*e-X*tra*

Naturally Occurring Broad-Spectrum Powdery Mildew Resistance in a Central American Tomato Accession Is Caused by Loss of *Mlo* Function

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The resistant cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) line LC-95, derived from an accession collected in Ecuador, harbors a natural allele (*ol-2*) that confers broad-spectrum and recessively inherited resistance to powdery mildew (*Oidium neolycopersici*). As both the genetic and phytopathological characteristics of *ol-2*–mediated resistance are reminiscent of powdery mildew immunity conferred by loss-of-function *mlo* alleles in barley and *Arabidopsis*, we initiated a candidate-gene approach to clone *Ol-2*. A tomato *Mlo* gene (*SlMlo1*) with high sequence-relatedness to barley *Mlo* and *Arabidopsis AtMLO2* mapped to the chromosomal region harboring the *Ol-2* locus. Complementation experiments using transgenic tomato lines as well as virus-induced gene silencing assays suggested that loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by *ol-2*. In progeny of a cross between a resistant line bearing *ol-2* and the susceptible tomato cultivar Moneymaker, a 19-bp deletion disrupting the *SlMlo1* coding region cosegregated with resistance. This polymorphism results in a frameshift and, thus, a truncated nonfunctional SlMlo1 protein. Our findings reveal the second example of a natural *mlo* mutant that possibly arose postdomestication, suggesting that natural *mlo* alleles might be evolutionarily short-lived due to fitness costs related to loss of *mlo* function.

Additional keywords: comparative genetics, co-orthologs, introgression lines, pleiotropic effects.

Powdery mildew is a common disease of higher plant species that is caused by obligate biotrophic Ascomycete fungi belonging to the order Erysiphales. Worldwide, approximately 500 powdery mildew species are able to colonize about 10,000 distinct plant species (Takamatsu 2004). The disease is particularly prevalent in temperate and humid climates, where it frequently causes significant yield losses in agricultural settings, including greenhouse and field tomato (*Solanum lycopersicum*) farming. Powdery mildew caused by *Oidium neolycopersici* has recently been recognized as a worldwide emerging pathogen on tomato (Jones et al. 2001). There are two known tomato powdery mildew species in the *Oidium* genus, *O. lycopersici* occurring in Australia and *O. neolycopersici* in the rest of the world (Kiss et al. 2001). Several powdery mildew (*Oidium neolycopersici*, *Ol*) resistance genes have been described and mapped in tomato, of which most confer dominantly inherited, isolate-specific resistance (Bai et al. 2005). However, *ol*-2 mediates recessively inherited broad-spectrum (non-isolate-specific) resistance to *O. neolycopersici* (Bai et al. 2005; Ciccarese et al. 1998). The *ol*-2 allele originates from a wild accession of *S. lycopersicum* var. *cerasiforme* (cherry tomato), a close relative of common cultivated tomato that is thought to represent an admixture of wild and cultivated tomatoes (Ciccarese et al. 1998; Nesbitt and Tanksley 2002). The respective accession, LA-1230, was originally collected in 1970 in Ecuador (University of California Davis, Tomato Genetics Resource Center database) in a home garden and park area. Based on genetic mapping, *ol*-2 was found to reside around the centromere of tomato chromosome 4 (De Giovanni et al. 2004). Histological analysis of the host-pathogen interaction of an *ol*-2–containing line revealed an early abortion of fungal pathogenesis associated with the formation of plant cell-wall appositions (papillae) at the attempted fungal entry sites (Bai et al. 2005). Taken together, these characteristics (recessive inheritance, broad-spectrum resistance profile, early termination of fungal pathogenesis) are reminiscent of powdery mildew resistance in barley (*Hordeum vulgare*) and thale cress (*Arabidopsis thaliana*) conferred by loss-of-function *mlo* (*mildew resistance locus o*) alleles (Consonni et al. 2006; Jørgensen 1992).

In land plants, *Mlo* genes comprise small to medium-sized families encoding a novel type of plant-specific integral membrane proteins with one or more as-yet-unknown biochemical functions (Devoto et al. 1999, 2003). In the monocot barley and the dicot *Arabidopsis*, mutations in particular *Mlo* genes result in broad-spectrum powdery mildew resistance (Büschges et al. 1997; Consonni et al. 2006). It is thought that the respective protein isoforms modulate vesicle-associated defense responses at the cell periphery and that the powdery mildew

pathogen possibly exploits these proteins for successful host-cell entry (Panstruga 2005a). While, in barley, loss-of-function of a single *Mlo* gene suffices to confer full resistance, unequal genetic redundancy (Briggs et al. 2006) between three phylogenetically closely related *Mlo* co-orthologs (*AtMLO2*, *AtMLO6* and *AtMLO12*; Panstruga 2005b) results in a more complex scenario in *Arabidopsis*; absence of *AtMLO2* confers partial powdery mildew resistance that becomes enhanced in *Atmlo2 Atmlo6* or *Atmlo2 Atmlo12* double mutants. Full resistance requires loss-of-function of all three co-orthologs, i.e., an *Atmlo2 Atmlo6 Atmlo12* triple mutant (Consonni et al. 2006). Barley *mlo* mutants have been known for more than 60 years (Freisleben and Lein 1942) and have been successfully employed in European barley agriculture for more than 25 years (Lyngkjaer et al. 2000), emphasizing the principal durability of *mlo*-mediated disease resistance under agricultural conditions.

In case of barley, in addition to a broad range of induced mutations (Büschges et al. 1997; Panstruga et al. 2005; Piffanelli et al. 2002), one natural allele (*mlo*-11) has been described (Piffanelli et al. 2004). This allele derives from a barley landrace, an intermediate between wild and cultivated barley, initially found in Ethiopia. At the molecular level, it is characterized by the presence of a complex tandem repeat array, comprising *Mlo* 5′ regulatory and partial coding sequences, that localize upstream of a fully intact *Mlo* wild-type copy (Piffanelli et al. 2004). Experimental evidence points to a transcriptional 'read-through' phenomenon caused by the presence of the upstream repeat units, interfering with proper transcription of the downstream *Mlo* wild-type copy (Piffanelli et al. 2004). Interestingly, the *mlo*-11 allele is meiotically unstable, resulting in rare meiotic reversion events that give rise to susceptible progeny. Reversion to susceptibility was found to coincide with a loss of the repeat array and restoration of wild-type-like *Mlo* transcript and Mlo protein levels (Piffanelli et al. 2004).

Here, we used a targeted approach based on comparative genetics to clone the tomato *Ol*-2 gene. We selected and mapped two *SlMlo* candidate genes. The chromosomal location as well as genetic gain- and loss-of-function experiments suggested that *SlMlo1* is *Ol*-2. Sequence analysis revealed a short deletion in the *Ol*-2 coding region of the resistant tomato accession that cosegregated with resistance.

