *mlo* resistance mechanism involves actin cytoskeleton, proteins involved in cell exocytosis, such as PEN1, PEN2 and PEN3, and possibly Receptor-Like Kinases (RLKs) (Collins *et al*., 2003; Lipka *et al*., 2005; Stein *et al*., 2006; Humphry *et al*., 2011; Feechan *et al*., 2013). It was hypothesized that strong resistance of *mlo* mutants is either based on perturbed MAMP responses (Lorek *et al*., 2013) or on missed defence suppression by PM fungi (Kim *et al*., 2002). In the latter scenario, wild-type functional MLO proteins should be targeted by fungal effector proteins to interfere with the plant immune system. The availability of complete plant genomes allowed the identification of *MLO* family members in several plant species (e.g. Appiano *et al*., 2015; Devoto *et al*., 2003; Feechan *et al*., 2009; Iovieno *et al*., 2015; Liu *et al*., 2008; Pessina *et al*., 2014; Zheng et *al*., 2016). Moreover, the expression of *MLO* genes has been shown to occur in different plant organs, tissues and cell types, and to be affected by multiple biotic or abiotic stresses (Piffanelli *et al*., 2002; Chen *et al*., 2006). In this study, we investigated the *MLO*-family within twenty-three and two genomes of plants and algae, respectively. Furthermore, we conducted a phylogenetic analysis in order to address questions about evolutionary history of MLO proteins and to provide a list of candidates for breeding purpose.

Putative MLO amino acid sequences of twenty-five different species (*Volvox carteri*, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, *Brassica rapa*, *Capsella rubella*, *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*,

Corresponding author: G. Andolfo
Fax: +39.081.2539431
E-mail: giuseppeandolfo@hotmail.com

Table 1. *MLO* sequences annotated in 25 green plant genomes.

Family	Specie	Novel MLOs	Previously annotated *MLO*s	Total *MLO*s	References of previously annotated *MLOs*
Volvocaceae	*V. carteri*	3	–	3	–
Chlamydomonadaceae	*C. reinhardtii*	4	–	4	–
Brassicaceae	*A. thaliana*	–	15 (15)[a]	15	Consonni *et al*., 2006
	B. rapa	23	–	23	–
	C. rubella	16	–	16	–
Cucurbitaceae	*C. lanatus*	–	14 (14)	14	Iovieno *et al*., 2015
	C. melo	–	16 (16)	16	Iovieno *et al*., 2015
	C. sativus	–	13 (13)	13	Schouten *et al*., 2014
Euphorbiaceae	*M. esculenta*	19	–	19	–
Fabaceae	*G. max*	2	39 (39)	41	Deshmukh *et al*., 2014
	M. truncatula	16	–	16	–
	P. vulgaris	20	–	20	–
Poaceae	*B. distachyon*	2	11 (11)	13	Ablazov and Tombuloglu, 2015
	O. sativa	–	12 (12)	12	Liu *et al*., 2007
	S. bicolor	2	13 (13)	15	Singh *et al*., 2012
	T. aestivum	47	8 (8)	55	Elliot, 2005
Rosaceae	*F. vesca*	–	17 (17)	17	Pessina *et a*l., 2014
	M. domestica	–	21 (21)	21	Pessina *et al*., 2014
	P. persica	–	19 (19)	19	Pessina *et al*., 2014
Solanaceae	*S. lycopersicum*	–	17 (17)	17	Chen *et al*., 2014
	N. tabacum	–	14 (14)	14	Appiano *et al*., 2015
	C. annuum	14	1 (1)	15	Kim *et al*., 2012
	S. melongena	17	1 (1)	18	Appiano *et al*., 2015
	S. tuberosum	–	12 (12)	12	Appiano *et al*., 2015
Vitaceae	*V. vinifera*	2	17 (17)	19	Feechan *et al*., 2009
Total		**187**	**260**	**447**	

[a]In brackets the number of previously annotated *MLO* genes that were confirmed in this work.

