Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2011) 12(9), 866–878 DOI: 10.1111/J.1364-3703.2011.00718.X

Durable broad-spectrum powdery mildew resistance in pea *er1* plants is conferred by natural loss-of-function mutations in *PsMLO1*

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SUMMARY

Loss-of-function alleles of plant-specific *MLO* (M*ildew Resistance Locus* O) genes confer broad-spectrum powdery mildew resistance in monocot (barley) and dicot (*Arabidopsis thaliana*, tomato) plants. Recessively inherited powdery mildew resistance in pea (*Pisum sativum*) *er1* plants is, in many aspects, reminiscent of *mlo*-conditioned powdery mildew immunity, yet the underlying gene has remained elusive to date. We used a polymerase chain reaction (PCR)-based approach to amplify a candidate *MLO* cDNA from wild-type (*Er1*) pea. Sequence analysis of the *PsMLO1* candidate gene in two natural *er1* accessions from Asia and two *er1*-containing pea cultivars with a New World origin revealed, in each case, detrimental nucleotide polymorphisms in *PsMLO1*, suggesting that *PsMLO1* is *Er1*. We corroborated this hypothesis by restoration of susceptibility on transient expression of *PsMLO1* in the leaves of two resistant *er1* accessions. Orthologous legume *MLO* genes from *Medicago truncatula* and *Lotus japonicus* likewise complemented the *er1* phenotype. All tested *er1* genotypes showed unaltered colonization with the arbuscular mycorrhizal fungus, *Glomus intraradices*, and with nitrogen-fixing rhizobial bacteria. Our data demonstrate that *PsMLO1* is *Er1* and that the loss of *PsMLO1* function conditions durable broad-spectrum powdery mildew resistance in pea.

INTRODUCTION

Fungi are responsible for many of the world's most devastating plant diseases and are a major threat for agriculture with regard to both product yield and quality. Members of the order Erysiphales, phylum Ascomycota, comprise the causal agents of the widespread powdery mildew disease of higher plant species. The disease is particularly prevalent in temperate and humid climates, where it frequently causes significant yield losses and quality reductions in agricultural settings, including greenhouse and field farming. This applies to key cereals (e.g. barley and wheat), horticultural crops (e.g. grapevine, pea and tomato) and economically important ornamentals (e.g. roses). Limited access to natural resources of resistance to powdery mildews, rapid changes in virulence in the pathogen populations and the time-consuming introgression of suitable resistance genes into elite varieties account for the widespread use of fungicides to control the disease. The challenge is compounded by increasing problems caused by the evolution and spread of fungicide resistance, which is especially dramatic amongst the most economically important powdery mildews (Wyand and Brown, 2005).

In the monocot barley (*Hordeum vulgare*) and the dicots *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), loss-of-function mutations in *MLO* (M*ildew Resistance Locus* O) genes confer highly effective broad-spectrum powdery mildew resistance (Bai *et al*., 2008; Büschges *et al*., 1997; Consonni *et al*., 2006). Resistance conditioned by *mlo* alleles acts early and typically terminates fungal pathogenesis before invasion of the first host cell (Jørgensen, 1992; Lyngkjaer *et al*., 2000). This type of immunity was originally discovered in barley following the mutagenesis of wild-type germplasm, as well as via a natural source of resistance in Ethiopian landraces (Jørgensen, 1992; Piffanelli *et al*., 2004). In barley, *mlo* resistance is of great agronomic importance given that approximately one-half of the current European acreage occupied by spring varieties is of the *mlo* genotype and thus highly resistant against *Blumeria graminis* f.sp. *hordei*, the causal agent of barley powdery mildew disease (Lyngkjaer *et al*., 2000). In tomato, powdery mildew resistance conferred by *ol*-2 is also a result of a loss of *MLO*

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function, conditioned by a natural polymorphism resulting in a small deletion within the *MLO* coding region (Bai *et al*., 2008).

MLO genes code for a plant-specific type of integral membrane protein with as yet unknown biochemical function(s) (Devoto *et al*., 1999; Panstruga, 2005b). The genes occur in small- to medium-sized families with approximately 10–20 members per plant species (Devoto *et al*., 2003; Feechan *et al*., 2008; Konishi *et al*., 2010; Liu and Zhu, 2008); however, only members of a specific phylogenetic clade play a role in powdery mildew susceptibility/resistance (Bai *et al*., 2008). These members are characterized by the presence of clade-specific peptide motifs in cytoplasmic regions of the heptahelical MLO protein, of which the D/E-F-S/T-F tetrapeptide at the distal end of the C-terminal cytoplasmic tail is probably the most diagnostic (Feechan *et al*., 2008; Panstruga, 2005a). In Arabidopsis and barley, these MLO family members are part of larger sets of coexpressed genes that define a conserved functional module in antifungal immunity (Humphry *et al*., 2010). It has been hypothesized previously that MLO proteins may serve as targets for defence suppression or as modulators of plant defence (Panstruga and Schulze-Lefert, 2003). Although the exact mechanism of *mlo*-conditioned resistance remains obscure, it seems to rely largely on the plant's basal defence machinery (Collins *et al*., 2003; Consonni *et al*., 2006, 2010; Humphry *et al*., 2006).

In pea (*Pisum sativum*), two recessively inherited genes (*er1* and *er2*) represent the major natural sources of resistance against the respective powdery mildew pathogen, *Erysiphe pisi* (Heringa *et al*., 1969; Kumar and Singh, 1981; Vaid and Tyagi, 1997). Both *er1* and *er2* originate from wild accessions (Harland, 1948; Heringa *et al*., 1969), have been introgressed into elite pea varieties and, in particular, *er1* has been found to provide durable broad-spectrum protection from powdery mildew disease (Cousin, 1997; Tiwari *et al*., 1997). The analysis of a range of pea accessions revealed the presence of *er1* in multiple lines of different geographical origin (Fondevilla *et al*., 2006; Tiwari *et al*., 1997), and it has also been identified recently in chemically induced pea mutants (Pereira and Leitao, 2010; Pereira *et al*., 2010). The *er1* gene has been mapped to pea linkage group 6 (Dirlewanger *et al*., 1994; Janila and Sharma, 2004; Timmerman *et al*., 1994; Tiwari *et al*., 1998; Tonguc and Weeden, 2010), whereas *er2* maps to linkage group 3 (Katoch *et al*., 2010). At the cellular level, resistance conferred by *er1* is characterized by a failure of successful invasion of epidermal pea cells, whereas *er2* resistance acts post-penetration and is associated with the execution of host cell death (Fondevilla *et al*., 2006). Taken together, the features of *er1* resistance (monogenic trait, recessive inheritance, broad-spectrum resistance, durability under agricultural conditions and pre-invasive termination of fungal pathogenesis) are reminiscent of *mlo*-conditioned resistance in barley, Arabidopsis and tomato (Bai *et al*., 2008; Büschges *et al*., 1997; Consonni *et al*., 2006). We thus previously hypothesized that *er1* resistance could be caused by loss of *MLO* gene function (Bai *et al*., 2008).