RESULTS

We first assessed whether the tomato powdery mildew pathogen, *O. neolycopersici*, is principally amenable to *mlo*-mediated resistance. Besides tobacco and other host plants, *O. neolycopersici* has been reported to be virulent on *A. thaliana* (Xiao

et al. 2001). We thus inoculated susceptible Col-0 wild-type plants and the previously described set of resistant *Arabidopsis mlo* mutants with *O. neolycopersici*. In *Arabidopsis*, three phylogenetically closely related *Mlo* co-orthologs, *AtMLO2*, *AtMLO6*, and *AtMLO12*, (Panstruga 2005b), exhibit unequal genetic redundancy with respect to powdery mildew infection phenotypes. *Atmlo2* mutants are partially resistant to adapted powdery mildews, while *Atmlo2 Atmlo6* and *Atmlo2 Atmlo12* double mutants exhibit increased, and the *Atmlo2 Atmlo6 Atmlo12* triple mutant full resistance (Consonni et al. 2006). Reminiscent of this scenario, *Atmlo2* plants were partially resistant and its derived double (*Atmlo2 Atmlo6*, *Atmlo2 Atmlo12*) and triple (*Atmlo2 Atmlo6 Atmlo12*) mutants showed full resistance to *O. neolycopersici* (Fig. 1; Table 1). We conclude that *O. neolycopersici,* similar to powdery mildews colonizing barley (*Blumeria graminis* f. sp. *hordei*) or *Arabidopsis* (e.g., *Golovinomyces cichoracearum* and *Golovinomyces orontii*), is sensitive to *mlo*-based resistance.

Given the fact that *O. neolycopersici* requires host *Mlo* function for pathogenesis and based on the genetic and phytopathological similarities between *ol*-2–mediated powdery mildew resistance and the well-characterized loss-of-function *mlo* mutants in barley and *Arabidopsis*, we initiated a candidate approach to clone *Ol*-2. We inspected the publicly available tomato expressed sequence tag (EST) collections by BLAST analysis for cDNAs with a significant sequence relatedness to *AtMLO2*-encoded transcripts. We identified two EST contigs comprising 12 and three EST sequences, respectively, that correspond to cDNAs of two distinct genes, which we designated *SlMlo1* and *SlMlo2* (Fig. 2A). While multiple overlapping EST sequences enabled the inference of a conceptual full-length *SlMlo1* cDNA sequence (National Center for Biotechnology Information [NCBI] UniGene database, tomato Les.746), *SlMlo2* was only represented as a partial cDNA covering the 3′ end of the coding sequence (Fig. 2). Tissue origin of the respective EST indicates that *SlMlo1* is ubiquitously expressed

Table 1. Infection phenotypes of *Arabidopsis mlo* mutants upon challenge with *Oidium neolycopersici*

| Genotype | Disease index (DI)[a] | | |
	8 dpi[b]	13 dpi	15 dpi
Col-0	1.6 ± 0.5[c]	2.8 ± 0.4	2.8 ± 0.4
Atmlo2	0.0	1.0 ± 0.0	1.0 ± 0.0
Atmlo6	0.0	2.0 ± 0.0	1.5 ± 0.0
Atmlo12	0.0	2.0 ± 0.0	2.0 ± 0.0
Atmlo2 Atmlo6	0.0	0.0	0.0
Atmlo2 Atmlo12	0.0	0.0	0.0
Atmlo6 Atmlo12	0.0	2.0 ± 0.0	1.5 ± 0.5
Atmlo2 Atmlo6 Atmlo12	0.0	0.0	0.0

[a] To semiquantitatively assess infection phenotypes, the following DI scale was used: 0 = no visible fungal sporulation, 1 = few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies.
[b] dpi = days postinoculation.
[c] DI mean ± standard deviation of five plants per genotype are presented.

Fig. 1. *Oidium neolycopersici* requires *AtMLO2* function for pathogenesis on *Arabidopsis thaliana*. Exemplary infection phenotypes of rosette leaves of 7-week-old *A. thaliana* wild-type (Col-0) plants and T-DNA insertion mutants (*Atmlo2*, *Atmlo6*, *Atmlo12* single, double, and triple mutants) inoculated with *O. neolycopersici*. The photograph was taken 15 days postinoculation. The experiment was repeated once with similar results.

(leaf, callus, flower), while expression of *SlMlo2* appears to be predominant in tomato breaker fruits (data not shown). We designed oligonucleotide primers flanking the deduced sequence contigs and performed reverse transcription polymerase chain reaction (RT-PCR) on RNA obtained from wild-type tomato leaves to verify the predicted *SlMlo1* and *SlMlo2* cDNA sequences. This confirmed the expression of either gene in tomato leaves and validated the nucleotide sequences of the respective EST-derived cDNA contigs (data not shown). Phylogenetic analysis comprising all *Arabidopsis* MLO protein sequences as well as SlMlo1 and SlMlo2 revealed that the two deduced tomato proteins cluster in the same clade as AtMLO2, AtMLO6, and AtMLO12 (Fig. 3). Based on this finding and the presence of a shared distinctive polypeptide motif at the C-terminus of the encoded polypeptides (Fig. 2B), which is considered to be diagnostic of an orthologous phylogenetic relationship (Panstruga 2005b), *SlMlo1* and *SlMlo2* likely represent co-orthologs of *AtMLO2*, *AtMLO6*, and *AtMLO12*.

A collection of *S. lycopersicum* × *S. pennellii* introgression lines (IL; Eshed and Zamir 1995) provides an efficient tool for the rapid mapping of genes to tomato chromosomal segments. Using a core set of 50 IL covering each of the 12 tomato chro-
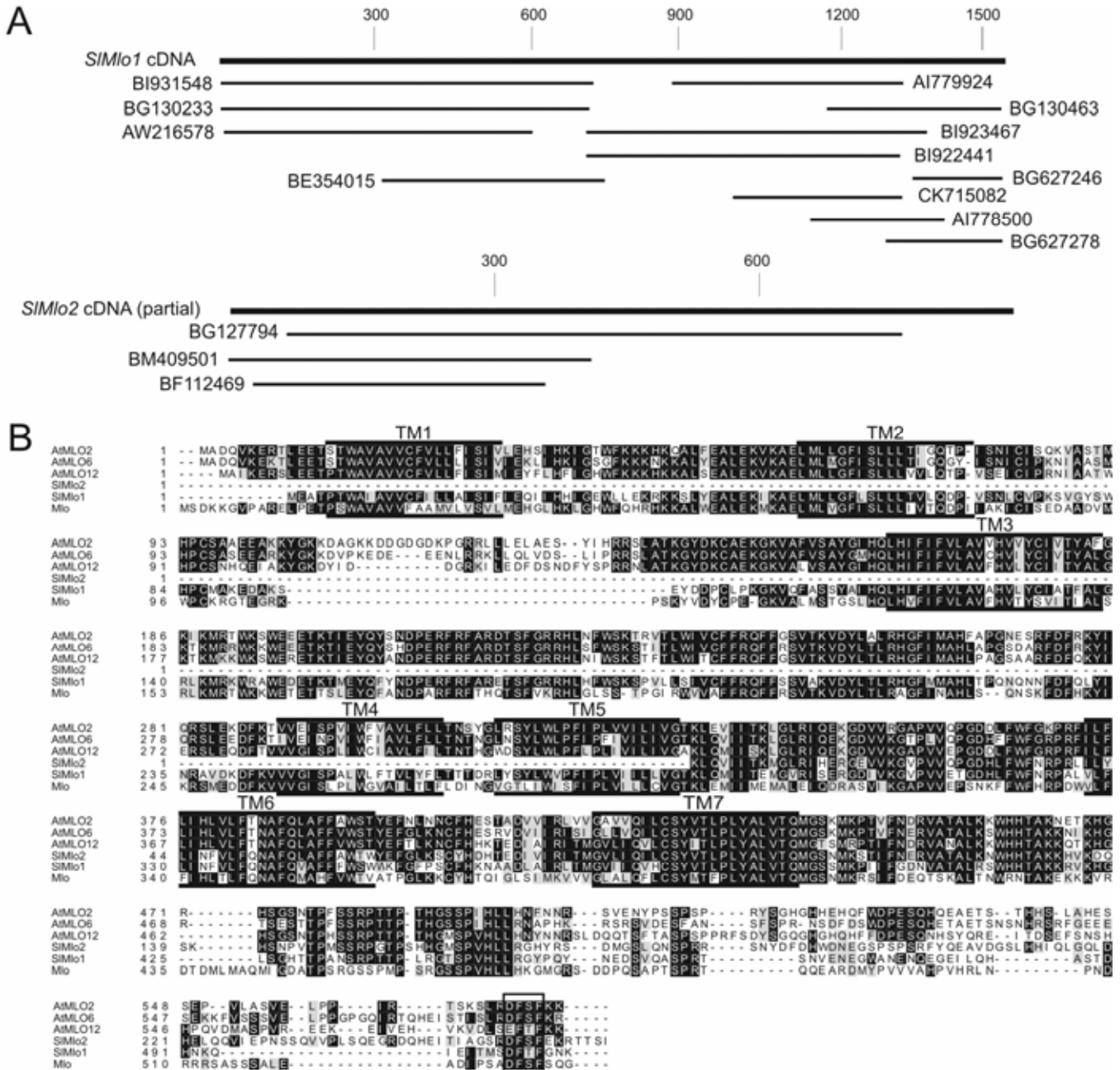
Fig. 2. *SlMlo1* and *SlMlo2* encode polypeptides that are highly sequence related to barley Mlo, *Arabidopsis* AtMLO2, AtMLO6, and AtMLO12. **A,** The scheme represents the coverage of *SlMlo1* (top bold line) and *SlMlo2* (bottom bold line) cDNAs by expressed sequence tag (EST) sequences (thin lines) as revealed by BLASTN analysis using the *SlMlo1* or *SlMlo2* cDNA contig as query sequences, respectively. Designations next to the EST-signifying lines specify GenBank accession numbers. Scales above cDNAs indicate length in nucleotides. Note the different scales for *SlMlo1* and *SlMlo2*. **B,** Multiple amino-acid sequence alignment of barley Mlo, tomato SlMlo1 and SlMlo2 as well as *Arabidopsis* AtMLO2, AtMLO6, and AtMLO12 polypeptide sequences. The alignment was generated by CLUSTALW using the default parameter. Positions of the seven transmembrane regions (TM1 through TM7) inferred from the experimentally determined topology of barley Mlo (Devoto et al. 1999) are indicated by bars above and below the sequences. The C-terminal D/E-F-S/T-F tetra-peptide sequence, one of several motifs characteristic of barley Mlo orthologs (Panstruga 2005b), is boxed.

