Exploring recessive resistance to the powdery mildew disease

Stefano Pavan

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General Introduction

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General introduction

Stefano Pavan[1,2], Luigi Ricciardi[2], Richard G. F. Visser[1] and Yuling Bai[1]

[1]Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

1. Plant immunity

Although plants are exposed to a plethora of potential pathogens, disease onset is most of the times prevented by innate mechanisms of immunity, acting either constitutively or upon pathogen recognition.

Constitutive barriers to pathogen colonization include physical elements, such as waxy cuticle layers and rigid cell walls, and anti-microbial secondary metabolites, collectively referred to as phytoanticipins (van Etten et al. 1994). Not surprisingly, it has been shown that adapted pathogens evolved methods to detoxify phytoanticipins. For example, the tomato fungal pathogen *Fusarium oxysporum* hydrolyzes the constitutively expressed steroidal glicoalkaloid α-tomatine (Ito et al. 2004).

Pathogens overcoming constitutive barriers can be recognized by plant receptors, leading to the induction of defence responses. At the molecular level, these receptors may fall into two main classes (Chisholm et al. 2006). The most abundant class is intracellular and contains a nucleotide binding site (NBS), which is thought to function in signal transduction, and a leucine-rich repeat (LRR) domain, involved in the recognition process. The other is characterized by an extracellular LRR domain. In the frame of plant-pathogen co-evolution, Jones and Dangl (2006) proposed a model distinguishing two possible types of induced immunity. One, termed PAMP-triggered immunity or PTI, follows the recognition of molecular patterns (pathogen-associated molecular patterns or PAMPs) which are conserved within a pathogenic Kingdom (Bacteria, Stramenopila, Fungi) and have no role in virulence (i.e. are not important for the infection process). The other, referred to as effector-triggered immunity or ETI, derives from the recognition of effector proteins which are evolved by particular strains of a pathogen species in order to suppress previously effective PTI or ETI. Virulence activity of effectors has been in many cases demonstrated to occur through the interaction with cognate host proteins, referred to as plant virulence targets.

Thomma et al. (2011) pointed out that Jones and Dangl's strict distinction between PAMPs and effectors cannot be maintained. Indeed, classically defined PAMPs, such as bacterial flagellin or lipopolysaccharides, have been shown to play a direct role in virulence and in suppressing plant defence (Taguchi et al. 2010; Tellström et al. 2007;). In addition, there are proteins that can be regarded as PAMPs and effectors at the same time. For instance, Ecp6 and Avr4 proteins can be considered effectors, as they are able to suppress plant defence, but also PAMPs, as they are widely conserved across different fungal species (Bolton et al. 2008; de Jonge and Thomma, 2009). Besides the lack of a clear border between PAMPs and effectors (and therefore between PTI and ETI), another complication to the Jones and

Dangl's model is given by evidence for the existence of receptors perceiving modifications in plant molecular patterns derived from the infection process (damage-associated molecular patterns or DAMPs, Zipfel and Robatzek, 2010). Taken together, evidence above reported leads to a model for molecular mechanisms at the basis of inducible immunity in which a battery of plant receptors senses danger signals from both microbial and host origin and triggers appropriate defence responses (Thomma et al. 2011). On the other hand, during co-evolution with plants, pathogens have differentiated a series of effectors which can be both conserved and strain-specific proteins promoting pathogenesis.

Following the recognition event, phytormones are pivotal in tuning plant defence to pathogens with different lifestyles. Salicylic acid (SA) has a major role in defence signalling towards biotrophs, whereas jasmonic acid (JA) and ethylene (ET) have this role towards necrotrophs. An antagonistic cross-talk between SA and JA/ET signalling pathways is generally accepted (Glazebrook, 2005). Additional hormones, namely abscissic acid, auxin, gibberellic acid, cytokinin, brassinosteroids and peptide hormones have also recently been implicated in the plant defence signalling network, indicating that the control of growth, development and defence is interconnected and allows plants to use their resources in a cost-efficient manner (Pieterse et al. 2009).

Besides locally at the infection sites, plant defence responses can also be induced systemically (i.e. from a site of infection to undamaged distal tissues). Two kinds of induced immunity are known to occur in plants: systemic acquired resistance (SAR), mediated by SA and effective against biotrophs, and induced systemic resistance (ISR), dependent on the JA/ET signalling pathway and effective against necrotrophs.

Common responses to biotrophs include the formation of cell-wall appositions, polarized secretion at plant-pathogen interaction sites, accumulation of particular pathogen-related proteins (e.g. PR1) and reactive oxygen species (ROS), and finally programmed cell death (hypersensitive response or HR, Jones and Dangl, 2006). Obviously, cell death is not an effective defence mechanism against necrotrophic pathogens (Lorang et al. 2007). Defence reactions to necrotrophs, reviewed by Lazniewska et al. (2010), include callose and lignin apposition and the production of antimicrobial compounds such as phytoalexins (e.g. Arabidopsis camalexin) and defensins (e.g. PDF1.2).

2. Breeding for disease resistance

The availability of cultivars which are resistant to main diseases is a fundamental pre-requisite

for sustainable agriculture, providing good qualitative and quantitative production standards by using low environmental impact practices. In addition, it allows to reduce costs due to pesticide applications. Therefore, the identification and use of disease resistance genes is a pivotal activity of plant breeding.

2.1. Dominant R-genes

More than fifty years ago, Harold Henry Flor showed that the outcome of the interaction between flax and the rust pathogen *Melampsora lini* was dependent on the allelic situation at two *loci*. Indeed, disease resistance only occurred upon simultaneous presence of a dominant plant allele, termed R-gene, and a matching dominant pathogen allele, termed Avr-gene (Flor, 1955). Along the years, "gene-for-gene" relationships similar to the one studied by Flor have been documented in many interactions involving crop species and biotrophs, the latter being viruses, bacteria, oomycetes, fungi and even parasitic plants. It is now clear that gene-for-gene resistance is referable to the recognition process which takes place between plant receptors and cognate pathogen counterparts. As these are often rapidly evolving effectors, an important practical consequence is that immunity conferred by R-genes is generally not durable in the field.

Nowadays, breeding for resistance to biotrophs is still mostly referable to the pioneering work of Flor. R-genes are continuously searched in the germplasm of cultivated or wild species, and used, through traditional or biotechnological approaches, for cultivar development. Pyramiding of different R-genes in the same genotype has been proposed as a breeding tool to increase durability of resistance (Tan et al. 2010).

2.2. Recessive resistance genes

A number of recessive alleles conferring disease resistance has been reported. When characterized at the molecular level, they have been shown to derive from loss-of-function mutations of genes which are thus required for susceptibility (in Chapter 6 of this thesis referred to as susceptibility genes or S-genes). Notably, all the susceptibility genes which have been isolated in cultivated species have been predicted to encode for virulence targets manipulated by effectors in order to promote pathogenesis.

2.2.1 Recessive resistance to viruses and bacteria

Recessive genes conferring resistance to viruses, especially to those belonging to the family of Potyviridae, are quite common (Diaz-Pendon et al. 2004). Remarkably, their isolation in barley, melon, lettuce, pepper, pea and tomato has invariantly revealed loss-of-function mutations in isoforms of the component of the eukaryotic initiation factor eIF4E. It is thought that these mutations prevent the interaction of eIF4E with the viral effector VPg, which is responsible for recruiting the translation initiation complex to the viral RNA (Robaglia and Caranta, 2006).

Two rice recessive genes, *xa5* and *xa13*, are associated to resistance to the bacterium *Xanthomonas oryzae* pv. *oryzae*. *xa5* carries a single amino acid substitution in the subunit TFIIAγ of the transcription factor IIA, which is likely to hamper effector-mediated transcription of genes important for nutrient availability (Iyer-Pascuzzi and McCouch, 2007). The *xa13* allele contains a mutation in the promoter region of a gene encoding a plasma membrane protein. This hampers gene up-regulation by the bacterial effector PthXo1, which normally leads to disease establishment through a still unknown mechanism (Yang et al. 2006).

2.2.2 mlo recessive resistance to powdery mildew fungi

The powdery mildew disease is caused by obligate biotrophic fungi of the Ascomycete order of Erysiphales, encompassing one family (Erysiphaceae), 16 genera and about 650 species. Erysiphales imperfect fungi (i.e. those whose teleomorph is not known) are grouped in the genus *Oidium.* Worldwide, powdery mildew fungi are able to colonize about 10,000 distinct dicotyledonous and monocotyledonous plant species and cause severe losses in many agricultural systems (Takamatsu 2004).

Among cultivated species, recessively inherited resistance to powdery mildew fungi has been reported to occur in barley (*mlo* resistance), tomato (*ol-2* resistance) and pea (*er1* resistance) (Büschges et al. 1997; Bai et al. 2005; Fondevilla et al. 2006). Molecular characterization of *mlo* resistance revealed it derives from loss-of-function mutations of a gene (*MLO*) encoding for a plasma membrane protein with seven transmembrane domains, topologically reminiscent of metazoan G-protein coupled receptors.

MLO proteins accumulate in plasma membrane microdomains at sites of interactions with powdery mildew fungi (Bhat et al. 2005), possibly under the control of the FERONIA receptor-like kinase (Kessler et al. 2010). MLO activity is able to modulate the arrangement of cell actin cytoskeleton, since *mlo* mutant epidermal cells exhibit strong and fast focusing of

actin cytoskeleton at interaction sites with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) at different time points (14, 18, 24 and 36 hours) after artificial inoculation (Opalski et al. 2005). In turn, actin cytoskeleton focusing is believed to be essential to provide routes for polarized defense responses, associated to the formation of cell wall apposition, limiting fungal entry in *mlo* mutants (Opalski et al. 2005; Underwood and Somerville, 2008). Studies with the model species Arabidopsis suggest two actin-dependent polarized secretion systems which are modulated by a pathway including MLO proteins, small monomeric G-proteins of the Rho of plants (ROP) family and actin-binding proteins of the actin-depolymerizing factor (ADF)/cofilin family (Miklis et al. 2007; Underwood and Somerville 2008; Fig. 1). One involves vesicle exocytosis through the formation of a ternary SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) complex between the plasma membrane resident proteins PEN1 and SNAP33 and one of the vesicle-associated proteins VAMP721 and VAMP722. The other involves transport across the plasma membrane, mediated by the ATP binding cassette (ABC) transporter PEN3, of a toxic compound produced by the enzymatic activity of the peroxisome-associated glycosil hydrolase PEN2.
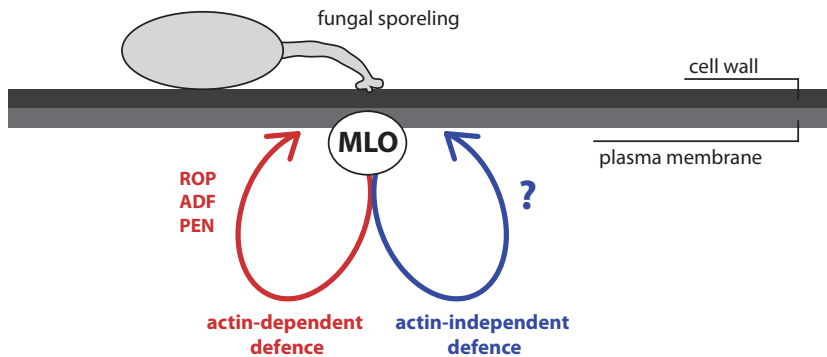
Fig. 1 Model for the role of MLO proteins in plant defense (adapted from Miklis et al. 2007).
MLO modulates two defense pathways in epidermal cells in contact with the powdery mildew fungus. One is dependent on actin cytoskeleton and involves plant ROP G-proteins, actin-binding proteins of the actin-depolymerizing factor (ADF)/cofilin family, and PEN proteins. The actin-independent defence pathway is unknown. *Blumeria graminis* f. sp. *hordei* might target MLO in order to suppress defense responses at the cell periphery

An important finding is that in an early infection stage, 4 hours after *Bgh* inoculation, actin dynamics at plant-fungus interaction sites is similar in *MLO* and *mlo* genotypes (Opalski et al. 2005). This suggests that MLO is either directly or indirectly targeted by fungal effectors in order to interfere with cytoskeleton-associated defense responses and promote pathogenesis.

As complete genetic and pharmacological interference with actin cytoskeleton only partially compromises *mlo* resistance, MLO is thought to be involved in actin independent defense mechanisms as well (Miklis et al. 2007) (Fig. 1).

2.3. Use of recessive resistance in breeding

Although it is not as exploited in breeding as dominant resistance, recessive resistance appears to be generally very durable. Pepper *pvr1* allele, associated to resistance to potyviruses, is for example still effective more than 50 years after its introduction in agriculture (Kang et al. 2005). Concerning plant-powdery mildew interactions, *mlo* resistance in barley and *er1* resistance in pea are used in breeding for several decades. Tomato *ol-2* resistance has been only recently identified, nonetheless it has been shown to be effective towards several isolates of the powdery mildew fungus *Oidium neolycopersici* (Bai et al. 2005). Several authors pointed out that *mlo* resistance is mechanistically similar to the most durable form of plant immunity, referred to as non-host resistance, which is exhibited by plant species outside the host range of a given pathogen species (Trujillo et al. 2004; Ellis et al. 2006; Humpry et al. 2006; Miklis et al. 2007). Common features between *mlo* and non-host powdery mildew immunities include the histology of the defense mechanism, which occurs before fungal penetration and is associated to the formation of cell wall appositions (papillae), independence from SA, JA and ET signaling pathways and dependence on actin cytoskeleton and PEN genes.

3. Thesis outline

This thesis was initially aimed to fine-map and isolate the tomato recessive gene *ol-2,* conferring resistance to powdery caused by the fungus *Oidium neolycopersici*. In Chapter 2, we provide evidence for genetic and physical association between the *ol-2* locus and tomato *MLO* homolog sequences. Chapter 3 reports the characterization of *ol-2* as a loss-of-function allele of a tomato *MLO* homolog, *SlMLO1*, on tomato chromosome 4. Chapter 4 focuses on the identification and characterization of recessive powdery mildew resistance in pea.

We describe the obtainment of a chemically-induced mutant at the *er1* locus, associated to resistance to the powdery mildew fungus *Erysiphe pisi*. In addition, we report evidence associating *er1* resistance to loss-of-function mutations of another *MLO* homolog, *PsMLO1*.

Results in Chapter 3 and 4 clearly suggest the possibility that *mlo*-based resistance can be generally pursued in plants. Therefore, in the last experimental part of the thesis (Chapter 5) we address the identification of candidate MLO susceptibility isoforms in several Solanaceae species affected by the powdery mildew disease. Furthermore, we perform a domain-restricted comparative analysis in dicots MLO homologs putatively involved in powdery mildew susceptibility, resulting in the identification of conserved motifs in transmembrane protein regions.

Based on the results obtained, in Chapter 6 we review literature on plant genes required for disease susceptibility and discuss the opportunities and perspectives of a breeding strategy based on their inactivation.

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Chapter 2

Map- vs. homology-based cloning for the recessive
gene *ol-2* conferring resistance to tomato powdery mildew

Chapter 2

Map- vs. homology-based cloning for the recessive gene *ol-2* conferring resistance to tomato powdery mildew

Stefano Pavan[1,2]**, Zheng Zheng**[1]**, Mariya Borisova**[3]**, Petra van den Berg**[1]**, Concetta Lotti**[4]**, Claudio De Giovanni**[2]**, Pim Lindhout**[1]**, Hans de Jong**[3]**, Luigi Ricciardi**[2]**, Richard G. F. Visser**[1]**,Yuling Bai**[1,5]

[1]Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Environmental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, Bari 70126, Italy

[3]Laboratory of Genetics, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

[4]Department of Agro-Environmental Science, Chemistry and Crop Protection, University of Foggia, Via Napoli 25, Foggia 71100, Italy

Abstract

The recessive gene *ol-2* confers papilla-associated and race-non-specific resistance to tomato powdery mildew caused by *O. neolycopersici*. In order to facilitate marker assisted selection (MAS) in practical breeding programmes, we identified two simple sequence repeat (SSR) markers and one cleaved amplified polymorphic sequence (CAPS) marker which are linked to the resistance locus and co-dominantly inherited. Aiming to provide a base for *ol-2* positional cloning, we used a large segregating F_2 population to merge these markers with all the *ol-2* linked amplified fragment length polymorphism (AFLP®) markers previously identified in an integrated genetic map. By screening a tomato bacterial artificial chromosome (BAC) library, we detected two BAC clones containing two expressed sequence tags (ESTs) homologous to the gene *mlo*, responsible for powdery mildew resistance in barley, as well as an *ol-2*-linked marker. Chromosomal mapping by Fluorescence *in situ* Hybridization (FISH) revealed major signals of the two BAC DNAs in the pericentromeric heterochromatin of the short arm of chromosome 4, in the same region where the *ol-2* gene was previously mapped. The genetic and cytogenetic co-localisation between *ol-2* and tomato *mlo*-homologue(s), in addition to the similarity of *ol-2* and *mlo* resistances for both genetic and phytopathological characteristics, suggests that *ol-2* is likely a *mlo*-homologue. Thus, a homology-based cloning approach could be more suitable than positional cloning for *ol-2* isolation.

Introduction

Tomato powdery mildew caused by *Oidium neolycopersici* has recently been recognized as a worldwide emerging pathogen on tomato (Jones et al. 2001, Kiss et al. 2001). Several dominant genes have been characterized, which confer resistance to *O. neolycopersici* and originate from wild tomato species (Bai et al. 2005; Kozik 1993; Lindhout et al. 1994). The exploitation of these genes in plant breeding is limited due to linkage drag phenomena, consisting in unfavourable wild alleles closely linked to the resistance loci (Zeven et al. 1983; Young and Tanksley 1989). In addition, resistance conferred by the dominant *Ol*-genes is based on race-specific hypersensitive response (HR), thus is likely not durable. In contrast, the recessive gene *ol-2*, located around the centromere of chromosome 4 (De Giovanni et al. 2004), derives from the germplasm of the cultivated tomato species, *Solanum lycopersicum* (Ciccarese et al. 1998), and confers race-non-specific resistance via papilla formation, a non-HR-based mechanism (Bai et al. 2005).

