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ORIGINAL PAPER

Pea powdery mildew *er1* resistance is associated to loss-of-function mutations at a *MLO* homologous locus

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Abstract The powdery mildew disease affects several crop species and is also one of the major threats for pea (*Pisum sativum* L.) cultivation all over the world. The recessive gene *er1*, first described over 60 years ago, is well known in pea breeding, as it still maintains its efficiency as a powdery mildew resistance source. Genetic and phytopathological features of *er1* resistance are similar to those of barley, *Arabidopsis*, and tomato *mlo* powdery mildew resistance, which is caused by the loss of function of specific members of the *MLO* gene family. Here, we describe the obtainment of a novel *er1* resistant line by experimental mutagenesis with the alkylating agent diethyl sulfate. This line was found to carry a single nucleotide polymorphism in the *PsMLO1* gene sequence, predicted to result in premature termination of translation and a non-functional protein. A cleaved amplified polymorphic sequence (CAPS) marker was developed on the mutation site and shown to be fully co-segregating with resistance in F_2 individuals. Sequencing of *PsMLO1* from three powdery mildew resistant cultivars also revealed the presence of loss-of-function mutations. Taken together, results reported in this study strongly indicate the identity between *er1* and *mlo* resistances and are expected to be of great breeding importance for the development of resistant cultivars via marker-assisted selection.

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Introduction

The powdery mildew disease, caused by obligate biotrophic fungi belonging to the ascomycete order of Erysiphales, is common among higher plant species and severely affects the yield and the quality of many crops. The powdery mildew fungus *Erysiphe pisi* D. C. (*Ep*) causes yield losses up to 50% in pea (*Pisum sativum* L.), the most widely grown grain legume in Europe and the fourth-most in the world (Rubiales et al. 2009; Warkentin et al. 1996). The biological cycle of the fungus includes germination of asexual (conidia) or sexual (ascospores) spores, differentiation of appressoria and haustoria, development of colonies growing epiphytically on host epidermal cells and spore production.

Most pea powdery mildew resistant cultivars rely on the presence of the recessive gene *er1*, which was first reported by Harland (1948) following the screening of germplasm collected in the town of Huancabamba, in the northern Peruvian Andes. The *er1* locus has been mapped on linkage group VI of the pea consensus map (Timmerman et al. 1994). Aiming to aid selection in breeding programs, a number of studies identified several molecular markers linked to the *er1* locus (Dirlewanger et al. 1994; Ek et al. 2005; Janila and Sharma 2004; Timmerman et al. 1994; Tiwari et al. 1998; Tonguç and Weeden 2010).

The *MLO* gene family encodes for seven transmembrane domain proteins in plants, topologically reminiscent of G-protein coupled receptors (GPCRs) present in animals and fungi (Devoto et al. 2003). Specific *MLO* homologs have been demonstrated to act as susceptibility factors towards epiphytic powdery mildew fungi, as their inactivation, through loss-of-function mutations, results in recessively inherited disease immunity (*mlo* resistance) (Pavan et al. 2010). It is thought that functional MLO proteins modulate vesicle-associated defense responses at the cell periphery and their targeting by virulent pathogen effectors promotes fungal penetration in epidermal cells (Jones and Dangl 2006; Panstruga 2005). So far, *mlo* resistance has been reported to occur in barley, *Arabidopsis*, and tomato (Bai et al. 2008; Büschges et al. 1997; Consonni et al. 2006; Devoto et al. 2003).

In a previous study, we pointed out that *er1* and *mlo* resistances share genetic and phytopathological features (Bai et al. 2008). Indeed, besides being recessively inherited, they are both based on a defense mechanism independent from hypersensitive response and associated to the early abortion of pathogenesis after the differentiation of fungal appressoria (Bai et al. 2005; Consonni et al. 2006; Fondevilla et al. 2006; Hückelhoven et al. 2000). In addition, they do not show specificity towards particular fungal isolates, in contrast with most other powdery mildew resistance sources reported in crop species (Bai et al. 2005; Lyngkjaer et al. 2000; Tonguç and Weeden 2010).