mosomes with overlapping introgressed segments of *S. pennellii*, we determined a likely localization of *SlMlo1* at the center of chromosome 4 in a small region that also harbors the *ol-2*–containing interval (De Giovanni et al. 2004), while *SlMlo2* was found to reside on top of chromosome 6 (Fig. 4). Since *ol-2* was previously unequivocally found to be a single recessive locus located on tomato chromosome 4 (De Giovanni et al. 2004), we focused in the following on *SlMlo1* as a candidate for *Ol-2* and excluded *SlMlo2* from further analysis.

We performed semiquantitative RT-PCR analyses in a long-term timecourse experiment following powdery mildew challenge, to study *SlMlo1* expression in susceptible *Ol*-2 wild-type and resistant *ol*-2 (line R28) mutant plants. We observed moderately reduced *SlMlo1* transcript accumulation in line R28 as compared with wild-type plants at all tested time points (Fig. 5A). Similar results were obtained upon comparison of *SlMlo1* transcript levels at early timepoints following *O. neolycopersici* inoculation in homozygous susceptible (*Ol-2/Ol-2* genotype) and resistant (*ol-2/ol-2* genotype) F$_2$ progeny of a cross between the susceptible cv. Moneymaker (MM) and the resistant line R28 (Fig. 5B). Consistent with previous findings in barley (Piffanelli et al. 2002), both *Ol*-2 and *ol*-2 genotypes exhibited a noticeable increase in *SlMlo1* transcript levels at early timepoints following powdery mildew challenge (Fig. 5B).

To directly assess a potential role of *SlMlo1* in conferring powdery mildew susceptibility in wild-type tomato, we initiated both genetic gain- and loss-of-function experiments. We reasoned that, if loss of *SlMlo1* function was responsible for resistance conferred by the *ol-2* gene, then strong constitutive expression of a *SlMlo1* transgene should restore susceptibility in resistant plants bearing the *ol-2* allele. Vice versa, *SlMlo1* gene silencing in susceptible tomato wild-type plants should result in enhanced powdery mildew resistance. For this and the following experiments, we used a resistant F$_3$ line, R26, of a cross between the susceptible cv. Super Marmande (SM) and the original resistant line *S. lycopersicum* var. *cerasiforme* LC-95 (De Giovanni 2004). We generated a binary vector harboring *SlMlo1* under control of the strong constitutive *Cauliflower mosaic virus* 35S promoter and transformed tomato line R26 with the respective construct. Upon selection on kanamycin, we obtained 11 independent transgenic lines. We performed semiquantitative as well as real-time RT-PCR, using RNA extracted from leaf material of T$_1$ plants to assess expression levels of the *SlMlo1* transgene. Both types of analysis revealed that transgenic lines 3, 10, and 11 expressed the highest levels of wild-type *SlMlo1* cDNA (Fig. 6A and B). We challenged selfed progeny (T$_2$ plants) of the individual transgenic lines with *O. neolycopersici* and observed restoration of macroscopically visible powdery mildew growth among the offspring of the two lines (lines 10 and 11) that, beforehand, exhibited strongest transgene expression in the T$_1$ generation (Fig. 6C and D; T$_1$ seeds of transgenic lines 1, 3, 6, and 7 did not germinate, preventing analysis of the respective T$_2$ progeny). Powdery mildew infection phenotypes of susceptible segregants derived from transgenic lines 10 and 11 were comparable to the fully susceptible cv. MM (disease index [DI] score = 3). PCR fingerprint analysis employing codominant markers TOM316 and U3-2*Hha*I, closely linked to *ol*-2 (Pavan et al. in press), confirmed the identity of the parental line (R26) used for transformation and excluded the possibility of a seed contamination (data not shown). In total, 28 T$_2$ plants (12 from line 10 and 16 from line 11) were tested, and susceptibility to *O. neolycopersici* segregated in both these T$_2$ generations in an approximately 3:1 (susceptible/resistant) ratio, suggesting the presence of single-transgene loci in both of the transgenic lines (Table 2). Occurrence of susceptible T$_2$ plants and segregation in the T$_2$ progeny indicates that restored susceptibility is a heritable genetic trait upon transgenic expression of *SlMlo1*.