In this study, we used a polymerase chain reaction (PCR)-based approach to obtain a *MLO* candidate gene for pea *Er1* plants. Sequence analysis revealed deleterious nucleotide polymorphisms in *PsMLO1* in four pea accessions harbouring *er1* resistance. Complementation analysis based on transient gene expression in detached pea leaves of the *er1* genotype corroborated that *PsMLO1* is *Er1*. The availability of *mlo* mutants in a legume provided the exciting possibility to test whether bacterial (rhizobial) and fungal (arbuscular mycorrhizal, AM) symbionts, which, like powdery mildews, have an intracellular lifestyle, also require MLO for invasion of plant cells.

RESULTS

Reverse transcription-polymerase chain reaction (RT-PCR)-based amplification of *PsMLO1* cDNA

We have previously cloned the tomato *MLO* gene, *SlMLO1*, based on the presence of candidate expressed sequence tag (EST) sequences in public databases (Bai *et al*., 2008). In the case of pea, however, BLAST searches did not reveal any EST sequence with significant homology to any *MLO* gene. We thus opted for a PCR-based approach by taking advantage of known sequence information from other plant species. First, we performed an alignment of diverse dicotyledonous *MLO* coding sequences employing genes with a suspected orthologous relationship to Arabidopsis *AtMLO2* (Fig. S1, see Supporting Information). Aligned genes were derived from *A. thaliana* (*AtMLO2*), *Solanum lycopersicum* (tomato; *SlMLO1*), *Capsicum annuum* (pepper; *CaMLO1*), *Lotus japonicus* (*LjMLO1*) and *Medicago truncatula* (*MtMLO1*). On the basis of this multiple sequence alignment, we selected conserved regions to deduce oligonucleotide sequences for PCR-based amplification of the respective part of the orthologous pea *MLO* cDNA. In total, we designed three oligonucleotides (PsMLO1–PsMLO3; Table S1, see Supporting Information), whose sequences at the nonconserved positions were adjusted according to the legume *MtMLO1* sequence as, of the species under consideration, *M. truncatula* is that which is most closely related to *P. sativum* (Young *et al*., 2003).

We performed RT-PCR with two distinct primer combinations (PsMLO1 + PsMLO2, PsMLO1 + PsMLO3) using pea RNA derived from the susceptible wild-type cultivar JI 502 as a template. In each case, we obtained a single distinct product of the expected size range (data not shown). BLASTX analysis of the sequenced PCR products against the National Center for Biotechnology Information nonredundant (NCBInr) database revealed that the respective cDNA fragments encode part of a MLO-like protein. We designed new pairs of internal oligonucleotides and performed 5′ and 3′ rapid amplification of cDNA ends (RACE) to

obtain full-length sequence information of this cDNA. The *in silico*-assembled overlapping nucleotide sequences from 5′ and 3′ RACE reactions yielded a cDNA that encodes a predicted protein (designated *Ps*MLO1) with high sequence relatedness to *Ca*MLO1, *Lj*MLO1, *At*MLO2 and *Sl*MLO1 (Fig. 1). To determine whether *Ps*MLO1 represents a genuine orthologue of these proteins, we performed phylogenetic analysis. The resulting dendrogram indicates that *Ps*MLO1 resides in the same phylogenetic clade as *At*MLO2, *Hv*MLO and *Sl*MLO1 (Fig. 2), each of which has been shown previously to be required for powdery mildew susceptibility (Bai *et al*., 2008; Büschges *et al*., 1997; Consonni *et al*., 2006). Taken together, we identified a cDNA that represented the full-length transcript of an *Er1* candidate gene.

PsMLO1 is defective in four independent *er1* lines

We focused our subsequent molecular analysis on two pea accessions and two pea cultivars originating from various regions of the world that have been reported previously to harbour *er1* resistance: JI 210, JI 1559, JI 1951 and JI 2302 (Tiwari *et al*., 1997) (Table 1). First, we macroscopically and microscopically analysed powdery mildew infection phenotypes of these lines and compared them with the susceptible (*Er1* genotype) cultivar JI 502 using our domestic *E. pisi* isolate. Consistent with a previous report (Fondevilla *et al*., 2006), we found near-complete pre-invasive immunity in these lines (Fig. 3). Next, we determined the nucleotide sequence of *PsMLO1* cDNAs of the four resistant lines by direct sequencing of full-length RT-PCR products. This revealed single-nucleotide deletions resulting in frame shifts in lines JI 210 and JI 1951, a nucleotide substitution resulting in a premature stop codon in JI 1559, as well as multiple overlapping sequence traces in JI 2302. The latter often results from a defect in intron splicing, leading to the co-presence of multiple distinct transcript variants. We thus cloned and sequenced individual *PsMLO1* cDNAs derived by RT-PCR from RNA of line JI 2302. On the basis of nine clones, this analysis revealed three distinct types of mis-spliced cDNAs, two of which are characterized by the occurrence of ectopic sequences in the cDNA that are not present in the genomic wild-type sequence (Fig. 4A). We assumed that these extra sequences stem from the insertion of an unrelated DNA fragment into the *PsMLO1* genomic sequence. Consistent with this hypothesis, conventional and long-range PCR amplification of the genomic region under investigation failed only in accession JI 2302, possibly owing to the large size of the inserted DNA fragment (Fig. 4B,C). BLASTN analysis against the NCBInr database revealed that the part of the insertion that is present in aberrant cDNAs of line JI 2302 is nearly identical (~93% identity) to a sequence that occurs five times in a pea genomic BAC clone (GenBank accession number CU655882). In addition, an ~170-bp stretch of this sequence is highly similar (~86% identity) to part of the long terminal repeats (LTRs) of the pea *Ogre* retrotransposon (GenBank accession number AY299398). The *Ogre* retroelement is an exceptionally large (22 kb) and transcriptionally active Ty/*gypsy*-like LTR-type retrotransposon that makes up 5% of the pea genome (Neumann *et al*., 2003). We conclude that *PsMLO1* function is disrupted through insertional mutation in accession JI 2302, possibly by integration of a (retro-)transposon that is similar to the *Ogre* element. Taken together, the identification of four independent mutational events in powdery mildew-resistant *er1* lines of distinct geographical origin suggests that *PsMLO1* is identical to *Er1* (Table 1).

Complementation of *er1* resistance by transient gene expression

To further support our assumption that *PsMLO1* is *Er1*, we aimed to complement the defect in *er1* plants by transient expression of *PsMLO1* in *er1* genotypes. We opted for an established assay based on particle bombardment-mediated transformation of single leaf epidermal cells to test the functionality of *PsMLO1* (Panstruga, 2004; Shirasu *et al*., 1999). We generated a construct that drives the expression of epitope-tagged *Ps*MLO1 under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. This construct, together with a plasmid harbouring the β-glucuronidase (*GUS*) reporter gene, was ballistically transformed into leaf epidermal cells of *er1* powdery mildew-resistant accessions JI 1559 and JI 2302. Transformation of the *GUS* reporter gene alone did not allow fungal entry into epidermal cells in the resistant accessions (Fig. 5 and Table S2, see Supporting Information). In contrast, transient expression of *PsMLO1* conferred substantial levels (58%–80%) of host cell entry in these lines, indicating that each of these genes is capable of complementing the *er1* resistance phenotype (Fig. 5 and Table S2). Successful host cell penetration mediated by the expression of *PsMLO1* was restricted to transformed (GUS-stained) cells, whereas nontransformed cells retained resistance against the fungal pathogen (Fig. 5C). Transient expression of epitope-tagged versions of the phylogenetically closely related legume *MLO* genes *LjMLO1* and *MtMLO1* likewise complemented the *er1* geneotype, although with somewhat lower efficiencies (*LjMLO1*, 28%–54%; *MtMLO1*, 52%–69%; Table S2). Collectively, these findings further corroborate the claim that *PsMLO1* is *Er1*.