Here, we report the identification of a novel chemically induced *er1* allele, shown to be co-segregating with a cleaved amplified polymorphic sequence (CAPS) marker developed on a loss-of-function point mutation of the pea *MLO* homolog *PsMLO1*. In addition, we detected *PsMLO1* loss-of-function mutations in three powdery mildew resistant cultivars, further suggesting that *er1* resistance is mediated by a *mlo*-based mechanism.

Materials and methods

Experimental mutagenesis and selection for powdery mildew resistance

Approximately 4,000 seeds of a pea breeding line derived from the old processing cultivar Sprinter [germplasm collection of the Department of Agroforestry, Environmental Biology and Chemistry (DiBCA), University of Bari] were immersed in a 0.2% solution of diethyl sulfate for 2 h, rinsed with distilled water and planted immediately in paper pots filled with sterilized soil. About 2,200 M_1 plants reached maturity and about 2,000 set seeds. M_1 seeds were separately collected from each plant and sown at the experimental farm "P. Martucci" of the University of Bari (Italy), to give an M_2 generation of approximately 27,000 individuals. In order to ensure a high level of powdery mildew infection, M_2 plants were artificially inoculated by spraying a water suspension of conidia (about 5×10^4 conidia/ml) of a local *Ep* isolate maintained on susceptible plants at the University of Bari, with the addition of 0.0025% volume of Tween20. The line ROI3/02 was obtained by four selfings of a single individual free of disease symptoms detected in a segregating M_2 family. Response of ROI3/02 to *Ep* was assessed both in open field and controlled greenhouse conditions (20 ± 1°C, 60 ± 10 RH and 16 h photoperiod), following pathogen spray-inoculation as above described.

Histological studies

The resistant line ROI3/02 and the susceptible cultivar Sprinter were artificially inoculated with the same *Ep* isolate used for the selection program by tapping heavily infected plant parts over the leaves. After 48 h, during which plants were kept at 20 ± 1°C, 60 ± 10 RH and 16 h photoperiod, leaf samples from three individuals of each genotype were cleared for 2 days in an acetic acid:ethanol (1:3) solution and stained with trypan blue, according to

the method described by Huang et al. (1998). For each individual, 30 infection units were observed, an infection unit being a germinated spore that produced at least a primary appressorium.

Inference of the genetic control of resistance in the line ROI3/02

The F_1 ($n = 15$) and F_2 ($n = 110$) populations generated by the cross between the resistant line ROI3/02 (female parent) and the susceptible cultivar Progress9 (Asgrow) were grown in a greenhouse compartment and 1-month-old plants were spray-inoculated, according to the methods specified in the previous paragraphs. The outcome of the plant–pathogen interaction was visually assessed 15 days after inoculation, based on the presence/absence of disease symptoms. A χ^2 test was used to examine the goodness-of-fit of the Mendelian 3:1 ratio for segregation.

Genetic mapping of resistance at the *er1* locus

DNA from ROI3/02, Progress9 and their F_2 progeny was extracted according to a cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle 1990). Parental lines were tested with three sequence characterized amplified region (SCAR) molecular markers known to be linked to the *er1* locus, ScOPD-10_{650}, ScOPO-18_{1200} and ScOPO-06_{1100} (Pereira et al. 2010; Timmerman et al. 1994; Tiwari et al. 1998). Polymorphic markers ScOPO-18_{1200} and ScOPO-06_{1100} were next tested on the F_2 progeny. Genetic distances between markers and resistance locus were estimated by using the JoinMap 4.0 software (van Ooijen 2006) with a LOD score threshold value of three. Evidence for correspondence between the resistance locus controlling resistance in ROI3/02 and *er1* was obtained by carrying out a new greenhouse disease test based on the spray-inoculation of the F_1 progeny ($n = 15$) derived from the complementation cross between the ROI3/02 (female parent) and the resistant cultivar Franklin (USDA-ARS, conserved at the DiBCA germplasm collection), known to be homozygous for the *er1* allele (Ondřej et al. 2008).