The introduction of *ol-2* resistance into tomato cultivars via traditional breeding methods is complicated by its recessive nature, as the selection of the *ol-2* allele needs to be tested in the selfing progeny of each backcross generation. Although marker assisted selection (MAS) with co-dominant molecular markers would allow the breeder to perform the selection of the *ol-2* allele in backcross generations, the low genetic variation within *S. lycopersicum* (Miller and Tanksley 1990) hindered the identification of polymorphic co-dominant markers linked to the resistance locus. So far, mainly amplified fragment length polymorphism (AFLP®) markers have been identified for the *ol-2* region (De Giovanni et al. 2004; Ricciardi et al. 2007). In practical breeding, AFLP® markers cannot offer efficient MAS as they are dominant and require advanced equipment. Moreover, AFLP® markers are patented and thus not utilizable by most breeding companies. Although several efforts were made, the conversion of *ol-2* linked AFLP® markers into PCR markers failed (Ricciardi et al. 2007).

Similarly to *ol-2*, the barley gene *mlo* (Büschges et al. 1997) is recessively inherited and confers durable resistance to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) through the apposition of papillae at the cell wall (Piffanelli et al. 2002). The similarity of *ol-2* and *mlo* resistances for both genetic (recessive) and phytopathological (papilla-based mechanism) characteristics may indicate that *ol-2* confers *mlo*-based durable resistance.

In the present work, several attempts were carried out in order to obtain new simple PCR co-dominant markers linked to *ol-2*, allowing selection of the resistance via MAS. Aiming at *ol-2* positional cloning, a segregating F$_2$ population of 316 individuals was analyzed to generate an integrated map of the *ol-2* region, including all the dominant AFLP® markers

reported so far. Bacterial artificial chromosome (BAC) library screening and Fluorescence *in situ* Hybridization (FISH) were used to investigate the possibility that *ol-2* resistance is mediated by a *mlo*-based mechanism.

Materials and methods

Plant material

A F_2 mapping population of 316 individuals was used, which was derived from a cross between the resistant F_3 line R28 and the susceptible *S. lycopersicum* cv Moneymaker (MM). The R28 line homozygous for the *ol-2* allele was developed from a cross between the resistant line LC-95 of *S. lycopersicum* var. *cerasiforme* and the susceptible *S. lycopersicum* cv Super Marmande (SM) (Ciccarese et al. 1998).

Fungal material and disease test

The *O. neolycopersici* used in this study was collected from infected tomato plants in The Netherlands. One-month old plants were inoculated with a suspension of conidia as described by Bai et al. (2003). The inoculated plants were grown in a greenhouse compartment at 20 ± 2°C with 70±15% relative humidity (RH) and day-length of 16 hours. Disease evaluation was carried out twice, 12 and 15 days post inoculation (dpi), on the basis of presence or absence of fungal sporulation.

Marker analysis

Total DNA was extracted from plant leaves two days before inoculation by using a rapid cetyl trimethyl ammonium bromide (CTAB) isolation method (Brugmans et al. 2003).

Seven simple sequence repeat (SSR) markers on tomato chromosome 4 were chosen among the ones published by Suliman-Pollatschek et al. (2003) (Table 1). PCR reactions (10 µl) contained 50 ng of genomic DNA, 1x PCR buffer, 0.5 pmol of each forward and reverse primer (one of which was labelled with IRD700 or IRD800), 0.2 mM dNTPs and 0.5 units of Taq-polymerase in demi water. PCR conditions were: 1 cycle at 94°C for 3 min followed by 30 cycles of 30s at 94°C, 45s at the optimal annealing temperature, 45s at 72°C, and a final extension of 7min at 72°C. Amplified products were scored on 6% polyacrylamide gel with a

LICOR 4200 DNA sequencer and on 2.5% agarose gel stained with ethidium bromide.

The cleaved amplified polymorphic sequence (CAPS) marker U3-2*Hha*I (Table 1) was obtained by designing primers on the SCAU3$_{1500}$ sequence characterized amplified region (SCAR) sequence reported by De Giovanni et al. (2004) with the software Primer3 (Rozen and Skaletsky 2000) and digesting the amplification products with different restriction enzymes. PCR and digestion were carried out according to the conditions reported by Bai et al. (2003).

Nine markers with known sequence mapped on tomato chromosome 4 and two tomato expressed sequence tag (EST) sequences (cLEC-80-N18 and cTOC-20-K10) homologous to the barley *mlo* gene were selected from the Sol Genomics Database (http://www.sgn.cornell. edu, Table 1). Locus specific primers were designed to amplify genomic DNA as described by Bai et al. (2003). All the amplification products were subsequently digested with several restriction enzymes as described by Bai et al. (2004). AFLP® fingerprints were generated on a LICOR 4200 DNA sequencer as described by Bai et al. (2003).

Linkage analysis

The software JoinMap 3.0 (van Ooijen and Voorrips 2001) was used to generate an integrated linkage map of the *ol-2* chromosomal region. A logarithm of odds (LOD) score of 4.0 was used as linkage threshold and the Kosambi mapping function was used to convert recombination frequency into genetic map distance.

BAC screening and Fluorescence in situ Hybridization

The *Hind*III BAC library of *S. lycopersicum* cv Heinz 1706 (Budiman et al. 2000) was screened for the presence of the tomato *mlo*-homologous EST sequences cLEC-80-N18 and cTOC-20-K10 and the *ol-2* linked SCAR sequence U3-2. The screening was performed by PCR with corresponding primers and conditions presented in Table 1.

FISH of the BACs 184L18 and 205G16 on pachytene complements was performed as described by Zhong et al. (1999) and Chang (2004). In brief: flower buds of tomato cv Tiny Tim containing anthers at meiotic prophase I were fixed in ethanol acetic acid (1:3). After digesting the cell walls with a pectolytic enzyme mix, cells were spread in acetic acid 45% and post-fixed in ethanol-acetic acid (3:1). BAC clones 184L18 and 205G16 were directly labelled with FITC-dUTP and Cy3.5-dCTP, respectively. The probes were hybridised on the cell complements together with excess of Cot100 genomic DNA for blocking repetitive DNA sequences. The slides were counterstained with DAPI and studied under a fluorescence

Table 1: Markers tested to reveal polymorphisms between the parental lines *Solanum lycopersicum* cv. MM and R28.

Marker name	Type	Source	Primers sequence (5'→3')	Size (bp)	Tm(℃)
Tom316	SSR	Suliman-Pollatschek et al 2002	GAGTTGTTCTTTGGTTGTTT TAGATTTTTCGTGTAGATGT	134+ 138	50
Tom332	SSR	Suliman-Pollatschek *et al.* 2002	GATACCATTAAAGCTCATTC GGTTTCCGTCATTATGTCAG	115+ 120	50
Tom 95-96	SSR	Suliman-Pollatschek *et al.* 2002	GTGGATGGATATGTGTGA GCACGGTAGGTCGCAGGCA	-	46
Tom268-269	SSR	Suliman-Pollatschek *et al.* 2002	AGGGTATGAGATGAGACAAT TTTTACCTTCTTTACTTGGA	-	48
Tom292-293	SSR	Suliman-Pollatschek *et al.* 2002	ATGTACTCTAATGGATGTTT ATTCATAGCATTGTCATAGG	-	47
Tom160-161	SSR	Suliman-Pollatschek *et al.* 2002	TGCTGAAGAATACAATGTTA CC ATTGTTGGATGCTCAGTTTG	-	48
Tom61-62	SSR	Suliman-Pollatschek *et al.* 2002	GGCAAAGAAGGACCCAGAG C GGTGCCTAAAAAAGTTAAAT	-	48
U3-2	SCAR	Present work	AGTGGTTGGCGGATAGGTG TTGGCAAGGTGGGAAAACT	1200	55
T0707	COS	http://www.sgn.cornell.edu	TCGTGGATTATGGGCTTTCT T GGTAAGGCTGCAACACATCA	458	50
TG339	RFLP	http://www.sgn.cornell.edu	GAAACCTTACCCCTCTA CGCTGTTTCTTGCCATTT	436	46
TG652	RFLP	http://www.sgn.cornell.edu	CGGGGATCTGATAAGGAAG G ATAAGGGATCGTCAAGGAGT AACA	542	59
T1405	COS	http://www.sgn.cornell.edu	CACCAACAACTAGCCCTTGA AAGCAATTCCTCCAGCTTCA	535	55
cLEC7B23	EST	http://www.sgn.cornell.edu	GGAGAACACGGCTACCTCA G GGAGAACACGGCTACCTCA G	600	55
CT173	RFLP	http://www.sgn.cornell.edu	TGAGCACGGTATTACGCAG TTAGTAGTATGTGTGGCAGT CAC	500	55
TG2FS/R	RFLP	http://www.sgn.cornell.edu	TGTTGAAGAAGGCTCAGCAA TGCCCCTGTATATCCCTCAG	241	55

Table 1 (continued)

Marker name	Type	Source	Primers sequence (5'→3')	Size (bp)	Tm(℃)
TG2FL/R	RFLP	http://www.sgn.cornell.edu	CCTCAATCCCAGGAATTTGT TGCCCCTGTATATCCCTCAG	403	57
TG516	RFLP	http://www.sgn.cornell.edu	GGCACTTCGCAAAACTGAAT GGTACGGCGGGTTTAAGACT	349	55
cTOC-20-K10	EST	http://www.sgn.cornell.edu	TGTGGTTTGCTTCATCTTGC ACCAACACTCTTGGGGACAC	360	56
cLEC-80-N18	EST	http://www.sgn.cornell.edu	CGTATCTTTGGGTGCCATTT CAGGGCGATTAAACCAGAAA	260	56

microscope with a high-sensitive CCD camera. Image capturing and analysis followed the procedures as described by Chang (2004).

Results

Disease test

A disease test was performed on the 316 individuals of the F_2 population of MM x R28. Ten MM and ten R28 plants were used as susceptible and resistant controls, respectively. Ten days post-inoculation (dpi) all the MM plants showed powdery mildew sporulation on the upper side of the leaves, whereas no symptoms could be detected on R28 individuals. The F_2 population was rated for resistance at 12 and 15 dpi. In total, there were 240 susceptible and 76 resistant F_2 individuals, which fit into the expected 3:1 ratio for a single recessive gene model ($\chi^2=0.152$).

An integrated map for the chromosomal region harbouring ol-2

Aiming to fine-map the *ol-2* gene, we designed primers for nine chromosome 4 specific sequences located in the region bearing *ol-2* (Table 1). All the primer pairs amplified monomorphic fragments between MM and R28, which were then digested with different
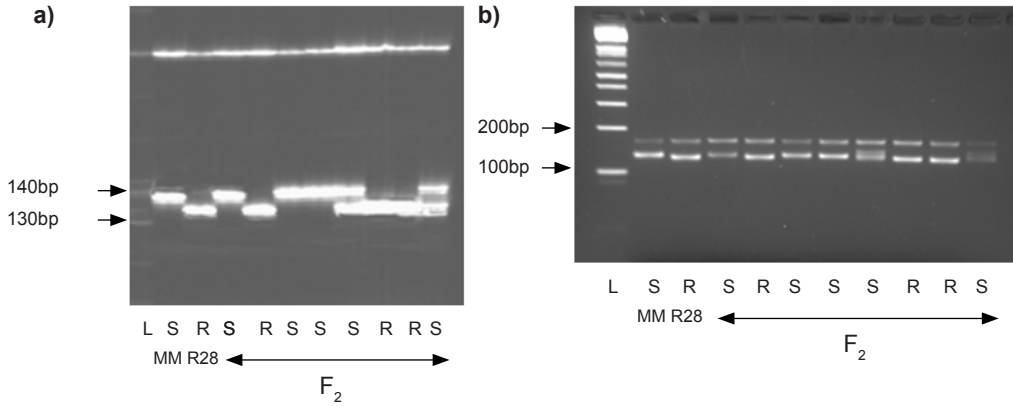
Fig. 1. SSR marker Tom316 tested on the susceptible (S) parent Moneymaker (MM), the resistant (R) parent R28 and eight F_2 individuals. Co-dominant length polymorphism is revealed on both 6% polyacrylamide gel (a) and 2.5% agarose gels (b). Lane L (ladder) points the molecular weight (bp).
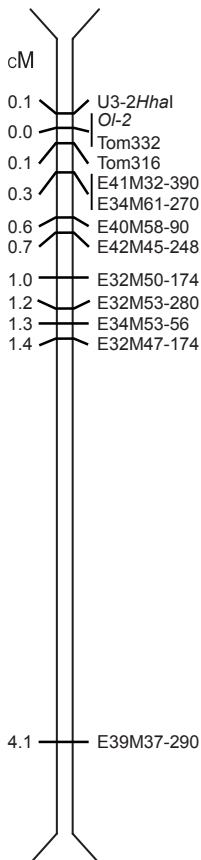

Fig. 2 Integrated linkage map of the *Ol-2* locus region on tomato chromosome 4. Genetic distances were estimated by using Kosambi's mapping function.

restriction enzymes. Unfortunately, no CAPS polymorphism was detected.

SSR markers have been reported to be powerful in detecting polymorphisms within *S. lycopersicum*. Therefore, we selected seven SSRs mapped on chromosome 4 and two of them, Tom316 and Tom332, revealed length polymorphisms between MM and R28 on 6% polyacrylamide gel. Polymorphism of the SSR marker Tom316 was also detectable on 2.5% agarose gel (Fig. 1).

All the *ol-2* linked markers previously identified were used to search for polymorphisms between MM and R28. We failed to amplify the SCAU3$_{1500}$ SCAR sequence with the primers published by De Giovanni et al. (2004), thus new primers were designed. The amplification product, named U3-2, was subsequently digested and converted into a co-dominant CAPS marker, U3-2*Hha*I. Three out of the eight *ol-2* linked AFLP® markers so far reported (Table 1), E34M61-270, E40M56-90 and E41M32-390, showed polymorphism between MM and R28.

By screening the F$_2$ population of MM and R28 with the three polymorphic AFLP® markers, the CAPS marker U3-2*Hha*I and the two SSR Tom316 and Tom332, a map of the *ol-2* chromosomal region was constructed and integrated with the *ol-2* maps reported by De Giovanni et al. (2004) and Ricciardi et al. (2007) (Fig. 2). Remarkably, almost identical segregation patterns were observed for nearly all the markers and the resistance, indicating that the *ol-2* gene is located in a chromosomal region characterized by recombination suppression.

Physical co-localisation of the ol-2 gene and tomato mlo-homologue(s)

As both the genetic and phytopathological features of the *ol-2*-associated resistance mimic the well-characterised *mlo*-based resistance in barley, it is logical to argue that *ol-2* is likely a *mlo* homologue. By inspecting the tomato EST collection, two tomato ESTs (cLEC-80-N18 and cTOC-20-K10) were identified that are homologous to barley *mlo*. Unfortunately, genetic mapping of these two ESTs in the F$_2$ population was hampered by the lack of polymorphism between MM and R28. To visualise the relative chromosomal location of the *ol-2* gene and tomato *mlo*-homologue(s), we first screened the Heinz BAC library with PCR primers amplifying the tomato *mlo*-homologous ESTs cLEC-80-N18 and cTOC-20-K10 (Table 1). Two BAC clones, 184L18 and 205G16, were identified, which were positive for both the ESTs. These two BAC clones were further inspected for the presence of the *ol-2* linked SCAR sequence U3-2. Remarkably, both BACs were positive for the U3-2 sequence, suggesting the co-localization of the *ol-2* gene with *mlo* homologue(s). In addition to the expected PCR fragment of 1200bp for the U3-2 sequence, other amplification products were obtained,

indicating that the two BACs contain repetitive sequences.

FISH of 184L18 and 205G16 BACs on pachytene chromosomes of tomato revealed in both cases major signals on chromosome 4, in the heterochromatin block of the short arm near the centromere (Fig. 3). In spite of blocking the BAC probes with Cot100 repetitive DNA, we also found small signals in heterochromatic regions of other chromosomes, which indicate
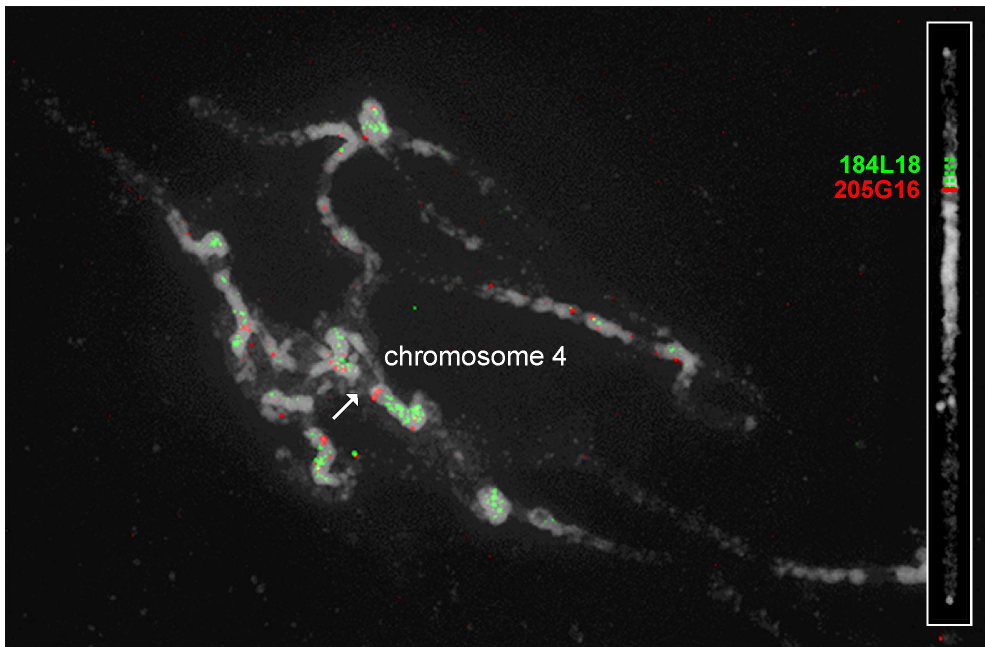
Fig. 3. FISH signals of the BAC clones 184L18 (labelled with FITC-dUTP) and 205G16 (labelled with Cy3.5-dCTP). The BACs display small and variable signals, close together, in the heterochromatic area near the centromere of chromosome 4. 205G16 gives a major focus next to the centromere and few minor signals more distally, whereas 184L18 produces a variable number of smaller foci in the whole pericentromeric heterochromatic region. Some of the green and red signals overlap giving yellow fluorescence. Both BACs also give some background signals in heterochromatin areas of other chromosomes, due to cross hybridisation of the repetitive sequences in the labelled BAC probe.

that the BACs are rich in pericentromeric heterochromatic specific dispersed repeats. Repeat signals were more abundant for 184L18 (Fig. 3). As the *ol-2* gene is located on chromosome 4 around the centromere (De Giovanni et al. 2004), the presence of tomato *mlo*-homologue(s) in the pericentromeric heterochromatin of the same chromosome further suggests that *ol-2* is a *mlo*-homologue.

