1 **Identification of candidate *MLO* powdery mildew susceptibility**
2 **genes in cultivated Solanaceae and functional characterization of**
3 **tobacco *NtMLO1***

4

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Abstract

Specific homologs of the plant *Mildew Locus O* (*MLO*) gene family act as susceptibility factors towards the powdery mildew (PM) fungal disease, causing significant economic losses in agricultural settings. Thus, in order to obtain PM resistant phenotypes, a general breeding strategy has been proposed, based on the selective inactivation of *MLO* susceptibility genes across cultivated species. In this study, PCR-based methodologies were used in order to isolate *MLO* genes from cultivated solanaceous crops that are hosts for PM fungi, namely eggplant, potato and tobacco, which were named *SmMLO1*, *StMLO1* and *NtMLO1*, respectively. Based on phylogenetic analysis and sequence alignment, these genes were predicted to be orthologs of tomato *SlMLO1* and pepper *CaMLO2*, previously shown to be required for PM pathogenesis. Full-length sequence of the tobacco homolog *NtMLO1* was used for a heterologous transgenic complementation assay, resulting in its characterization as a PM susceptibility gene. The same assay showed that a single nucleotide change in a mutated *NtMLO1* allele leads to complete gene loss-of-function. Results here presented, also including a complete overview of the tobacco and potato *MLO* gene families, are valuable to study *MLO* gene evolution in Solanaceae and for molecular breeding approaches aimed at introducing PM resistance using strategies of reverse genetics.

Keywords: MLO, Solanaceae, powdery mildew, resistance, plant breeding

49 **Introduction**

50 Powdery mildew (PM) is a major fungal disease affecting thousands of plant species, caused
51 by ascomycete fungi belonging to the order of Erysiphales (Glawe 2008). Chemical control of
52 PM accounts for a large proportion of fungicides used in agricultural settings (Hewitt 1998).
53 Therefore, the use of cultivars harbouring genetic sources of PM resistance is generally
54 envisaged as a valuable strategy to reduce farming costs and to cope with public concerns
55 related to environmental pollution and human health.

56 The *Mildew Locus O* (*MLO*) gene family encodes for plant-specific proteins harbouring
57 several transmembrane domains, topologically reminiscent of metazoan G-protein coupled
58 receptors (Devoto et al. 2003). Specific homologs of the *MLO* family act as susceptibility genes
59 towards PM fungi. Indeed, their inactivation, through loss-of-function mutations or silencing,
60 has been associated with a peculiar form of PM resistance, referred to as *mlo* resistance (Pavan
61 et al. 2010). This is associated with the enhancement of exocytosis defence pathways at plant-
62 pathogen interaction sites, which are thought to contribute to the prevention of fungal
63 penetration into host cells. Initially discovered in barley, *mlo* resistance has been later shown to
64 occur in other plant species as well, specifically Arabidopsis, tomato, pea, pepper and bread
65 wheat (Bai et al. 2008; Büschges et al. 1997; Consonni et al. 2006; Humphry et al. 2011; Pavan
66 et al. 2011; Wang et al. 2014; Zheng et al. 2013). This eventually led to the formalization of a
67 breeding approach based on the systematic inactivation of *MLO* susceptibility genes across
68 cultivated species affected by the PM disease (Dangl et al. 2013; Pavan et al. 2010; Pavan et al.
69 2011). Proof of concept for this strategy has been recently provided by the work of Wang et al.
70 (2014), reporting the introduction of PM resistance in bread wheat following targeted
71 mutagenesis of three *MLO* homoeoalleles. In contrast with most genetic sources of PM
72 resistance, experimental data clearly indicate that *mlo* immunity is not specific towards
73 particular fungal isolates and is extremely durable. For example, loss-of-function mutations of

74 barley *HvMLO* confer resistance to all known isolates of the PM fungus *Blumeria graminis* f.
75 sp. *hordei*, and is successfully employed in barley breeding since 1979 (Lyngkjaer et al. 2000).
76 Similarly, pea *er1* PM resistance, originating from the loss of function of *PsMLO1*, was first
77 reported more than sixty years ago and is the only resistance source worldwide used for
78 breeding purposes (Harland 1948; Humphry et al. 2011; Pavan et al. 2013).

79 Following the completion of the respective genome sequencing projects, a number of
80 *MLO* homologs variable between 12 and 19 has been identified in the diploid species
81 Arabidopsis, rice, grapevine, peach, woodland strawberry and cucumber (Devoto et al. 2003;
82 Feechan et al. 2008; Liu and Zhu 2008; Pessina et al. 2014; Schouten et al. 2014). Remarkably,
83 when placed in MLO protein family phylogenetic trees, all dicot MLO isoforms experimentally
84 shown to be required for PM susceptibility group in the same clade, referred to as clade V in
85 scientific literature (e.g. Feechan et al. 2008; Pavan et al. 2011; Acevedo-Garcia et al. 2014).
86 This shows that evolutionary studies on MLO proteins may predict candidates for being PM
87 susceptibility factors.