MLO family comparative analysis and assessment of association between *er1* resistance and loss-of-function mutations in *PsMLO1*

The cDNA sequence of the pea *MLO* homolog *PsMLO1* was retrieved in the GenBank Database of the National Center for Biotechnology Information (NCBI, accession number FJ463618.1). The corresponding protein (PsMLO1) amino acid sequence was imported together with those of the 15 *Arabidopsis* AtMLO homologs and tomato SlMLO1 in the CLC sequence viewer software (http://clcbio.com) for Clustal alignment (gap open cost and gap extension cost were respectively set equal to 10 and 4) and the obtainment of an UPGMA-based comparative tree (bootstrap value was set equal to 100).

Total RNA from the five genotypes Sprinter, ROI3/02, Franklin, Dorian (Asgrow) and Nadir (DiBCA germplasm collection) was extracted with the SV Total RNA Isolation System Kit (Promega) and corresponding cDNAs were synthesized by using the QuantiTect Reverse Transcription Kit (Qiagen) with oligo(dT) primers. The *PsMLO1*-specific primer pair 5́-AAAATGGCTGAAGAGGGAGTT-3́/5́-TCCACAAATCAAGCTGCTACC-3́, was selected by using the Primer3 software (Rozen and Skaletsky 2000) and used for a PCR reaction with annealing temperature of 54°C. Amplicons were purified by using the Nucleospin Extract II kit (Macherey–Nagel) and ligated (molar ratio 1:1) into the pGEM-T easy vector (Promega). Recombinant plasmids were cloned in *E. coli* DH10β chemically competent cells and recovered by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed by using universal T7 and SP6 primers (Eurofins MWG Operon).

The CAPS marker GIM-300/*Sml*I, discriminating the mutation site identified in the *PsMLO1* sequence of ROI3/02, was obtained by amplification with the primer pair 5́-TCTGCATATGGAATTCACCAA-3́/5́-AATTGATATT CAACTGTTCTTGTC-3́ (annealing temperature 54°C), digestion of the amplification product with *Sml*I for 2 h at 50°C and visualization on a 2.5% GellyPhor agarose gel (EuroClone). Marker and phenotype segregation data in the ROI3/02 × Progress9 F_2 progeny were compared.

Results

Identification and histological characterization of the ROI3/02 powdery mildew resistant mutant line

A field screening of approximately 2,000 M_2 families allowed the identification of one family which was segregating for response to powdery mildew infection. The resistant line ROI3/02 was next selected by means of successive self-pollinations of a single resistant individual.

In field conditions, the line ROI3/02 appeared to be completely resistant to *Ep*, as no symptoms could be detected (Fig. 1). However, under greenhouse conditions, particularly favorable for fungal development, a low level of fungal sporulation could be occasionally observed.

At the histological level, 2 days after artificial inoculation, most germinated *Ep* spores developed mycelium on the susceptible control cultivar Sprinter. In contrast, at the same time point no spores developed secondary hyphae on ROI3/02. Similarly to the previous observations reported

Fig. 1 Powdery mildew infected leaves on the pea susceptible cultivar Sprinter and the resistant line ROI3/02 obtained by mutagenesis with diethyl sulfate

for *er1*- and *mlo*-based immunities (Bai et al. 2005; Consonni et al. 2006; Fondevilla et al. 2006), in ROI3/02 pathogenesis was found to terminate after the differentiation of the primary appressorium and was not associated with the host epidermal cell hypersensitive response.