Discussion

Although several powdery mildew resistance genes have been reported, most of the current commercial tomato cultivars are susceptible to powdery mildew. The *ol-2* source of resistance, which originates from the cultivated tomato species *S. lycopersicum*, might allow circumvention of linkage drag problems which are encountered during the transfer of wild tomato resistance genes into cultivated backgrounds. On the other hand, the genetic relatedness between the donor parent and elite genotypes complicates the identification of polymorphic markers to be used for MAS breeding programmes. This was demonstrated by the failure in trying to convert several sequences located on the *ol-2* chromosomal region into PCR markers.

Suliman-Pollatschek et al. (2002) reported percentages of polymorphism within *S. lycopersicum* were 52% for SSR, 10.8% for AFLP® and 5% for RFLP markers. The higher variation of SSR loci was demonstrated by the present study, in which two out of seven SSR markers tested revealed length polymorphisms between MM and R28.

The three markers identified in this work can be easily obtained (the SSR marker Tom316 requires a simple PCR reaction and visualization on agarose gel) and are not patented, thus can be freely and conveniently applied for MAS in breeding programmes. Furthermore, their co-dominant nature will allow breeders to select directly for the presence of the recessive *ol-2* allele.

In this study, BAC library screening and FISH analyses were used to demonstrate the co-localisation of an *ol-2* linked marker with *mlo* orthologue(s) in the pericentromeric heterochromatic region of the short arm of tomato chromosome 4. Amplification of the U3-2 sequence by using BAC DNA as a template was invariably accompanied by the presence of multiple PCR amplification. However, when genomic DNA was used as PCR template, only one PCR fragment was observed. It seems that primer mismatches occur during PCR amplification only with the BAC DNA as template. Multiple PCR amplifications obtained by using the U3-2 primer pair suggests that the BACs contain repetitive sequences, which are known to occur with very high frequency in heterochromatic regions. The abundance

of repetitive sequences also explains the presence of multiple weak hybridization signals obtained with FISH analyses. On the other hand, the presence of discrete PCR fragments with the expected molecular weight for the two tomato *mlo* ESTs suggests the presence of only one intact *mlo*-like gene sequence. Although heterochromatin has been classically depicted as a gene poor region of the genome, our findings are in accordance with a growing body of evidence which shows that genes resident in heterochromatin can indeed encode essential functions (Nagaki et al. 2004; Rossi et al. 2007).

In tomato, the actual ratio of genetic and physical distance varies considerably depending on the chromosomal region. In the *Tm-2a* region, close to the centromere of chromosome 9, one cM corresponds to more than five million base pairs (Pillen et al. 1996), whereas for the *chloronerva* gene region, located in the euchromatin of chromosome 1, the ratio of genetic to physical distance is only 160 kb per 1 cM (Ling et al. 1999). By determining frequency and distribution of recombination nodules on tomato synaptonemal complexes, Sherman and Stack (1995) observed a much lower frequency of recombination nodules in heterochromatic regions around the centromeres compared to euchromatin. Occurrence of recombination suppression near the centromere is reported also for other plant species (Tanksley et al. 1992; Droaud et al. 2006). Therefore, the observed clustering of *ol-2* linked markers can well be explained by their localization in the pericentromeric heterochromatin of chromosome 4. The low ratio between genetic and physical distance does not favour *ol-2* positional cloning, as it could require the screening of very large segregating populations and extensive chromosome walking.

In addition to the similarity of *ol-2* and *mlo* resistances for both genetic (recessive) and phytopathological (papilla-based mechanism) characteristics, BAC screening and FISH data in this study indicated the genetic and cytogenetic co-localisation of the *ol-2* gene with tomato *mlo*-homologue(s). All of this evidence strongly suggests that *ol-2* is likely a *mlo* homologue. We are currently following a homology-based cloning approach for *ol-2* isolation as an alternative to positional cloning.

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Chapter 3

Naturally occurring broad-spectrum powdery mildew resistance in a central American tomato accession is caused by loss of *MLO* function

Chapter 3

Naturally occurring broad-spectrum powdery mildew resistance in a central American tomato accession is caused by loss of *MLO f*unction

Yuling Bai[1], Stefano Pavan[1,2], Zheng Zheng[1], Nana Zappel[3], Anja Reinstädler[3], Concetta Lotti[2], Claudio De Giovanni[2], Luigi Ricciardi[2], Pim Lindhout[1], Richard Visser[1], Klaus Theres[4], Ralph Panstruga[3]

[1]Laboratory of Plant Breeding, Plant Sciences Group, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, Bari 70126, Italy

[3]Max-Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

[4]Max-Planck Institute for Plant Breeding Research, Department of Plant Breeding and Genetics, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

Abstract

The resistant cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) line LC-95, derived from an accession collected in Ecuador, harbours a naturally occurring 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 harbouring the *Ol-2* locus. Complementation experiments using transgenic tomato lines as well as virus-induced gene silencing (VIGS) 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 co-segregated with resistance. This polymorphism results in a frame shift and, thus, a truncated non-functional SlMLO1 protein. Our findings reveal the second example of a natural *mlo* mutant that possibly arose post-domestication, suggesting that natural *mlo* alleles might be evolutionarily short-lived due to fitness costs related to loss of *mlo* function.

Introduction

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*, <u>*Ol*</u>) 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 defence 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 et al. 2005b) results in a more complex scenario in Arabidopsis: absence of *AtMLO2* confers partial powdery mildew resistance which 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, which 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 reversion events which 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 which co-segregated 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* and *Atmlo2 Atmlo12*) and triple (*Atmlo2 Atmlo6 Atmlo12*) mutants showed full resistance to *O. neolycopersici* (Fig. 1, Table 1). We conclude that *O. neolycopersici*, similarly 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 twelve and three EST sequences, respectively, which correspond

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 post inoculation. The experiment was repeated once with similar results.

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

Genotype	Disease index (DI)[a]		
	8dpi[b]	13dpi	15dpi
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	2.00.0
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 post-inoculation.

[c]*DI mean*± standard deviation of five plants per genotype are presented.

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 ESTs indicates that *SlMLO1* is ubiquitously expressed (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 presence of either transcript 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

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.

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 chromosomes 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
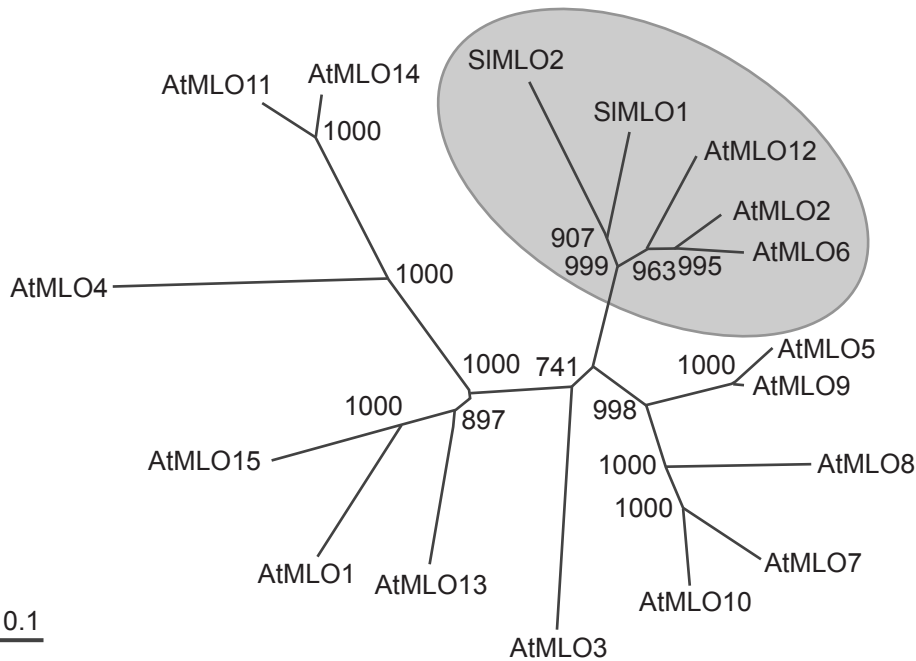

Fig. 3. A neighbor-joining phylogenetic tree of *Arabidopsis* and selected tomato MLO proteins. SlMLO1 and SlMLO2 are co-orthologs of AtMLO2, AtMLO6, and AtMLO12. The unrooted radial tree comprises all 15 *Arabidopsis* MLO paralogs (AtMLO1 to AtMLO15; Devoto et al. 2003) as well SlMLO1 and SlMLO2 and 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.

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
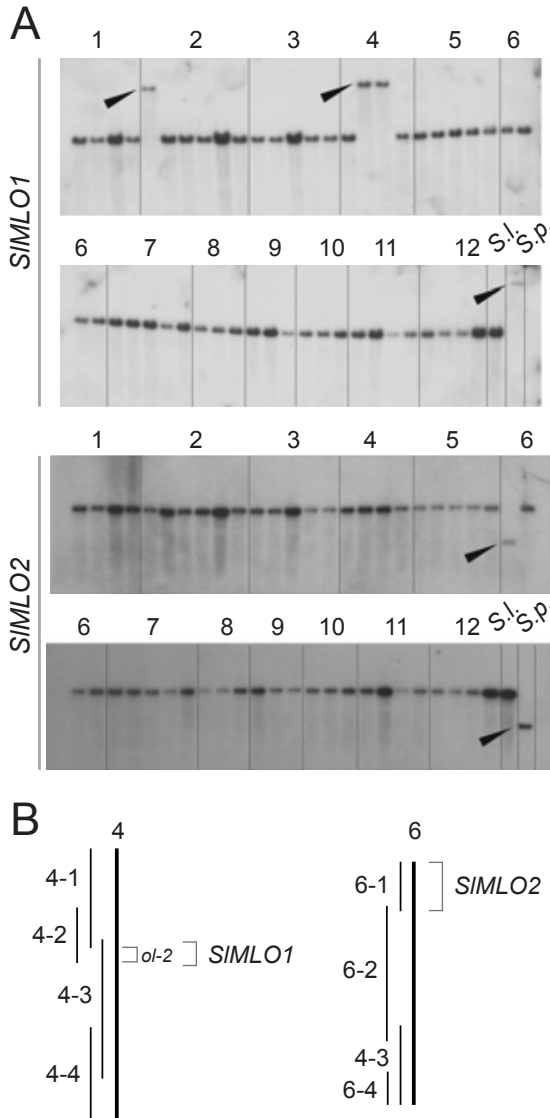

Fig. 4. Introgression line(IL)-based mapping of *SlMLO1* and *SlMLO2*. A, Gel blot hybridization of labelled *SlMLO1* (top panels) or *SlMLO2* (bottom panels) cDNA fragments on filters comprising restriction-digested genomic DNAs of a core set of *Solanum lycopersicum* x *Solanum pennellii* ILs and respective parental lines. Genomic DNAs were either digested with restriction enzyme *Bgl*II (*SlMLO1*) or *Eco*RV (*SlMLO2*). Parental polymorphisms in *S. pennellii* and ILs 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 absence of a second hybridizing band in any lane of this blot suggests that this polymorphism is most likely due to presence of an ectopic genomic fragment of chromosome 4 in line 2-1 (an impurity of the ILs; 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 ILs, bold numbers highlighting segments harbouring a sequence polymorphism of *SlMLO1* or *SlMLO2*. The previously determined map position of *ol*-2 (De Giovanni et al. 2004) as well as the deduced intervals harbouring *SlMLO1* and *SlMLO2* are indicated.

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 *ol-2*. 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 harbouring *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 semi-quantitative as well as real-time RT-PCR, using RNA extracted from leaf material of T$_1$ plants to assess expression levels of the 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. 2008, Chapter 2 of this PhD thesis], 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
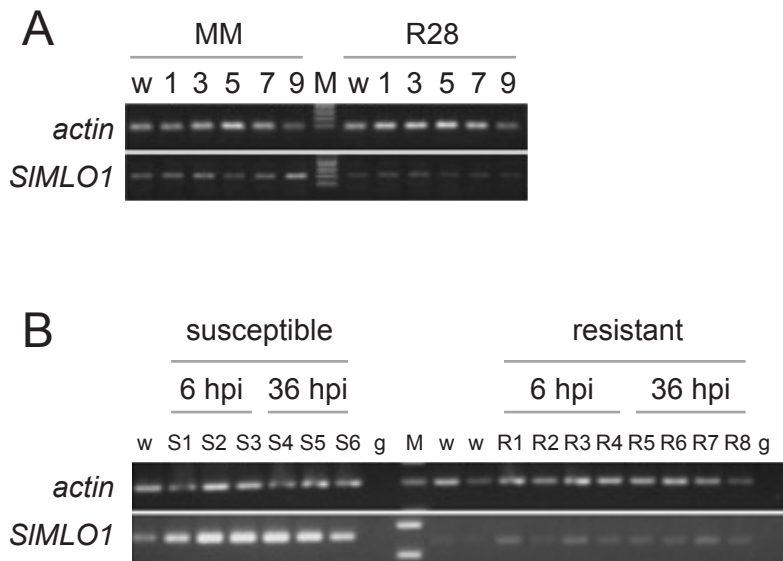

Fig. 5. ***SlMLO1* transcript accumulation upon powdery mildew challenge. A,** Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *SlMLO1* and *actin* transcript levels in susceptible wild-type tomato (cv. Moneymaker [MM]) and the powdery mildew resistant F3 line R28. 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) F2 progeny of a cross between cv. MM and the powdery mildew–resistant F3 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.
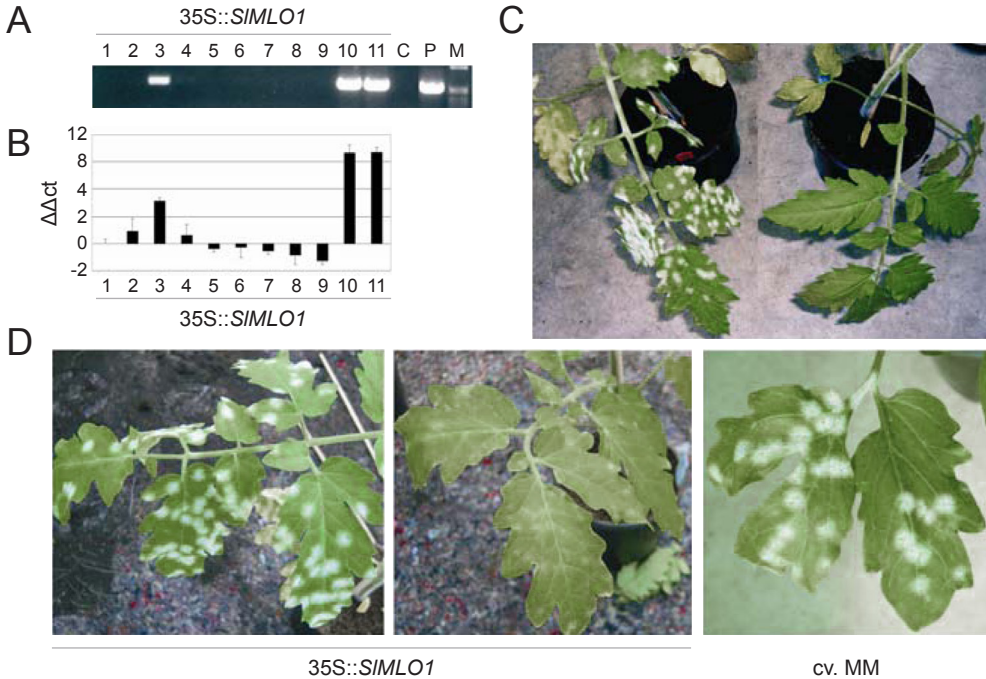
Fig. 6. Complementation of *ol-2*–mediated resistance in transgenic tomato lines overexpressing *SlMLO1*. A, Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (25 cycles) of eleven (1 to 11) transgenic T1 lines expressing 35S::*SlMLO1* derived from the powdery mildew 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 T2 progeny of a transgenic line. Depicted are two descendants of T1 line 11 (described above), one being susceptible (left, DI = 3) and one being largely resistant (right, DI = 0). The photograph was taken 17 days post-inoculation. **D**, Close-up views of powdery mildew–infected leaves of susceptible (left panel) and resistant (middle panel) progeny of a susceptible transgenic line (T2 descendants of T1 line 11 expressing 35S::*SlMlo1*) and the susceptible tomato wild-type control (cv. Moneymaker [MM]; right panel). Photographs were taken 17 days post-inoculation. Note that infection phenotypes of the susceptible transgenic line and cv. MM are comparable (DI = 3).

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*.

Table 2. Segregating analysis of transgenic tomato lines.

T_1 generation	T_2 generation		
Line	Susceptible[a]	Resistant[a]	$\chi^2 (3:1)$[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 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.

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 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.

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 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

TRV:: *SlMlo1*   TRV (empty vector)
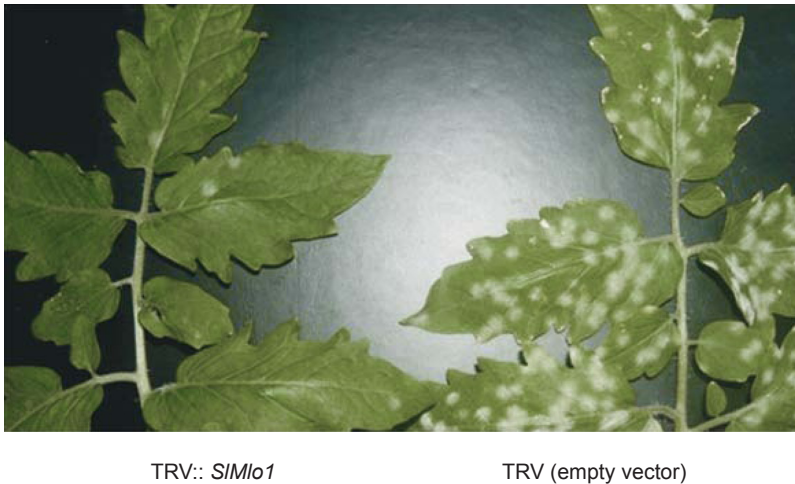
Fig. 7. VIGS of *SlMLO1* confers enhanced powdery mildew resistance in wild-type tomato. Leaves of wild-type tomato (cv. MoneyMaker) were infiltrated with agrobacteria harbouring either a T-DNA with *Tobacco rattle virus* (TRV) carrying no insert (right) or a T-DNA with a TRV derivative harbouring a *SlMLO1* fragment (left). At 17 days after bacterial inoculation, leaves were challenged with *Oidium neolycopersici*. The photograph was taken 13 days after fungal inoculation.