Mutations in *PsMLO1* do not affect the invasion of plant cells by arbuscular mycorrhiza or nitrogen-fixing rhizobial bacteria

On the basis of the previously determined resistance spectrum of barley and Arabidopsis *mlo* mutants against various phytopathogens, *mlo* resistance seems to be rather

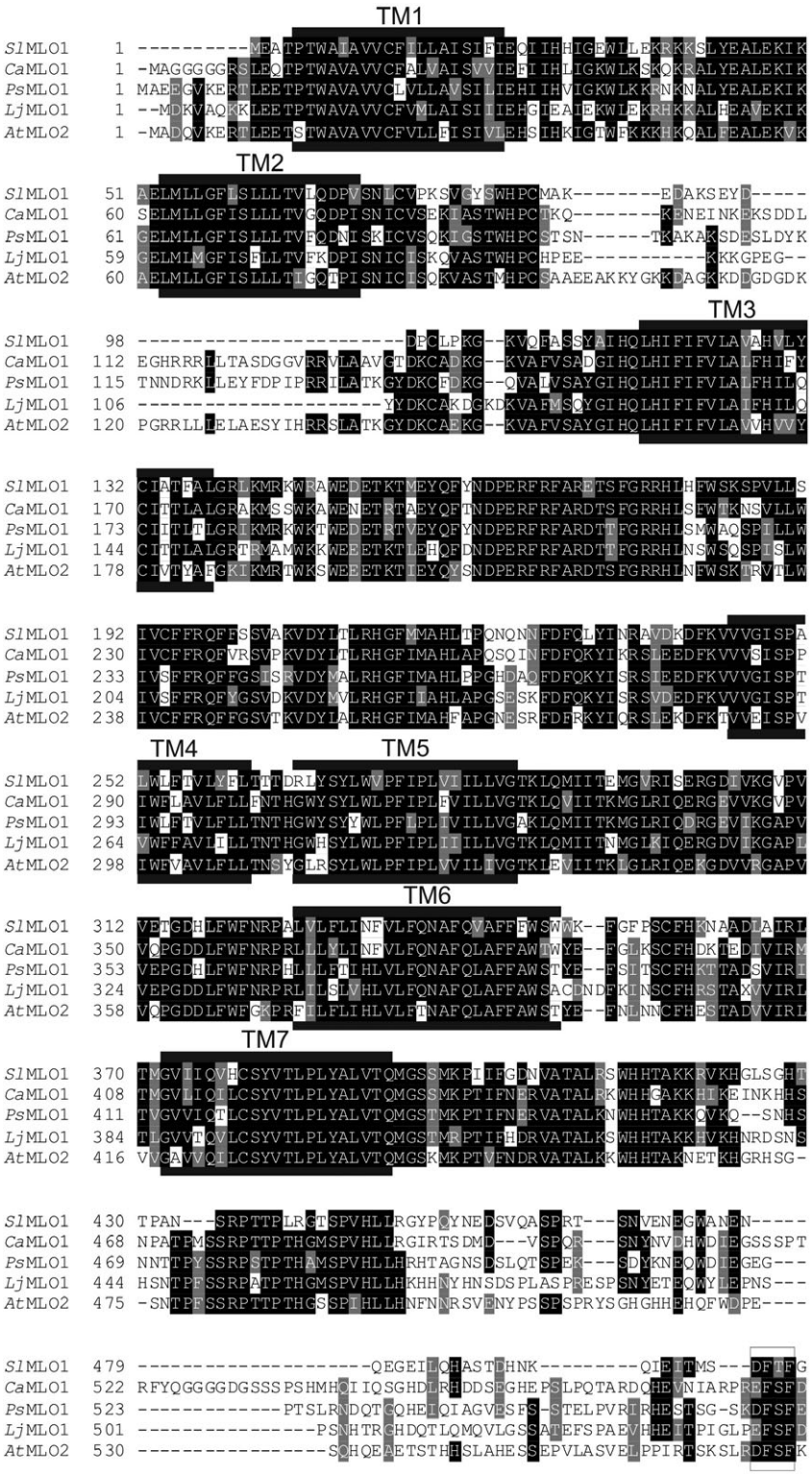

Fig. 1 Multiple amino acid sequence alignment of MLO (*M*ildew Resistance *L*ocus *O*) proteins. Amino acid sequences of Arabidopsis (*At*MLO2), *Capsicum annuum* (*Ca*MLO1), *Lotus japonicus* (*Lj*MLO1), tomato (*Sl*MLO1) and pea (*Ps*MLO1) were aligned with CLUSTALW using the default parameters. The positions of the seven transmembrane domains (TM1–TM7), as inferred from the experimentally determined topology of barley MLO (Devoto *et al*., 1999), are indicated by the bars above and below the sequence alignment. The C-terminal D/E-F-S/T-F tetrapeptide, which is diagnostic for the phylogenetic clade harbouring proteins with a function in powdery mildew interactions (Panstruga, 2005a), is boxed.