Genetic analysis of the ROI3/02 mutation

The cross between the resistant line ROI3/02 and the susceptible cultivar Progress9 generated a susceptible F_1 population ($n = 15$) and an F_2 population ($n = 110$) segregating according to a 3:1 (susceptible:resistant) ratio ($\chi^2 = 1.43$; $0.3 < P < 0.2$), thus indicating that resistance was due to a recessive mutation event at one locus. Two out of three tested *er1* linked SCAR markers, ScOPO-18_{1200}, and ScOPO-06_{1100}, were polymorphic between the parents and were, therefore, used for F_2 linkage analysis. Both marker loci were found to be associated with the resistance locus, with an estimated distance of 1.1 cM for ScOPO-18_{1200} and 3.1 cM for ScOPO-06_{1100}.

The cross between the line ROI3/02 and the *er1* resistant cultivar Franklin resulted in F_1 individuals ($n = 15$) all showing a powdery mildew resistant phenotype, providing final evidence for the identification of a new mutant at the *er1* locus.

er1 resistance is associated with loss-of-function mutations of pea *PsMLO1*

As *er1* and *mlo* resistances share genetic and phytopathological similarities, we investigated the possibility that resistance of the ROI3/02 line could be due to the loss of function of a *MLO* homolog. The interrogation of the NCBI database allowed the identification of the pea *MLO* homolog full-length sequence *PsMLO1*. The corresponding PsMLO1 protein was found to cluster together with all known dicot MLO isoforms associated with the powdery mildew susceptibility (AtMLO2, AtMLO6, AtMLO12, and SlMLO1) in a MLO protein family comparative tree (Fig. 2), suggesting a functional role of PsMLO1 as susceptibility factor for *Ep*.

PsMLO1 full-length coding sequence was amplified from the cDNAs of Sprinter and ROI3/02. *PsMLO1* sequence in ROI3/02 was found to contain a point mutation (A in place of G), predicted to cause a premature termination of translation in correspondence of the second N-proximal MLO protein intracellular loop and thus, a severely truncated protein (Fig. 3). As the mutation occurring in the *PsMLO1* sequence of ROI3/02 was found to result in a cutting site for the restriction enzyme *Sml*I, we designed a primer pair flanking the mutation site and developed a polymorphic CAPS marker, GIM-300/*Sml*I. Remarkably, all the resistant individuals in the ROI3/02 × Progress9 F_2 population were found to be homozygous for the G → A *PsMLO1* transition, whereas susceptible phenotypes were either homozygous for the wild-type allele or heterozygous (Fig. 4).

We next used the same primer pair used for the amplification of Sprinter and ROI3/02 *PsMLO1* in PCR reactions using cDNAs from the cultivar Franklin, known to be homozygous for an *er1* resistance allele, and from the powdery mildew resistant cultivars Dorian and Nadir, whose genotype at the *er1* locus was unknown. For each of these three genotypes, PCR resulted in the amplification of two severely mutated *PsMLO1* transcripts, predicted to be associated with non-functional proteins. Compared to the *PsMLO1* transcript of the susceptible cultivar Sprinter, the two transcripts of Dorian and Nadir were characterized, one by a 129 bp deletion and the other by an insertion/deletion mutation, resulting in a net gain of 155 bp (Fig. 5). Franklin *PsMLO1* transcripts were identical to those of Dorian and Nadir, except for the presence of an additional mutation, i.e. a frameshift-associated 5 bp deletion (Fig. 5). Since, Franklin, Dorian and Nadir are homozygous lines, we suppose that the two transcripts identified in these genotypes are the result of aberrant splicing events.

Discussion

In this study, we provide the following evidence indicating that *er1* powdery mildew resistance is due to the loss of function of a pea *MLO* homolog: (1) two different *er1* resistant genotypes (ROI3/02 and Franklin) and two powdery mildew resistant cultivars (Dorian and Nadir) are