88 Concerning solanaceous crops, we have functionally characterized the two *MLO*
89 orthologs *SlMLO1* in tomato and *CaMLO2* in pepper, whose inactivation is causally associated
90 with PM resistance (Bai et al. 2008; Zheng et al. 2013). In this work, we report the isolation,
91 through a PCR-based approach, of three *MLO* genes from other cultivated Solanaceae, namely
92 eggplant, potato and tobacco, which are likely to share a relation of orthology with *SlMLO1*
93 and *CaMLO2*. The tobacco *MLO* homolog *NtMLO1* was chosen for a transgenic
94 complementation assay, resulting in its functional characterization and identification of a loss-
95 of-function mutant allele. Finally, newly available tobacco and potato genome sequences
96 (Sierro et al. 2014; The Potato Genome Consortium 2011) were exploited to provide a
97 comprehensive overview of the *MLO* gene families in these species.

98

99 **Materials and methods**

100 *PCR-based isolation and phylogenetic characterization of MLO putative orthologs*

101 Young leaves of eggplant (*Solanum melongena* cv. Half Lange Violette), potato (*Solanum*
102 *tuberosum* cv. Desiree) and tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) were collected
103 for RNA extraction, which was performed using the Trizol reagent (Invitrogen). After RNA
104 purification with the NucleoSpin RNA II kit (Macherey-Nagel), cDNA was synthesized using
105 the SuperScript III RT first-strand cDNA synthesis kit (Invitrogen) with oligo(dT) primers.

106 Aiming to identify sequences of *SlMLO1* putative orthologs, the primer pairs Sol-F1 (5'-
107 CATTTGACATTTCCCCTTCTTC-3') / Sol-R1 (5'-GCACCATGCATGAGTACCTCT-3')
108 and Sol-F2 (5'-TTGGCAGTTGCTCATGTATTG-3') / Sol-R2 (5'-
109 ATGGTGCCAGCTTCTAAGAG-3') were designed on the untranslated and coding sequences
110 of the *SlMLO1* gene (GeneBank accession number NM_001247885), respectively, (Primer3,
111 Rozen and Skaletsky 2000) and used for PCR amplification of cDNAs. Amplicons obtained
112 with the Sol-F2/Sol-R2 primer pair were purified using the NucleoSpin Extract II kit
113 (Macherey-Nagel) and ligated (molar ratio 1:1) into the pGEM-T easy vector (Promega).
114 Recombinant plasmids were cloned in *E. coli* DH10β chemically competent cells and recovered
115 by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed using
116 universal T7 and SP6 primers (Eurofins MWG Operon).

117 In order to obtain full-length coding sequences of potato and tobacco *MLO* genes,
118 sequences overlapping with those of the amplicons above mentioned were retrieved by BLAST
119 search, using the tomato *SlMLO1* coding sequence as query against expressed sequence tags
120 (ESTs) and predicted coding sequence repositories, both available at the Sol Genomic Network
121 (SGN) database (http://solgenomics.net), and then used for local alignment. The expression and
122 sequence of candidate genes was verified by PCR amplification of cDNAs, using the primer
123 pairs StMLO1-F (5'- ATGGCTAAAGAACGGTCG -3') / StMLO1-R (5'-

124 TTATTTGTTTCCAAAAGT-3’) and NtMLO1-F (5’-ATGGAGGCAACTCCGACTTG-3’) /
125 NtMLO1-R (5’-TCAACTCATTTTGTTGCCAAATG-3’), cloning and sequencing, which
126 were performed as above described.

127 In order to amplify a full-length *MLO* sequence in eggplant, the following primer pair
128 was used: SmMLO1-F2 (5’-ATGGCTAAAGAACGGTCG-3’) / SmMLO1-R1 (5’-
129 TTATTTGTTTCCAAAAGTAAAATCTGA-3’). The corresponding PCR product was cloned
130 and sequenced as indicated above.

131 Full-length eggplant, potato and tobacco *MLO* genes (named *SmMLO1*, *StMLO1* and
132 *NtMLO1*, respectively) were translated *in silico*. Corresponding protein sequences were used,
133 together with those of dicot MLO proteins experimentally associated with PM susceptibility
134 [*Arabidopsis thaliana* AtMLO2 (GenBank accession code NP172598), AtMLO6 (NP176350)
135 and AtMLO12 (NP565902), *Solanum lycopersicum* SlMLO1 (NP001234814), *Capsicum*
136 *annuum* CaMLO2 (AFH68055), *Pisum sativum* PsMLO1 (ACO07297), *Lotus japonicus*
137 LjMLO1 (AAX77015) and *Medicago truncatula* MtMLO1 (ADV40949)] and those of the
138 remaining twelve homologs of the *Arabidopsis thaliana* AtMLO protein family, for ClustalW
139 alignment and the construction of a Unweighted Pair Group Method with Arithmetic Mean
140 (UPGMA) phylogenetic tree. Bootstrap values were calculated from 100 replicates. All of these
141 bioinformatic analyses were performed using the CLC sequence viewer software
142 (http://www.clcbio.com/).