For genetic loss-of-function experiments, we engineered a transgenic *Tobacco rattle virus* (TRV) harboring a *SlMlo1* fragment for virus-induced gene silencing (VIGS) experiments. Upon inoculation of the modified virus (TRV-*SlMlo1*) on susceptible wild-type tomato plants of *S. lycopersicum* cv. MM and subsequent challenge with *O. neolycopersici*, we observed a dramatic reduction of macroscopically visible powdery mildew colonies as compared with inoculation with wild-type TRV (Fig. 7). Two independent VIGS assays yielded
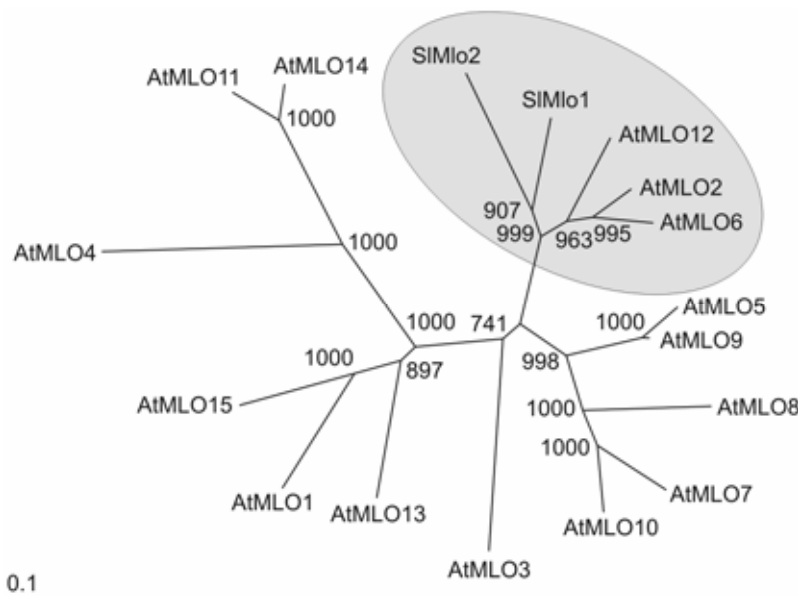

Fig. 3. A neighbor-joining phylogenetic tree of *Arabidopsis* and selected tomato MLO proteins. SlMlo1 and SlMlo2 are co-orthologs of AtMLO2, AtMLO6, and AtMLO12. The unrooted radial tree comprises all 15 *Arabidopsis* MLO paralogs (AtMLO1 to AtMLO15; Devoto et al. 2003) as well as SlMlo1 and SlMlo2 and was established on the basis of an optimized multiple-sequence aligment using the PHYLIP software package. Numbers above nodes indicate bootstrap values (based on 1,000 replicates) that support the respective branch. The scale (left bottom corner) indicates the number of amino-acid exchanges per site. The circled clade shaded in light gray harbors potential tomato (SlMlo1 and SlMlo2) and *Arabidopsis* (AtMLO2, AtMLO6, and AtMLO12) co-orthologs.

comparable results. Taken together with the complementation experiments described above, this finding suggests that, indeed, a loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by the recessively inherited *ol-2* gene.
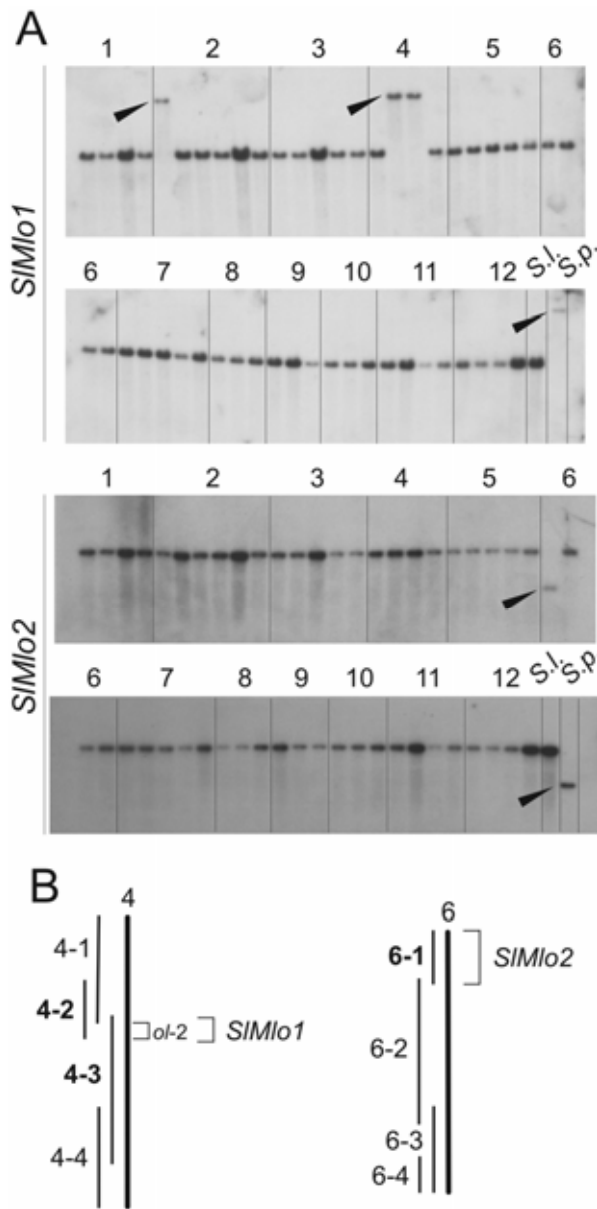
Fig. 4. Introgression line (IL)-based mapping of *SlMlo1* and *SlMlo2*. **A,** Gel blot hybridization of labeled *SlMlo1* (top panels) or *SlMlo2* (bottom panels) cDNA fragments on filters comprising restriction-digested genomic DNAs of a core set of *Solanum lycopersicum* × *Solanum pennellii* IL and respective parental lines. Genomic DNAs were either digested with restriction enzyme *Bgl*II (*SlMlo1*) or *Eco*RV (*SlMlo2*). Parental polymorphisms in *S. pennellii* and IL showing the particular polymorphism are highlighted by black arrowheads. Numbers above gel sections indicate the lines representing the respective tomato chromosome. *S.l.* = *Solanum lycopersicum*; *S.p.* = *Solanum pennellii* (parental lines). Note the additional *SlMlo1* polymorphism in line 2-1 (upper panel). Occurrence of two overlapping chromosomal segments of chromosome 4 (lines 4-2 and 4-3) and the absence of a second hybridizing band in any lane of this blot suggests that this polymorphism is most likely due to the presence of an ectopic genomic fragment of chromosome 4 in line 2-1 (an impurity of the IL; Bonnema et al. 2002). **B,** Scheme depicting the interpretation of the IL-based mapping shown in A. Solid thick lines represent tomato chromosome 4 (left) and 6 (right). Thin lines designate chromosomal segments covered by the respective IL, bold numbers highlight segments harboring a sequence polymorphism in *SlMlo1* or *SlMlo2*. The previously determined map position of *ol-2* (De Giovanni et al. 2004) as well as the deduced intervals harboring *SlMlo1* and *SlMlo2* are indicated.