A

SIMLO1 (*ol-2*)
SIMLO1 (wild type)

B

SIMLO1 wild type sequence

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MEATPTWAIAVVCFILLAISIFIEQIIHHIGEWLLEKRKKSLYEALEKIK  50
AELMLLGFLSLLLTVLQDPVSNLCVPKSVGYSWHPCMAKEDAKSEYDDPC  100
LPKGKVQFASSYAIHQLHIFIFVLAVAHVLYCIATFALGRLKMRKWRAWE  150
DETKTMEYQFYNDPERFRFARETSFGRRHLHFWSKSPVLLSIVCFFRQFF  200
SSVAKVDYLTLRHGFMMAHLTPQNQNNFDFQLYINRAVDKDFKVVVGISP  250
ALWLFTVLYFLTTTDRLYSYLWVPFIPLVIILLVGTKLOMIITEMGVRIS  300
ERGDIVKGVPVVETGDHLFWFNRPALVLFLINFVLFQNAFQVAFFFWSWW  350
KFGFPSCFHKNAADLAIRLTMGVIIQVHCSVVTLPLYALVTOMGSSMKPI  400
IFGDNVATALRSWHHTAKKRVKHGLSGHTTPANSRPTTPLRGTSPVHLLR  450
GYPQYNEDSVQASPRTSNVENEGWANENQEGEILQHASTDHNKQIEITMS  500
DFTFGNK[+]
```
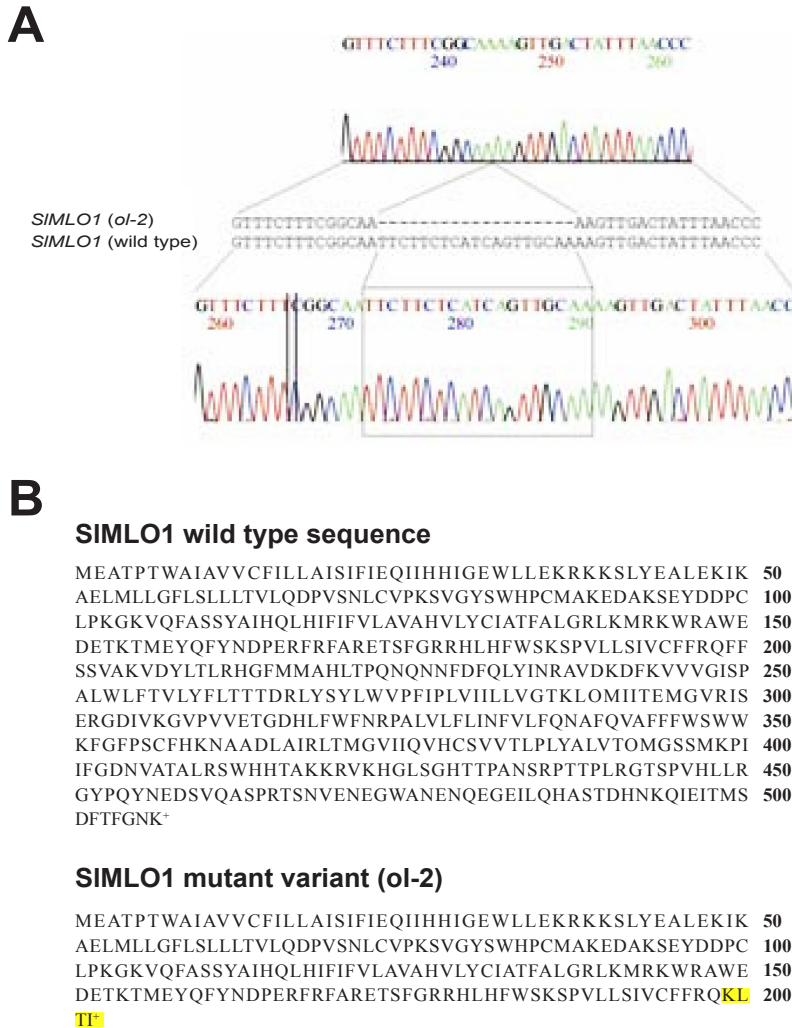
SIMLO1 mutant variant (ol-2)

```
MEATPTWAIAVVCFILLAISIFIEQIIHHIGEWLLEKRKKSLYEALEKIK  50
AELMLLGFLSLLLTVLQDPVSNLCVPKSVGYSWHPCMAKEDAKSEYDDPC  100
LPKGKVQFASSYAIHQLHIFIFVLAVAHVLYCIATFALGRLKMRKWRAWE  150
DETKTMEYQFYNDPERFRFARETSFGRRHLHFWSKSPVLLSIVCFFRQKL  200
TI[+]
```

Fig. 8. The *ol-2* allele represents a *SIMLO1* 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 *SIMLO1* 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 *SIMLO1* wild-type cDNA and the mutant variant emphasize the 19-bp deletion in the *SIMLO1* coding region of *ol-2* plants (boxed in the *SIMLO1* wild-type sequence). **B**, Consequence of the 19-bp deletion on the conceptual SIMLO1 amino-acid sequence. Upper panel, predicted wild-type SIMLO1 amino-acid sequence; lower panel, predicted mutant SIMLO1 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 SIMLO1 wild-type polypeptide.

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 analyzed F_2 individuals), further substantiating that *SlMLO1* is *Ol-2* (Fig. 9).
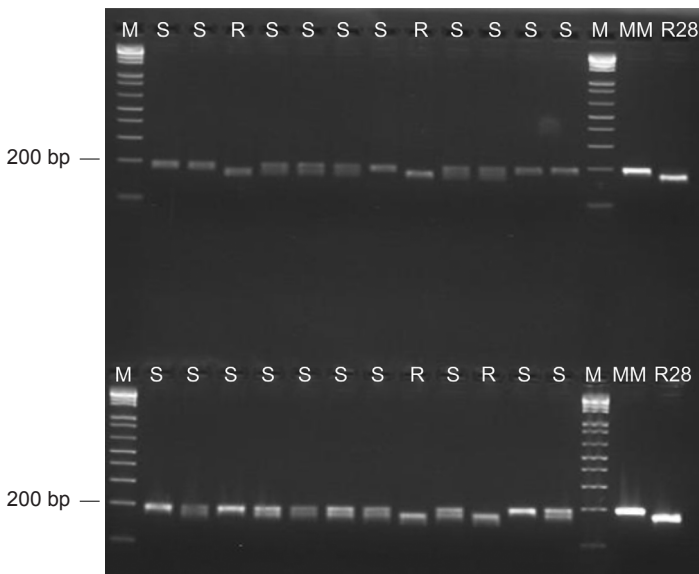

Fig. 9. The 19bp polymorphism in *SlMLO1* co-segregates with powdery mildew resistance. F_2 progeny of a cross of wild-type tomato (cv. MM) and the resistant line R28 were analyzed for *O. neolycopersici* infection phenotypes and the respective genotype of the co-dominant M/SlMLO1 marker. PCR products representing the marker were amplified from genomic DNA as a template and resolved on a 2.5% agarose gel. Like R28, resistant (R) F_2 plants are homozygous for the mutant *ol-2* allele while susceptible (S) F_2 plants are either homozygous for the wild-*Ol-2* allele (like cv. MM) or heterozygous (indicated by the presence of a double band). M=DNA size marker.

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 co-segregation of a small (19 bp) deletion in the *SlMLO1* coding region with the resistant phenotype (Figs. 8 and 9). 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.

In tomato, currently available ESTs 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 as 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 non-expressed 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 also confers resistance in the genetic background of cultivated round tomatoes (*S. lycopersicum*, e.g. cv. 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 phenotype(s) 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 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 which 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 *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 thus 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 effects 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. Homozygous 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 co-segregation 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)/19 °C (night) with 60 to 70% RH (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 milliliter, while five week-old Arabidopsis plants were sprayed with an inoculum of 1×10^5 spores/ml. 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. 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 ILs 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 labelled and hybridized to filters harbouring 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 ILs 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′-gtgacggatccCGTATCTTTGGGTGCCATTT-3′ and 5′-gtgacggtaccCAGGGCGATTAAACCAGAAA-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 RNA was extracted using Trizol (Invitrogen Life Technologies Co., Carlsbad, CA, U.S.A.) and purified with a NucleoSpin RNA II kit (MACHEREY-NAGEL GmbH & Co. KG. Düren, Germany). cDNA were synthesized with olig(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′-GCTCCACCAGAGAGGAAATACAGT-3′ and 5′-CATACTCTGCCTTTGCAATCCA-3′. The expression of *SlMLO1* was monitored by marker cTOC20 by amplifying a transcript with primers 5′-TGTGGTTTGCTTCATCTTGC-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′-ACAATCGATCGGTAGTAGTCAGA-3′.

For analysis of *SlMLO1* expression in T1 transgenic lines (Fig. 6A and B), oligonucleotides actin1 (5′-TGAGCCTCATTTTAAGCTC-3′) and actin4 (5′-GAAATACAACTCTTCTACATA-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′-TTCCCATCTTGCTTTCATAAG-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 ΔΔct method.

Co-segregation 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.

Acknowledgements

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Chapter 4

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

Chapter 4

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

Stefano Pavan[1,2], Adalgisa Schiavulli[3], Michela Appiano[2], Angelo R. Marcotrigiano[2], Fabrizio Cillo[4], Richard G. F. Visser[1], Yuling Bai[1], Concetta Lotti[3], Luigi Ricciardi[2]

[1]Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Environmental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

[3]Department of Agro-Environmental Science, Chemistry and Crop Protection, University of Foggia, Via Napoli 25, 71100 Foggia, Italy

[4]National Research Council - Institute of Plant Virology, Via Amendola 165/A, 70126 Bari, Italy

Abstract

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

Introduction

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

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

The *MLO* gene family encodes for seven transmembrane domain proteins in plants, topologically reminiscent of G-protein coupled receptors (GPCRs) present in animals and fungi (Devoto et al. 2003). Specific *MLO* homologs have been demonstrated to act as susceptibility factors towards epiphytic powdery mildew fungi, as their inactivation, through loss-of-function mutations, results in recessively inherited disease immunity (*mlo* resistance) (Pavan et al. 2010, Chapter 1 and 6 of this PhD thesis). It is thought that functional MLO proteins modulate vesicle-associated defense responses at the cell periphery and their targeting by virulent pathogen effectors promotes fungal penetration in epidermal cells (Jones and Dangl 2006; Panstruga 2005). So far, *mlo* resistance has been reported to occur in barley, *Arabidopsis* and tomato (Bai et al. 2008, Chapter 3 of this PhD thesis; Büschges et al. 1997; Consonni et al. 2006; Devoto et al. 2003).

In a previous study, we pointed out that *er1* and *mlo* resistances share genetic and phytopathological features (Bai et al. 2008, Chapter 3 of this PhD thesis). Indeed, besides being recessively inherited, they are both based on a defense mechanism independent from hypersensitive response and associated to the early abortion of pathogenesis after the differentiation of fungal appressoria (Bai et al. 2005; Consonni et al. 2006; Fondevilla et al. 2006; Hückelhoven et al. 2000). In addition, they do not show specificity towards particular fungal isolates, in contrast with most other powdery mildew resistance sources reported in

crop species (Bai et al. 2005; Lyngkjaer et al. 2000; Tonguç and Weeden 2010).

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

Materials and methods

Experimental mutagenesis and selection for powdery mildew resistance

Approximately 4000 seeds of a pea breeding line derived from the old processing cultivar Sprinter [germplasm collection of the Department of Agroforestry, Environmental Biology and Chemistry (DiBCA), University of Bari] were immersed in a 0,2% solution of diethyl sulfate for 2 h, rinsed with distilled water and planted immediately in paper pots filled with sterilized soil. About 2200 M_1 plants reached maturity and about 2000 set seeds. M_1 seeds were separately collected from each plant and sown at the experimental farm "P. Martucci" of the University of Bari (Italy), to give an M_2 generation of approximately 27000 individuals. In order to ensure a high level of powdery mildew infection, M_2 plants were artificially inoculated by spraying a water suspension of conidia (about 5 x 10^4 conidia/ml) of a local *Ep* isolate maintained on susceptible plants at the University of Bari, with the addition of 0,0025% volume of Tween20. The line ROI3/02 was obtained by four selfings of a single individual free of disease symptoms detected in a segregating M_2 family. Response of ROI3/02 to *Ep* was assessed both in open field and controlled greenhouse conditions (20±1°C, 60±10 RH and 16 h photoperiod), following pathogen spray-inoculation as above described.

Histological studies

The resistant line ROI3/02 and the susceptible cultivar Sprinter were artificially inoculated with the same *Ep* isolate used for the selection program by tapping heavily infected plant parts over the leaves. After 48 h, during which plants were kept at 20±1 °C, 60±10 RH and 16 h photoperiod, leaf samples from three individuals of each genotype were cleared for 2 days in an acetic acid:ethanol (1:3) solution and stained with trypan blue, according to the method described by Huang et al. (1998). For each individual, 30 infection units were observed, an

infection unit being a germinated spore that produced at least a primary appressorium.

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

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

Genetic mapping of resistance at the er1 locus

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

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

The cDNA sequence of the pea *MLO* homolog *PsMLO1* was retrieved in the GenBank Database of the National Center for Biotechnology Information (NCBI, accession number FJ463618.1). The corresponding protein (PsMLO1) amino acid sequence was imported together with those of the 15 *Arabidopsis* AtMLO homologs and tomato SlMLO1 in the CLC sequence viewer software (http://clcbio.com) for Clustal alignment (gap open cost and gap

extension cost were respectively set equal to 10 and 4) and the obtainment of an UPGMA-based comparative tree (bootstrap value was set equal to 100).

Total RNA from the five genotypes Sprinter, ROI3/02, Franklin, Dorian (Asgrow) and Nadir (DiBCA germplasm collection) was extracted with the SV Total RNA Isolation System Kit (Promega) and corresponding cDNAs were synthesized by using the QuantiTect Reverse Transcription Kit (Qiagen) with oligo(dT) primers. The *PsMLO1*-specific primer pair 5'-AAAATGGCTGAAGAGGGAGTT-3'/5'-TCCACAAATCAAGCTGCTACC-3', was selected by using the Primer3 software (Rozen and Skaletsky 2000) and used for a PCR reaction with annealing temperature of 54° C. Amplicons were purified by using the Nucleospin Extract II kit (Macherey-Nagel) and ligated (molar ratio 1:1) into the pGEM-T easy vector (Promega). Recombinant plasmids were cloned in *E. coli* DH10β chemically competent cells and recovered by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed by using universal T7 and SP6 primers (Eurofins MWG Operon).

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

Results

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

A field screening of approximately 2000 M_2 families allowed the identification of one family which was segregating for response to powdery mildew infection. The resistant line ROI3/02 was next selected by means of successive self-pollinations of a single resistant individual.

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

At the histological level, 2 days after artificial inoculation, most germinated *Ep* spores developed mycelium on the susceptible control cultivar Sprinter. In contrast, at the same

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

time point no spores developed secondary hyphae on ROI3/02. Similarly to the previous observations reported for *er1*- and *mlo*-based immunities (Bai et al. 2005; Consonni et al. 2006; Fondevilla et al. 2006), in ROI3/02 pathogenesis was found to terminate after the differentiation of the primary appressorium and was not associated with the host epidermal cell hypersensitive response.

Genetic analysis of the ROI3/02 mutation

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

used for F_2 linkage analysis. Both marker loci were found to be associated with the resistance locus, with an estimated distance of 1.1 cM for ScOPO-18$_{1200}$ and 3.1 cM for ScOPO-06$_{1100}$.

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

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

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

PsMLO1 full-length coding sequence was amplified from the cDNAs of Sprinter and ROI3/02. *PsMLO1* sequence in ROI3/02 was found to contain a point mutation (A in place of G), predicted to cause a premature termination of translation in correspondence of the second N-proximal MLO protein intracellular loop and thus, a severely truncated protein (Fig. 3). As the mutation occurring in the *PsMLO1* sequence of ROI3/02 was found to result in a
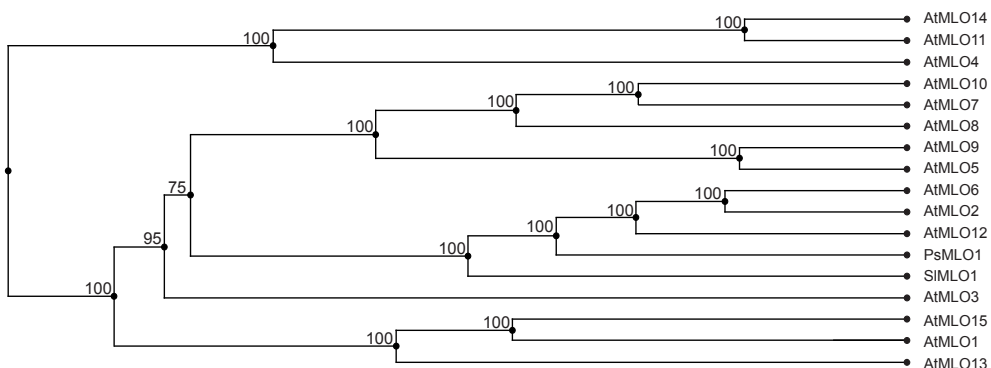

Fig. 2 UPGMA-based comparative analysis of a dataset of full-length MLO proteins composed of tomato SlMLO1, pea PsMLO1, and the 15 *Arabidopsis* AtMLO homologs. Numbers at each node represent bootstrap support values (out of 100 replicates)

SPRINTER PsMLO1