143

144 *Generation of transgenic plants overexpressing NtMLO1*

145 Two different *NtMLO1* PCR products, differing for a single nucleotide polymorphism, were
146 inserted into the Gateway-compatible vector pENTR D-TOPO (Invitrogen) and cloned in *E.*
147 *coli* competent cells. Presence of the inserts was assessed by colony PCR, restriction enzyme
148 digestion and sequencing using the universal M13 primer pair. Inserts were then transferred by

149 LR recombination into the binary plasmid vector pK7WG2, harboring the 35S Cauliflower
150 Mosaic Virus (CaMV) promoter for constitutive expression and the marker gene *nptII* for
151 kanamycin resistance selection. Plasmids were inserted into *E. coli* competent cells and positive
152 colonies were again screened by colony PCR and sequencing, as above. Recombinant vectors
153 were finally extracted and transferred to the AGL1-*vir*G strain of *A. tumefaciens* by
154 electroporation. A selected PM resistant tomato line, named Slmlo1, described by Bai et al.
155 (2008) and carrying a loss-of-function deletion in the *SlMLO1* coding sequence, was used for
156 transformation. This was performed according to the method described by McCormick et al.
157 (1986). Briefly, seeds were surface-sterilized and sown on half-strength Murashige and Skoog
158 (MS) agar supplemented with sucrose (10 g/l). Cotyledons were excised from 10-day-old
159 seedlings, cut in two parts and submerged in an *A. tumefaciens* suspension with an OD_{600} value
160 of about 0.125. Infected cotyledonary explants were placed abaxially on the GCF10 medium
161 (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 108.73 mg/l Nitsch vitamins, 1.5 mg/l
162 zeatin riboside, 0.2 mg/l indole-3-acetic acid, pH 5.8) supplemented with 1 ml/l acetosyringone
163 at 25°C for 48 h. Then, they were transferred to the GCF10 medium to which 100 mg/ml
164 timentin and 50 mg/ml kanamycin were added and sub-cultured onto fresh medium every 3
165 weeks until shoot buds were observed. These were excised from the callus and transferred to
166 the GCF11 medium (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 108.73 mg/l
167 Nitsch vitamins, 1.9 mg/l zeatin riboside, pH 5.8) with 100 mg/ml timentin and 50 mg/ml
168 kanamycin. After meristem development, the explants were transferred to the root-inducing
169 medium MS30B5 (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 112 mg/L vitamin
170 B5, 50 mg/ml kanamycin, pH 5.8). Once roots were developed, plantlets were finally located
171 on woolen rock and grown in a greenhouse compartment.

172 For each of the two transformations with a different *NtMLO1* gene sequence, twenty T_1 plants
173 and two T_2 families (each composed by fifteen individuals derived from self-pollination of

174 individual T_1 plants) were assayed for the presence of the construct, using the primer pair ntpIIF
175 (5'- TCGGCTATGACTGGGCACAAC-3') / ntpIIR (5'-AAGAAGGCGATAGAAGGCGA-
176 3'), designed on the *ntpII* gene sequence, and the primer pair 35S-F (5'-
177 GCTCCTACAAATGCCATCA-3') / 35S-R (5'- GATAGTGGGATTGTGCGTCA-3'),
178 designed on the 35S promoter sequence. Expression of the transgene was assessed by qPCR
179 using the primer pair NtMLO1_qFw (5'-GTGGAAATAAGTCCAGCATTATG-3')/
180 NtMLO1_qRev (5'- CACCCAAAGGTACGAGTACAATC- 3').
181
182 *Disease tests and Oidium neolycopersici quantification on transgenic plants*
183 Cuttings of T_1 individuals and plants of the T_2 families mentioned above were challenged with
184 an isolate of the tomato PM fungus *Oidium neolycopersici* maintained at the Plant Breeding
185 Department of the University of Wageningen, The Netherlands. The Slmlo1 mutant line and
186 the susceptible cultivar Moneymaker (MM) were used as controls. Inoculation was performed
187 as described by Pavan et al. (2008), by spraying plants with a suspension of conidiospores
188 obtained from freshly sporulating leaves of heavily infected plants and adjusted to a final
189 concentration of 4 x 10^4 spores/ml. Inoculated plants were grown in a greenhouse compartment
190 at 20 ± 2°C with 70 ± 15% relative humidity and day-length of 16 hours. Disease evaluation
191 was carried out fifteen days after inoculation, based on a visual scoring as described by Bai et
192 al. (2008) and/or analytically, by the relative quantification of the ratio between fungal and
193 plant gDNAs. The latter was performed by the qPCR assay reported by Huibers et al. (2013).
194 Specifically, plant and fungal genomic DNAs were extracted from *O. neolycopersici* infected
195 tomato leaves (Qiagen DNeasy Plant Mini Kit) and used for amplification with the primer pairs
196 On-F (5'-CGCCAAAGACCTAACCAAAA-3') / On-R (5'-
197 AGCCAAGAGATCCGTTGTTG-3'), designed on *O. neolycopersici* internal transcribed
198 spacer (ITS) sequences (GenBank accession number EU047564), and Ef-F (5'-

199 GGAACTTGAGAAGGAGCCTAAG-3') / Ef-R (5'-CAACACCAACAGCAACAGTCT-3'),

200 designed on the tomato *Elongation Factor 1α* (*Ef1α*) gene (Løvdal and Lillo 2009). Relative

201 quantification was performed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Pfaffl 2001).

202

203 *In silico* characterization of the tobacco and potato *MLO* gene families

204 In order to retrieve tobacco and potato MLO homologs, nucleotide sequences of *NtMLO1* and

205 *StMLO1* and corresponding translated sequences were used as query for BLAST (BLASTn and

206 tBLASTn) search against the Sol Genomics Network (SGN) and the Potato Genomics Resource

207 (Spud DB) databases, using default parameters.

208 The number of transmembrane domains was predicted using the online software TMHMM

209 (http://www.cbs.dtu.dk/services/TMHMM/). The putative number of introns was obtained

210 using the online service FGENESH of Softberry (http://www.softberry.com/). Chromosomal

211 localization and gene position of potato *MLO* genes were inferred by the annotations of the

212 Potato Genome Consortium. Finally, the MEME (http://meme.nbcr.net/) (Bailey et al. 2009)

213 package was used to predict functional motifs in the NtMLO and StMLO protein families.

