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Conserved requirement for a plant host cell protein in powdery mildew pathogenesis

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In the fungal phylum Ascomycota, the ability to cause disease in plants and animals has been gained and lost repeatedly during phylogenesis[1]. In monocotyledonous barley, loss-of-function *mlo* alleles result in effective immunity against the Ascomycete *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew disease[2,3]. However, *mlo*-based disease resistance has been considered a barley-specific phenomenon to date. Here, we demonstrate a conserved requirement for MLO proteins in powdery mildew pathogenesis in the dicotyledonous plant species *Arabidopsis thaliana*. Epistasis analysis showed that *mlo* resistance in *A. thaliana* does not involve the signaling molecules ethylene, jasmonic acid or salicylic acid, but requires a syntaxin, glycosyl hydrolase and ABC transporter[4–6]. These findings imply that a common host cell entry mechanism of powdery mildew fungi evolved once and at least 200 million years ago, suggesting that within the Erysiphales (powdery mildews) the ability to cause disease has been a stable trait throughout phylogenesis.

Immunity of barley (*Hordeum vulgare*) to the biotrophic grass powdery mildew (*Blumeria graminis* f. sp. *hordei* (*Bgh*)), is predominantly controlled by resistance (R) proteins that are presumed to detect the presence of isolate-specific fungal effectors[3]. However, because of its narrow spectrum and ephemerality, *R* gene resistance to powdery mildews is of limited agronomic value. In contrast, induced[3] and natural[7] loss-of-function barley *mlo* alleles provide durable broad-spectrum powdery mildew resistance. Owing to the exceptional efficacy and longevity of *mlo* resistance, elite barley lines carrying introgressed *mlo* alleles have been successfully used in European agriculture for about three decades[2]. The barley MLO protein is thought to modulate defense responses to *Bgh* via a vesicle-associated and SNARE protein–dependent mechanism[8].

To test whether *mlo*-based resistance may occur in other plant species, we selected homozygous insertion lines for 14 of the 15 *A. thaliana MLO* genes[9] and challenged them with a virulent powdery mildew species, *Golovinomyces orontii*. Macroscopic inspection demonstrated that all mutants retained susceptibility to the fungal pathogen except *Atmlo2*, which showed no disease symptoms (**Supplementary Fig. 1** online and **Fig. 1a**). Microscopic examination showed that resistance in *Atmlo2* was incomplete and characterized by a diminished rate of entry into host epidermal cells and substantially reduced conidiophore formation (**Fig. 1b**).

AtMLO2 belongs to a phylogenetic clade of three *A. thaliana* genes (*AtMLO2*, *AtMLO6* and *AtMLO12*) that represent co-orthologs of barley *Mlo*[10]. We generated double and triple mutants by intermutant crosses of respective insertion lines (**Supplementary Fig. 1**). When challenged with *G. orontii*, *Atmlo6* and *Atmlo12* single mutant lines and *Atmlo6 Atmlo12* (*Atmlo6/12*) double mutant lines supported wild-type levels of secondary hyphae formation and conidiophore production, whereas *Atmlo2 Atmlo6* (*Atmlo2/6*) and *Atmlo2 Atmlo12* (*Atmlo2/12*) double mutant lines supported lower levels of fungal growth than *Atmlo2*. Reminiscent of barley *mlo* mutants, the *Atmlo2 Atmlo6 Atmlo12* (*Atmlo2/6/12*) triple mutant was fully resistant to the fungal pathogen (**Fig. 1**). Similar results were obtained with another virulent powdery mildew species, *Golovinomyces* (formerly *Erysiphe*) *cichoracearum* (**Supplementary Fig. 1** and data not shown). These results indicate a partial functional redundancy among the three co-orthologs, with a predominant role for *AtMLO2* in the establishment of compatibility with two powdery mildew species.