```
                    20                    40                    60
                    |                     |                     |
MAEEGVKERTLEETPTWAVAVVCLVLLAVSILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLL
          80                   100                   120
          |                     |                     |
GFISLLLTVFQDNISKICVSQKIGSTWHPCSTSNTKAKAKSDESLDYKTNNDRKLLEYFDPIPRRI
      140                   160                   180
      |                     |                     |
PATKGYDKCFDKGQVALVSAYGIHQLHIFIFVLALFHILQCIITLTLGRIKMRKWKTWEDETRTVE
  200                   220                   240                   260
  |                     |                     |                     |
YQFYNDPERFRFARDTTFGRRHLSMWAQSPILLWIVSFFRQFFGSISRVDYMALRHGFIMAHLPPG
          280                   300                   320
          |                     |                     |
HDAQFDFQKYISRSIEEDFKVVVGISPTIWLFTVLFLLTNTHGWYSYYWLPFLPLIVILLVGAKLQ
      340                   360                   380
      |                     |                     |
MIITKMGLRIQDRGEVIKGAPVVEPGDHLFWFNRPHLLLFTIHLVLFQNAFQLAFFAWSTYEFSIT
  400                   420                   440                   460
  |                     |                     |                     |
SCFHKTTADSVIRITVGVVIQTLCSYVTLPLYALVTQMGSTMKPTIFNERVATALKNWHHTAKKQV
          480                   500                   520
          |                     |                     |
KQSNHSNNTTPYSSRPSTPTHAMSPVHLLHRHTAGNSDSLQTSPEKSDYKNEQWDIEGEGPTSLRN
      540                   560
      |                     |
DQTGQHEIQIAGVESFSSTELPVRIRHESTSGSKDFSFEKRHLGSN*
```

ROI3/02 PsMLO1

```
                    20                    40                    60
                    |                     |                     |
MAEEGVKERTLEETPTWAVAVVCLVLLAVSILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLL
          80                   100                   120
          |                     |                     |
GFISLLLTVFQDNISKICVSQKIGSTWHPCSTSNTKAKAKSDESLDYKTNNDRKLLEYFDPIPRRI
      140                   160                   180
      |                     |                     |
LATKGYDKCFDKGQVALVSAYGIHQLHIFIFVLALFHILQCIITLTLGRIKMRKWKT*
```

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

cutting site for the restriction enzyme *Sml*I, we designed a primer pair flanking the mutation site and developed a polymorphic CAPS marker, GIM-300/*Sml*I. Remarkably, all the resistant individuals in the ROI3/02 x Progress9 F_2 population were found to be homozygous for the G→A *PsMLO1* transition, whereas susceptible phenotypes were either homozygous for the wild-type allele or heterozygous (Fig. 4).

We next used the same primer pair used for the amplification of Sprinter and ROI3/02 *PsMLO1* in PCR reactions using cDNAs from the cultivar Franklin, known to be homozygous for an *er1* resistance allele, and from the powdery mildew resistant cultivars Dorian and
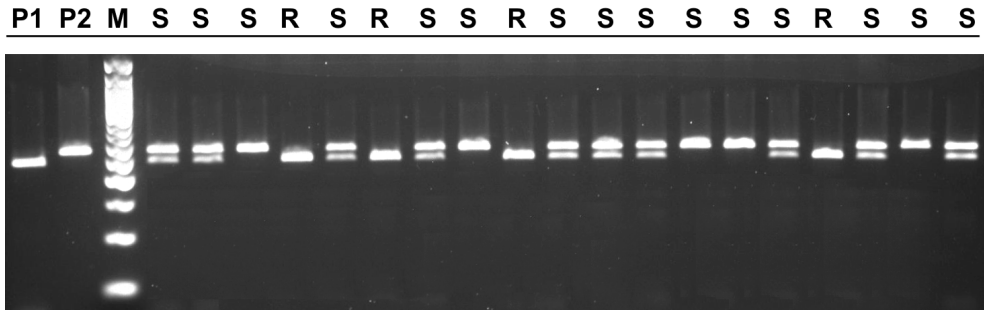
P1 P2 M S S S R S R S S R S S S S S S R S S S

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

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

Discussion

In this study, we provide the following evidence indicating that *er1* powdery mildew resistance is due to the loss of function of a pea *MLO* homolog: 1) two different *er1* resistant genotypes (ROI3/02 and Franklin) and two powdery mildew resistant cultivars (Dorian and Nadir) are characterized by mutations in the sequence of the *PsMLO1* gene, predicted to result in non-functional proteins; 2) full co-segregation occurs between *er1* powdery mildew resistance and

```
Sprinter PsMLO1       TTTTATAATGATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCAC 660
Franklin PsMLO1 cDNA1 TTTTATAATGATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCAC 660
Franklin PsMLO1 cDNA2 TTTTATAATGATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCAC 660

Sprinter PsMLO1       TTGAGCATGTGGGCTCAGTCACCTATTTTGTTATGGATTGTTAGCTTCTTCAGACAATTC 720
Franklin PsMLO1 cDNA1 TTGAGCATGTGGGCTCAGTCACCTATTTTGTTATGGATT-----CTTCTTCAGACAATTC 715
Franklin PsMLO1 cDNA2 TTGAGCATGTGGGCTCAGTCACCTATTTTGTTATGGATT-----CTTCTTCAGACAATTC 715

Sprinter PsMLO1       TTTGGATCTATCAGTAGAGTTGATTATATGGCTCTTAGGCATGGATTTATCATGGCTCAT 780
Franklin PsMLO1 cDNA1 TTTGGATCTATCAGTAGAGTTGATTATATGGCTCTTAGGCATGGATTTATCATGGCTCAT 775
Franklin PsMLO1 cDNA2 TTTGGATCTATCAGTAGAGTTGATTATATGGCTCTTAGGCATGGATTTATCATGGCTCAT 775

Sprinter PsMLO1       CTTCCTCCAGGACATGATGCACAATTTGATTTCCAAAAGTATATAAGTAGATCAATTGAA 840
Franklin PsMLO1 cDNA1 CTTCCTCCAGGACATGATGCACAATTTGATTTCCAAAAGTATATAAGTAGATCAATTGAA 835
Franklin PsMLO1 cDNA2 CTTCCTCCAGGACATGATGCACAATTTGATTTCCAAAAGTATATAAGTAGATCAATTGAA 835

Sprinter PsMLO1       GAGGATTTTAAAGTTGTTGTAGGAATAAGTCCAACTATCTGGCTCTTCACAGTGCTTTTC 900
Franklin PsMLO1 cDNA1 GAGGATTTTAAAGTTGTTGTAGGAATAAGTCCAACTATCTGGCTCTTCACAGTGCTTTTC 895
Franklin PsMLO1 cDNA2 GAGGATTTTAAAGTTGTTGTAGGAATAAGTCCAACTATCTGGCTCTTCACAGTGCTTTTC 895

Sprinter PsMLO1       CTTCTTACAAATACTCATGGGTGGTATTCTTATTATTGGCTTCCATTTCTTCCACTAATT 960
Franklin PsMLO1 cDNA1 CTTCTTACAAATACTCATGGGTGGTATTCTTATTATTGGCTTCCATTTCTTCCACTAATT 955
Franklin PsMLO1 cDNA2 CTTCTTACAAATACTCATGGGTGGTATTCTTATTATTGGCTTCCATTTCTTCCACTAATT 955

Sprinter PsMLO1       GTAATCTTATTAGTTGGTGCTAAGTTACAAATGATCATAACAAAAATGGGATTAAGGATT 1020
Franklin PsMLO1 cDNA1 GTAATCTTATTAGTTGGTGCTAAGTTACAAATGATCATAACAAAAATGGGATTAAGGATT 1015
Franklin PsMLO1 cDNA2 GTAATCTTATTAGTTGGTGCTAAGTTACAAATGATCATAACAAAAATGGGATTAAGGATT 1015

Sprinter PsMLO1       CAAGACAGAGGAGAAGTAATCAAGGGTGCACCTGTGGTTGAGCCTGGAGATCACCTTTTC 1080
Franklin PsMLO1 cDNA1 CAAGACAGAGGAGAAGTAATCAAGGGTGCACCTGTGGTTGAGCCTGGAGATCACCTTTTC 1075
Franklin PsMLO1 cDNA2 CAAGACAGAGGAGAAGTAATCAAGGGTGCACCTGTGGTTGAGCCTGGAGATCACCTTTTC 1075

Sprinter PsMLO1       TGGTTCAATCGTCCTCACCTTCTTCTCTTCACGATTCATCTTGTTCTCTTTCAGAATGCC 1140
Franklin PsMLO1 cDNA1 TGGTTCAATCGTCCTCACCTTCTTCTCTTCACGATTCATCTTGTTCTCTTTCAGAATGCC 1135
Franklin PsMLO1 cDNA2 TGGTTCAATCGTCCTCACCTTCTTCTCTTCACGATTCATCTTGTTCTCTTTCAGAATGCC 1135

Sprinter PsMLO1       TTTCAACTTGCATTTTTTGCTTGGAGTACATATGAGTTTTCCATAACCTCTTGCTTCCAC 1200
Franklin PsMLO1 cDNA1 TTTCAACTTGCATTTTTTGCTTGGAGTACATATGAGTTTTCCATAACCTCTTGCTTCCAC 1195
Franklin PsMLO1 cDNA2 TTTCAACTTGCATTTTTTGCTTGGAGTACA------------------------------ 1165

Sprinter PsMLO1       AAAACAACTGCAGATAGTGTCATTAGAATCACTGTAGGGGTTGTAATACAAACTCTATGT 1260
Franklin PsMLO1 cDNA1 AAAACAACTGCAGATAGTGTCATTAGAATCACTGTAGGGGTTGTAATACAAACTCTATGT 1255
Franklin PsMLO1 cDNA2 ------------------------------------------------------------ 1165

Sprinter PsMLO1       AGCTATGTGACTTTGCCTCTTTATGCTCTAGTCACA------------------------ 1296
Franklin PsMLO1 cDNA1 AGTGTCGCAATCTGAAAAATACAGTGCGCGAAAAAAACAACCGGCGAAAGAAAATGACAG 1315
Franklin PsMLO1 cDNA2 ------------------------------------------------------------ 1165

Sprinter PsMLO1       ------------------------------------------------------------ 1296
Franklin PsMLO1 cDNA1 AAGAGTCGCCACCGTGCGTTATTCATCCCAAAGGAGGGAAAGGAAACGCTCGAAGTAAAC 1375
Franklin PsMLO1 cDNA2 ------------------------------------------------------------ 1165

Sprinter PsMLO1       ------------------------------------------------------------ 1296
Franklin PsMLO1 cDNA1 CTGAAAAGAGGAAAGGAAAAGACAAGGTCTCGCAACCAAATCTTGGGTTCGGGAGTCGGT 1435
Franklin PsMLO1 cDNA2 ------------------------------------------------------------ 1165

Sprinter PsMLO1       -----------CAGATGGGATCAACCATGAAACCAACCATTTTCAACGAAAGAGTGGCAA 1345
Franklin PsMLO1 cDNA1 TATGCGAAGGGAAGATGGGATCAACCATGAAACCAACCATTTTCAACGAAAGAGTGGCAA 1495
Franklin PsMLO1 cDNA2 -------------ATGGGATCAACCATGAAACCAACCATTTTCAACGAAAGAGTGGCAA 1211

Sprinter PsMLO1       CAGCGCTTAAGAACTGGCACCACACAGCCAAAAAGCAGGTAAAACAGAGCAACCACTCAA 1405
Franklin PsMLO1 cDNA1 CAGCGCTTAAGAACTGGCACCACACAGCCAAAAAGCAGGTAAAACAGAGCAACCACTCAA 1555
Franklin PsMLO1 cDNA2 CAGCGCTTAAGAACTGGCACCACACAGCCAAAAAGCAGGTAAAACAGAGCAACCACTCAA 1271
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Fig. 5 Nucleotide alignment between a segment of the wild-type *PsMLO1* coding sequence detected in the cultivar Sprinter (Sprinter *PsMLO1* cDNA) and homologous regions of the two *PsMLO1* cDNA sequences identified in the cultivar Franklin (Franklin *PsMLO1* cDNA 1 and 2). Mutation sites are boxed. The two *PsMLO1* cDNAs identified in the genotypes Dorian and Nadir only retain the mutation event in the down box.

PsMLO1 loss-of-function. Aiming to the functional characterization of *er1* resistance, we are currently carrying out a complementation experiment consisting of the expression of wild-type *PsMLO1* in *er1* mutants. Importantly, during the revision of this paper, another study was released providing additional experiments indicating the identity between *er1* and *mlo* immunities (Humphry et al. 2011).

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

In the past years, several authors focused on the identification of *er1*-linked molecular markers, differing with respect to: distance from the resistance locus, repeatability, ease of obtainment, possibility to score heterozygous individuals and capacity to reveal polymorphism in different segregating populations (Dirlewanger et al. 1994; Ek et al. 2005; Janila and Sharma 2004; Pereira et al. 2010; Timmerman et al. 1994; Tiwari et al. 1998; Tonguç and Weeden 2010). Here, we report the identification of a simple agarose-based co-dominant CAPS marker fully co-segregating with *er1* resistance and thus, expected to be of great interest for breeding activities using the line ROI3/02 as donor parent. In addition, we mapped two previously reported *er1*-linked SCAR markers, ScOPO-18$_{1200}$ and ScOPO-06$_{1100}$, which showed polymorphism within the segregating F$_2$ population of 110 individuals used in this study. A genetic distance of 3.1 cM between the marker locus ScOPO-06$_{1100}$ and the *er1* locus was estimated. By using a different F$_2$ population of 94 individuals, Pereira et al. (2010) estimated the distance between the two loci to be 1.2 cM. The reason for this slight discrepancy could be referable to the moderate size of the F$_2$ populations used for linkage analysis. The marker ScOPO-18$_{1200}$, which was co-segregating with the *er1* locus in a previous genetic analysis carried out with 57 F$_3$ individuals (Tiwari et al. 1998), was mapped at a distance of 1.1 cM from *er1*, as one recombination event was detected.

Here, we show that barley *mlo* and pea *er1* immunities, which are still effective in the field several decades after their introduction in cultivation (Fondevilla et al. 2006; Lyngkjaer et al. 2000), are likely to share the same molecular basis. In previous studies, we showed that a third recessive resistance source referable to a loss-of-function mutation of a *MLO* homolog, tomato *ol-2*, was effective towards several isolates of the powdery mildew fungus *Oidium neolycopersici* L. Kiss tested worldwide (Bai et al. 2005; Bai et al. 2008, Chapter 3 of this PhD thesis; Pavan et al. 2008, Chapter 2 of this PhD thesis). Taken together, these data suggest that forward and reverse genetic approaches aiming at the mutagenesis or silencing of *MLO*

susceptibility genes could represent a valid breeding strategy to provide broad-spectrum and durable powdery mildew resistance across agricultural crops.

In greenhouse experimental conditions, extremely favorable to *Ep* development, we could detect moderate fungal sporulation on the line ROI3/02. This is consistent with the observations of Pereira and Leitão (2010) on another *er1* mutant. If *er1* is due to the loss of function of *PsMLO1*, this finding would be in accordance with the presence of low levels of powdery mildew colonization in *Arabidopsis Atmlo2* and tomato *Slmlo1* mutants when plants are artificially inoculated (Bai et al. unpublished data; Consonni et al. 2006). In *Arabidopsis*, full resistance to the compatible powdery mildew species *Golovinomyces orontii* (Castagne) V.P. Heluta, *Golovinomyces cichoracearum* (D.C.) V.P. Heluta and *Oidium neolycopersici* L. Kiss is provided by the simultaneous loss of function of the three phylogenetically related homologs *AtMLO2*, *AtMLO6* and *AtMLO12* (Bai et al. 2008, Chapter 3 of this PhD thesis; Consonni et al. 2006). If a similar scenario holds true, it should be possible to retrieve other *PsMLO* homologs for susceptibility and provide complete powdery mildew immunity in pea.

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Chapter 5

MLO proteins involved in powdery mildew susceptibility: identification of candidate isoforms in cultivated Solanaceae and conserved transmembrane motifs

Chapter 5

MLO proteins involved in powdery mildew susceptibility: identification of candidate isoforms in cultivated Solanaceae and conserved transmembrane motifs

Stefano Pavan[1,2], Domenico Catalano[3], Michela Appiano[2], Zheng Zheng[1], Concetta Lotti[4], Richard G. F. Visser[1], Yuling Bai[1], Luigi Ricciardi[2]

[1]Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

[3]National Research Council - Institute of Plant Genetics, Via Amendola 165/A, 70126 Bari, Italy

[4]Department of Agro-Environmental Science, Chemistry and Crop Protection, University of Foggia, Via Napoli 25, 71100 Foggia, Italy

Abstract

Loss of function of specific homologs of the *MLO* gene family has been associated to broad-spectrum resistance to powdery mildew fungi in barley, Arabidopsis, tomato and pea. This suggests that identification and inactivation of *MLO* susceptibility genes could represent a valuable breeding strategy for the development of resistant cultivars across cultivated species. In this study, several comparative analyses were carried out by using a dataset of dicot MLO protein sequences, allowing the detection of three intracellular and one extracellular domains which are diagnostic to predict candidate powdery mildew susceptibility isoforms. Based on this, novel *MLO* sequences identified by PCR in eggplant, petunia, potato and tobacco were associated to genes expected to favour pathogenesis in these species. In addition, fourteen *MLO* homologs, among which three candidates for being involved in disease susceptibility, were identified in tomato following an *in silico* approach. Finally, four conserved amino acid motifs, likely having an isoform-specific role, were detected in transmembrane domains of MLO homologs with ascertained or predicted role in powdery mildew susceptibility.

Introduction

The *MLO* gene family encodes for seven transmembrane domain proteins in plants, reminiscent of G-protein coupled receptors (GPCRs) present in animals and fungi (Devoto et al. 2003). After the completion of the respective genome sequencing projects, 15 *MLO* homologs have been identified in Arabidopsis, 12 in rice and 17 in grapevine (Devoto et al. 2003; Feechan et al. 2008; Liu and Zhu 2008). Only a few other *MLO* homolog sequences have been reported for other monocot and dicot species (Bai et al. 2008, Chapter 3 of this PhD thesis; Devoto et al. 2003; Panstruga 2005a).