214 Predicted tobacco NtMLO and potato StMLO proteins were used to integrate the phylogenetic

215 tree described in the previous section, according to the same methodologies above mentioned.

216

217 **Results**

218 *Identification of MLO gene sequences from cultivated Solanaceae*

219 Two primer pairs, one designed on the untranslated sequence and the other on the coding

220 sequence of tomato *SlMLO1*, were used to amplify homologous sequences from eggplant,

221 potato and tobacco cDNAs. PCRs performed with the Sol-F1/R1 primer pair failed, thus

222 suggesting the occurrence of polymorphic sequences in untranslated regions. In contrast, PCR

223 performed with the Sol-F2/R2 primer pair, designed within the *SlMLO1* coding sequence,

224 resulted in single amplification products of 876 bp. Full-length sequences of a 1560 bp tobacco
225 gene, named *NtMLO1,* and a 1557 bp potato gene, named *StMLO1,* were obtained by
226 assembling partial gene sequences of PCR products with overlapping sequences retrieved by
227 the interrogation of the SGN database. Amplification and sequencing of *StMLO1* and *NtMLO1*
228 from potato and tobacco cDNAs provided evidence for their actual expression in leaves and
229 validated their sequences. These were deposited in the GenBank database with the accession
230 codes KM244715 (*StMLO1*) and KM244716 (*NtMLO1*).

231 In order to clone an eggplant *MLO* gene putatively involved in PM susceptibility,
232 several primers were designed, based on the identification of conserved regions from the
233 alignment of *SlMLO1*, *StMLO1* and *NtMLO1*. These primers were then tested on eggplant
234 cDNA. The SmMLO1-F2/SmMLO1-R1 primer pair produced a single PCR amplification
235 product. The corresponding sequence of 1572 bp was named *SmMLO1* and deposited in the
236 GenBank database with the accession code KM244717.

237

238 *Bioinformatic analyses support the identification of solanaceous MLO functional orthologs*
239 *required for PM susceptibility*

240 StMLO1, NtMLO1 and SmMLO1 protein sequences were used to perform a phylogenetic
241 analysis. With strong bootstrap support, they were found to group in the phylogenetic clade V,
242 containing all the dicot MLO homologs so far experimentally shown to be required for PM
243 susceptibility (AtMLO2, AtMLO6, AtMLO12, SlMLO1, CaMLO2, PsMLO1, LjMLO1 and
244 MtMLO1) (Fig. 1), thus indicating they could possibly be functionally related.

245 Previous studies highlighted the presence of amino acid residues highly conserved either
246 in the whole MLO protein family or in MLO orthologs involved in the interaction with PM
247 fungi, which are predicted to play a key functional role (Elliott et al. 2005; Panstruga 2005).
248 All of these residues were found to be present in the StMLO1, NtMLO1 and SmMLO1 protein

249 sequences (Supplementary Fig. 1), providing further evidence for the identification of *MLO*
250 genes required for PM susceptibility.

251 Finally, another strong bioinformatic indication for the identification of solanaceous *MLO*
252 susceptibility genes was provided by aligning the coding sequences of *StMLO1*, *NtMLO1* and
253 *SmMLO1* with those of the PM susceptibility genes *SlMLO1* and *CaMLO2*, functionally
254 characterized in tomato and pepper, respectively (Bai et al. 2008; Zheng et al. 2013)
255 (Supplementary Fig. 2). Indeed, this revealed a very high percentage of nucleotide identity
256 (81,4% between tomato and tobacco, 87,5% between tomato and eggplant and 94,8% between
257 tomato and potato), suggesting that all of these solanaceous *MLO* genes are orthologs.

258

259 *Tobacco NtMLO1 complements tomato SlMLO1 in a functional complementation assay*

260 In order to characterize *NtMLO1* at the functional level, we set up an assay based on its
261 transgenic overexpression in the previously described tomato line Slmlo1, which carries a loss-
262 of-function mutation in the tomato *SlMlo1* homolog and is thus resistant to the PM fungus *O.*
263 *neolycopersici* (Bai et al. 2008). We hypothesised that overexpression of *NtMLO1* would have
264 restored PM susceptibility in the tomato Slmlo1 mutant line, thereby demonstrating functional
265 conservation between *NtMLO1* and *SlMLO1*.

266 After transformation, cuttings of twenty T_1 transgenic individuals were challenged with *O.*
267 *neolycopersici*. Fifteen of them showed restoration of PM symptoms (not shown). In order to
268 confirm this result, two T_2 families of fifteen individuals (T_2_a and T_2_b) derived from self-
269 pollination of two different T_1 plants were also inoculated, together with MM (the susceptible
270 control) and the Slmlo1 mutant line (the resistant control). The presence of the overexpression
271 construct in segregating T_2 families was assessed by PCR amplification with primer pairs
272 designed on the *nptII* gene and the 35S promoter (Supplementary Fig. 3). T_2 individuals not
273 carrying the overexpression construct [T_2(-)], as well as individuals of the Slmlo1 mutant line,