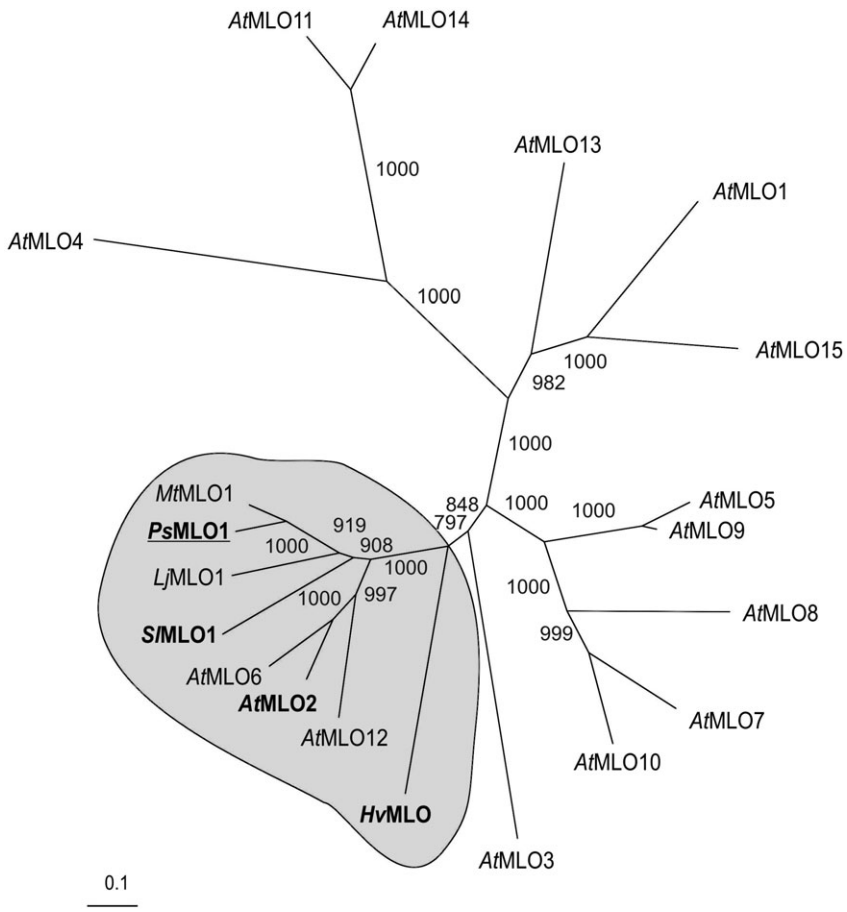

Fig. 2 Phylogenetic relationship of selected MLO (*M*ildew Resistance *L*ocus *O*) proteins. Phylogenetic (neighbour-joining) tree of Arabidopsis (*At*MLO1–*At*MLO15), barley (*Hv*MLO), tomato (*Sl*MLO1), *Medicago truncatula* (*Mt*MLO), *Lotus japonicus* (*Lj*MLO1) and pea (*Ps*MLO1) MLO proteins. The clade harbouring presumptive (co-)orthologues is highlighted in grey. Proteins known to play an important role in conferring powdery mildew susceptibility are highlighted in bold; the pea MLO protein is underlined. The numbers at the edges designate the bootstrap support based on 1000 replicates. The scale bar indicates the number of amino acid exchanges per site.

Table 1 Pea lines used in this study.

JI accession number*	Alternative name	Country of origin	Genotype	Mutational event at *PsMLO1*†	*er1* allele designation	Reference
JI 502	cv. Rondo	The Netherlands	*Er1 Er2*	None	n.a.	Heringa *et al*. (1969)
JI 1559	Mexique 4	Mexico	*er1 Er2*	$C^{680}G$ (Ser → stop)	*er1*-1	Tiwari *et al*. (1997)
JI 2302	Stratagem	USA	*er1 Er2*	Insertion of unknown size and identity	*er1*-2	Tiwari *et al*. (1997)
JI 210	Unnamed landrace	India	*er1 Er2*	ΔG^{862} (frame shift)	*er1*-3	Tiwari *et al*. (1997)
JI 1951	Unnamed landrace	China	*er1 Er2*	ΔA^{91} (frame shift)	*er1*-4	Tiwari *et al*. (1997)
JI 2480	SVP951, CGN3027	Peru	*Er1 er2*	None	n.a.	Tiwari *et al*. (1997)

*Accession code of John Innes *Pisum* collection (http://data.jic.bbsrc.ac.uk/cgi-bin/germplasm/pisum/).
†According to the *PsMLO1* coding sequence.
n.a., not applicable.

specific to powdery mildew fungi (Consonni *et al*., 2006; Jarosch *et al*., 1999; Jørgensen, 1977). However, despite the fact that a common mechanistic basis for biotrophic parasitism and endosymbiosis has been discussed (Parniske 2000), the effect of mutations in *Mlo* genes on mutualistically beneficial plant–microbe interactions, which involve an intracellular lifestyle, has been less thoroughly examined (Ruiz-Lozano *et al*., 1999). As the legume species pea is able to engage in both rhizobial (bacterial) and mycorrhizal (fungal) symbiosis, it represents a well-suited model to study the effect of *mlo* mutations on the invasion of cells in symbiosis. In legume root nodules, rhizobial bacteria are hosted intracellularly in host-derived membrane compartments, whereas AM fungi form so-called intracellular arbuscules that are surrounded by a host membrane. We infected roots of pea lines JI 210, JI 502, JI 1559, JI 1951 and JI 2302 with *Rhizobium leguminosarum* bv. *viciae* pMW1071(*nodX*), which harbours an extrachromosomal copy of the *nodX* gene that broadens the host range of this symbiotic bacterium. In roots of all tested pea lines,

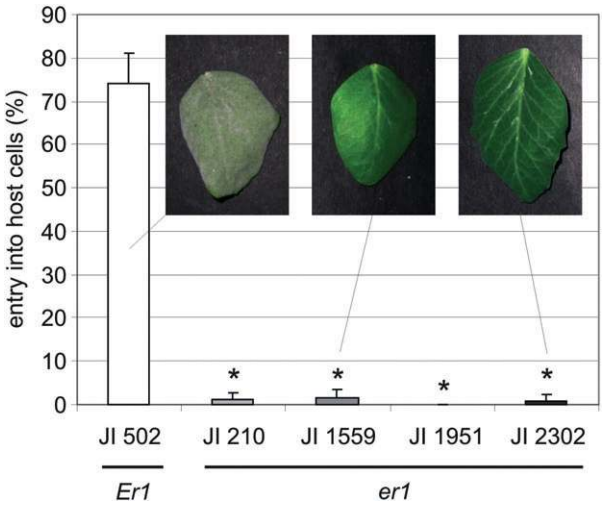

Fig. 3 Pre-invasive resistance terminates powdery mildew pathogenesis in *er1* pea accessions. Leaves of accessions JI 502 (wild-type), JI 210, JI 1559, JI 1951 and JI 2302 (*er1* genotypes; Table 1) were inoculated with *Erysiphe pisi* conidia and harvested at 48 h post-inoculation. Powdery mildew penetration success was quantified as the average of at least 100 interaction sites per leaf on four distinct individuals per line. Error bars indicate standard deviation. Inset photographs depict the macroscopic infection phenotypes of selected genotypes at 7 days post-inoculation. Asterisks indicate a statistically significant difference from JI 502 ($P < 0.000001$; two-tailed Student's *t*-test).