 Specific MLO isoforms are necessary for susceptibility to epiphytic fungi causing the powdery mildew disease, as their loss results in reduced fungal penetration in plant epidermal cells. Likely, these proteins act as negative regulators of defence responses at the cell periphery and/or as molecular targets exploited by virulent fungal effectors in order to induce pathogenesis (Jones and Dangl 2006; Kim et al. 2002; Pavan et al. 2010, Chapter 1 and 6 of this PhD thesis). Dicot susceptibility isoforms have been shown to cluster together in MLO protein family phylogenetic trees (Devoto et al. 2003; Feechan et al. 2008; Panstruga 2005a). Conserved amino acid residues or motifs specific for presumptive MLO orthologues involved in powdery mildew susceptibility have been identified in the first (i.e. N- proximal) extracellular loop, the second and third intracellular loops and the C-terminus of the MLO protein (Panstruga 2005a).

 Powdery mildew resistance due to the loss of function of *MLO* homologs (*mlo* resistance) has been reported in barley, Arabidopsis, tomato and pea (Bai et al. 2008, Chapter 3 of this PhD thesis; Büschges et al. 1997; Consonni et al. 2006; Humphry et al. 2011; Pavan et al. 2011, Chapter 4 of this PhD thesis). In contrast to other kinds of immunities, experimental data clearly indicate that *mlo* resistance is not specific towards particular fungal isolates and is extremely durable. For example, mutation of barley *HvMLO* confers resistance against all known isolates of *Blumeria graminis* f. sp. *hordei* and is used in barley cultivars since 1979 (Lyngkjaer et al. 2000; Panstruga 2005b). Similarly, pea *er1* resistance, originating from the loss of function of pea *PsMLO1*, was first reported more than sixty years ago and is still widely used in pea breeding (Harland 1948; Humphry et al. 2011; Pavan et al. 2011, Chapter 4 of this PhD thesis). Tomato *ol-2* resistance, due to a loss-of-function mutation of *SlMLO1*, is not yet commercially exploited, but it has been shown to be effective towards several isolates of *O. neolycopersici* tested worldwide (Bai et al. 2005; Bai et al. 2008, Chapter 3 of this PhD thesis). It has been suggested that the inactivation of *MLO* susceptibility genes could be conveniently used to provide broad-spectrum powdery mildew resistance across cultivated species (Pavan

et al. 2010, Chapter 6 of this PhD thesis). Indeed, epiphytic powdery mildew fungi affect the yield and/or the quality of a very large range of crops and in the Solanaceae family cause significant economic losses in tomato, potato, eggplant, tobacco and petunia (Glawe et al. 2004; Kiss et al. 2008).

Here, we present a comparative analysis involving dicot MLO homologs, allowing the identification of domains and transmembrane motifs which are characterizing isoforms involved in powdery mildew susceptibility. Furthermore, we report the identification of sequences of novel *MLO* genes predicted to favour powdery mildew pathogenesis in five Solanaceae species of agronomic interest.

Materials and methods

Comparative analysis of MLO domains in dicots

Thirty-four dicotyledonous MLO homolog protein sequences were extracted from the PFAM database (PFAM accession: PF03094) and used as dataset in the CLC sequence viewer software (http://www.clcbio.com) for Clustal alignment (the gap open cost and the gap extension cost were set equal to 10 and 4, respectively) and the obtainment of an UPGMA-based comparative tree (bootstrap value was set equal to 100). Based on the MLO protein domains boundaries indicated by Feechan et al. (2008), the following MLO domains were extracted from the sequences above mentioned: the first and third extracellular loops; the three intracellular loops; the seven transmembrane domains; the C-terminus. Next, these domains were used to obtain additional alignments and trees according to the same parameters used for the study of full-length proteins. Finally, identical analyses were performed by using a chimeric sequence obtained by joining the seven MLO transmembrane domains.

Identification of putative SlMLO1 orthologs in Solanaceae

RNA from potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*) and petunia (*Petunia hybrida*) was extracted by using the Trizol reagent (Invitrogen) and purified with the NucleoSpin RNA II kit (Macherey-Nagel). cDNAs were synthesized by using the SuperScript III RT first-strand cDNA synthesis kit (Invitrogen) with oligo(dT) primers. In order to amplify *SlMLO1* putative orthologs, the following primer pairs were assayed: F1(5'-CATTTGACATTTCCCCTTCTTC-3')/R1(5'-GCACCATGCATGAGTACCTCT-3'), designed on

the *SlMLO1* 5' and 3' UTR regions, respectively; F2(5'-TTGCTGTGGTTTGCTTCATC-3')/ R2(5'-ATGGTGCCAGCTTCTAAGAG-3') and F3(5'-TTGGCAGTTGCTCATGTATTG-3')/ R3(5'-CCACCCTTCATTTTCGACAT-3') designed on the *SlMLO1* coding sequence. All primer pairs were selected by using the Primer3 software with default parameters (Rozen and Skaletsky 2000). PCR products were purified by using the NucleoSpin Extract II kit (Macherey-Nagel) and ligated (molar ratio 1:1) into the pGEM-T easy vector (Promega). Recombinant plasmids were cloned in *E. coli* DH10β chemically competent cells and recovered by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed by using universal T7 and SP6 primers (Eurofins MWG Operon). Nucleotide sequences were translated *in silico* and amino acids corresponding to the second and third MLO intracellular domains were extracted. Two independent domain-restricted comparative analyses, also including amino acid sequences of the 15 Arabidopsis AtMLO homologs, SlMLO1 and PsMLO1, were performed according to the methodology above described.

Database searching and characterization of tomato MLO homologs

Full-length gene models of tomato *MLO* homologs were extracted from the SOL genomic network (SGN) database (http://solgenomics.net/) by using a BioPerl script. Corresponding protein sequences were aligned with those of the whole Arabidopsis AtMLO family, SlMLO1 and PsMLO1 and a comparative tree was constructed, according to the methods described in the previous paragraphs.

Results

Identification of diagnostic domains and conserved transmembrane motifs in MLO homologs associated to powdery mildew susceptibility

A comparative analysis was performed with thirty-four full-length MLO protein sequences of dicot species, resulting in a phylogenetic tree consistent with the ones reported by other authors (Devoto et al. 2003; Panstruga 2005a) (Fig. 1a). One of the tree clades was found to contain all the dicot MLO isoforms proven to be important for powdery mildew susceptibility (Arabidopsis AtMLO2, AtMLO6, AtMLO12, tomato SlMLO1 and pea PsMLO1) together with four grapevine MLO homologs (VvMLO3, VvMLO4, VvMLO13 and VvMLO17), whose corresponding loss-of-function phenotype is unknown. However, the genes *VvMLO3*, *VvMLO4* and *VvMLO17* have
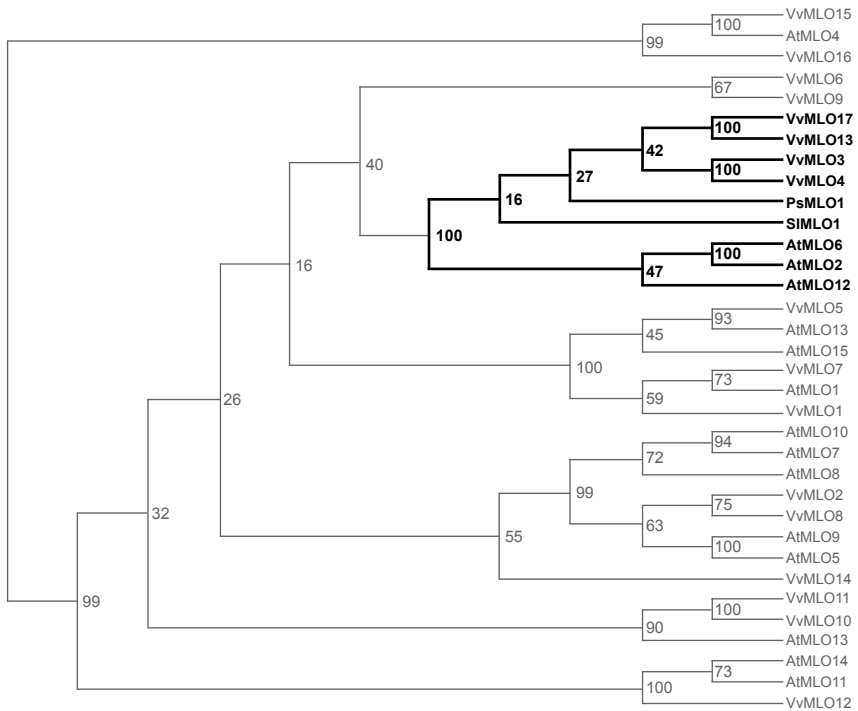
Fig. 1.

a)
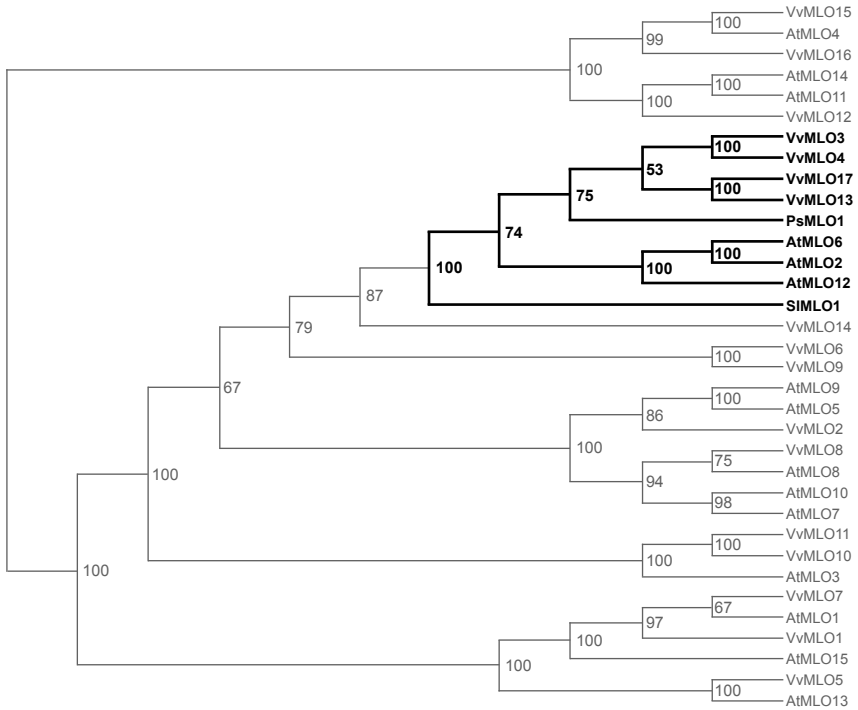
b)

Fig. 1. (on page 88) Comparative analysis of dicot MLO full-length proteins and transmembrane domains. UPGMA-based trees were obtained from a dataset composed of tomato SlMLO1, pea PsMLO1 and the Arabidopsis (At) and grape (Vv) MLO families. a) Tree resulting from the analysis of full-length protein sequences. b) Tree resulting from the analysis of chimeric proteins obtained by joining in silico all transmembrane domains. Numbers at each node represent bootstrap support values (out of 100 replicates). In both figures a clade containing all ascertained or presumptive MLO powdery mildew susceptibility isoforms is highlighted.

been also associated to powdery mildew susceptibility based on their up-regulated expression following artificial inoculation with the fungus *Uncinula necator* (Feechan et al. 2008).

Strong bootstrap value support for the presence of a clade composed as above resulted from the analysis of the first extracellular loop, the second and third intracellular loops and the intracellular C-terminus, suggesting that candidate MLO susceptibility isoforms can be inferred by the comparative analysis of partial sequences corresponding to these domains. Interestingly, the tree resulting from the analysis of an *in silico*–derived chimeric protein obtained by joining all the transmembrane domains also clearly discriminated the same clade (Fig. 1b). Therefore, transmembrane MLO sequences were inspected in search for amino acid regions specifically conserved in the clade containing powdery mildew susceptibility isoforms. Eventually, four conserved motifs were identified in the first, third, fifth and sixth transmembrane domains (Fig. 2).

PCR-identification of sequences of putative MLO powdery mildew susceptibility genes in cultivated Solanaceae

Three different primer pairs designed on the *SlMLO1* sequence were used to amplify homolog sequences in eggplant, petunia, potato and tobacco. PCRs performed with the F1/R1 primer pair, designed on the *SlMLO1* untranslated regions, failed, whereas the coding sequence-based F2/R2 primer pair allowed to get a single amplification product only when potato cDNA was used as template. In contrast, PCR performed with the coding sequence-based F3/R3 primer pair resulted, for all the Solanaceae tested, in single amplification products of 876bp. Corresponding nucleotide sequences were deposited in the GenBank database with the accession numbers HQ880607, HQ880608, HQ880609 and HQ880610. The obtainment of single amplicons from the cDNA of all the species under investigation and their sequence similarity with *SlMLO1* (the lowest percentage of nucleotide identity was 90,6% for the tobacco
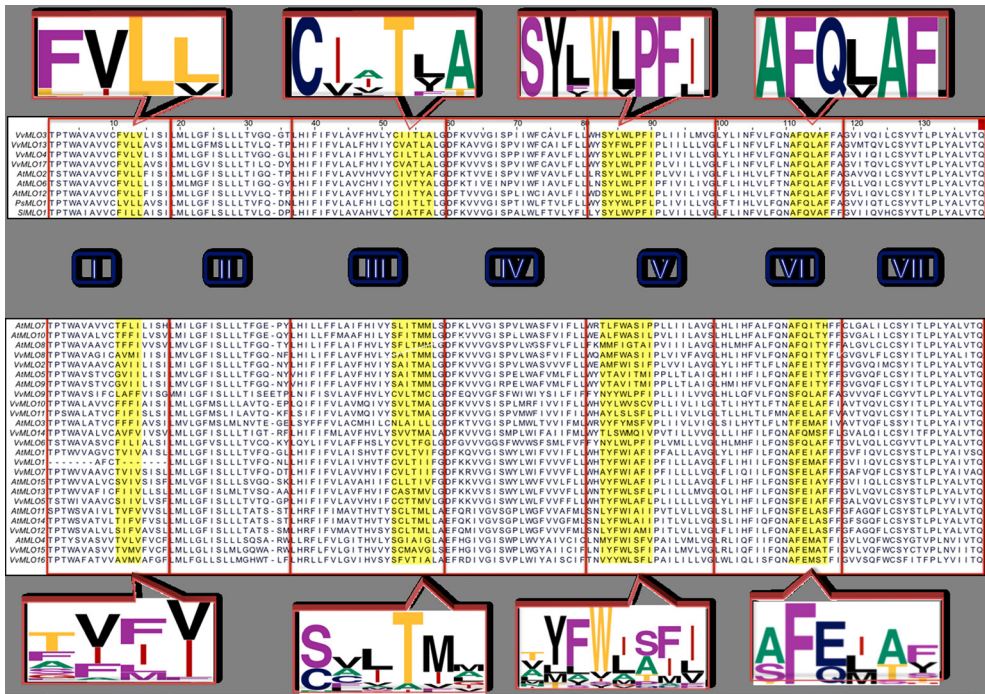
Fig. 2. Alignment of transmembrane domains of dicot MLO proteins. The analysis was performed by using chimeric proteins obtained *in silico* by joining the seven transmembrane domains of a dataset composed of tomato SlMLO1, pea PsMLO1 and the Arabidopsis (At) and grape (Vv) MLO families. Sequence logos are reported for amino acid patterns characterizing MLO homologs with ascertained or predicted role in powdery mildew susceptibility (AtMLO2, AtMLO6, AtMLO12, SlMLO1, PsMLO1, VvMLO3, VvMLO4, VvMLO13 and VvMLO17) and for corresponding regions of other MLO homologs.

MLO homolog, the highest was 97,5% for the potato *MLO* homolog) strongly suggest that they belong to *SlMLO1* orthologues, which are thus likely to be involved in disease susceptibility.

The amino acid sequences resulting from the *in silico* translation of the amplification products above mentioned spanned the second and third MLO intracellular loops, previously shown to be meaningful to discriminate a MLO clade containing dicot powdery mildew susceptibility genes. Therefore, we carried out two additional comparative analyses restricted to these domains. In both cases, with high bootstrap value support sequences fit in a clade containing all the dicot isoforms known to be involved in the interaction with powdery mildew fungi (Fig. 3).
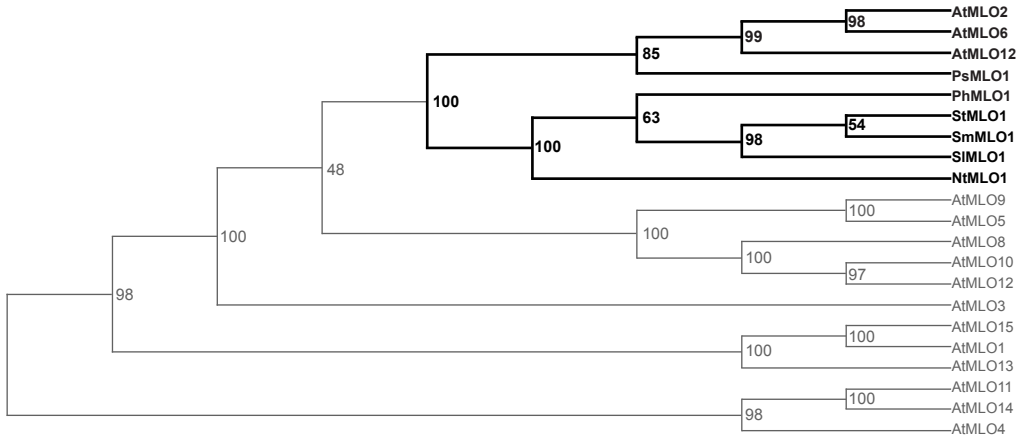
Fig. 3. Comparative analysis of the second intracellular loop of dicot MLO proteins. Amino acid sequences used to obtain the UPGMA-based tree were extracted by a protein dataset composed of tomato SlMLO1, pea PsMLO1, the Arabidopdis (At) MLO family and the potato (St), eggplant (Sm), tobacco (Nt) and petunia (Ph) MLO homologs identified in this study. Numbers at each node represent bootstrap support values (out of 100 replicates). Similar results were obtained from the analysis of the third intracellular loop. A clade containing all known dicot MLO powdery mildew susceptibility isoforms is highlighted.