Manihot esculenta, *Glycine max*, *Medicago truncatula*, *Phaseolus vulgaris*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum*, *Fragaria vesca*, *Malus domestica*, *Prunus persica*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Capsicum annuum*, *Solanum melongena*, *Solanum tuberosum* and *Vitis vinifera*) were retrieved from the portal Phytozome v10 (https://phytozome.jgi.doe.gov/). A BLASTp (E-value < 1e-6) search in twenty-four proteomes, using the Arabidopsis MLO protein dataset reported by Consonni *et al*. (2006) was performed. The domain composition of proteins was assessed through a domain detection analysis using InterProScan (Zdobnov and Apweiler, 2001), and a total of 447 proteins containing the MLO domain (Pfam ID: PF03094) was identified (Supplementary Table 1, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). Sequences were examined manually to verify completeness and correctness of characteristic protein regions (extracellular N-terminal, transmembrane, intracellular/extracellular loops and intracellular C-terminus), using Geneious v6 (Kearse *et al*., 2012).

As shown in Table 1, with respect to previous scientific literature our analysis allowed the identification of two novel *MLO* members in *G. max* (Deshmukh *et al*., 2014), two in *B. distachyon* (Ablazov and Tombuloglu, 2015), two in *S. bicolor* (Singh *et al*., 2012), 47 in *T. aestivum* (Konishi *et al*., 2010) and two in *V. vinifera* (Feechan *et al*., 2009) and provided manual curated MLO protein catalogues. Among the twenty-five *MLO* families used in this study, a vast difference was found in terms of member numbers (ranging from three in *V. carteri* to fifty-five in *T. aestivum*) (Fig. 1). Differently from previous unsuccessful search in the *Chlorophyta phylum*, three and four MLO proteins were identified for the first time in *V. carteri* and *C. reinhardtii*, respectively. This result is plausibly obtained due to an increasing number of available ESTs (Devoto *et al*., 2003). Normalization of the total number of *MLOs* identified in each species with respect to the number of genes/haploid genome revealed that the proportion of each *MLO* family varies considerably (Fig. 1). In detail, the highest k-values were found in *P. vulgaris*, *V. vinifera*, *P. persica* and *G. max* (>7e-4) whereas the lowest was found in *N. tabacum* genome (1.6e-4). However, the *MLO* family is poorly represented in algae (k-value *ca*. 2e-4). At the family level, the highest expansion of MLO proteins was observed in Vitaceae with average k-values of 7.2e-4. In contrast, in Solanaceae genome this gene family seems contracted (average k-value *ca*. 4e-4). In the genome of Brassicaeae and Poaceae, the MLO protein family size seems to be expanded since it shows an average k-value of *ca*. 6e-4. Our analysis displayed a slight reduction of the number of *MLO* genes in function of Rosaceae, Fabaceae and in Poaceae size genomes (average k-value of *ca*. 4e-4).