274 showed no *NtMLO1* expression and an average of disease score of about 0.5. In contrast, T_2
275 individuals of the two families positive for the presence of the construct [T_2(+)_a and T_2(+)_b]
276 showed *NtMLO1* expression and an average disease score of 1.8 and 1.7, respectively (Fig. 2
277 and Supplementary Fig. 4).
278
279 *A NtMLO1 point mutation causing the substitution of a conserved glutamine residue results in*
280 *gene loss of function*
281 During the preparation of the 35S::*NtMLO1* overexpression vector, we accidentally cloned
282 another insert, carrying a single nucleotide polymorphism in the tobacco *NtMLO1* gene. This
283 resulted in the substitution of a glutamine residue, located in the protein second intracellular
284 loop and previously reported to be invariable throughout the whole MLO protein family, with
285 arginine (Q198R, Fig. 3). We could not get the same arginine-coding insert by repeating the
286 cloning procedure several times from tobacco cDNA, so we assumed that this resulted from a
287 mutation due to an error by the Taq polymerase used for amplification. Nonetheless, in order
288 to study the effect of this substitution on protein function, we developed transgenic lines
289 carrying an overexpression construct for this insert. Following *O. neolycopersici* inoculation,
290 none of 20 individual T_1 plants developed disease symptoms. Individuals of two independent
291 T_2 families positive for the presence of the construct [T_2(+)_Q198R-a and b] were found to
292 express the transgene, as assessed by qPCR (Supplementary Fig. 4). Nevertheless, following
293 *O. neolycopersici* challenge, no PM symptoms were visible on [T_2(+)_Q198R] individuals,
294 which were phenotypically undistinguishable from those of the Slmlo1 line (Fig. 4A). In order
295 to test whether the mutated *NtMLO1* sequence maintained some residual functional activity,
296 even so still resulting in a macroscopically resistant phenotype, we quantified, in transgenic
297 individuals of the two T_2 families, the fold-change in the ratio between *O. neolycopersici* and
298 tomato gDNAs, relatively to the Slmlo1 line. No significant difference was found (Fig. 4B),

299 indicating that the point nucleotide mutation causing the substitution of glutamine with arginine
300 in the NtMLO1 protein sequence leads to complete gene loss of function.
301
302 *In silico* characterization of tobacco and potato *MLO* families
303 Recently released sequences from potato (group *Phureja DM1*) and tobacco (cv. *Basma Xanthi*)
304 prompted us to perform a genome-wide search aiming to characterize the *MLO* gene families
305 in these species. This search revealed a total of 15 and 13 predicted tobacco *NtMLO* and potato
306 *StMLO* loci, respectively, which were named according to the nomenclature specified in Tables
307 1 and 2. A predicted tobacco coding sequence, referred to as mRNA_127718_cds in the Sol
308 Genomics Database, was found to be identical to *NtMLO1*. No sequence fully matching with
309 *StMLO1* could be identified by the interrogation of the Potato Genomics Resource database,
310 but in its place a partial gene sequence showing 100% of identity with the same gene.
311 For tobacco and potato MLO proteins, amino acid length and number of transmembrane
312 domains were inferred (Table 1 and Table 2). In addition, information on chromosomal
313 localization and intron number was available for predicted *StMLO* genes (Table 2).
314 The tobacco NtMLO and potato StMLO protein families were used as input to search
315 for conserved motifs, using an approach similar to the one previously reported by Deshmukh et
316 al. (2014). We looked for motifs with length ranging from 40 to 70 residues and shared by at
317 least three homologs. For each of the two families, seven motifs were identified. Of these, five
318 were found to be at least partially matching with those identified in the soybean protein family
319 (Deshmukh et al. (2014) (Table 3).
320 A comparative analysis was carried out in order to establish phylogenetic relationships
321 between the NtMLO and the StMLO protein families and MLO proteins from other dicot plant
322 species. The analysis resulted in the distinction of five clades, designated with Roman numbers
323 based on the position of Arabidopsis AtMLO homologs, according to the nomenclature

324 indicated by Feechan et al. (2008) (Fig. 1). Besides NtMLO1 and StMLO1, additional NtMLO
325 (NtMLO2, NtMLO3, NtMLO4 and NtMLO5) and StMLO (StMLO9 and StMLO12) homologs
326 were found to group in clade V together with all dicot MLO proteins previously associated with
327 PM susceptibility.
328

329 **Discussion**

330 In previous studies, we functionally characterized tomato *SlMLO1* and pepper *CaMLO2* as two
331 solanaceous *MLO* susceptibility genes, as their inactivation was causally associated with PM
332 resistance (Bai et al. 2008; Zheng et al. 2013). Starting from this information, we followed here
333 a combined approach based on database search and PCR amplification, which resulted in the
334 isolation of three *MLO* genes from other widely distributed solanaceous species affected by the
335 PM disease, namely eggplant (*SmMLO1*), potato (*StMLO1*) and tobacco (*NtMLO1*). PM disease
336 represents one of the most important fungal diseases of tobacco and eggplant (Bubici and Cirulli
337 2008; Darvishzadeh et al. 2010) and in conducive environments may lead to important
338 economic losses in potato cultivation (Glawe et al. 2004).