A previous screen for powdery mildew resistant (*pmr*) *A. thaliana* mutants identified six *PMR* loci, five of which are required for full susceptibility to both *G. cichoracearum* and *G. orontii*[11–13]. Notably, *PMR2* resides in the same genomic region as *AtMLO2* (ref. 11), suggesting that *PMR2* might be allelic to *AtMLO2*. Analysis of the *AtMLO2* genomic sequence uncovered nucleotide changes in all *pmr2*

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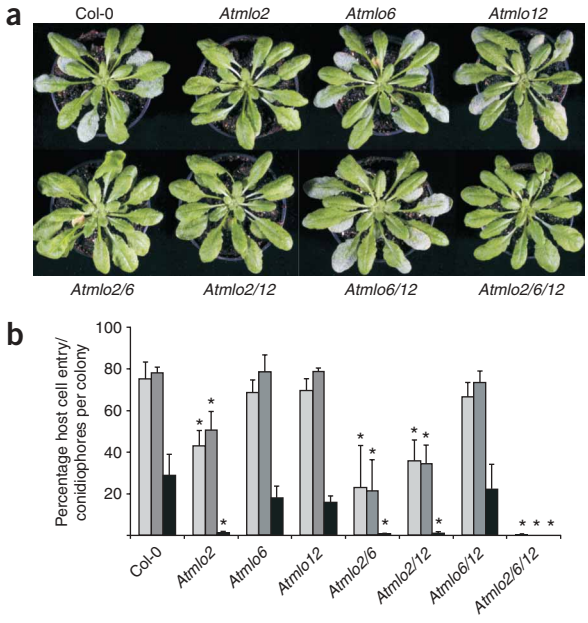

Figure 1 Loss of *AtMLO2* function confers powdery mildew resistance in *A. thaliana*. (**a**) Infection phenotypes of representative Col-0 wild-type and *Atmlo* mutant individuals at 10 d post-inoculation with *G. orontii*. (**b**) Quantitative analysis of host cell entry (determined at 48 h (light gray bars) and 72 h post-inoculation (dark gray bars)) and conidiophore formation (6 d post inoculation; black bars) on wild-type Col-0 and *Atmlo* mutant plants. Results represent mean ± s.d. of seven (host cell entry) and four (conidiation) independent experiments, respectively. Asterisks indicate a significant difference from Col-0 ($P < 0.01$; Student's *t*-test). Similar results were obtained upon inoculation with *G. cichoracearum*.

alleles (**Supplementary Fig. 1** and **Supplementary Table 1**), demonstrating that *AtMLO2* is allelic to *PMR2*.

To test whether efficient *mlo*-based resistance can be engineered via dsRNA interference (dsRNAi)–mediated gene silencing, we constructed a vector designed to simultaneously silence the three *A. thaliana MLO* co-orthologs (**Supplementary Fig. 1**). Among progeny (T2 families) of 25 transgenic lines, one was fully resistant to *G. orontii*, whereas others supported varying levels of fungal growth (**Supplementary Fig. 1** and data not shown). Disease resistance in the strongly resistant line was characterized by early termination of fungal infection owing to failed host cell invasion (**Supplementary Fig. 1**). *AtMLO2*, *AtMLO6* and *AtMLO12* transcript levels were much lower in the resistant line than in wild-type and noneffective dsRNAi plants, as shown by RT-PCR (**Supplementary Fig. 1**). These data illustrate that *mlo* resistance may, in principle, be extended to crop species for which genetic or mutational approaches are not practical.

We challenged the *Atmlo* mutant lines (including the double and triple mutants) with compatible pathogens unrelated to powdery mildew, such as the biotrophic oomycete *Hyaloperonospora parasitica* and the bacterium *Pseudomonas syringae*. Like wild-type plants, *Atmlo* mutants were fully susceptible to *P. syringae* and to *H. parasitica* as assessed by disease symptoms, bacterial growth or sporangiophore production, respectively (data not shown). In extension of findings reported in ref. 11, these data indicate that even mutations in multiple *MLO* genes do not interfere with compatible *A. thaliana–P. syringae* and *A. thaliana–H. parasitica* interactions.