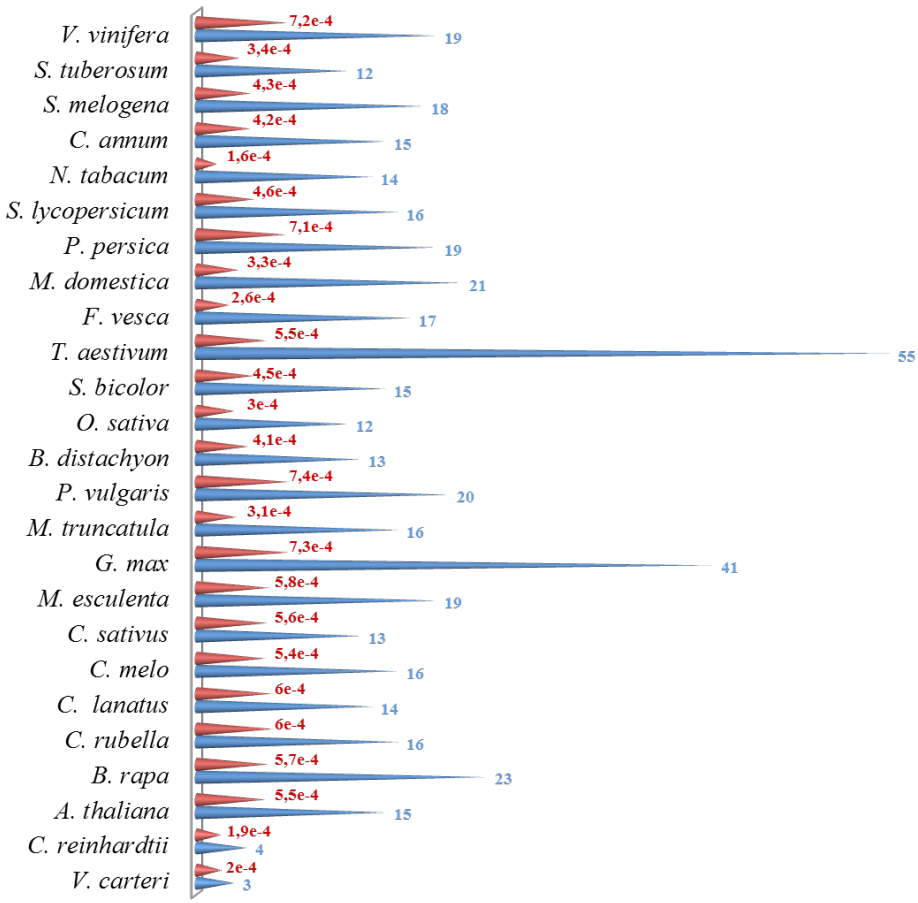

Fig. 1. Composition of twenty-five *MLO* gene families and corresponding normalized profiles on genome size. The dataset analysed include twenty-three plant (*A. thaliana*, *B. distachyon*, *B. rapa*, *C. lanatus*, *C. melo*, *C. rubella*, *C. sativus*, *F. vesca*, *G. max*, *S. lycopersicum*, *M. esculenta*, *M. domestica*, *M. truncatula*, *N. tabacum*, *O. sativa*, *C. annuum*, *P. persica*, *P. vulgaris*, *S. melongena*, *S. bicolor*, *S. tuberosum*, *T. aestivum*, *V. vinifera*) and two green algae (*V. carteri* and *C. reinhardtii*) genomes. *k-value*: ratio between number of *MLO* members for each species and the number of genes/haploid genome for each species.

In order to address questions about evolutionary diversification of *MLO* gene families, predicted protein sequences of twenty-five different genomes were aligned by MUSCLE v3.6 (Edgar *et al*., 2004). *MLO* sequences with less than 50% of the full-length MLO domain were not included in the analysis. The corresponding MLO domain, predicted from DB-Pfam (ID: PF03094), of a final dataset of 396 MLO proteins was used for a multi alignment (Fig. 2). Evolutionary analyses were conducted using MEGA6 (Tamura *et al*., 2011). A consensus phylogenetic tree was obtained using the Maximum Likelihood method and setting the bootstrap value as 100. We considered significant clades those having a bootstrap value support ≥ 69. These were designated with the Roman numerals from I to VIII (Fig. 2). In order to simplify this complex phylogenetic analysis, a subsequently partitioning of clades in subgroups was performed. This highlighted subgroups indicated as "subclades", shown in the Supplementary Fig. 1-8 (http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). Seven out of the eight clades included homologs in accordance with those characterized by previous phylogenetic studies carried out in monocots and dicots (Devoto *et al*., 2003). In addition, we identified a new clade, indicated as clade VIII. The algal MLO homolog group is separated from the rest of sequences (Fig. 2). The presence of this 'outgroup' is probably due to a real low similarity with the protein dataset or to the incompleteness of the retrieved amino acid sequences (Fig. 2).