339 A chain of evidence, based on phylogenetic relatedness (Fig. 1) and sequence
340 conservation with other known PM susceptibility genes and proteins (Supplementary Fig. 1)
341 was provided, suggesting the identification of solanaceous orthologs of *SlMLO1* and *CaMLO2*.
342 Aiming at the functional characterization of *NtMLO1*, we set up an assay based on its
343 heterologous overexpression in a tomato *mlo*-mutant genetic background, taking advantage
344 from the availability of a tomato resistant line and routine protocols for tomato genetic
345 transformation (Bai et al. 2008). Success of such an assay, as demonstrated by the restoration
346 of symptoms in transgenic plants (Fig. 2), provides a final evidence for the role of *NtMLO1* as
347 a PM susceptibility gene. Although it was not proven at the functional level, we speculate that

348 both *StMLO1* and *SmMLO1* are involved in PM susceptibility in potato and eggplant, as they
349 are, at the nucleotide level, even closer than *NtMLO1* to *SlMLO1* and *CaMLO2*.
350 While completing this work, newly released sequences of potato and tobacco became
351 available. Thus, a genome-wide search was performed, which allowed to retrieve additional
352 *MLO* homologs and, presumably, to characterize the complete tobacco and potato *MLO* gene
353 families. Phylogenetic analysis using these sequences highlighted the presence of additional
354 NtMLO and StMLO proteins in clade V, previously shown to group dicot MLO homologs
355 acting as PM susceptibility factors (Fig. 1). Functional redundancy of MLO homologs
356 belonging to this clade has been shown to occur in *Arabidopsis thaliana*, as the simultaneous
357 inactivation of the three homolog genes *AtMLO2*, *AtMLO6* and *AtMLO12* is required to result
358 in complete PM resistance. Thus, functional analyses, such as the transgenic complementation
359 test above mentioned, might lead to the identification of additional solanaceous MLO homologs
360 playing a role in the interaction with PM fungi.
361 Interestingly, due to a polymerase error during the cloning procedure, we also had the
362 opportunity to verify the crucial role of a glutamine residue localized in the second intracellular
363 MLO domain. This amino acid has been shown to be invariable throughout the whole MLO
364 protein family and therefore predicted to be fundamental for the role of MLO proteins as PM
365 susceptibility factors (Elliott et al. 2005). Indeed, its replacement with arginine in tobacco
366 NtMLO1 (Fig. 3) resulted in complete failure of transgenic complementation, as inferred by
367 visual scoring and relative quantification of fungal gDNA with respect to plant gDNA (Fig. 4).
368 This result represents a complement to earlier investigations addressed to the functional
369 characterization of MLO proteins (Reinstädler et al. 2010; Pavan et al. 2013).
370 A growing body of experimental evidence supports the view that *mlo*-based resistance
371 can be conveniently pursued as a strategy to cope with the PM disease in practical breeding
372 (Pavan et al. 2010). Therefore, we predict that results here provided might be of great interest

373 for future activities aimed at the introduction of PM resistance in Solanaceae. Targeted
374 identification of mutations of *MLO* susceptibility genes can be achieved through conventional
375 approaches of TILLING (targeted induced local lesions in genomes) or RNA interference
376 (McCallum et al. 2000; Matthew 2006). In addition, cutting-edge technologies of genome
377 editing are also available to the breeder, based on zinc finger nucleases (ZFNs), clustered
378 regularly interspaced short palindromic repeat (CRISPR) and transcription activator-like
379 effector nucleases (TALEN) (Gaj et al. 2013, Terns 2014). Noteworthy, a TALEN-based
380 approach has been recently successfully applied to introduce PM resistance in bread wheat
381 through simultaneous targeting of three homoelog *MLO* alleles, as mentioned in Wang et al.
382 (2014).
383

384 **Acknowledgements**
385 This work was supported by the Italian Ministry of University and Research through the project
386 GenHort PON R&C.
387

388 **Conflict of interest**
389 The authors declare that they have no conflict of interest.
390

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499

500 **Figure captions**

501

502 **Fig. 1** UPGMA-based tree of full-length MLO proteins. The dataset includes the tobacco
503 NtMLO, potato StMLO and Arabidopsis AtMLO protein families, tomato SlMLO1, pepper
504 CaMLO2, eggplant SmMLO1, pea PsMLO1, lotus LjMLO1 and barrel clover MtMLO1.
505 Phylogenetic clades are designated with Roman numbers based on the position of AtMLO
506 homologs, according to the nomenclature indicated by Feechan et al. (2008). Homologs
507 identified by means of a PCR-based approach in this study (SmMLO1, StMLO1 and NtMLO1)
508 are indicated in bold red. Numbers at each node represent bootstrap support values (out of 100
509 replicates).

510

511 **Fig. 2** Effects of the transgenic expression of *NtMLO1* in a tomato *mlo* loss-of-function genetic
512 background. Panel A) refers from left to right as follows: one individual of a T_2 family (T_2_a)
513 positive for the presence the *NtMLO1* overexpression construct; one individual of another
514 independent T_2 family (T_2_b) positive for the presence of the *NtMLO1* overexpression
515 construct; one T_2 individual negative for the presence of the overexpression construct; one
516 individual of the tomato Slmlo1 mutant line, carrying a loss of function deletion in the *SlMLO1*
517 gene; one individual of the susceptible cultivar Moneymaker (MM). Panel B) reports the
518 average visual scoring of disease incidence observed on: individuals of the same two T_2 families
519 positive for the presence of the 35S::*NtMLO1* construct [T2_a(+) and T_2_b(+)]; individuals of
520 the T2_a and T_2_b families negative for the presence of the 35S::*NtMLO1* construct [T_2(-)];
521 individuals of the Slmlo1 mutant line; individuals of the cultivar MM. The scale from 0
522 (completely resistant) to 3 (fully susceptible) reported by Bai et al. (2008), was used for scoring.
523 Bars and standard errors refer to 11 T_2(+)_a plants, 10 T_2(+)_b plants, 9 T_2(-) plants, 10 Slmlo1
524 plants and 10 MM plants.