Table 1. Characteristics of *SlMLO1* and other fourteen tomato *MLO* homologs extracted by the Sol Genomics Network database.

SGN locus name	Chromosome	ORF lenght (aa)	Introns
SlMLO1	4	507	14
Solyc00g007200	n.a.	554	14
Solyc01g102520	1	475	13
Solyc02g077570	2	375	10
Solyc02g082430	2	553	14
Solyc02g083720	2	533	14
Solyc03g095650	3	517	14
Solyc06g010010	6	477	14
Solyc06g010030	6	591	14
Solyc06g082820	6	511	13
Solyc07g063260	7	563	14
Solyc08g015870	8	504	13
Solyc08g067760	8	532	14
Solyc10g044510	10	558	14
Solyc11g069220	11	506	13

In sum, our findings indicate that we identified sequences of several cultivated Solanaceae *SlMLO1* orthologues predisposing for powdery mildew susceptibility.

In silico characterization of MLO homologs in tomato

Search in the Sol Genomic Network (SGN) database resulted in the identification fourteen *MLO* homolog gene models, of which thirteen with known chromosomal location (Table 1). Protein sequences of three homologs (Solyc03g095650, Solyc06g010030 and Solyc11g069220) grouped in a clade containing AtMLO2, AtMLO6, AtMLO12, SlMLO1 and PsMLO1 (Fig.4), suggesting a role for them as powdery mildew compatibility factors. The gene *Solyc06g010030*, localized on chromosome 6, was found to include *SlMLO2*, a partial cDNA sequence reported and mapped on the same chromosome in a previous study (Bai et al. 2008).

Fig. 4. Comparative characterization of tomato MLO homologs. The UPGMA-based tree was obtained from a dataset composed of tomato SlMLO1, pea PsMLO1, the Arabidopsis (At) MLO family and fourteen tomato MLO homolog sequences extracted by the Sol Genomic network database. Numbers at each node represent bootstrap support values (out of 100 replicates).

Discussion

Comparative analyses restricted to the MLO first extracellular loop, the second and third intracellular loops and the C-terminus were sufficient to discriminate a clade containing all dicot isoforms known to act as powdery mildew susceptibility factors. This finding, which is in accordance with the presence in the same domains of amino acid residues specifically conserved in presumptive orthologs of barley HvMLO (Panstruga, 2005a), allowed us to relate partial *MLO* sequences isolated in eggplant, petunia, potato and tobacco to candidate powdery mildew susceptibility genes in these species. Full-length sequences of other three candidate tomato susceptibility genes were retrieved following database search.

Aiming to the obtainment of resistant genotypes, we are currently carrying out a project in which sequences identified in this study are being used for gene silencing through RNA interference (Waterhouse and Helliwell 2003). Alternatively, resistant genotypes could be also identified by using non-transgenic technologies such as TILLING (targeted induced lesions in genomes) and nuclease zinc-finger site-specific mutagenesis (Colbert et al. 2001; Marton et al. 2010). Powdery mildew resistance would be particularly relevant in eggplant, petunia and tobacco, as most commercial cultivars are susceptible to the disease and severe epidemics have been reported worldwide (Kiss et al. 2008).

The presence of multiple tomato homologs putatively involved in powdery mildew susceptibility (SlMLO1 and the three identified in this study) is in accordance with the existence of three Arabidopsis AtMLO isoforms (AtMLO2, AtMLO6 and AtMLO12) associated to increased fungal penetration (Consonni et al. 2006) and with the presence of four grapevine VvMLO homologs in the powdery mildew susceptibility clade reported in Fig. 1. In Arabidopsis, complete powdery mildew penetration resistance is only achieved through the simultaneous absence of all three susceptibility isoforms (Consonni et al. 2006). If a similar scenario holds true in tomato, *ol-2* resistance, which is still associated to some fungal entry in epidermal cells and sporulation (Bai et al. 2005; Li et al. 2007), could be complemented by reverse genetic approaches targeting other *MLO* loci.

This is the first report of conserved transmembrane amino acid motifs specifically conserved in MLO susceptibility isoforms. The motif detected in the fifth transmembrane domain shows invariable proline and serine residues. Transmembrane prolines provide regions of α-helix distortion (Pro-kinks) that, in the case of seven-transmembrane domains GPCRs, play a pivotal role for protein conformational changes, transmitting extracellular signals into the cytoplasmic space (Samson and Weinstein 2000; Deupi et al. 2004). Transmembrane serine or threonine residues, alone or in proximity of Pro-kinks, have been also shown to

produce significant variations in the α-helix structure, which are thought to be important for signalling of integral membrane proteins (Ballesteros et al. 2000). Therefore, we could have detected a protein region involved in the activation of isoform-specific signalling pathways.

Interestingly, the invariable clade-specific proline in the fifth transmembrane domain forms a PXXP motif with the successive proline, which is conserved for the whole MLO family. As in animal GPCR proteins PXXP motifs have been associated to binding sites for signal transduction proteins carrying Src homology 3 (SH3) domains (Saksela et al. 1995), a similar molecular mechanism could be hypothesized to occur in plants.

Functional studies on the conserved transmembrane patterns identified in this research could help in revealing their actual biochemical role in plant-pathogen interactions. Mutagenesis targeted to these protein regions could be also investigated to cause complete/ partial loss of function and, therefore, powdery mildew resistance.

Arabidopsis *AtMLO4* has been recently related to the physiological phenomenon of root thigmomorphogenesis (Chen et al. 2009). A similar role could be retained by the tomato putative homolog *Solyc02g083720*, as it was found to be mostly related to *AtMLO4* (Fig. 4). Analogously, recent findings from Kessler et al. (2010) suggest a possibile role of at least one between *Solyc00g007200* and *Solyc02g082430*, closely related to *AtMLO7,* in pollen tube receptivity. Other tomato genes here reported show similarity with Arabidopsis *AtMLO* homologs with unknown biological role. Functional reverse genetics studies making use of these sequences could possibly unravel new physiological roles for the *MLO* gene family.

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Chapter 6

General discussion: loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance

Chapter 6

General discussion: loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance

Stefano Pavan[1, 2], Evert Jacobsen[1], Luigi Ricciardi[2], Richard G.F. Visser[1], Yuling Bai[1]

[1]Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy;

Breeding for disease resistance: R-genes or S-genes?

Over fifty years ago, Flor (1955) showed for the first time that plant resistance towards a pathogen was subordinated to the simultaneous presence of a dominant plant allele (termed resistance gene or R-gene) and a dominant pathogen allele (avirulence or Avr-gene). Since then, hundreds of similar gene-for-gene plant-pathogen interactions have been documented and breeding for disease resistance has focused on the search and exploitation of R-genes. However, R-gene mediated resistance is typically not durable in the field, as it can be easily overcome by the onset of pathogen variants harboring new alleles at the Avr locus. Furthermore, progressive loss of biodiversity makes it more and more difficult to identify new R-genes.

Based on the the characterization of recessive plant alleles associated to disease resistance, including those treated in this this thesis (Chapter 2 to 4), we introduce in this Chapter the concept that resistance to pathogens can also be pursued through the inactivation of genes necessary for pathogenesis, further referred to as susceptibility genes (S-genes). Eckardt (2002) first coined the term of "plant susceptibility gene", commenting the discovery that the Arabidopsis gene *PMR6* promotes the growth of powdery mildew fungi. From a genetic point, S-genes can be defined as genes whose loss-of-function mutations result in recessively inherited resistance. In cultivated species, recessive resistance sources have been known for many years. For example, the first reported resistance gene is recessively inherited, which was identified by Biffen in 1905 and confers resistance to wheat yellow rust (*Puccinia striiformis*) (Singh and Singh 2005).

R-genes and S-genes in the context of plant immunity

Similarly to metazoans, plants are characterized by an innate immune system which recognizes potential pathogens and addresses appropriate defense responses (Jones and Dangl 2006). Gene-for-gene resistance, first described in genetic terms, is due to an immune system response, originated by the recognition between the products of R- and Avr-genes. The isolation of several R-genes revealed that they mostly encode for intracellular receptor proteins with an N-terminal nucleotide binding (NB) site and C-terminal leucine rich repeats (LRRs) (Takken et al. 2006). On the other hand, it is now clear that Avr-genes encode for pathogen effectors that, in absence of cognate R-proteins, interact with specific plant

proteins, termed virulence targets, in order to suppress plant defense responses (Nomura et al. 2005; Chisholm et al. 2006). Once recognition between R-proteins and effectors has occurred, a cascade of defense pathways is triggered, in which a major role is played by the phytohormones salicylic acid, jasmonic acid and ethylene. Most R-proteins function in a tripartite module, where the R-protein guards a specific virulence target, and, in doing so, can detect modifications induced by the effector (van der Hoorn et al. 2002).

Notably, all S-genes characterized in cultivated species have been predicted to encode for virulence targets used by effectors to suppress plant defense responses (Table 1). Therefore, recessive resistance due to their loss-of-function mutations originates from the lack of interaction between pathogen effectors and cognate host virulence targets. One well-characterized S-gene in a cultivated species is barley *MLO*, which is required for susceptibility to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Büschges et al. 1997; Piffanelli et al. 2004). In loss-of-function *mlo* mutants, pathogenesis stops prior to fungal penetration, in correspondence of cell-wall appositions (papillae) formed at plant-pathogen interaction sites. Wild-type *MLO* encodes for a transmembrane protein modulating different kinds of defense pathways at the cell periphery, dependent and independent from actin cytoskeleton (Miklis et al. 2007). Evidence that actin cytoskeleton dynamics of *MLO* and *mlo* genotypes in response to powdery mildew infection become distinguishable only several hours after pathogen inoculation strongly suggests that MLO proteins are targeted by fungal effectors in order to suppress plant defense responses (Opalski et al. 2005). In this thesis (Chapters 2 to 4), we demonstrate that the recessive alleles *ol-2* and *er1*, associated to powdery mildew resistance in tomato and pea, respectively, are due to loss-of-function mutations of *MLO* gene orthologs (Table 1).

Other plant susceptibility genes (reported in Table 2) have been characterized in the model species *Arabidopsis thaliana* following experimental approaches like induced mutagenesis or gene silencing. Some of these genes, like *TOM1* and *TOM3*, have been predicted to encode for virulence targets (Tsujimoto et al. 2003). Many others have been shown to act as negative regulators of plant defense pathways, whose inactivation leads to resistance through enhanced defense responses. Examples of this kind are *EDR1*, *MPK4* and *PMR4*, which negatively regulate the defense pathway mediated by the salicylic acid, and *CEV1*, negatively regulating the jasmonic acid defense pathway (Petersen et al. 2000; Ellis and Turner 2001; Frye et al. 2001; Nishimura et al. 2003).

It should be stressed that not all the genes encoding for virulence targets act as S-genes, as their loss is not associated to resistance to pathogens. This can be explained by

one of the following scenarios: 1) the same effector acts on multiple virulence targets, so that loss-of-function of one of them does not suffices to lead to resistance; 2) the aim of the effector is to inactivate a virulence target which has a role in enhancing plant defense responses. In this case, the loss of the virulence target would indeed mimic the activity of the effector. This holds true for example for the effector HopAI1 of *Pseudomonas syringae*, which suppresses plant defenses by inactivating Arabidopsis mitogen-activated protein kinases MPK3 and MPK6, two key components of the plant immune response signaling cascade (Zhang et al. 2007).

Susceptibility genes and fitness

Despite the fact that they promote pathogen proliferation and disease establishment, S-genes have not been excluded by evolution. On the contrary, they prevail in nature over their corresponding non-functional mutations, thus indicating they play a major role in plant fitness. Evidence suggests that certain S-genes, besides being involved in plant-pathogen interactions, are required for the correct functioning of other important aspects of plant physiology. For example, the rice gene *Xa13* is required for both the growth of *X. oryzae* pv. *oryzae* and plant pollen development (Chu et al. 2006). The dual function of S-genes such as *Xa13* provides a unique opportunity for exploring the functional overlap between pathways involved in plant development and in pathogen-induced susceptibility.

Susceptibility genes with a role in the negative regulation of defense pathways presumably confer a selective advantage by optimizing the distribution of energetic resources among various physiological processes. Indeed, mutations of these kinds of genes are often accompanied by adverse pleiotropic effects due to unregulated defense activation, such as reduced growth and lesion-mimic phenotypes (Table 2).

It is clear, however, that genes with a negative impact on plant fitness in natural ecosystems may be desirable for agricultural purposes. Many successes obtained in breeding can be referred to the introduction of allelic variants which are rare or even absent in natural germplasm. Thus, it is not surprising that S-genes mutant alleles have been in some cases successfully introduced in cultivation. Although pleiotropic effects have been reported together with the *mlo* resistant phenotype (Büschges et al. 1997 and Table 1), *mlo* resistance is by far the most used powdery mildew resistance source in spring barley grown in Europe (Lyngkjaer et al. 2000). Similarly, resistances conferred by mutations of *Er1* in pea, *Xa5* and *Xa13* in rice, and *eIF4E* orthologs in barley, pepper, lettuce, melon and pea are also widely employed in

agricultural systems (Candresse et al. 2002; Nicaise et al. 2003; Gao et al. 2004a and 2004b; Kang et al. 2005; Morales et al. 2005; Nieto et al. 2006; Iyer-Pascuzzi and McCouch, 2007; Rakotomalala et al. 2008; Tyrka et al. 2008; Humphry et al. 2011; Chapter 4 of this thesis). In addition, phenotypes associated with the loss of function of *EDR1*, *PMR4*, *DMR1* and *Ol-2*, seem to be devoid of deleterious pleiotropic effect and compatible with their commercial exploitation (Frye et al. 2001; Nishimura et al. 2003; van Damme, 2007; Bai et al. unpublished, Tables 1 and 2).

Nonhost-like resistance conferred by S-genes loss of function

Nonhost resistance is defined as the immunity exhibited by plant species outside the host range of a given pathogen species (Thordal-Christensen 2003; Lipka et al. 2008). When not dependent on pre-formed barriers to pathogen colonization (e.g. cuticle or wax layers, antimicrobial compounds), nonhost resistance has been shown to arise from active defense mechanisms induced by the immune system (Thordal-Christensen, 2003; Lipka et al. 2005). The recognition event leading to nonhost resistance is likely to involve plant plasma membrane receptors perceiving chemical patterns from microbial origin (e.g. fungal chitin) or host origin (e.g. patterns resulting from enzymatic degradation of the host cell wall) (Ellis 2006). Nonhost resistance, besides being the most common kind of immunity in nature, is extremely stable as demonstrated by the fact that pathogens have rarely altered their host species range over recorded history (Heath, 2000; Lipka et al. 2008). Several authors pointed out that the ideal genetic resistance should exploit molecular mechanism of nonhost resistance (Thordal-Christensen, 2003; Lipka et al. 2008).

Analogies between *mlo* resistance and nonhost resistance towards powdery mildew fungi have been repeatedly pointed out (Trujillo et al. 2004; Ellis 2006). Humphry et al. (2006) even stated that *mlo* resistance and nonhost resistance can be considered as "two faces of the same coin". Common points between the two kinds of immunity include: 1) the histology of the defense mechanism, which occurs before fungal penetration and is associated to the accumulation of callose and other compounds in cell-wall papillae at plant-pathogen interaction sites; 2) independence from defense pathways commonly associated to R-gene mediated resistance, such as those associated to the hormones salicylic acid, jasmonic acid and ethylene; 3) dependence on actin cytoskeleton functioning and on defense pathways involving *PEN* genes, encoding for particular kinds of syntaxins, glycosyl hydrolases and ABC

transporters.

Data reported in literature strongly suggest loss of susceptibility has the potential to result in resistance that shares the "hallmarks of nonhost resistance" (Humphry et al. 2006): broad-range of action and durability. As reported in Table 2, loss of S-genes is often effective towards pathogen belonging to different pathogenic Kingdoms. In addition, when tested against different genetic variants of a pathogen, it has been often proven to be race non-specific (e.g. Bai et al. 2005; Kang et al. 2005). Little information is available relative to the durability of the immunity conferred by the lack of many S-genes. However, resistances conferred by mutations of *MLO* genes in barley and pea and *eIF4E* in pepper are still effective in the field, several decades after their introduction in agriculture (Lyngkjaer et al. 2000; Kang et al. 2005; Fondevilla et al. 2006). Extensive information on the stability of resistance due the loss of susceptibility genes will derive from their future exploitation in plant breeding.

Conclusions and future perspectives

In this thesis, we show that broad-spectrum and nonhost-like *mlo* resistance not only occurs in barley but also in other two cultivated species, tomato and pea, thus suggesting that it is generally possible to breed for *mlo* resistance in crops affected by the powdery mildew disease. In addition, in this Chapter we review a series of resistance sources, mainly characterized in Arabidopsis, which, similarly to *mlo*, originate from the loss-of function of S-genes (Tables 1 and 2). Many S-genes are likely to be identified in the next few years, as, in the field of plant-microbe interactions, the characterization of host virulence targets manipulated by pathogen effectors has moved to a central stage (Hoefle and Hückelhoven 2008).

To exploit S-genes for resistance breeding, two questions need to be addressed: (1) Are there S-gene orthologues across cultivated plant species? (2) How to obtain and apply S-genes loss-of-function in resistance breeding? A targeted approach based on comparative genetics/genomics would provide answers to these questions. Firstly, sequence homology to characterized S-genes should be identified, for example, as followed in Chapter 5, by the aid of available sequence databases. Secondly, potential S-gene candidates should be functionally characterized, in order to observe altered phenotypes in relation to interaction with pathogens as well as to other agricultural traits. Rapid functional analyses could be carried out by using virus-induced gene silencing (VIGS), a technique which has been today optimized for several crop species (e.g. Ratcliff et al. 2001; Liu et al. 2002). Once genes of interest have been identified, their stable silencing could be achieved by using other techniques based

on RNA interference (RNAi), such as short hairpin RNA interference (shRNAi) (Waterhouse and Helliwell, 2003). Loss-of-function mutations of S-genes can be achieved by targeted approaches, such as insertional mutagenesis (Krysan et al. 1999; Parinov et al. 1999; Speulman et al. 1999), TILLING (targeting induced local lesions in genomes) (Colbert et al. 2001) and recently introduced mutagenesis mediated by zinc-finger nucleases (Marton et al. 2010). The application of the last two approaches is particularly promising, as they combine high efficiency in the identification of mutations of interest with the advantage of being non-transgenic technologies.