Clade I is characterized by the presence of two Arabidopsis homologs, AtMLO4 (AT1G110000) and AtMLO11 (AT5G53760), involved in root responses to mechanical stimuli (thigmomorphogenesis) (Jaffe, 1973; Chen *et al*., 2009). In our tree this clade contains 75 members that can be partitioned into three subclades (Ia, Ib, Ic) (Supplementary Fig. 1, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). Thus, homologs of this clade could be further investigated for their role in thigmomorphogenesis. Other roles of clade I homologs are also possible since AtMLO14 (AT1G26700), not involved in root thigmorphogenesis, is also included in clade I. Interestingly, the subclade Ib differs from the others for not containing members of the Brassicaceae family. Evolutionarily, clade I is

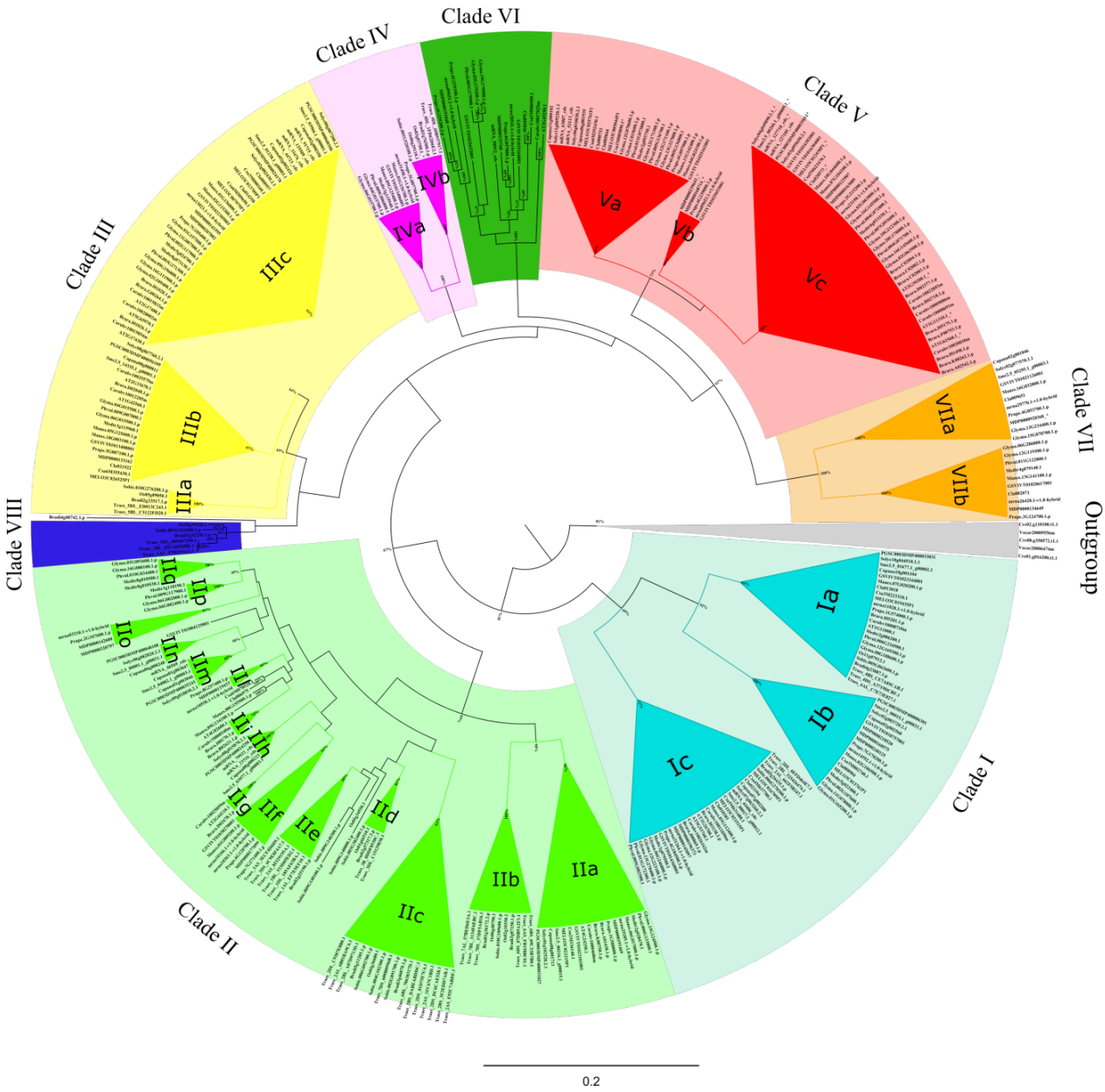

Fig. 2. Maximum likelihood analysis of twenty-five Viridiplantae MLO-families. Bootstrap values > 50% are indicated above branches. The tree is drawn to scale, with branch lengths proportional to the number of substitutions per site. Identified clades are indicated by Roman numbers and underlined by coloured boxes (clades I-VIII). To facilitate the tree description, the clades were split in "subclades" (subgroups described in more detail).

reported by Jiwan *et al.* (2013) to be the most ancient clade since they hypothesized that this clade was present before the separation of vascular and nonvascular plants.

Clade II, exhibiting 113 members, is the most numerous MLO proteins group characterized in this study. It is divided in 15 subclades (IIa-IIq), most of which seems to be class or even family-specific (Supplementary Fig. 2, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). In more detail, the subclades IIb, IIc, IId, IIe (with 44 members in total) are exclusive for monocots; the subclades IIg and IIi include 7 members of the Brassicaceae family; IIh, IIm and IIn include 16 Solanaceae-specific members; IIl and IIo contain 7 homologs specific of Rosaceae; IIp and IIq group 9 homologs of the Fabaceae family.

Clade III includes the Arabidopsis homolog AtMLO7 (AT2G17430), previously shown to be involved in pollen tube reception from the synergid cells during fertilization (Kessler *et al.*, 2010). In this study, it grouped 69 members divided in three subclades (Supplementary Fig. 3, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). Interestingly, the subclade IIIa is specific for monocots.

Clade IV is primarily but not exclusively represented by monocot MLO proteins (Acevedo-Garcia *et al.*, 2014; Várallyay *et al.*, 2012) and contains all the