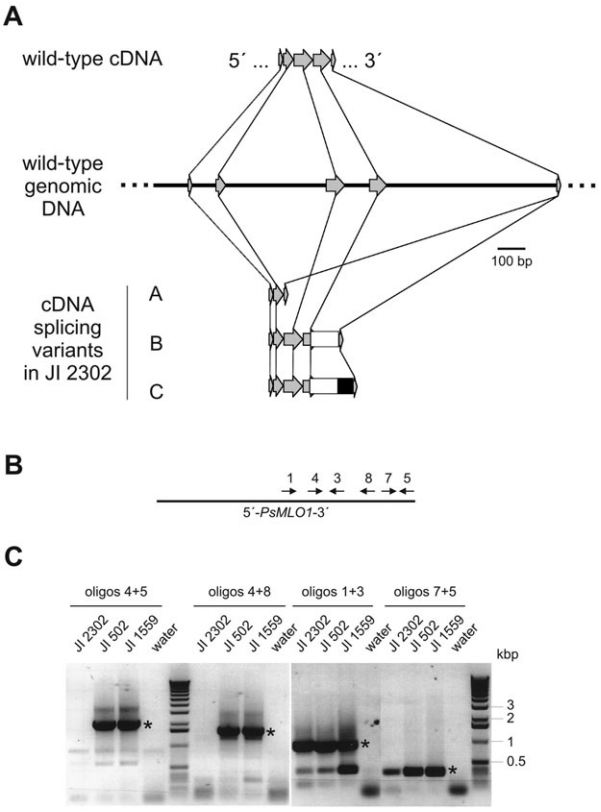

Fig. 4 Evidence for the presence of a large DNA insertion in *PsMLO1* in line JI 2302. (A) Aberrant transcripts and deduced organization of the genomic *PsMLO1* locus in pea line JI 2302. The scheme depicts the organization of part of the wild-type genomic *PsMLO1* locus and its derived cDNA, as well as aberrant splice variants found in line JI 2302. Light grey arrows indicate exons; black lines indicate introns. The white and black boxes symbolize ectopic DNA sequences, most probably derived from the insertional event in *PsMLO1* in line JI 2302. (B, C) Lack of polymerase chain reaction (PCR)-based amplification of particular *PsMLO1* fragments in line JI 2302. (B) Scheme depicting the relative position and orientation of oligonucleotides 1, 3, 4, 5, 7 and 8 with respect to the *PsMLO1* genomic sequence (full line). (C) Agarose electropherograms of PCR products obtained with genomic DNA of lines JI 2302, JI 502 and JI1559 (templates, plus water control) and various oligonucleotide combinations. Please note the presence of JI 2302-derived PCR products using oligonucleotide combinations 1 + 3 and 7 + 5, but the absence of amplicons using oligonucleotide combinations 4 + 5 and 4 + 8. Asterisks in the water control lane mark the expected product size for the respective primer combination.

nodules were formed and, except for JI 210, the nodule number was 10 or more per root system (Fig. 6A). For each genotype, several nodules were well developed and had a red colour (owing to leghaemoglobin accumulation), indicating that they were functional. Sectioning also showed that infected cells of nodules were, like wild-type nodular cells, fully filled with rhizobial bacteria. A reduced number of nodules in the root system of line JI 210 was not seen on inoculation with a different *Rhizobium* strain (*R. leguminosarum* bv. *viciae* CIAM 1026) (Safronova and Novikova, 1996), suggesting that the inability to colonize JI 210 is not caused by the mutation in *PsMLO1* but, rather, an incompatibility with *R. leguminosarum* bv. *viciae* pMW1071(*nodX*). Similar to the interaction with the bacterial symbionts, quantitative analysis of the interaction with *Glomus intraradices*, a fungus that establishes symbiotic relationships with a broad range of plant hosts, revealed essentially unaltered colonization and formation of intracellular arbuscules in all four tested *er1* genotypes (Fig. 6B).

DISCUSSION

In this study, we have shown that resistance in pea lines JI 210, JI 1559, JI 1951 and JI 2302 is caused by loss of function in *PsMLO1*. This claim is supported by detrimental lesions (JI 210, JI 1559 and JI 1951) and a large insertion (JI 2302) in the *PsMLO1* gene of the accessions, as well as by complementation of the resistance phenotype (i.e. restoration of susceptibility) by ectopic *MLO* gene expression. As lines JI 210, JI 1559, JI 1951 and JI 2302 harbour the *er1* resistance gene, we propose that *PsMLO1* is *Er1*. We did not find any sequence polymorphism in the *PsMLO1* cDNA of line JI 2480, which reportedly harbours the *er2* resistance gene. Features of powdery mildew resistance in line JI

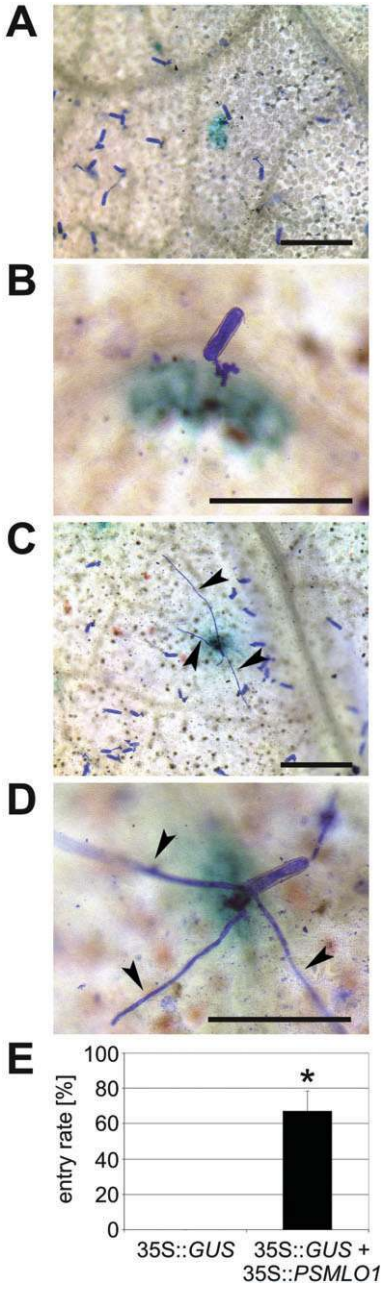

Fig. 5 Single-cell complementation of the *er1* phenotype by transient expression of *PsMLO1*. Pea leaves (line JI 2302; *er1* genotype) were bombarded with either 35S::*GUS* alone or co-bombarded with 35S::*GUS* plus 35S::*PsMLO1*. Leaves were inoculated with *Erysiphe pisi* conidiospores at 4 h after bombardment and stained for β-glucuronidase (GUS) activity at 48 h post-inoculation. (A–D) Micrographs of failed (A, B) and successful (C, D) penetration attempts of *E. pisi* on GUS-stained epidermal cells. Successful host cell entry and haustorium formation are indicated by the growth of secondary hyphae (arrowheads). Please note that successful invasion in (C) is restricted to the GUS-stained cell, whereas the surrounding cells retain resistance. Size bars are 200 μm in (A, C) and 100 μm in (B, D). (E) Quantitative assessment of *E. pisi* entry into GUS-stained cells. Results represent the mean ± standard deviation from three independent experiments and are based on the data shown in Table S2. The asterisk indicates a statistically significant difference from 35S::*GUS* ($P < 0.001$; two-tailed Student's *t*-test).

2480 differ at the cellular level from resistance in lines JI 210, JI 1559, JI 1951 and JI 2302. For example, in contrast with these lines, powdery mildew resistance in JI 2480 acts at the post-invasive level, is associated with host cell death and is temperature sensitive (Fondevilla *et al*., 2006). We thus conclude that resistance in line JI 2480 is unlikely to be mediated by a loss of *PsMLO1* function, but is probably a result of the malfunction of a distinct gene. This conclusion is consistent with previous linkage analysis, which genetically separated *er1*- from *er2*-conditioned resistance (Heringa *et al*., 1969; Tiwari *et al*., 1997). Similarly, a recently discovered gene (*Er3*) in *Pisum fulvum*, a wild relative of *P. sativum*, confers dominantly inherited post-penetration resistance to *E. pisi* (Fondevilla *et al*., 2007), maps elsewhere in the genome (Fondevilla *et al*., 2008) and therefore is unlikely to relate to *PsMLO1*.

We were unable to analyse in detail the molecular nature of the insertion in line JI 2302. The size of the insertion exceeds the capacity of conventional and long-range PCR amplification, suggesting that it is at least several kilobases in size. In addition, BLAST searches suggest a sequence relationship of part of the inserted DNA to the LTRs of the *Ogre* retrotransposon. It is therefore likely that the large insertion in pea line JI 2302 represents a (retro-)transposon, which are known to range in size from less than 100 bp (in the case of miniature transposable elements, MITEs) to 25 kb (in the case of some exceptional LTR-type retrotransposons (Wicker *et al*., 2007). Despite the seemingly considerable size of the insertion, the cellular splicing machinery is capable of removing most of it during transcript processing, as evidenced by the presence of rather small ectopic DNA sequences in part of the aberrant *PsMLO1* cDNAs derived from line JI 2302 (Fig. 4). This polymorphism, as well as the other mutational events at *PsMLO1* in *er1* mutant plants revealed in this study, promise to be useful in marker-assisted selection of powdery mildew-resistant seedlings in future pea breeding programmes.