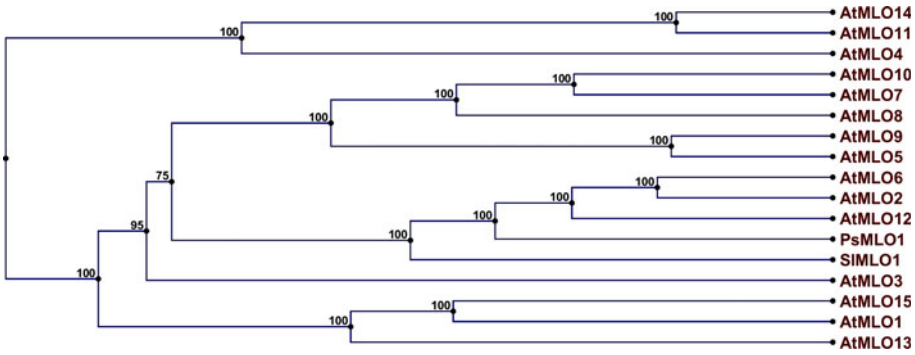

Fig. 2 UPGMA-based comparative analysis of a dataset of full-length MLO proteins composed of tomato SlMLO1, pea PsMLO1, and the 15 *Arabidopsis* AtMLO homologs. Numbers at each node represent bootstrap support values (out of 100 replicates)

SPRINTER PsMLO1

MAEEGVKERTLEETPTWAVAVVCLVLLAVSILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLL
GFISLLLTVFQDNISKICVSQKIGSTWHPCSTSNTKAKAKSDESLDYKTNNDRKLLEYFDPIPRRI
PATKGYDKCFDKGQVALVSAYGIHQLHIFIFVLALFHILQCIITLTLGRIKMRKWKTWEDETRTVE
YQFYNDPERFRFARDTTFGRRHLSMWAQSPILLWIVSFFRQFFGSISRVDYMALRHGFIMAHLPPG
HDAQFDFQKYISRSIEEDFKVVVGISPTIWLFTVLFLLTNTHGWYSYYWLPFLPLIVILLVGAKLQ
MIITKMGLRIQDRGEVIKGAPVVEPGDHLFWFNRPHLLLFTIHLVLFQNAFQLAFFAWSTYEFSIT
SCFHKTTADSVIRITVGVVIQTLCSYVTLPLYALVTQMGSTMKPTIFNERVATALKNWHHTAKKQV
KQSNHSNNTTPYSSRPSTPTHAMSPVHLLHRHTAGNSDSLQTSPEKSDYKNEQWDIEGEGPTSLRN
DQTGQHEIQIAGVESFSSTELPVRIRHESTSGSKDFSFEKRHLGSN*

ROI3/02 PsMLO1

MAEEGVKERTLEETPTWAVAVVCLVLLAVSILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLL
GFISLLLTVFQDNISKICVSQKIGSTWHPCSTSNTKAKAKSDESLDYKTNNDRKLLEYFDPIPRRI
LATKGYDKCFDKGQVALVSAYGIHQLHIFIFVLALFHILQCIITLTLGRIKMRKWKT*

Fig. 3 PsMLO1 protein sequence predicted for the susceptible cultivar Sprinter and the resistant line ROI3/02. Protein translation in ROI3/02 prematurely terminates due to a point mutation

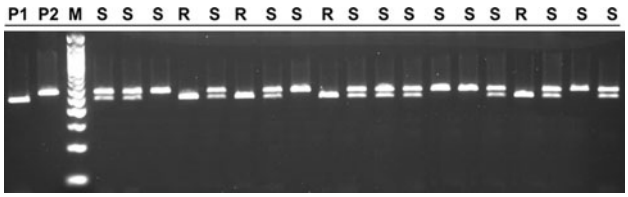

Fig. 4 Genotyping of the powdery mildew resistant line ROI3/02 (P1), the susceptible cultivar Progress9 (P2), and 19 F_2 segregating individuals with the co-dominant cleaved amplified polymorphic sequence marker GIM-300/*Sml*I. Susceptible and resistant F_2 individuals are indicated with S and R, respectively. *Lane M* contains the size marker 50 bp DNA ladder (New England Biolabs). All resistant genotypes are homozygous for a G → A transition in the *PsMLO1* coding sequence that originates a cutting site for the restriction enzyme *Sml*I and a short fragment of about 250 bp. Similar results were obtained with the analysis of additional 91 F_2 individuals

characterized by mutations in the sequence of the *PsMLO1* gene, predicted to result in non-functional proteins; (2) full co-segregation occurs between *er1* powdery mildew resistance and *PsMLO1* loss-of-function. Importantly, during the revision of this paper, another study was released providing additional experiments indicating the identity between *er1* and *mlo* immunities (Humphry et al. 2011). Aiming to the functional characterization of *er1* resistance, we are currently carrying out a complementation experiment consisting of the expression of wild-type *PsMLO1* in *er1* mutants.