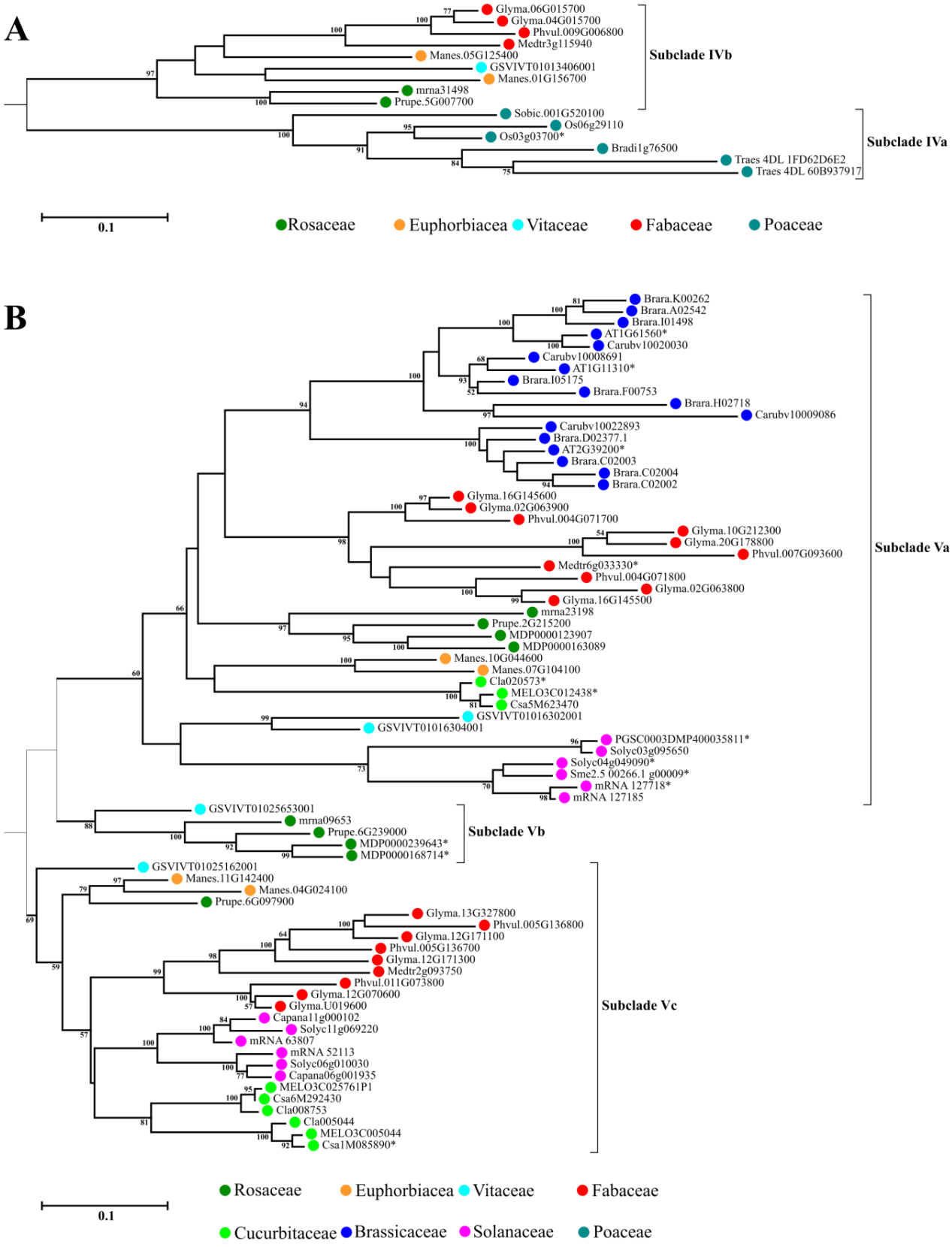

Fig. 3. Close-up view on IV and V phylogenetic clades. Botanic families corresponding to each homolog are indicated by coloured spots. Gene-IDs of MLO-homologs functionally involved in powdery mildew susceptibility are marked with an asterisk (*). A) Clade IV known to be the clade of monocot MLO homologs acting as susceptibility factors for Powdery Mildew. Subgroups IVa are exclusively composed by monocots members and IVb by dicots ones. B) Clade V including some of the homologues functionally characterized for their involvement in PM interaction.

monocot homologs involved in the interaction with PM fungi (namely barley HvMLO, rice OsMLO3 and wheat TaMLO_A1 and TaMLO_B1) (Fig. 3A). For the first time, we divided the clade IV in the two subclades IVa and IVb, which group monocot and dicot members, respectively. In further detail, subclade IVa is composed by 6 members, whereas subclade IVb contains 9 genes of the Eurosids I Cladus (Fig. 3A). Many eudicot species seem to have lost the clade IV, as we found that 13 out of 19 of the dicot species considered in this study do not show any MLO homolog in clade IV. Probably, this MLO group plays a minor role in these species.