To determine whether *Atmlo*-mediated resistance was also involved in immunity to nonadapted powdery mildew species, we analyzed the interaction of *Atmlo* mutants with two powdery mildew fungi that show low levels of invasion on *A. thaliana* wild-type plants, *E. pisi* (a pathogen of pea) and *Bgh*[5]. Microscopic analysis demonstrated a pattern of disease susceptibility and resistance comparable to the interactions with the adapted mildews (**Supplementary Fig. 2**). In all genotypes and in both plant-fungus interactions, the incidence of cell death roughly correlated with the frequency of successful host cell entry by the nonadapted powdery mildews. We assume that the cell death response is a second line of defense that is activated when the nonadapted fungi are able to successfully invade epidermal cells[5]. These data demonstrate that AtMLO proteins are essential for fungal entry of not only adapted powdery mildew species but also for two nonadapted fungal species.

Besides resistance to *Bgh*, barley *mlo* mutants also show enhanced susceptibility to the hemibiotroph *Magnaporthe grisea* and the necrotroph *Bipolaris sorokiniana*[14,15]. To investigate whether *Atmlo* mutants were similarly more susceptible to such pathogens, we challenged them with *Alternaria alternata*, *A. brassicicola* and *Phytophthora infestans*, which have a necrotrophic (*Alternaria* spp.) or a hemibiotrophic lifestyle (*P. infestans*), respectively. This consistently resulted in enhanced disease symptoms and cell death in *Atmlo2/6* double and *Atmlo2/6/12* triple mutants compared with the wild-type lines (**Supplementary Fig. 2**). These data strengthen the notion that MLO proteins influence the infection outcomes of diverse pathogen species, promoting susceptibility to powdery mildews and resistance to some necrotrophs and hemibiotrophs.

When grown under axenic conditions, barley *mlo* plants show developmentally controlled phenotypes, including spontaneous cell wall appositions, cell death and senescence-like chlorosis and necrosis[16–18]. We observed developmentally controlled callose deposition in unchallenged *Atmlo2*, *Atmlo2/6*, *Atmlo2/12* and *Atmlo2/6/12* mutants from six weeks onwards (**Fig. 2a**,**b**). Callose deposition coincided with the production of reactive oxygen species (**Fig. 2c**). In addition, beginning at 8 weeks, *Atmlo2* mutants also had leaf chlorosis and necrosis, which was enhanced in *Atmlo2/6*, *Atmlo2/12* and *Atmlo2/6/12* mutants (**Fig. 2d**). Reminiscent of barley *mlo* mutants, the extent of

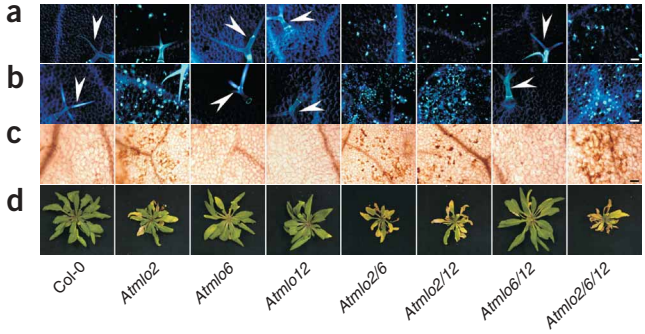

Figure 2 *Atmlo2* plants show developmentally controlled callose deposition and early senescence-like phenotypes. Micrographs showing callose accumulation (as shown by Aniline blue staining) in rosette leaves of Col-0 wild-type and *Atmlo* mutants grown under powdery mildew–free conditions at 6 weeks (**a**) and 7 weeks (**b**). Fluorescent needle-like structures highlighted by white arrowheads represent leaf hairs (trichomes). Bars = 100 µm. (**c**) 3,3-Diaminobenzidine tetrahydrochloride (DAB) stain of 7-week-old plants showing sporadic accumulation of H_2O_2 in wild-type and *Atmlo* mutants. Bar = 100 µm. (**d**) Macroscopic phenotypes of representative unchallenged Col-0 wild-type and *Atmlo* mutant plants at 8 weeks.