In principle, lack of the *SlMlo1* transcript or alterations in the encoded cDNA or polypeptide sequence could lead to the observed disease-resistance phenotype. In barley, all induced mutants analyzed to date were found to confer changes in the *Mlo* coding sequence (Büschges et al. 1997; Panstruga et al. 2005; Piffanelli et al. 2002), while the sole known natural *mlo* allele, *mlo*-11, is characterized by severely perturbed *Mlo* transcript levels (Piffanelli et al. 2004). Since RT-PCR analysis revealed *SlMlo1* transcript accumulation in both *Ol-2* and *ol-2* genotypes (discussed above), we analyzed *SlMlo1* cDNA sequences of tomato wild-type plants (cv. SM) and the resistant progeny (F$_3$ line R26) of *S. lycopersicum* var. *cerasiforme* LA-1230. While the cDNA sequence of SM was identical to the wild-type *SlMlo1* reference sequence (GenBank accession number AY967408; NCBI UniGene tomato Les.746), we found
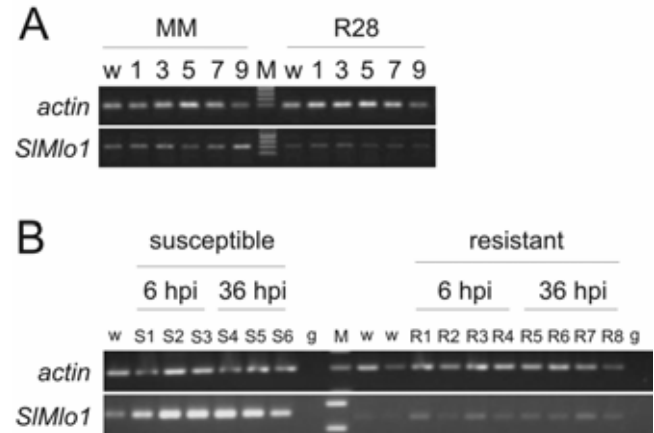
Fig. 5. *SlMlo1* transcript accumulation upon powdery mildew challenge. **A,** Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *SlMLO1* and *actin* transcript levels in susceptible wild-type tomato (cv. Moneymaker [MM]) and the powdery mildew resistant F$_3$ line R28. RNAs were extracted from leaves of either unchallenged (w = water control) or powdery mildew–challenged plants at 1, 3, 5, 7, and 9 days after inoculation with *Oidium neolycopersici*. Lane 'M' indicates DNA size marker. **B,** Semiquantitative RT-PCR analysis of *SlMlo1* and *actin* transcript levels in homozygous susceptible (*Ol-2/Ol-2* genotype; lines S1 to S6) and resistant (*ol-2/ol-2* genotype; lines R1 to R8) F$_2$ progeny of a cross between cv. MM and the powdery mildew–resistant F$_3$ line R28. RNAs were extracted from leaves of either unchallenged or powdery mildew–challenged plants at 6 and 36 h after inoculation with *O. neolycopersici*. Lane g = amplification using genomic DNA as template, lane M = DNA size marker. Note the slight size difference (owing to the 19-bp deletion) between *SlMLO1* cDNAs derived from susceptible (*Ol-2*) or resistant (*ol-2*) lines. Absence of products derived from genomic DNA as template likely is due to either the presence of multiple introns in the respective genomic sequences, the potential location of primer binding sites on exon or intron junctions, or both.

Table 2. Segregation analysis of transgenic tomato lines

T$_1$ generation	T$_2$ generation		
Line	Susceptible[a]	Resistant[a]	χ^2 (3:1)[b]
2	0	22	n.a.
4	0	16	n.a.
5	0	16	n.a.
8	0	12	n.a.
9	0	11	n.a.
10	7	5	P > 0.5
11	12	4	P > 0.9

[a] Susceptible plants with a disease index (DI) score of 2 or 3 and resistant plants with a DI of 0 or 1 (0 = no visible fungal sporulation, 1 = few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies).

[b] n.a. = not applicable.

a 19-bp deletion in the cDNA of line R26. This sequence alteration leads to a frameshift in the coding region, predicted to result in premature translational termination in the second cytoplasmic loop of SlMlo1 and, thus, a severely truncated protein (Fig. 8). We found the same DNA sequence polymorphism in the genomic sequence of the independent resistant F_3 line R28 (data not shown). Notably, apart from this difference, the nucleotide sequences of the *SlMlo1* open reading frames of the susceptible SM and the resistant R26 lines were identical (data not shown). We conclude that the resistant natural accession is expected to encode a *SlMlo1* null allele conditioned by a single mutational event in the *SlMlo1* coding region.

We designed oligonucleotide primers flanking the 19-bp deletion in the *SlMlo1* cDNA and analyzed the *SlMlo1* genotype in an F_2 population segregating for *ol-2*–mediated powdery mildew resistance. As judged from PCR data, presence of the 19-bp deletion fully correlated with resistance (*n* = 106 ana-lyzed F_2 individuals), further substantiating that *SlMlo1* is *Ol*-2 (Supplementary Fig. 2).

DISCUSSION

We have shown here that the recessively inherited *ol*-2–mediated powdery mildew resistance originally identified in a natural accession (LA-1230) of *Solanum lycopersicum* var. *cerasiforme* collected in Ecuador is mediated by loss of *SlMlo1* function. The chain of evidence consists of a common map position (Fig. 4B), genetic gain- and loss-of-function experiments (Figs. 6 and 7), as well as cosegregation of a small (19 bp) deletion in the *SlMlo1* coding region with the resistant phenotype (Fig. 8). Taken together, these findings indicate that the resistant mutant derived from the cherry tomato accession LA-1230 encodes a *SlMlo1* null allele, caused by a small nucleotide polymorphism in the coding region of the gene.
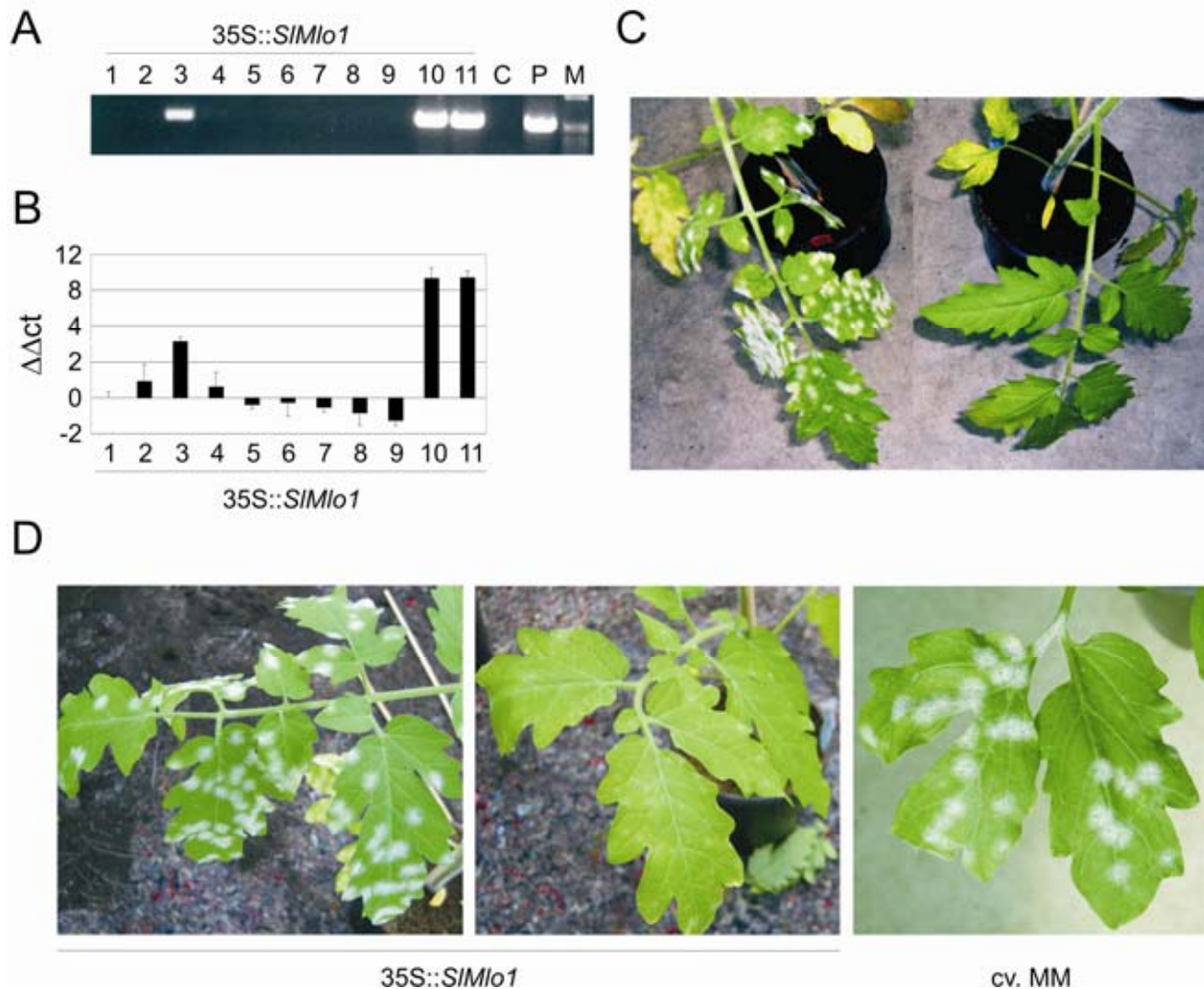
Fig. 6. Complementation of *ol*-2–mediated resistance in transgenic tomato lines overexpressing *SlMlo1*. **A,** Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (25 cycles) of eleven (1 to 11) transgenic T_1 lines expressing 35S::*SlMlo1* derived from the powdery mildew resistant line R26. RT-PCR reactions were performed using the *SlMlo1* transgene-specific oligonucleotide combination SlMlo2/35S term. Note that the panel has been composed of various sections of a larger photograph of the respective agarose gel. C = water instead of template negative control, P = plasmid positive control, and M = size marker. **B,** Quantitative real-time PCR analysis of 35S::*SlMlo1* transgene expression. Real-time PCR reactions were performed with the *SlMlo1* transgene-specific oligonucleotide combination SlMlo2/35Sterm (*SlMlo1* expression) and the *actin*-specific primer combination actin1/actin4 (*actin* expression). Relative expression levels based on the ΔΔct values (average ± standard deviation of four replicates) are indicated for the 11 transgenic lines in relation to line 1. Note that ΔΔct values are logarithmic indicators of differences in transcript accumulation. A second experiment yielded similar results. **C,** Exemplary infection phenotypes upon powdery mildew (*Oidium neolycopersici*) challenge of T_2 progeny of a transgenic line. Depicted are two descendants of T_1 line 11 (described above), one being susceptible (left, DI = 3) and one being largely resistant (right, DI = 0). The photograph was taken 17 days postinoculation. **D,** Close-up views of powdery mildew–infected leaves of susceptible (left panel) and resistant (middle panel) progeny of a susceptible transgenic line (T_2 descendants of T_1 line 11 expressing 35S::*SlMlo1*) and the susceptible tomato wild-type control (cv. Moneymaker [MM]; right panel). Photographs were taken 17 days postinoculation. Note that infection phenotypes of the susceptible transgenic line and cv. MM are comparable (DI = 3).