All the *MLO* susceptibility genes functionally characterized in dicots, namely Arabidopsis AtMLO2, AtMLO6, AtMLO12 (Consonni *et al*., 2006), tomato SlMLO1 (Bai *et al*., 2008), grapevine VvMLO3 and VvMLO4 (Feechan *et al*., 2013), tobacco NtMLO1 (Appiano *et al*., 2015), pepper CaMLO2 (Zheng *et al*., 2013) and barrel clover MtMLO1 (Humphry *et al*., 2011) group in clade V. In the present study, 74 putative homologs clustered in clade V and were further separated into three subclades, named Va, Vb and Vc (Fig. 3B). The Va subclade presents 6 homologs belonging to Solanaceae, 2 to Vitaceae, 3 to Cucurbitaceae, 2 to Euphorbiaceae, 4 to Rosaceae, 10 for Fabaceae and 17 for Brassicaceae species, the latter possibly as the result of a recent duplication event. The subclade Vb, except for a *Vitis vinifera* homolog, seems to be typical of the Rosaceae family. The subclade Vc includes 6 members belonging to Solanaceae, 6 to Cucurbitaceae, 9 to Fabaceae, 5 to Rosaceae, 2 to Euphorbiaceae and 1 to Vitaceae. We identified 19 and 5 putative novel PM susceptibility factors in subclades Va and Vc, respectively. In more detail, the subclade Va presents 10, 4, 2 and 3 novel *MLO* candidate genes identified in *B. rapa*, *C. rubella*, *M. esculenta* and *P. vulgaris* genomes, respectively (Fig. 3B). The subclade Vc presents 2 and 3 PM susceptibility candidates in *M. esculenta* and *P. vulgaris*, respectively. This phylogenetic analysis confirmed that clade V**,** emerging after the divergence of monocots from dicots (Jiwan *et al*., 2013), likely replaced the function of clade IV. Indeed, Appiano *et al.* (2015) demonstrated that, despite the phylogenetic distance of monocot and dicot MLO proteins involved in the interaction with PM fungi are functionally conserved.

As shown in supplementary Fig. 4 (http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5), clade VI is composed of 17 dicot members and does not contain homologs of the Euphorbiaceae family. It presents 3 homologs of Fabaceae, 7 of Rosaceae, 2 of Cucurbitaceae, 2 of Vitaceae and 2 of Brassicaceae.

Our study expanded the clade VII to 21 dicot members, including the tomato SlMLO2 (Solyc02g77570) as previously proposed (Acevedo-Garcia *et al*., 2014) (Supplementary Fig. 5, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5). The subclade VIIa includes 4 homologs of Fabaceae, 1 of Euphorbiaceae, 1 of Vitaceae, 1 of Cucurbitaceae and 3 of Rosaceae. The subclade VIIb includes 11 MLO proteins from the same species of the subclade VIIa and Solanaceae species. Differently from the findings of Acevedo-Garcia *et al.* (2014), there are no members of *C. sativus* in this clade since we used full-length sequence genes (Schouten *et al*., 2014) from a new genome version.

Moreover, three MLO genes (VvMLO6, ClMLO1 and ClMLO8), previously reported in the Clade V (Iovieno *et al*., 2015; Feechan *et al*., 2008), were grouped into the phylogenetic clade VII. Interestingly, in this study we identified a clade (VIII) including six monocot-specific members (Supplementary Fig. 6, http://sipav.org/main/jpp/index.php/jpp/article/downloadSuppFile/3699/5).

In conclusion, in this study we present the identification of 183 novel *MLO* genes in several plant genomes and confirm 264 *MLO* homologs previously reported. In addition, we identified *MLO* genes in two algal species for the first time. We report a detailed investigation on phylogenetic reconstruction of the *MLO* gene family in twenty-five genomes. Furthermore, we refine previous phylogenetic work reporting two new clades in which no Arabidopsis members are present. We confirm that *MLO* homologues functionally characterized for their involvement in plant-PM interactions belong to clade IV and clade V, and we identified new putative PM susceptibility factors. The information here retrieved significantly increases our understanding of *MLO* gene family organization and diversification and is a basis for further functional investigations on the role of *MLO* genes. The functional characterization of the putative PM susceptibility factors and the identification or generation of *mlo* mutant (using genome editing technologies, for instance) could be used as a breeding strategy to introduce PM resistance across cultivated species.

ACKNOWLEDGEMENTS

We thank the anonymous reviewers for their constructive comments that contributed to improving the final version of the paper. This research was carried out within the GenoPOM-Pro and GenHort projects funded by the Italian Ministry of Education, University and Research. We acknowledge Prof. Luigi Frusciante and Prof. Maria Raffaella Ercolano for proofreading the manuscript.

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Received January 28, 2016
Accepted July 12, 2016