A new chemically induced *er1* resistance allele, the third reported so far after those recently described by Pereira and Leitão (2010), was found to be associated to a G → A transition in the *PsMLO1* coding sequence. This kind of mutational event was expected, since diethyl sulfate is known to cause base mispairing through guanine alkylation (Holwitt and Krasna 1974).

In the past years, several authors focused on the identification of *er1*-linked molecular markers, differing with respect to: distance from the resistance locus, repeatability,

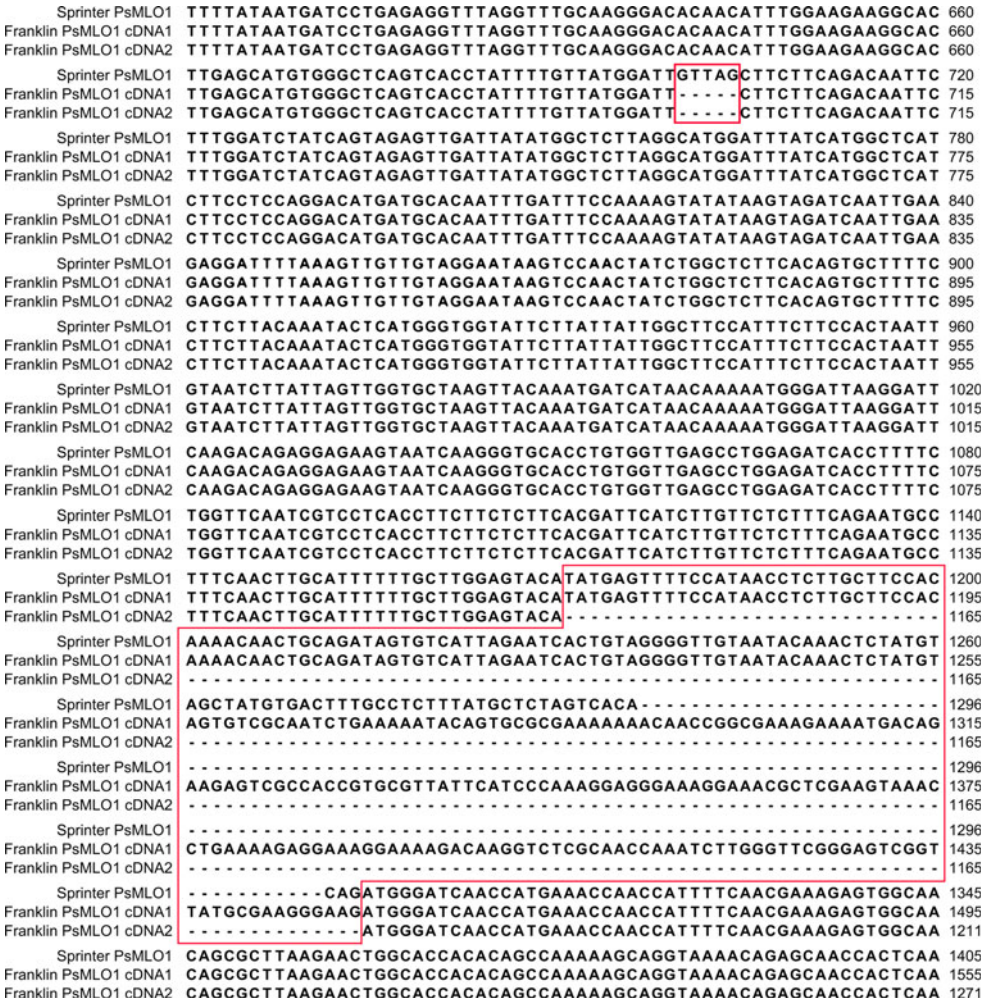

Fig. 5 Nucleotide alignment between a segment of the wild-type *PsMLO1* coding sequence detected in the cultivar Sprinter (Sprinter *PsMLO1* cDNA) and homologous regions of the two *PsMLO1* cDNA sequences identified in the cultivar Franklin (Franklin *PsMLO1* cDNA 1 and 2). Mutation sites are boxed. The two *PsMLO1* cDNAs identified in the genotypes Dorian and Nadir only retain the mutation event in the down box

ease of obtainment, possibility to score heterozygous individuals, and capacity to reveal polymorphism in different segregating populations (Dirlewanger et al. 1994; Ek et al. 2005; Janila and Sharma 2004; Pereira et al. 2010; Timmerman et al. 1994; Tiwari et al. 1998; Tonguç and Weeden 2010). Here, we report the identification of a simple agarose-based co-dominant CAPS marker fully co-segregating with *er1* resistance and thus, expected to be of great interest for breeding activities using the line ROI3/02 as donor parent. In addition, we mapped two previously reported *er1*-linked SCAR markers, ScOPO-18_{1200} and ScOPO-06_{1100}, which showed polymorphism within the segregating F_2 population of 110 individuals used in this study. A genetic distance of 3.1 cM between the marker locus ScOPO-06_{1100} and the *er1* locus was estimated. By using a different F_2 population of 94 individuals, Pereira et al. (2010) estimated the distance between the two loci to be 1.2 cM. The reason for this slight discrepancy could be referable to the moderate size of the F_2 populations used for linkage analysis. The marker ScOPO-18_{1200}, which was co-segregating with the *er1* locus in a previous genetic analysis carried out with 57 F_3 individuals (Tiwari et al. 1998), was mapped at a distance of 1.1 cM from *er1*, as one recombination event was detected.