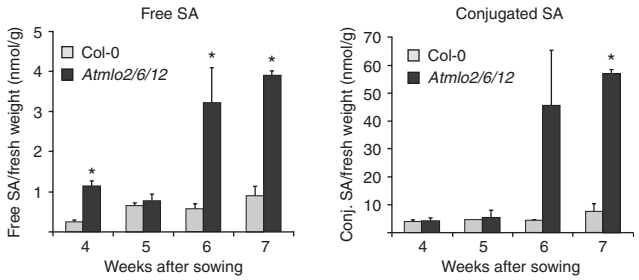

Figure 3 *Atmlo2/6/12* mutants show a developmentally controlled increase in salicylic acid levels. Time-course analysis of free and conjugated salicylic acid (SA) levels in rosette leaves of wild-type and *Atmlo2*/6/12 triple mutant plants grown in powdery mildew–free conditions. Data represent mean ± s.d. of three experiments. Asterisks indicate a significant difference from Col-0 ($P < 0.01$; Student's *t*-test).

this senescence-like phenotype was modulated by unknown conditions, as it varied between Germany and the USA. These data suggest a negative regulatory function for these *MLO* co-orthologs in senescence and defense mimic phenotypes.

In dicots, salicylic acid is an essential signaling molecule that has a major role in disease resistance[19]. We measured free and conjugated salicylic acid levels of 4-, 5-, 6- and 7-week-old wild-type and triple mutant plants (**Fig. 3**). Although younger, fully resistant *Atmlo2/6/12* plants did not possess higher levels of free and conjugated salicylic acid than wild-type plants, these levels markedly increased in older plants. Salicylic acid levels were highly variable in 6-week-old plants, possibly owing to intrinsic changes of salicylic acid levels at around this time. Six-week-old *Atmlo2* single mutants as well as *Atmlo2/6* and *Atmlo2/12* double mutants also showed higher levels of conjugated and free salicylic acid than wild-type plants (**Supplementary Fig. 3**) These results indicate that in the mutants containing *Atmlo2*, there is a developmentally controlled increase of constitutive salicylic acid levels that correlates approximately with the onset of spontaneous callose deposition (**Fig. 2a**).

To determine directly if the powdery mildew resistance in *Atmlo2* was dependent on salicylic acid, ethylene or jasmonate signaling, we crossed *Atmlo2* plants to mutants that had defects in each of these signaling pathways. Whereas double mutants affecting the ethylene or jasmonate pathway did not have altered infection phenotypes, salicylic acid double mutants and *NahG* transgenics showed a modest increase in visible powdery mildew growth (**Fig. 4a**). However, fungal growth was considerably enhanced only in *Atmlo2 NahG* double mutants (**Fig. 4b**), suggesting that the non–salicylic acid–dependent defenses associated with *NahG* expression and catechol production[20] are more important for *Atmlo2* resistance than is salicylic acid (**Supplementary Fig. 4**).

Given the role of salicylic acid in senescence[21] and the increased levels of salicylic acid in *Atmlo2* plants, we investigated the role of salicylic acid in callose accumulation and the senescence-like phenotype observed in *Atmlo2* plants, using double mutants. In uninfected plants, the accumulation of callose was suppressed in double mutants affected in *AtMLO2* and the salicylic acid signaling pathway (**Fig. 4c**). This was the same for the early senescence-like phenotype (**Fig. 4d**), demonstrating that, in contrast to previous belief[17,18], the pleiotropic effects can be uncoupled from *mlo*-based resistance. In contrast, double mutants affected in *AtMLO2* and the jasmonate signaling pathway had a more severe senescence-like phenotype, which may be due to the antagonistic effect of jasmonate on salicylic acid signaling[22].