525 **Fig. 3** Alignment of part of the second MLO intracellular loop from several MLO proteins
526 experimentally shown to be required for powdery mildew susceptibility (Arabidopsis AtMLO2,
527 AtMLO6 and AtMLO12, tomato SlMLO1, pepper CaMLO2, pea PsMLO1, lotus LjMLO1,
528 barrel clover MtMLO1 and barley HvMLO), and NtMLO1 proteins derived from the
529 conceptual translation of the two inserts obtained during the cloning procedure (NtMLO1 and
530 NtMLO1-Q198R). The latter is characterized by the substitution of an invariable glutamine
531 with arginine, whose position is indicated by an arrow.
532
533 **Fig. 4** Effects of the transgenic expression of a *NtMLO1* mutant sequence, resulting in the
534 substitution of a glutamine residue with arginine in the protein second intracellular loop
535 (Q198R)**.** Panel A) shows the phenotype of a plant of the tomato loss-of-function Slmlo1 line
536 (right) and transgenic individuals from two different T_2 families (left and centre) assessed for
537 transgene overexpression. Panel B) shows the relative quantification of the ratio between
538 *Oidium neolycopersici* and plant gDNAs in transgenic individuals of the same T_2 families
539 assessed for the presence or absence of the overexpression construct [T_2(+)_Q198R and T_2(-
540)_Q198R, respectively] and in the tomato Slmlo1 mutant line. Bars and standard errors refer to
541 11 and 7 transgenic individuals for NtMLO1_Q198R-a and b, respectively, and 10 Slmlo1
542 plants.
543
544 **Supplementary Fig. 1** Protein multiple alignment of a dataset composed of eggplant
545 SmMLO1, potato StMLO1, tobacco NtMLO1, the Arabidopsis AtMLO protein family and the
546 susceptibility proteins tomato SlMLO1, pepper CaMLO2, pea PsMLO1, lotus LjMLO1 and
547 barrel clover MtMLO1. Black shading shows amino acid residues reported to be conserved
548 throughout the whole MLO protein family, whereas grey shading highlights residues which are

549 shared by MLO proteins experimentally shown to be required for powdery mildew
550 susceptibility.
551
552 **Supplementary Fig. 2** Nucleotide multiple alignment of full-length coding sequences of
553 eggplant *SmMLO1*, potato *StMLO1*, tobacco *NtMLO1*, tomato *SlMLO1* and pepper *CaMLO2*.
554
555 **Supplementary Fig. 3** Segregation of the T_2_a (left) and T_2_b (right) families for the marker
556 sequences nptII (panels A and C) and 35S (panels B and D), indicating the presence of the
557 35S::*NtMLO1* construct. Wells at the right side of each gel adjacent to the ladder were loaded
558 with PCR-negative controls.
559
560 **Supplementary Fig. 4** Relative quantification of *NtMLO*1 expression levels in
561 complementation tests, assessed by qPCR. Data refer to eleven and ten individuals of two T_2
562 families positive for the presence of the overexpression construct harbouring wild-type
563 *NtMLO1* [T2(+)_a and T2(+)_b]; eleven and seven individuals of two T_2 families positive for
564 the presence of the overexpression construct harbouring a *NtMLO1* mutant sequence, resulting
565 in the substitution of a glutamine residue with arginine [T2(+)_Q198R-a and b]; eighteen non-
566 transgenic individuals from the four T_2 families above mentioned [T2(-)]; ten individuals of the
567 Slmlo1 mutant line, used as background genotype for transformation.
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576 **Table 1** Characteristics of the tobacco *NtMLO* gene family members identified in this study

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Gene	SGN database sequence ID	Predicted TM	Amino-acid length	Clade
NtMLO1	mRNA_127718_cds	6	525	V
NtMLO2	mRNA_127185_cds	6	532	V
NtMLO3	mRNA_63807_cds	7	597	V
NtMLO4	mRNA_106507_cds	6	605	V
NtMLO5	mRNA_52113_cds	7	520	V
NtMLO6	mRNA_44723_cds	6	554	III
NtMLO7	mRNA_90912_cds	7	489	VI
NtMLO8	mRNA_125509_cds	7	555	III
NtMLO9	mRNA_33476_cds	7	455	III
NtMLO10	mRNA_91715_cds	7	410	III
NtMLO11	mRNA_52133_cds	7	492	II
NtMLO12	mRNA_46569_cds	7	490	II
NtMLO13	mRNA_79933_cds	6	508	II
NtMLO14	mRNA_23316_cds	7	505	II
NtMLO15	mRNA_44406_cds	7	558	I

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580 **Table 2** Characteristics of the potato *StMLO* gene family members identified in this study