In tomato, currently available EST indicate the presence of at least two paralogs that likely represent co-orthologs of *AtMLO2*, *AtMLO6*, and *AtMLO12* in *Arabidopsis* (Figs. 2 and 3; Panstruga 2005b). Interestingly, unlike in *Arabidopsis*, loss-of-function of a single gene (*SlMlo1*) in tomato suffices to convey virtually full powdery mildew resistance (Bai et al., 2005). In *Arabidopsis*, *AtMLO2*, *AtMLO6*, and *AtMLO12* exhibit unequal genetic redundancy (Briggs et al. 2006), and a mutation in the gene with the major 'susceptibility conferring' activity, *AtMLO2*, results merely in partial powdery mildew resistance (Consonni et al. 2006). This indicates that the functional specialization of Mlo isoforms evolved differently in *Arabidopsis* and tomato. In barley, like in tomato, a mutation in a single *Mlo* gene is sufficient to confer full powdery mildew resistance. However, unlike in tomato, *Mlo* is the only expressed gene copy of this (co-)ortholog cluster in barley. A closely sequence-related paralog, *HvMlo2* (GenBank accession number Z95496) appears to represent a nonexpressed pseudogene (R. Panstruga, *unpublished findings*). In sum, it seems that the genetic complexity and functional specialization within the *Mlo* co-ortholog cluster related to powdery mildew susceptibility significantly differs between various monocot and dicot plant species. With respect to plant breeding aspects, this implies that the time and effort required for obtaining powdery mildew–resistant *mlo* mutants by either forward or reverse genetics or transgenic gene silencing approaches may also vary considerably between plant species.

Since in addition to cherry tomato, the *ol-2* allele confers resistance in cultivated round tomatoes (*S. lycopersicum*, e.g., cvs. MM or SM), *ol-2* might be useful for breeding in different types of tomatoes. Given the unusual durability of *mlo*-mediated powdery mildew resistance in barley (Lyngkjaer et al. 2000), the *ol-2* allele promises to represent indeed a precious resource for future tomato breeding, the 19-bp deletion being a valuable polymorphism for marker-assisted breeding. Interestingly, resistant tomato lines lack any obvious pleiotropic phenotypes, such as early senescence-like leaf chlorosis of unchallenged plants, which has been reported to occur in both barley

and *Arabidopsis mlo* mutants (data not shown; Consonni et al. 2006; Piffanelli et al. 2002). However, the extent of expression of pleiotropic effects in barley and *Arabidopsis* is considerably dependent on environmental conditions (Consonni et al. 2006; Jørgensen et al. 1992), leaving the possibility that lack of obvious pleiotropic phenotypes in *ol-2* lines is based on the particular growth settings used in this study.

We observed moderately lower *SlMlo1* transcript levels in *ol-2* as compared to *Ol-2* genotypes, both in unchallenged as well as in powdery mildew–inoculated plants (Fig. 5A and B). Given that *ol-2* likely is a null allele, one may hypothesize that the SlMlo1 protein exerts direct or indirect positive feedback regulation on the transcription of its own gene. Evidence for such a positive regulatory feedback loop has, however, neither been found in barley nor *Arabidopsis mlo* mutants (Piffanelli et al. 2002; C. Consonni and R. Panstruga, *unpublished data*). Unless this was a tomato-specific phenomenon, it is thus more likely that the observed difference in *SlMlo1* transcript levels is due to intrinsic genetic variation of tomato (sub)species, e.g., within the *SlMlo1* promoter region. Alternatively, the reduced transcript levels might result from one or more second-site mutational events in *SlMlo1* regulatory sequences following the inactivation of *SlMlo1* function by the 19-bp deletion in the coding region. The current unavailability of *SlMlo1* genomic sequences covering the 5′ regulatory region prevents testing these hypotheses at present. Intriguingly, despite lower *SlMlo1* transcript levels, the *ol-2* genotype retained rapid pathogen-responsiveness of *SlMlo1* transcript accumulation following powdery mildew challenge (Fig. 5B).

The *ol-2* allele is the second example, in addition to barley *mlo*-11, of a naturally occurring powdery mildew–resistant *mlo* mutant and one of few examples, besides the *Fer* gene controlling iron uptake (Ling et al. 2002) and the *Cnr* fruit-ripening locus (Manning et al. 2006), of a cloned natural tomato mutant gene. Identification of a second natural *Mlo* loss-of-function allele indicates that this type of mutant might be more prevalent in nature than previously anticipated. In pea, recessively inherited broad-spectrum *er-1* powdery mildew resistance is

TRV::*SlMlo1*   TRV (empty vector)

Fig. 7. Virus-induced gene silencing of *SlMlo1* confers enhanced powdery mildew resistance in wild-type tomato. Leaves of wild-type tomato (cv. Moneymaker) were infiltrated with agrobacteria harboring either a T-DNA with *Tobacco rattle virus* (TRV) carrying no insert (right) or a T-DNA with a TRV derivative harboring an *SlMlo1* fragment (left). At 17 days after bacterial inoculation, leaves were challenged with *Oidium neolycopersici*. The photograph was taken 13 days after fungal inoculation.

another candidate for natural *mlo*-based immunity (Fondevilla et al. 2006). Likewise, collections of novel barley accessions from various regions of the Old World represent further aspirants for natural *mlo* mutants (Czembor and Czembor 2002). It remains, however, to be seen whether *mlo*-11 and *ol*-2 signify the tip of the natural *mlo* mutant iceberg or whether they represent the rare exceptions.