PEN1, *PEN2* and *PEN3*, which encode a syntaxin, a glycosyl hydrolase and an ABC transporter, respectively, are required for limiting invasion by nonadapted powdery mildews in *A. thaliana*[4–6]. Previous results showed that the barley ortholog of PEN1, ROR2, is required for barley *mlo* penetration resistance[4,23]. To address the question of whether *PEN1*, *PEN2* or *PEN3* is required for *Atmlo2* resistance, we conducted double mutant analysis. Although *Atmlo2 pen1* plants supported near–wild-type levels of *G. cichoracearum* entry rates, there was no significant increase in conidiophore production, suggesting that a PEN1-independent mechanism restricts post-entry growth of this fungus in *Atmlo2* plants (**Fig. 5a,b**). In *Atmlo2 pen1* double mutants, elevated levels of host cell entry were observed only with *G. cichoracearum* but not with *G. orontii* (data not shown), suggesting that the latter species might be insensitive to PEN1-mediated defenses. Pathogen entry was restored to near–wild-type levels in *Atmlo2 pen2* and *Atmlo2 pen3* double mutants. Conidiation of *G. cichoracearum* on *Atmlo2 pen2* and *Atmlo2 pen3* was significantly greater than on *Atmlo2*, indicating that PEN2 and PEN3 may have additional roles in post-invasion host defenses. Notably, the spontaneous deposition of callose and the senescence-like phenotype were suppressed only in *Atmlo2 pen2*, not in *Atmlo2 pen1* or *Atmlo2 pen3* (**Fig. 5c,d**), suggesting that a mutation in *PEN2* compensates for all known phenotypes associated with loss of AtMLO2. This result is in contrast to findings in barley, in which pleiotropic phenotypes were found to be considerably suppressed in an *mlo ror2* genotype[17].

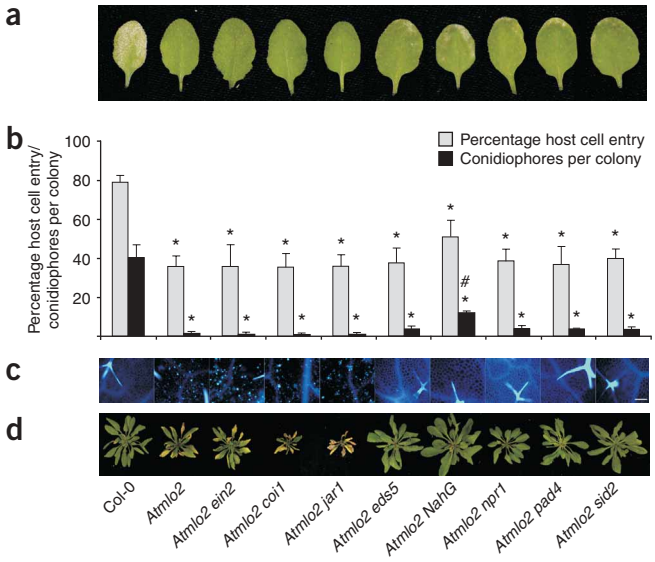

Figure 4 Salicylic acid has a role in the age-related phenotypes of *Atmlo2*. (**a**) Macroscopic phenotypes of Col-0 wild-type, *Atmlo2-11* and lines derived from crosses with mutants defective in ethylene, jasmonate or salicylic acid signaling pathways at 7 d post-inoculation with *G. cichoracearum*. Similar results were obtained upon inoculation with *G. orontii*. (**b**) Quantitative analysis of host cell entry (48 h post-inoculation) and conidiation (7 d post-inoculation) of *G. cichoracearum* on individuals assessed in **a**. Data represent mean ± s.d. of three independent experiments. Asterisks indicate a significant difference from Col-0, and hashes a significant difference from *Atmlo2* ($P < 0.01$; Student's *t*-test). Similar results were obtained upon inoculation with *G. orontii*. (**c**) Micrograph showing callose accumulation in rosette leaves at 7 weeks in powdery mildew–free conditions. Bar = 100 μm. (**d**) Macroscopic phenotypes of representative unchallenged plants at 8 weeks.