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Gene	Spud DB database sequence ID	Chr.	Position from – to (bp)	Predicted introns	Predicted TM	Amino-acid length	Clade
StMLO1*	-	-	-	-	6	519	V
StMLO2	PGSC0003DMG400013720	8	8,453,442-8,457,924	11	6	517	II
StMLO3	PGSC0003DMG400018975	10	17,809,877-17,818,901	13	3	456	I
StMLO4	PGSC0003DMG400020286	9	36,181,654-36,187,569	13	7	477	III
StMLO5	PGSC0003DMG400003574	2	39,242,676-39,247,920	13	4	455	I
StMLO6	PGSC0003DMG400012451	7	53,305,494-53,314,628	14	7	565	I
StMLO7	PGSC0003DMG400013667	2	38,189,087-38,196,233	13	7	552	III
StMLO8	PGSC0003DMG400018271	1	79,235,994-79,239,439	12	5	414	II
StMLO9	PGSC0003DMG400020605	3	36,041,611-36,048,004	13	6	366	V
StMLO10	PGSC0003DMG400023159	2	18,902,752-18,910,333	11	7	550	III
StMLO11	PGSC0003DMG400027665	6	57,855,801-57,859,958	12	7	507	II
StMLO12	PGSC0003DMG400030134	6	9,616,811-9,623,870	13	7	589	V
StMLO13	PGSC0003DMG400033623	8	42,249,518-42,256,102	14	7	532	III

582 * Features of StMLO1 refer to the homolog identified by a PCR-based approach for which no corresponding sequence is found in Spud DB
583 database .

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593 **Table 3** Features and distribution of motifs conserved in the potato StMLO and tobacco NtMLO protein families, as predicted by the MEME software
594 package. Correspondences with the motifs previously characterized by Deshmukh et al. (2014) in the soybean MLO protein family are reported.

	Width	**e-value**	**StMLO1**	**StMLO2**	**StMLO3**	**StMLO4**	**StMLO5**	**StMLO6**	**StMLO7**	**StMLO8**	**StMLO9**	**StMLO10**	**StMLO11**	**StMLO12**	**StMLO13**	**Sequence motif**	**Corresponding motif number in soybean (Deshmukh *et al.* 2014)**
MOTIF 1	70	1.6e-397	√	√	√	√	√	√	√	√	√	√	√	√	√	PTWAVAVVCTVIVAISLAIERIIHKLGKWLKKKNKKALYE ALEKIKEELMLLGFISLLLTVLQSYISKIC	4
MOTIF 2	70	8.4e-376	√	√	√	√	√	√	√	√	-	√	√	√	√	LIHFILFQNAFEIAFFFWIWWEYGFKSCFHDNFGFIIIRLVIG VIVQFLCSYSTLPLYALVTQMGSHMKK	3
MOTIF 3	70	1.6e-295	√	√	-	√	-	√	√	-	√	√	√	√	√	KFDFQKYIKRSLEDDFKVVVGISPVLWGFVVLFLLLNVH GWHAYFWIAFIPLIIILAVGTKLQHVITQMA	1
MOTIF 4	51	1.0e-245	√	√	√	√	√	√	√	√	√	√	√	√	√	GKVPLLSLEALHQLHIFIFVLAVFHVLYSAITMALGGLKIR QWKxWEDEIK	2
MOTIF 5	55	1.4e-176	√	√	-	√	-	√	√	√	√	√	√	√	√	RFTHETSFGRRHxSFWTKSPILFWIVCFFRQFFRSVxKSDY LTLRHGFIMAHLAP	5
MOTIF 6	40	3.9e-038	√	√	√	√	√	√	√	-	-	√	√	√	√	SIFDEQVQKALHGWHKKAKKRRGHKxxRSxTTxSTSSSx	-
MOTIF 7	40	2.00e-11	-	-	-	√	-	-	√	-	-	√	-	-	-	PESVADTLLPCPAKNKAAAEEEHRRRLLWEERRILAGAEP	-

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	Width	e-value	NtMLO1	NtMLO2	NtMLO3	NtMLO4	NtMLO5	NtMLO6	NtMLO7	NtMLO8	NtMLO9	NtMLO10	NtMLO11	NtMLO12	NtMLO13	NtMLO14	NtMLO15	Sequence motif	Corresponding motif number in soybean (Deshmukh *et al*. 2014)
MOTIF 1	70	1.1e-580	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	PSDDLFWFNRPQLVLFLIHFVLFQNAFQLAFFFWIW YEYGLKSCFHDNVEDIIIRLVMGVGIQFLCSYIT	3
MOTIF 2	70	3.9e-578	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	PTWAVAAVCFVIVAISIAIERIIHKLGKWLKKKHKK ALYEALEKIKAELMLLGFISLLLTVSQYPISKIC	4
MOTIF 3	70	5.4e-559	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	KFDFQKYIKRSLEDDFKVVVGISPPLWVFVVLFLLL NVHGWHAYFWIAFIPLIIILAVGTKLQHVITQMA	1
MOTIF 4	56	3.5e-408	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	PERFRFTRETSFGRRHLSFWTRSPILLWIGCFFRQFF RSVSKSDYLTLRHGFIMAH	5
MOTIF 5	56	1.3e-398	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	KGKVALISLDALHQLHIFIFVLAVLHVLYSALTMAL GRAKIRGWKAWEDETQTHEY	2
MOTIF 6	70	8.7e-246	√	√	√	√	√	√	√	√	-	-	√	√	√	√	√	LPLYALVTQMGSSMKKTIFDEHVATALKGWHHAA KKKKKLGGKHSNTTTGSSSPPATPGSQMEIIHLLRG	-
MOTIF 7	40	8.30e-48	-	-	-	-	-	√	-	√	√	√	√	√	√	√		IPESVANTMLPCPADEKxNETDEAEGHRRLLFELHR RGLA	-

600