The *mlo*-11 allele, originating from a barley landrace collected in Ethiopia, is characterized by the presence of a complex tandem-repeat array (Piffanelli et al. 2004). Resistance in *mlo*-11 is principally reversible due to a meiotic instability of the respective polymorphism. Based on this reversibility, it has been speculated that the barley *mlo*-11 allele might represent an adaptive balanced polymorphism that could dampen the adverse effects of *mlo* mutants and thus be advantageous in natural barley populations facing varying pathogen pressure (Piffanelli et al. 2004). Unlike barley *mlo*-11, the mutational event in *ol*-2 tomato is rather simple, considered to be essentially meiotically stable and is supposed to represent the result of a fortuitous incident during meiotic replication or recombination. The likely presence of this mutational event in a heterozygous state in the original *S. lycopersicum* var. *cerasiforme* accession LA-1230 (Ciccarese et al. 1998) may indicate that *ol*-2 is also maintained as a beneficial balanced polymorphism in natural tomato populations. Interestingly, the natural epigenetic mutation found at the *Cnr* fruit-ripening locus in the tomato hybrid cv. Liberto has also been reported to be subject to rare somatic reversion events and is also reversible (Manning et al. 2006).

The *SlMlo1* null mutant allele present in *ol*-2 genotypes is presumably free of any evolutionary constraints. This circumstance would allow for the accumulation of further nucleotide alterations in the defective *SlMlo1* gene over time. Absence of any additional sequence variation—besides the 19-bp deletion—in the *SlMlo1* coding region thus points to a recent origin of the *ol*-2 allele. The domestication history of tomato has not yet been fully resolved and is still controversially discussed (Nesbitt and Tanksley 2002; Peralta et al. in press). It was, however, recently proposed that *S. lycopersicum* var. *cerasiforme* represents an admixture of wild and cultivated tomatoes (Nesbitt and Tanksley 2002). Since *ol*-2 was originally identified in a Central American accession of *S. lycopersicum* var. *cerasiforme,* it seems that, reminiscent of barley *mlo*-11 (Piffanelli et al. 2004), the mutation in *ol*-2 originates from a domestication intermediate of a presently cultivated plant species. In addition, as in the case of *mlo*-11, the *ol*-2 mutational event possibly occurred after or coincident with the domestication of wild tomatoes. In the case of barley *mlo*-11, it has been speculated that the emergence of the natural allele might have compensated for the erosion of the natural genetic variation (including race-specific disease resistance) following barley domestication (Piffanelli et al. 2004). Occurrence of a second natural *mlo* allele derived from a domestication intermediate and of supposedly recent origin principally supports this hypothesis. Alternatively, owing to the known adverse affects of mutations in *mlo* genes, such as spontaneous callose deposition and early leaf senescence (Consonni et al. 2006; Piffanelli et al. 2002), *mlo* mutants might have a long-term selective disadvantage in natural settings, resulting in a selective sweep that prevents the discovery of evolutionary older *mlo* mutants that harbor additional DNA footprints. It appears that only the discovery and molecular analysis of more ancient *mlo* mutants will allow discrimination between these two possibilities.

MATERIALS AND METHODS

Plant and fungal material.

Powdery mildew–susceptible *S. lycopersicum* cvs. MM and SM were used as wild-type tomato lines in this study. Homozy-

gous resistant F$_3$ lines R26 and R28, of a cross of the susceptible parent SM and the resistant parental line LC-95 (derived from *S. lycopersicum* var. *cerasiforme* LA-1230), served as representatives of the *ol*-2 resistance locus (De Giovanni et al. 2004). An F$_2$ population of a cross between MM and R28 was used to verify the cosegregation of the *ol*-2 allele with resistance. *Arabidopsis Atmlo2*, *Atmlo6*, and *Atmlo12* single mutants as well as the respective double and triple mutants have been described before (Consonni et al. 2006). Plants were grown at 21°C (day) and 19°C (night) with 60 to 70% relative humidity. The Wageningen isolate of *O. neolycopersici* was maintained on cv. MM as described by Bai and associates (2005).

Infection assays with *O. neolycopersici*.

Disease tests were performed by spraying plants with a suspension of *O. neolycopersici* conidiospores (Bai et al. 2005). The inoculum was prepared from freshly sporulating leaves of heavily infected cv. MM plants. Conidiospores were washed in tap water and were used immediately. Four-week-old tomato plants were inoculated with an inoculum of 2×10^4 spores per
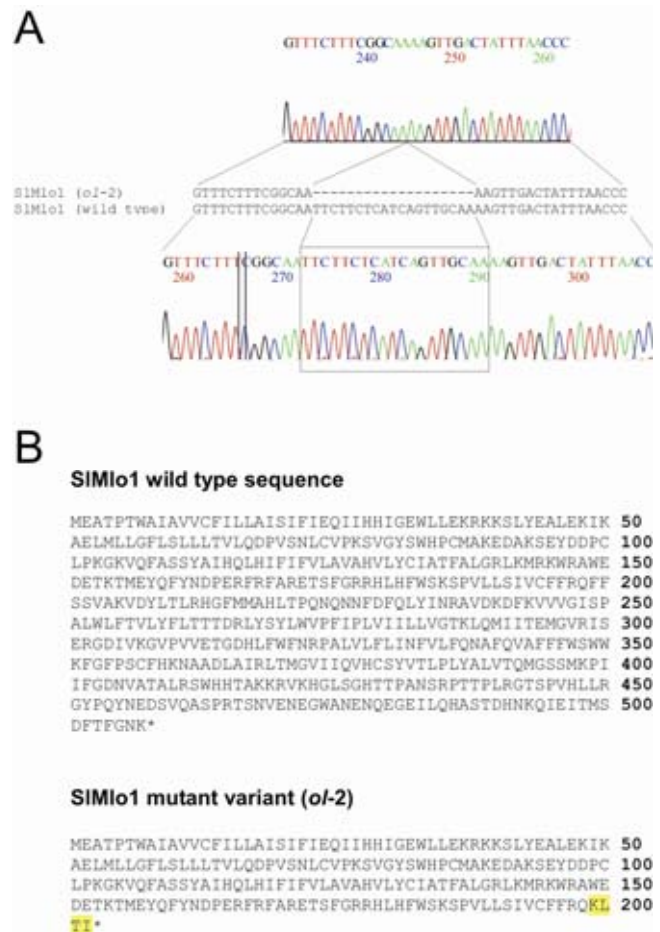
Fig. 8. The *ol*-2 allele represents a *SlMlo1* mutant variant with a 19-bp deletion in the coding region. **A,** Segment of sequence trace files obtained by direct sequencing of a full-size *SlMlo1* reverse transcription-polymerase chain reaction product using either RNA from leaves of resistant line R26 or RNA from wild-type tomato (cv. Super Marmande) as source material. Corresponding sequence alignments of *SlMlo1* wild-type cDNA and the mutant variant emphasize the 19-bp deletion in the *SlMlo1* coding region of *ol*-2 plants (boxed in the *SlMlo1* wild-type sequence). **B,** Consequence of the 19-bp deletion on the conceptual SlMlo1 amino-acid sequence. Upper panel, predicted wild-type SlMlo1 amino-acid sequence; lower panel, predicted mutant SlMlo1 amino-acid sequence. The last four amino acids of the mutant protein variant (highlighted in yellow) are due to the frameshift and are not present in the SlMlo1 wild-type polypeptide.

milliliter, while five-week-old *Arabidopsis* plants were sprayed with an inoculum of 1×10^5 spores per milliliter. A DI scale of 0, 1, 2, and 3 was used to semiquantitatively assess infection phenotypes: 0 = no visible fungal sporulation, 1 = few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies.