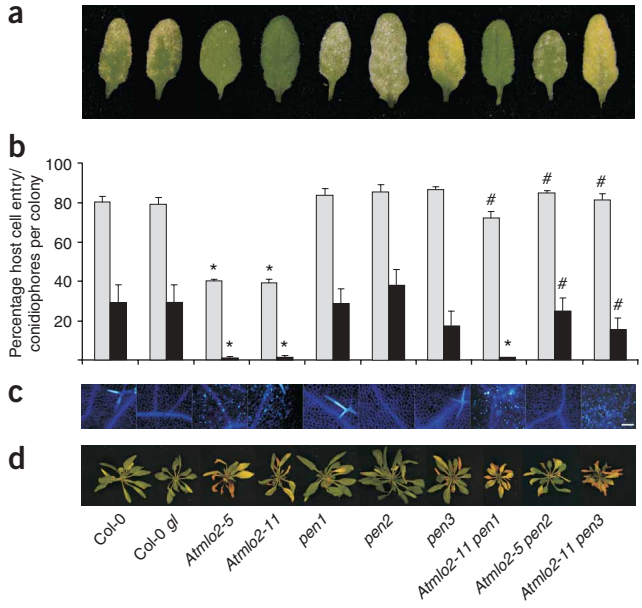

Figure 5 *Atmlo2*-mediated resistance requires components of non-host resistance. (**a**) Macroscopic phenotypes of *G. cichoracearum* growth on control plants, *Atmlo2* mutants and crosses with mutants defective in *PEN1*, *PEN2* or *PEN3*. Similar results were obtained upon inoculation with *G. orontii*. (**b**) Quantitative assessment of host cell entry (48 h post-inoculation; gray bars) and conidiation (7 d post inoculation; black bars). Data represent mean ± s.d. of three independent experiments. Asterisks indicate a significant difference from Col-0, hashes a significant difference from *Atmlo2* ($P < 0.01$; Student's *t*-test). Similar results were obtained upon inoculation with *G. orontii*, with one exception; *G. orontii* host cell entry and conidiation was similar on *Atmlo2* and *Atmlo2 pen1* plants. (**c**) Micrograph showing callose accumulation in rosette leaves at 7 weeks in powdery mildew–free conditions. Bar = 100 μm. (**d**) Macroscopic phenotypes of representative unchallenged plants at 8 weeks.

Both barley MLO and the PEN1 and ROR2 orthologous syntaxins colocalize and become concentrated in plasma membrane microdomains at attempted pathogen entry sites[24]. Thus, specific isoforms of MLO and syntaxins represent ancient and antagonistically acting components, promoting or restricting powdery mildew ingress, respectively. In contrast, the peroxisome-associated PEN2 glycosyl hydrolase[5] seems to be a recent innovation of *A. thaliana* (ref. 25 and **Supplementary Fig. 5**). PEN2 seems to act together with the plasma membrane-localized PEN3 ABC transporter in a pathway distinct from PEN1 (refs. 5,6). Therefore, *pen1* mutants might directly suppress *Atmlo2* resistance, and mutations in PEN2 or PEN3 may open bypass routes for powdery mildew host cell entry.

For several decades, *mlo* resistance has been envisaged as a unique feature of the monocot barley. Our results demonstrate that broad spectrum immunity against powdery mildews based on loss-of-function *mlo* alleles can be achieved in at least one additional, distantly related species, the dicot *A. thaliana*. This finding has several implications. First, it uncovers a role for these MLO co-orthologs as antagonists of a resistance mechanism(s) preventing fungal ingress at the cell periphery, which has been conserved over a time span of at least 200 million years (the approximate time of the monocot-dicot split; ref. 26). Second, the requirement for MLO proteins for host cell entry of diverse powdery mildews suggests that at least one aspect of pathogenesis is invariant and likely evolved before the monocot-dicot split in an ancestral Ascomycete adapted to colonize an angiosperm progenitor(s) of mono- and dicotyledonous plants. This hypothesis is supported by data on the molecular phylogeny of the Erysiphales, indicating that the evolution of powdery mildews has paralleled that of angiosperm plants[27]. The durability of this mechanism seems unusual given the assumed gains and losses of pathogenicity during evolution of pathogenic fungi in the phylum Ascomycota[1] in which species that are pathogenic and non-pathogenic on animals and plants occur within a single genus[28].

Our data imply that it might be feasible to engineer broad spectrum and potentially durable powdery mildew resistance in any higher plant species either by conventional mutagenesis or via *MLO* gene silencing. Finally, our findings indicate that, in principle, disease resistance can be fully uncoupled from the unwanted pleiotropic effects by second-site mutations. However, because double mutants in *AtMLO2* and key mediators of salicylic acid–dependent defense are likely to have enhanced susceptibility to other pathogens, further analysis will be required to determine whether it is possible to uncouple the pleiotropic trait from the resistance trait in an agronomically beneficial manner.

