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 (Büsches 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,
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 Brassicaceae and Poaceae, the MLO protein family size seems to be expanded since it shows an average k-value of 7.2e-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. 2e-4). In the genome of Brassicaceae and Poaceae, the MLO protein family size seems to be expanded since it shows an average k-value of ca. 4e-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).
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 thigmomorphogenesis, 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
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; IIb, IIm and IIi include 16 Solanaceae-specific members; III and Ilo 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; Váralyay 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 SIMLO1 (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 SIMLO2 (Solyco2g77570) 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, CIMLO1 and CIMLO8), 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.

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