Phylogenetic analysis.

For phylogenetic analysis of Mlo proteins, the Phylip 3.66 software package was used (Felsenstein 1989). First, a multiple sequence alignment (generated by CLUSTALW) was established and manually optimized (Supplementary Fig. 1). A distance matrix was calculated by PROTDIST from the multiple sequence alignment and was then transformed into a tree using the neighbor-joining method (NEIGHBOR). The resulting phylogenetic tree was visualized by TREEVIEW. For bootstrap support, SEQBOOT (1,000 replicates), PROTDIST, NEIGHBOR, and CONSENSE algorithms were sequentially applied. All programs were run with standard parameters.

SlMlo mapping.

A core set of 50 tomato IL covering overlapping segments of all 12 tomato chromosomes (Eshed and Zamir 1995) was used for mapping the *SlMlo* genes. Full-size *SlMlo1* and partial *SlMlo2* cDNAs were radioactively labeled and hybridized to filters harboring genomic DNAs of both parental lines digested with a variety of restriction enzymes to detect polymorphisms between the two parental lines. Subsequently, filters representing genomic DNAs of the IL cut with the selected restriction enzyme were used for the actual mapping process.

Transgenic tomato lines.

Tomato EST clones cLEC80N18 (GenBank accession BI923467) and cTOC20K10 (GenBank accession BI931548) representing either incomplete or disordered *SlMlo1* cDNAs were recombined to obtain a bona fide full-length *SlMlo1* cDNA clone. The respective cDNA was shuttled via plasmid pRT101 (harboring *Cauliflower mosaic virus* 35S promoter and terminator sequences; Töpfer et al. 1987) into binary vector pPZP211 (GenBank accession number U10490; Hajdukiewicz et al. 1994), using appropriate restriction sites. *Agrobacterium*-mediated transformation of resistant line R26 and subsequent selection of transgenic lines was performed as described (Knapp et al. 1994).

VIGS.

The TRV vectors, derived from the bipartite TRV virus, were previously described (Liu et al. 2002). The VIGS *SlMlo1* construct was engineered by cloning a 293-bp genomic DNA fragment from the central part of the *SlMlo1* coding region into pTRV2. This DNA fragment, corresponding to a central part of the *SlMlo1* cDNA (bp 806 to 973; Fig. 2) harboring an intron of 94 bp, was amplified with primers 5′-gtgacggatcc CGTATCTTTGGGTGCCATTT-3′ and 5′-gtgacggtacccCAGGG CGATTAAACCAGAAA-3′ (lowercase letters indicate the overhang harboring the restriction sites of *Asp*718 and *Bam*HI) from genomic tomato DNA. The PCR product was digested with *Asp*718 and *Bam*HI and was ligated into pTRV2.

For *Agrobacterium tumefaciens*–mediated virus infection, cultures of *A. tumefaciens* GV3101 containing pTRV1, empty vector control, and each of the constructs derived from pTRV2 were grown and harvested as described (van der Hoorn et al. 2000). Equal volumes of cultures harboring pTRV1 and pTRV2 were mixed and were subsequently infiltrated into the lower (adaxial) side of cotyledons of 10-day-old tomato seedlings, using a 1-ml syringe lacking a needle. Two weeks after infiltration, plants were inoculated with *O. neolycopersici*.

RNA isolation and semiquantitative RT-PCR.

To monitor *SlMlo1* transcript accumulation, total RNAs were extracted using the Trizol reagent (Invitrogen Life Technologies Co., Carlsbad, CA, U.S.A.) and were purified with a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). cDNAs were synthesized with oligo(dT)$_{18}$ primers using the SuperScript III RTS first-strand cDNA synthesis kit (Invitrogen).

For RT-PCR with MM and R28 (Fig. 5A), actin cDNA was amplified with primers 5′-GCTCCACCAGAGAGGAAATAC AGT-3′ and 5′-CATACTCTGCCTTTGCAATCCA-3′. The expression of *SlMLO1* was monitored by marker cTOC20 by amplifying a transcript with primers 5′-TGTGGTTTGCTTCA TCTTGC-3′ and 5′-ACCAACACTCTTGGGGACAC-3′. For RT-PCR with F$_2$ plants (Fig. 5B), actin cDNA was amplified with primers published by Ditt and associates (2001). The expression of *SlMlo1* was monitored by amplifying sequences flanking the 19-bp deletion in the *SlMLO1* cDNA with primers 5′-ATGGGAGGATGAAACAAAAAC-3′ and 5′-ACAATCGA TCGGTAGTAGTCAGA-3′.

For analysis of *SlMlo1* expression in T$_1$ transgenic lines (Fig. 6A and B), oligonucleotides actin1 (5′-TGAGCCTCATT TTAAGCTC-3′) and actin4 (5′-GAAATACAACTCTTCTACA TA-3′) were used for the amplification of the 1.3-kb actin amplicon serving as internal reference. Transgenic *SlMlo1* was selectively amplified by oligos SlMlo2 (5′-TTCCCATCTTGC-TTTCATAAG-3′, binding at the 3′ end of SlMlo1, forward primer) and 35Sterm (5′-CTACTCACACATTATTCTGG-3′, binding in the *Cauliflower mosaic virus* 35S transcriptional terminator sequence present in the binary vector, reverse primer), yielding a 0.55-kb PCR fragment. Real-time PCR was performed using SYBR green chemistry (Brilliant SYBR Green QPCR core reagent kit; Stratagene, La Jolla, CA, U.S.A.) on a iQ5 Multicolor real-time PCR detection system (Bio-Red, Munched, Germany). Relative transcript levels were calculated on the basis of the $\Delta\Delta$ct method.

Cosegregation of the 19-bp deletion in *SlMlo1* with powdery mildew resistance.

Total DNA was extracted from leaves of F$_2$ plants using the cetyltrimethylammonium bromide DNA isolation method (Brugmans et al. 2003) and was used for genotyping the F$_2$ progeny with a codominant M/SlMlo1 marker. This PCR marker, spanning the mutant site in *ol-2*, was amplified with oligonucleotides 5′-ACCCTTAAGAAACTAGGGCAAA-3′ (forward primer) and 5′-ACCATCATGAACCCATGTCT-3′ (reverse primer) with an annealing temperature of 55°C. Product size is 197 bp for wild-type *SlMlo1* (*Ol-2* genotype) and 178 bp for the homozygous *ol-2* mutant genotype, while heterozygous individuals exhibit the presence of both amplicons.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CLUSTALW multiple sequence alignment: www.ebi.ac.uk/clustalw/
NCBI Unigene database: www.ncbi.nlm.nih.gov/sites/entrez?db=unigene
NCBI UniGene database, tomato Les.746:
www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Les&CID=746
PHYLIP phylogenetic analysis: evolution.gs.washington.edu/phylip.html
University of Califormia Davis Tomato Genetics Resource Center (TGRC):
tgrc.ucdavis.edu/Data/Acc/dataframe.aspx?start=GIS_dataoption.aspx&navstart=nav.html
TREEVIEW phylogenetic tree visualization:
taxonomy.zoology.gla.ac.uk/rod/treeview.html