Alleles of *er1* derive from pea accessions/cultivars that originate from geographically diverse habitats (USA, Mexico, China and India; Table 1). The lesions in *PsMLO1* of lines JI 210, JI 1559, JI 1951 and JI 2302 are each of natural origin and thus probably caused by spontaneous mutation events. Powdery mildew resistance conferred by natural *mlo* alleles has also been found in the case of barley (Piffanelli *et al*., 2004) and tomato (Bai *et al*., 2008). However, in these instances, the polymorphisms leading to resistance were either the presence of a complex repeat array perturbing *Mlo* transcript accumulation or a 19-bp deletion in the cDNA leading to premature termination of translation,

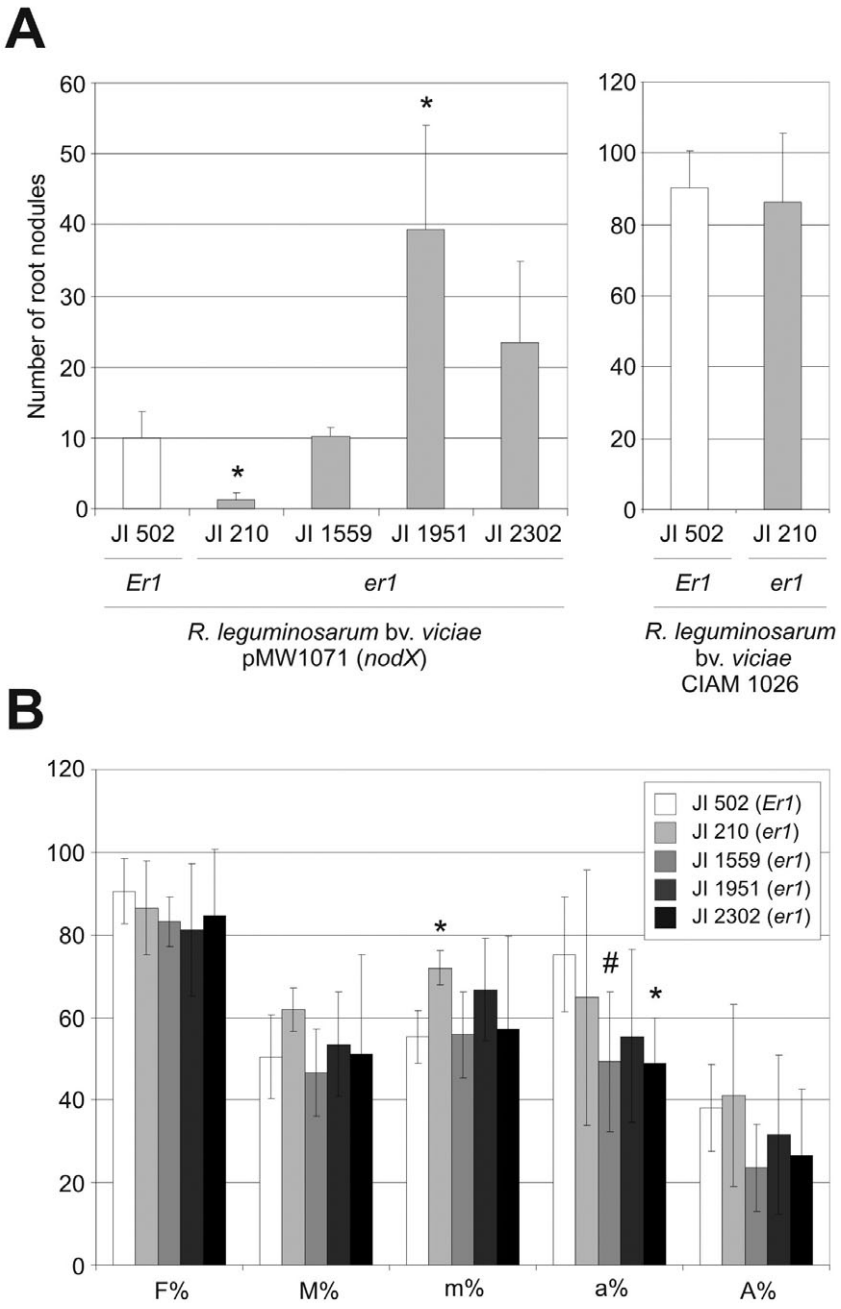

Fig. 6 Pea *er1* genotypes exhibit unaltered symbiotic interactions. Pea lines JI 502 (wild-type; *Er1* genotype), JI 210, JI 1559, JI 1951 and JI 2302 (*er1* genotypes; Table 1) were analysed for root nodule formation (A) and mycorrhization by *Glomus intraradices* (B). (A) Roots were infected with either *Rhizobium leguminosarum* bv. *viciae* pMW1071 (*nodX*) (left panel) or *Rhizobium leguminosarum* bv. *viciae* CIAM 1026 (right panel). Data represent the average ± standard deviation from four to seven plants per genotype. Asterisks indicate a statistically significant difference from JI 502 ($P < 0.01$; two-tailed Student's *t*-test). (B) Data represent the average ± standard deviation from three to six plants per genotype with approximately 75 cm of root system per plant analysed. F%, frequency of mycorrhiza in the root system; M%, intensity of mycorrhizal colonization in the root system; m%, intensity of the mycorrhizal colonization in the root fragments; a%, arbuscule abundance in mycorrhizal parts of the root fragments; A%, arbuscule abundance in the root system (according to Trouvelot *et al*. (1986)). Asterisks and the hash sign indicate a statistically significant difference from JI 502 ($P < 0.01$ and $P < 0.05$, respectively; two-tailed Student's *t*-test).

respectively (Bai *et al*., 2008; Piffanelli *et al*., 2004). In this study, we found single-base-pair deletions resulting in frame shifts in two cases, a nucleotide substitution resulting in a premature stop codon, and a large DNA insertion disrupting the *PsMLO1* gene. It is striking that, compared with the collection of induced barley *mlo* mutants, where the majority of alleles are characterized by single amino acid substitutions (Büschges *et al*., 1997; Piffanelli *et al*., 2002; Reinstädler *et al*., 2010), all natural *mlo* mutants identified to date are caused by more dramatic alterations of the coding sequence. This could indicate that a complete absence of the MLO protein provides an evolutionary advantage compared with the presence of a nonfunctional MLO protein variant. At present, it is unclear whether the occurrence of natural *mlo* alleles is linked to geographical regions in which the powdery mildew pathogen currently resides or was a recent prevalent parasite. Accessions of further plant species, such as *M. truncatula* (barrel medic) and *Beta vulgaris* (beet), exhibit powdery mildew resistance at the penetration level and thus might represent further candidates for natural *mlo* mutants (Fernández-Aparicio *et al*., 2009; Prats *et al*., 2007).