Here, we show that barley *mlo* and pea *er1* immunities, which are still effective in the field several decades after their introduction in cultivation (Fondevilla et al. 2006; Lyngkjaer et al. 2000), are likely to share the same molecular basis. In previous studies, we showed that a third recessive resistance source referable to a loss-of-function mutation of a *MLO* homolog, tomato *ol-2*, was effective towards several isolates of the powdery mildew fungus *Oidium neolycopersici* L. Kiss tested worldwide (Bai et al. 2005; Bai et al. 2008; Pavan et al. 2008). Taken together, these data suggest that forward and reverse genetic approaches aiming at the mutagenesis or silencing of *MLO* susceptibility genes could represent a valid breeding strategy to provide broad-spectrum and durable powdery mildew resistance across agricultural crops.

In greenhouse experimental conditions, extremely favorable to *Ep* development, we could detect moderate fungal sporulation on the line ROI3/02. This is consistent

with the observations of Pereira and Leitão (2010) on another *er1* mutant. If *er1* is due to the loss of function of *PsMLO1*, this finding would be in accordance with the presence of low levels of powdery mildew colonization in *Arabidopsis Atmlo2* and tomato *Slmlo1* mutants when plants are artificially inoculated (Bai et al. unpublished data; Consonni et al. 2006). In *Arabidopsis*, full resistance to the compatible powdery mildew species *Golovinomyces orontii* (Castagne) V.P. Heluta, *Golovinomyces cichoracearum* (D.C.) V.P. Heluta and *Oidium neolycopersici* L. Kiss is provided by the simultaneous loss of function of the three phylogenetically related homologs *AtMLO2*, *AtMLO6*, and *AtMLO12* (Bai et al. 2008; Consonni et al. 2006). If a similar scenario holds true, it should be possible to retrieve other *PsMLO* homologs for susceptibility and provide complete powdery mildew immunity in pea.

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