Table 1. Susceptibility genes cloned in cultivated species and features of resistance derived by their loss-of-function mutations

Gene	Plant species	Encoded protein	Pathogen	Reported pleiotropic phenotype	Reference
MLO orthologues	Barley	Transmembrane protein	*Blumeria graminis* f.sp. *hordei*	Early senescence and axenic cell death	Büschges et al. (1997)
	Tomato		*Oidium neolycopersici*	Not reported	Chapter 2 and 3 of this thesis
	Pea		*Erysiphe pisi*	Not reported	Chapter 4 of his thesis; Humphry et al. (2011)
eIF4E orthologues	Several species	Translation initiation factor	Potyviridae	Not reported	Diaz-Pendon et al. (2004); Robaglia and Caranta (2006)
Xa5	Rice	Transcription factor IIA-γ	*Xanthomonas oryzae*	Not reported	Iyer-Pascuzzi and McCouch (2007)
Xa13	Rice	Membrane-localized protein	*X. oryzae*	Pollen abortion	Chu et al (2006); Yang et al (2006); Sugio et al. (2007)

Table 2. Susceptibility genes cloned in Arabidopsis and features of resistance derived by their loss-of-function mutations or silencing

Gene	Encoded protein	Pathogen	Reported pleiotropic phenotype	Reference
BIK1	Membrane-anchored protein kinase	*P. syringae*	Enhanced susceptibility to *Botrytis cinerea* and *Alternaria brassicicola;* altered root growth	Veronese et al. (2006)
CEV1	Cellulose synthase	*G. orontii* *G. cichoracearum* *Oidium neolycopersici*	Reduced size, darker green leaf color, anthocyanins accumulation	Ellis and Turner (2001) Ellis et al. (2002)
CPR5	transmembrane protein	*Hyaloperonospora parasitica* *P. syringae*	Reduced growth; chlorotic lesions	Bowling et al. (1997) Kirik et al. (2001)
DMR6	2-oxoglutarate-Fe(II) oxygenase	*H. parasitica* *Colletotrichum higginsianum*	Slightly rounded leaves	van Damme et al. (2005) van Damme et al. (2008)
DND1	Cyclic nucleotide-gated ion channel	*P. syringae* *Xanthomonas campestris* Tobacco ringspot virus	Dwarf	Yu et al. (1998) Clough et al. (2000)
DND2	Cyclic nucleotide-gated ion channel	*P. syringae*	Dwarf	Jurkowski et al. (2004)
EDR1	Mitogen activated protein kinase kinase kinase	*P. syringae* *G. cichoracearum*	Stunted plants with spontaneous lesions under drought conditions	Asai et al. (2002) Frye et al. (2001) Tang et al. (2005)
LSD1	Zinc finger protein	*H. parasitica* *P. syringae*	Lesion formation	Dietrich et al. (1994) Kaminaka et al. (2006)
MAP65-3	Microtubule-associated protein	*Meloidogyne incognita*	Dwarf phenotype and reduced fertility	Caillaud et al. (2008)
MLO2	Transmembrane protein	*G.orontii* *G.cichoracearum* *O. neolycopersici*	Early senescence and axenic cell death	Consonni et al. (2006) Charter 3 of this thesis
MPK4	Mitogen activated protein kinase	*H. parasitica* *P. syringae*	Dwarf, curled leaves and reduced fertility	Petersen et al. (2000)

Table 2 (continued)

Gene	Encoded protein	Pathogen	Reported pleiotropic phenotype	Reference
PMR4	Callose synthase	G.orontii G.cichoracearum H. parasitica	Epinastic leaves	Vogel and Somerville (2000) Nishimura et al. (2003)
RAR1	Zinc-binding protein	P. syringae	Not reported	Shang et al. (2006)
SNI1	Leucine-rich nuclear protein	H. parasitica P. syringae	Reduced growth and fertility	Li et al. (1999); Mosher et al. (2006)
SON1	F-box protein	H. parasitica P. syringae	Not reported	Kim and Delaney (2002)
SR1	Ca^{2+}/calmodulin-binding transcription factor	Pseudomonas syringae	Chlorosis and autonomous lesions	Du et al. (2009)
SSI2	Stearoyl-acyl carrier protein desaturase	H. parasitica P. syringae	Small rosette, curled leaves, lesions	Kachroo et al. (2001) Shah et al. (2001)
WRKY family members	WRKY transcriptional factors	P. syringae G. orontii Blumeria graminis Xanthomonas axonopodis	Not reported	Eulgem and Somssich (2007) Oh et al. (2008) Peng et al. (2008) Shen et al. (2007) Xing et al. (2008)
DMR1	Homoserine kinase	H. parasitica	No effect or slightly smaller size, depending on the dmr1 allele	van Damme et al. (2005) van Damme et al. (2007)
PMR5	Unknown function protein	Golovinomyces orontii G. cichoracearum	Reduced growth, microlesions	Vogel et al. (2004)
PMR6	Pectate lyase-like protein	G. orontii G. cichoracearum	Reduced growth, microlesions	Vogel et al. (2002)
TOM and TOM3	Transmembrane proteins	Tobacco mosaic virus	Not reported	Diaz-Pendon et al. (2004)
TOM2A	Transmembrane protein interacting with TOM1	Tobacco mosaic virus	Not reported	Tsujimoto et al. (2003)

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Summary

The powdery mildew disease, caused by obligate biotrophic fungi belonging to the Ascomycete order of Erysiphales, is common among higher plants and represents one of the most important threats for the cultivation of many crops. Although powdery mildew resistance is usually a dominant trait, recessively inherited resistance has been reported to occur in Arabidopsis, barley, tomato and pea. In **Chapter 1** of this thesis, we provide a state of the art on the understanding of mechanisms underlying plant immunity and review monogenic dominant and recessive resistance sources used in breeding. In **Chapters 2 and 3,** we describe a successful homology-based cloning approach for the isolation of the recessive *ol-2* gene, naturally occurring in a tomato accession collected in Ecuador and conferring broad-spectrum resistance to the powdery mildew fungus *Oidium neolycopersici*. Resistance mediated by *ol-2* shares striking similarities with well-known barley and Arabidopsis *mlo* powdery mildew resistance, originating from loss-of-function mutations of genes encoding for specific (seven transmembrane domains carrying) MLO isoforms. The following chain of evidence is provided demonstrating that *ol-2* resistance is due to the loss of function of the tomato *MLO* homolog *SlMLO1*: a) *Ol-2* and *SlMLO1* loci share the same genetic and cytogenetic position; b) resistant *ol-2/ol-2* lines are homozygous for a loss-of-function deletion in the sequence of *SlMLO1*; c) *SlMLO1* transgenic expression in *ol-2/ol-2* individuals results in disease susceptibility; d) *SlMLO1* virus-induced silencing in *Ol-2/Ol-2* individuals is associated to increased powdery mildew resistance. In **Chapter 4**, we first illustrate a chemical mutagenesis program allowing the identification of a pea line showing recessive resistance towards the powdery mildew fungus *Erysiphe pisi*. Histological and genetic analyses revealed that the mutated gene is allelic to *er1*, commonly used in pea breeding for the development of resistant cultivars. As defense mechanisms associated to *er1* resistance are reminiscent of *mlo* immunity, we sequenced the pea *MLO* homolog *PsMLO1* and found a loss-of-function point mutation characterizing the resistant line. A polymorphic CAPS marker was developed on the mutation site and found to be fully co-segregating with resistance in a large F_2 population. Finally, *PsMLO1* sequencing in three *er1* resistant cultivars also resulted in the identification of aberrant alleles, further substantiating the identification of another case of *mlo*-based immunity. In **Chapter 5**, we report the identification (*in vitro* and *in silico*) of a series of *MLO* homolog sequences in five cultivated Solanaceae species affected by the powdery mildew disease. Comparative analyses using a dataset of several dicot MLO proteins allowed the identification of candidate isoforms for

disease susceptibility and the detection of cluster-specific transmembrane amino acid motifs. In **Chapter 6**, we look at disease resistance as a condition due to the lack of susceptibility genes like *MLO*. We review several susceptibility genes isolated in crop species and in Arabidopsis, with respect to their molecular characterization, their role in plant-pathogen interactions and the resistant phenotype deriving from their loss-of-function mutations/silencing. A breeding strategy based on the lack of plant susceptibility genes is discussed.

Samenvatting

Echte meeldauw, een ziekte veroorzaakt door obligaat biotrofe schimmels behorend tot de Ascomyceten orde van Erysiphales, komt vaak voor bij hogere planten en vertegenwoordigt een van de belangrijkste bedreigingen voor de teelt van veel gewassen. Hoewel meeldauw resistentie meestal een dominante eigenschap is, is ook recessief erfelijke resistentie in o.a. Arabidopsis, gerst, tomaat en erwt bekend. In **Hoofdstuk 1** van dit proefschrift, geven wij een beschouwing op het gebied van inzicht in de mechanismen die ten grondslag liggen aan plant immuniteit en toetsing van monogene dominante of recessieve bronnen die gebruikt worden in de veredeling. In **Hoofdstuk 2** en **3**, beschrijven we een succesvolle homologie gebaseerde klonerings aanpak voor de isolatie van het recessieve *ol-2* gen, dat van nature voorkomt in een wilde tomaten accessie verzameld in Ecuador, en die leidt tot een breed spectrum resistentie tegen de meeldauw schimmel *Oidium neolycopersici*. Resistentie gemedieerd door *ol-2* vertoont opvallende gelijkenissen met bekende gerst en Arabidopsis *mlo* meeldauw resistentie, afkomstig uit verlies-van-functie mutaties van de genen die coderen voor specifieke (zeven transmembraan domein bevattende) MLO iso-vormen. De volgende bewijzen zijn geleverd waaruit blijkt dat de *ol-2* resistentie te wijten is aan het verlies-van-functie van de tomaat *MLO* homoloog *SlMLO1*: a) *Ol-2* en *SlMLO1* loci delen dezelfde genetische en cytogenetische positie, b) resistente *ol-2/ol-2* lijnen zijn homozygoot voor een verlies-van-functie mutatie in de DNA volgorde van *SlMLO1*, c) *SlMLO1* transgene expressie in *ol-2/ol-2* individuen resulteert in ziektegevoeligheid, d) *SlMLO1* virus-geïnduceerde gene silencing in *Ol -2/Ol-2* individuen is gekenmerkt door een verhoogde weerstand tegen meeldauw. In **Hoofdstuk 4** zijn de resultaten beschreven van een chemisch mutagenese programma in erwt om resistentie te vinden tegen de erwten meeldauwschimmel *Erysiphe pisi*. Histologische en genetische analyses lieten zien dat het gemuteerde gen gelijk is aan *er1*, een gen dat al vaak gebruikt is in de erwten veredeling voor de ontwikkeling van resistente cultivars. Doordat de afweermechanismen gekoppeld aan de *er1* resistentie doen denken aan *mlo* immuniteit, hebben we het erwten *MLO* homoloog *PsMLO1* geïsoleerd en vonden we een punt mutatie in de resistente lijn die resulteerde in verlies-van-functie van het *MLO* gen. Een polymorfe CAPS marker werd ontwikkeld op de mutatie en deze merker co-segregeerde 100% met de resistentie in een grote F_2 populatie. Tenslotte bleek dat in drie *er1* resistente erwten rassen afwijkende allelen van het *MLO* gen konden worden aangetoond (allen verlies van functie) hetgeen een verdere bevestiging is van *mlo*-gebaseerde immuniteit in gewassen. In **Hoofdstuk 5** worden de resul-

taten beschreven van de identificatie (*in vitro* en *in silico*) van een serie van *mlo* homologe sequenties in vijf gecultiveerde Solanaceae soorten die allen lijden onder de meeldauwziekte. Vergelijkende analyses met behulp van een dataset van een aantal MLO eiwitten (afkomstig uit dicotyle gewassen) leidde tot de identificatie van kandidaat-isovormen voor vatbaarheid en de opsporing van cluster-specifieke transmembraan aminozuur motieven. In **Hoofdstuk 6**, de algemene discussie, wordt gespeculeerd over de reden van resistentie tegen ziektes door het ontbreken van zgn vatbaarheid of gevoeligheid genen, zoals *MLO.* We vergelijken een aantal vatbaarheids genen die geïsoleerd zijn uit gewassen en Arabidopsis, aan de hand van hun moleculaire samenstelling, hun rol in plant-pathogeen interacties en de resistente fenotypen die voortvloeien uit hun verlies-van-functie mutaties cq het plat leggen van hun expressie via 'silencing'. Een veredelings strategie gebaseerd op het ontbreken van vatbaarheid genen wordt besproken.

Acknowldgements

When I entered University, I considered studying just as a tool to obtain a degree as soon as possible. Fourteen years and several degrees later, I have to say that something has changed. I definitely enjoy studying; and I chose studying to be my job. This premise explains why I would like to acknowledge two categories of people: those who made me love studying and those who tolerated me while I had my mind too much into study.

I want first to thank my supervisors at Wageningen University, Prof. Richard Visser and Dr. Yuling Bai. They gave me trust and proposed me to start this PhD project; besides offering me excellent guidance, I was important to them. This helped me to increase self-confidence and face difficulties during these years. I thank Richard for the time he dedicated on me, for coming to Italy, for his precious suggestions. I thank Yuling for her patience, for our long discussions, for showing me her determination.

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During my stay in the Plant Breeding Department, I did not enjoy coffee (sorry, I am Italian), but I did enjoy coffee breaks because of enjoyable discussions with all my colleagues and

About the author

Stefano Pavan was born in Bari (Italy) on January 9th 1978. He obtained a first MSc degree in Biotechnology (Wageningen University, 2003), a second MSc degree in Agricultural Science (Bari University, 2004), and a PhD degree in Breeding and Pathology of agricultural and forestal plants (Bari University, 2008). Research presented in this PhD thesis is the result of a joint project between the Plant Breeding Departments of the Universities of Wageningen and Bari. He is currently working as Senior Researcher/Adjunct Professor in Agricultural Genetics at the University of Bari. His main interest is the study of genes required for susceptibility to powdery mildew fungi and parasitic plants.

CONTACT INFORMATION
Stefano Pavan

University of Bari
Faculty of Agriculture
Department of Agricultural, Forestal and Environmental Biology and Chemistry (DiBCA)
Section Genetics and Plant Breeding

Address: Via Amendola 165/A
70126 Bari, Italy
Tel: +39 080 5442998
Fax: +39 080 5442200
e-mail: stefano.pavan@agr.uniba.it

Full-paper publications

Pavan, S., Schiavulli, A., Appiano, M., Marcotrigiano, A. R.,Cillo, F., Visser, R. G. F., Bai, Y., Lotti, C., and Ricciardi, L. (2011). Pea powdery mildew *er1* resistance is associated to loss-of-function mutations at a *MLO* homologous locus. Theoretical and Applied Genetics, doi 10.1007/s00122-011-1677-6.

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Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School
EXPERIMENTAL PLANT SCIENCES
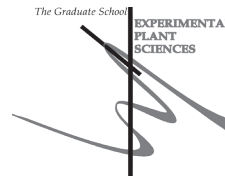
Issued to: Stefano Pavan
Date: 12 December 2011
Group: Laboratory of Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Comparative genomic analysis on the diversity and function(s) of Mlo orthologs in plant stress responses in tomato and Arabidopsis	Feb 27, 2007
► **Writing or rewriting a project proposal**	
Comparative genomic analysis on the diversity and function(s) of Mlo orthologs in plant stress responses in tomato and Arabidopsis	2008
► **Writing a review or book chapter**	
Solanum lycopersicum (Tomato), Published Online: 15 MAR 2009, IN: Encyclopedia of Life Sciences, DOI: 10.1002/9780470015902.a0003686	2008
Loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance, Molecular Breeding 25 (2010), 1-12	2008
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen	Sep 13, 2007
► **EPS theme symposia**	
EPS Theme 4 symposium 'Genome Biology'	Dec 09, 2011
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental Plant Sciences, Lunteren (two days meeting)	Apr 02-03, 2007
NWO-ALW Experimental Plant Sciences, Lunteren (two days meeting)	Apr 07-08, 2008
► **Seminars (series), workshops and symposia**	
attendance seminars organized by PBR	2007-2008
flying seminars organized in the Netherlands	2007-2008
► **Seminar plus**	
► **International symposia and congresses**	
XIII International Congress on Molecular Plant-Microbe Interaction Sorrento, Italy	Jul 21-27, 2007
51th SIGA Congress, Riva del Garda, Italy	Sept 23-26, 2007
XVI Eucarpia meeting of the tomato working group	May 13-15, 2008
Sol 2008 Cologne, Germany	Oct 12-16, 2008
54th SIGA Congress, Matera, Italy	Sept 27-30, 2010
55th SIGA Congress, Assisi, Italy	Sept 19-22, 2011
► **Presentations**	
Poster Presentation on XIII International Congress on Molecular Plant-Microbe Interaction	Jul 21-27, 2007
Oral Communication 51th SIGA Congress, Riva Del Garda, Italy	Sep 24, 2007
Oral Communication EPS school "On the Evolution of plant-Microbe interactions"	Jun 20, 2008
Oral communication 55th SIGA Congress, Assisi, Italy	Sep 19-22, 2011
► **IAB interview**	Dec 02, 2009
► **Excursions**	
Subtotal Scientific Exposure	*16.1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS Summer School "On the evolution of plant-pathogen interactions"	Jun 18-20, 2008
Bioinformatics (40 hours)	Apr-Jul 2009
Microscopy (10 hours)	Jun 2010
Plant Epigenetics (10 hours)	Jun-Jul 2011
► **Journal club**	
Plant Breeding seminars, Mondays	2006-2009
Resistance Group weekly seminars, Tuesdays	2006-2009
► **Individual research training**	
Subtotal In-Depth Studies	*6.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Responsible lecturer for the MSc course in 'Biotechnology for bioenergy crops' (40 hours), University of Bari	Oct-Dec 2009
Responsible lecturer for the MSc course in 'Biotechnology for bioenergy crops', (40 hours), University of Bari	Oct-Dec 2011
Lab safety (10 hours)	Apr 2011
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
	2009-2011
Subtotal Personal Development	*4.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40,3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.

Front cover and back covers:

Artistic representation of mycelium and conidiophores of *Oidium neolycopersici* developing on tomato leaves.