Resistance conferred by *er1* alleles has been reported to be highly effective and durable, but occasionally incomplete under field conditions (Fondevilla *et al*., 2006). This is surprising given the fact that *mlo* resistance in barley null alleles is known to be complete and *PsMLO1* lesions in pea lines JI 210, JI 1559, JI 1951 and JI 2302 are predicted to result in null mutations (Table 1). In *A. thaliana*, lesions in *AtMLO2*, one of three closely related *MLO* genes, are insufficient to confer full powdery mildew resistance and only a respective triple mutant mediates complete immunity (Consonni *et al*., 2006). Thus, in pea, other *MLO* paralogues may also contribute to powdery mildew susceptibility. However, in our experience, the above-mentioned pea lines showed rather complete resistance in mature leaves (Fig. 3). Likewise, in controlled inoculation experiments employing detached leaves and a range of *E. pisi* isolates, the above-mentioned pea mutants consistently showed strong resistance (Tiwari *et al*., 1997). This suggests that the reported incompleteness of powdery mildew resistance under field conditions might be a result of extraordinary biotic or abiotic stress conditions, rather than the principal genetic constitution of the mutant lines. An example of the latter is the temporary partial breakdown of barley *mlo* resistance as a consequence of the sudden relief of drought stress (Baker *et al*., 1998).

In barley and Arabidopsis, in addition to powdery mildew resistance, mutations in *MLO* result in a developmental abnormality that is manifested as early leaf senescence (Consonni *et al*., 2006, 2010; Piffanelli *et al*., 2002). We did not observe this undesired side-effect in the tested *er1* pea lines under our growth conditions, and also found no respective report in the literature. This is reminiscent of the situation in tomato, where powdery mildew resistance conditioned by a loss of *SlMLO1* function was also not associated with any obvious detrimental phenotype (Bai *et al*., 2008). There are several possible explanations for this seeming discrepancy. The expression of early leaf senescence might be modulated by as yet unidentified growth parameters (e.g. soil composition, light quality, water quality, use of fertilizers, etc.). Alternatively, the extent of this undesired phenomenon may vary between species and could be below the limit of visual detection in pea and tomato. Finally, the occurrence of early leaf senescence in *mlo* mutant plants could be a species-specific phenomenon. The last two scenarios would explain why natural *mlo* alleles appear to be more common in pea than in barley or Arabidopsis. It thus still remains to be investigated whether—and, if so, to what extent and in which conditions—*er1* plants suffer from any pleiotropic effects.

We observed cell-autonomous complementation of two *er1* genotypes (JI 1559 and JI 2302) by transient expression of *PsMLO1* as well as *MLO* genes from the closely related legume species *L. japonicus* and *M. truncatula* (*LjMLO1* and *MtMLO1*; Fig. 5 and Table S2). We noted a gene-specific gradation of complementation efficiency in both tested pea lines, ranging from *PsMLO1* (58%–80% entry rate), via *MtMLO* (46%–69% entry rate) to *LjMLO* (28%–54% entry rate). These incremental differences in complementation efficiency correlate with the phylogenetic relationship of the three *MLO* genes (Fig. 2) and the respective legume species (Young *et al*., 2003). Previously, we have reported similarly staggered complementation rates of the barley *mlo* mutant by transient expression of orthologous *MLO* genes from the monocots barley, wheat and rice (Elliott *et al*., 2002). Together, these data suggest that, with respect to powdery mildew susceptibility, orthologues from closely related taxa can substitute for *MLO* function in a given plant species. However, complementation efficiency seems to decrease with phylogenetic distance. A lack of full complementation by transiently expressed *PsMLO1* (compare the ~80% entry rate of *E. pisi* in JI 502 with ~60% in *PsMLO1*-transformed cells; Table S2) could be a result of the presence of the C-terminal haemagglutinin (HA) tag encoded by the constructs used in the assay, which might interfere with *Ps*MLO function. However, in the case of barley MLO, C-terminal tagging with green fluorescent protein (GFP) does not interfere with MLO functionality in transient gene expression studies (Shirasu *et al*., 1999).

Powdery mildew-resistant barley and Arabidopsis *mlo* mutants have been analysed previously for their interaction with a range of other phytopathogens. This revealed largely unaltered infection phenotypes for barley *mlo* mutants in response to the causal agents of stripe rust (*Puccinia striiformis*), leaf rust (*Puccinia hordei*), stem rust (*Puccinia graminis* f.sp. *tritici*), scald (*Rhynchosporium secalis*) and the take-all fungus (*Gaeumannomyces graminis*) (Jørgensen, 1977). Likewise, Arabidopsis *mlo2 mlo6 mlo12* triple mutants exhibited unaltered susceptibility to the bacterial pathogen *Pseudomonas syringae* (causing the

bacterial speck disease) and the oomycete *Hyaloperonospora arabidopsidis* (formerly known as *Hyaloperonospora parasitica*; the causal agent of downy mildew disease) (Consonni *et al*., 2006). However, barley *mlo* mutants were found to exhibit increased susceptibility to the rice blast pathogen *Magnaporthe oryzae* (formerly designated *Magnaporthe grisea*) (Jarosch *et al*., 1999). In contrast with pathogenic interactions, (endo-)symbiotic interactions of *mlo* mutants have been less thoroughly studied. This is possibly a result of the fact that barley is not a well-developed model system to study symbiosis, and Arabidopsis completely lacks rhizobial or mycorrhizal symbiosis. A preliminary study in barley employing a single *mlo* allele revealed a possible inhibitory effect of the *mlo* mutation on the colonization intensity (development within the root cortex) and arbuscule formation of the AM fungus *Glomus mossae* (Ruiz-Lozano *et al*., 1999). The identification of a collection of pea *mlo* mutant alleles now allows us to study the establishment of intracellular symbiotic infection structures in the context of a plant species that is able to engage in both common types of endosymbiotic interaction, namely rhizobial and AM symbiosis. In contrast with the preliminary data from barley (Ruiz-Lozano *et al*., 1999), we observed unaltered numbers of arbuscules in all tested *er1* genotypes (Fig. 6B). Similarly, all *er1* genotypes showed the formation of wild-type-like infected root nodules (Fig. 6). The variation in the number of root nodules probably can be attributed to the diverse genetic backgrounds of the tested *er1* plants. Together, these data strongly suggest that *PsMLO1* function is dispensable for the establishment of an intracellular lifestyle of the microbe in both mycorrhizal and rhizobial symbiosis.

EXPERIMENTAL PROCEDURES

Plant growth

The seeds of the pea lines used in this study were obtained from the John Innes *Pisum* collection (http://www.jic.ac.uk/germplas/pisum/). Pea plants for powdery mildew infection experiments and molecular analyses were grown in a controlled environment at 22 °C, 70% relative humidity and a 12-h light (80–90 μmol/m^2/s^2)/dark cycle. The MPIZ isolate of *E. pisi* was propagated on an anonymous susceptible pea variety.