METHODS

Plant material. Homozygous *A. thaliana* insertion mutants Garlic_0878_H12 (*Atmlo2-5*), Garlic_0523_D09 (*Atmlo6-2*) and SLAT 24-21 (*Atmlo12-1*) were used in this study. Homozygous double and triple mutants were selected from intermutant crosses using these lines as parents. *pmr2-1* (*Atmlo2-11*) was previously mapped to the top of chromosome 1 (ref. 11). We took a candidate gene approach to identify *PMR2* after *AtMLO2* was observed in the mapping interval. The identity of *PMR2* was confirmed by sequencing PCR-amplified genomic DNA containing *AtMLO2* coding sequence in each *pmr2* allele. *A. thaliana* mutant alleles *jar1-1*, *ein2-1*, *coi1-1*, *eds5-1*, *npr1-1*, *pad4-1*, *sid2-1*, *pen1-1*, *pen2-1* and *pen3-1* and the transgenic line expressing *NahG* were used for intermutant crosses with either *Atmlo2-5* or *Atmlo2-11*. All newly created materials will be provided by the authors upon request. Seeds of newly described *A. thaliana* single, double and triple mutants will be submitted to the appropriate stock centers.

Cytology. To visualize epiphytic fungal structures, specimens were stained with Coomassie brillant blue. For quantification of fungal host cell entry of *G. cichoracearum*, *G. orontii* and *E. pisi*, the proportion of germinated fungal sporelings that developed secondary hyphae served as an approximation of penetration success. The number of mature conidiophores per colony was counted at 6–7 d after inoculation. Penetration success of *Bgh* was quantified by visualizing haustoria with Coomassie brilliant blue and Aniline blue staining. To assay for *Alternaria* spp. and *P. infestans* disease symptoms, leaves were spray- or drop-inoculated and kept at saturating humidity until analysis. *P. infestans*–inoculated leaves were stained with trypan blue in lactophenol and ethanol. For visualization of callose, samples were stained with aniline blue. To assay for H_2O_2 accumulation, leaves were stained with 3,3-diaminobenzidine tetrahydrochloride (DAB).

Transgenic dsRNAi lines. PCR amplicons of the C-termini of *AtMLO2*, *AtMLO6* and *AtMLO12* were integrated into the binary dsRNAi vector pJawohl3, and the ecotype Col-0 was transformed with the resulting construct. Progeny of selected T1 lines were used for powdery mildew inoculations. Semiquantitative RT-PCR was performed to analyze transcript abundance with genomic DNA serving as a control to distinguish between cDNA and genomic DNA.

Quantification of salicylic acid levels. Frozen leaf tissue was used to extract salicylic acid via organic solvent-based extraction and subsequent acid hydrolysis. HPLC separation of salicylic acid was performed on a RP-C-18 Nucleosil column (Bischoff). Online measurements of salicylic acid were performed fluorimetrically at an excitation wavelength of 305 nm and an emission wavelength of 407 nm.

Statistical analysis. Statistical analysis of data was based on Student's *t*-test. Calculations were performed on a minimum of three independent data sets, assuming two-sample equal variance (homoscedastic) and a two-tailed distribution. We considered $P < 0.01$ to be a significant result.

A detailed description of the methods employed in this study is provided in **Supplementary Methods** online, and a list of primers is given in **Supplementary Table 2**.

Accession codes. GenBank: pJawohl3, AF404854.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHORS' CONTRIBUTIONS

C.C. and M.H. contributed equally to this work. C.C. identified the *Atmlo* mutants; M.H. cloned *PMR2*; C.C., J.V. and M.H. performed double mutant analysis; H.A.H. designed and generated the dsRNAi lines; M.L. determined salicylic acid levels; M.L., L.W. and B.K. performed phytopathology experiments shown in **Supplementary Fig. 2**; P.S.L., J.D., S.C.S. and R.P. designed experiments; V.L. performed PEN2 phylogenetic analysis and C.C., P.S.L., M.H., S.C.S. and R.P. wrote the paper. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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