RNA extraction, RT-PCR and RACE

RNA from pea leaves was extracted using the Qiagen RNAeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized by oligo-dT priming with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The oligonucleotides used in this study are listed in Table S1. The 5′ and 3′ RACE reactions were performed with the FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) following the kit protocol (60 °C annealing temperature, 35 cycles). RACE fragments were cloned via TOPO® cloning (Invitrogen) into vectors pCR4 (Invitrogen; 5′ RACE) and pCR2.1 (Invitrogen; 3′ RACE), respectively. Full-length *PsMLO1* cDNA was amplified (55 °C annealing temperature, 40 cycles) using the oligonucleotides PsMLO-GWF and PsMLO-GWR (which were designed on the basis of the sequence information obtained from the RACE products) and cloned by Gateway® BP reaction into the Gateway® entry vector pDONR201 (Invitrogen). The *PsMLO1* coding region of the various pea accessions was amplified by RT-PCR (55 °C annealing temperature, 35 cycles) using oligonucleotides PsMLO1-GWF and PsMLO5 (Table S1). PCR products were subjected to direct DNA sequencing, except for line JI 2302, for which amplicons were cloned into vector pCRII-Blunt Topo (Invitrogen) to enable DNA sequencing of individual clones. Genomic fragments of *PsMLO1* (Fig. 4C) were amplified by PCR (55 °C annealing temperature, 35 cycles) using the indicated oligonucleotide combinations.

Phylogenetic analysis

For the phylogenetic analysis of MLO proteins, the PHYLIP 3.63 software package was used (http://evolution.gs.washington.edu/phylip.html; Felsenstein, 1989). MLO protein sequences were aligned by CLUSTALW and the Protdist and Neighbor programs were employed to establish the neighbour-joining phylogenetic tree. To calculate the bootstrap support, the Seqboot, Protdist, Neighbor and Consense algorithms were applied sequentially, using 1000 replicates each. GenBank accession numbers of *At*MLO1–*At*MLO15 can be found in Devoto *et al*. (2003). The GenBank accession numbers of the *MLO* genes encoding the respective proteins are as follows: *Hv*MLO (Z83834), *Lj*MLO1 (AY967410), *Mt*MLO1 (HQ446457), *Ps*MLO1 (FJ463618) and *Sl*MLO1 (AY967408).

Transient gene expression

The procedure for transient gene expression was largely adopted from related experiments in barley (Elliott *et al*., 2002). Briefly, detached pea leaves of 3–4-week-old plants were placed on agar (Merck KGaA, Darmstadt, Germany), supplemented with 85 μM benzimidazole to delay leaf senescence, in Petri dishes. *MLO* genes (*PsMLO1*, *LjMLO1*, *MtMLO1*) lacking a stop codon were cloned into the pDONR201 entry vector by Gateway® BP reactions and subsequently shuttled by Gateway® LR reaction into the plant expression vector pAM-PAT-GWY-3xHA, a Gateway®-compatible derivative of the binary vector pPAM (GenBank accession number AY027531). This vector enables CaMV 35S promoter-driven expression of C-terminally tagged (3 × HA tag) proteins. Plasmids harbouring reporter (*GUS*) and test (*MLO*) genes were coated on gold particles (1 μm) by calcium

precipitation. Gene transfer was performed using a helium-driven PDS1000 gene gun system equipped with a Hepta adapter (Bio-Rad Laboratories, Hercules, CA, USA). Bombarded leaves were inoculated with a high density of *E. pisi* (isolate MPIZ) conidiospores using a settling tower 4 h post-bombardment, and staining for GUS activity was performed at 48 h post-inoculation. Specimens were cleared in ethanol and epiphytic powdery mildew infection structures were stained with Evan's Blue (Merck KGaA). Fungal entry rates were assessed by light microscopy on the basis of the presence of a haustorium and/or secondary hyphae. We noted that transient gene expression analysis in pea was less efficient than in barley owing to the smaller size of epidermal leaf cells and the lower inoculation density that we were able to achieve with *E. pisi*. In combination, these conditions led to a reduced number of transformed (GUS-stained) cells that were attacked by powdery mildew sporelings, thereby reducing the number of quantifiable cells.

Analysis of symbiotic interactions

For the analysis of rhizobial symbiosis, pea seedlings were germinated on agar plates, transferred after 1 week to pots with vermiculite [grown at 18 °C in a 16-h light (130–160 μmol/m^2/s^2)/8-h dark regime] and, 1 week later, inoculated with either *R. leguminosarum* bv. *viciae* pMW1071(*nodX*) or *R. leguminosarum* bv. *viciae* CIAM 1026 (Safronova and Novikova, 1996). The former, *nodX*-containing, rhizobium was used as some pea varieties have a *Sym2* allele that requires the *nodX* modification of the *nod* factor (Kozik *et al*., 1995). Plants were harvested at 3 weeks after inoculation and analysed by counting the number of root nodules per plant. For the analysis of AM symbiosis, pea seeds were initially incubated on agar plates for 2 days at 4 °C in the dark and germinated during 5 days at 18 °C in a 16-h light (130–160 μmol/m^2/s^2)/8-h dark regime. Mycorrhizal colonization of pea plants was analysed in a sand/hydrobeads culture [1 g of $Ca_3(PO4)_2$ per 1 kg of sand/hydrobeads mix (1:1, v/v)], supplemented with Hoagland solution (once per week) lacking phosphorus. Pea seedlings were transferred to pots with mycorrhizae, and the mycorrhization test was conducted at 21 °C in a 16-h light (130–160 μmol/m^2/s^2)/8-h dark regime. Mycorrhiza (*Glomus intraradices* BEG144) stock was propagated on chive (*Allium schoenoprasum*) and mixed with sand prior to transfer of the pea seedlings. Roots of pea plants were harvested at 3 weeks after inoculation, the latest time point that allows the detection of arbuscules on the roots before major developmental transitions (flowering) in some of the *er1* lines. Root samples were cleared in 2% (w/v) KOH and stained with trypan blue (0.05%, w/v) to detect intraradical mycelium; 1-cm fragments (~50) of randomly selected roots were mounted on glass slides and assessed by light microscopy. The levels of mycorrhizal colonization and arbuscule abundance were scored according to Trouvelot *et al*. (1986) using the computer program 'Mycocalc' (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

GenBank accession numbers

The sequences of *PsMLO1* and *MtMLO1* were deposited under GenBank accession numbers FJ463618 and HQ446457, respectively.

ACKNOWLEDGEMENTS

We wish to thank Mike Ambrose (John Innes Centre, Norwich, UK) for making available pea germplasm from the John Innes *Pisum* collection. This work was supported by funds from the Max-Planck Society. MH was partially supported by a postdoctoral fellowship from the Alexander-von-Humboldt Foundation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Sequence alignment of *MLO* coding sequences. Nucleotide sequences of *LjMLO1* (GenBank accession number AY967410), *SlMLO1* (AY967408), *CaMLO1* (AY934528) and *AtMLO2* (AF369563) were aligned by CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Conserved nucleotide stretches used for the design of oligonucleotide primers PsMLO1 (forward primer), PsMLO2 and PsMLO3 (reverse primers) are highlighted in light blue; the actual oligonucleotide sequences (highlighted in yellow) are shown below the respective regions. In the case of nucleotide positions that were divergent between the four *MLO* sequences, the oligonucleotide sequences were adjusted to the *LjMLO* sequence.

Table S1 Oligonucleotides used in this study.

Table S2 Single-cell complementation of the *er1* phenotype by transient expression of *MLO* genes.

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