

Serpentine plant MLO proteins as entry portals for powdery mildew fungi

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Abstract

In the dicotyledonous plant species *Arabidopsis* and the monocot barley, presence of specific isoforms of the family of heptahelical plasma membrane-localized MLO proteins is required for successful host-cell invasion by ascomycete powdery mildew fungi. Absence of these MLO proteins, either caused by natural polymorphisms or induced lesions in the respective *Mlo* genes, results in failure of fungal sporelings to penetrate the plant cell wall. As a consequence, recessively inherited cell-autonomous *mlo* resistance is effective against all known isolates of powdery mildew fungi colonizing either barley or *Arabidopsis*. Barley MLO interacts constitutively with the cytoplasmic calcium sensor calmodulin, but the strength of this interaction increases transiently during fungal pathogenesis. In addition, MLO as well as ROR2, a plasma membrane-resident syntaxin also implicated in *mlo* penetration resistance, focally accumulate at sites of attempted fungal attack, thereby defining a novel pathogen-triggered micro-domain. In conclusion, powdery mildew fungi appear to specifically corrupt MLO to modulate vesicle-associated processes at the plant cell periphery for successful pathogenesis.

Introduction

Even though plants are constantly threatened by a plethora of potentially pathogenic microbes, surprisingly, most of the vegetation appears generally healthy. Besides preformed physical barriers, this is most probably the consequence of an elaborate surveillance system of plasma membrane-anchored and possibly also cytoplasmic immune receptors that allow plants the early recognition of potential intruders by detection of conserved molecular structures, also termed PAMPs (pathogen-associated molecular patterns) [1]. PAMP recognition triggers a multitude of biochemical responses finally resulting in the activation of defence mechanisms that suffice to effectively arrest the attempted assaults of most microbes. This innate immune response is thought to be an integral part of the genetically complex and extraordinarily durable set of defence mechanisms collectively termed 'non-host resistance' (reviewed in [2]). However, few microorganisms appear to have evolved some means to subvert the basic defence system of individual plant species, and, as a consequence, have become pathogens of these species [3]. It is conceivable that targeting and manipulation of particular host proteins is a key step of this species-specific defence sabotage [4].

Mlo and the barley-powdery mildew interaction

Powdery mildew is a common fungal disease of many monocotyledonous and dicotyledonous plant species. In a

moderately temperate and humid climate, these ascomycete fungi cause severe yield losses in a wide range of crops. For successful pathogenesis, fungal sporelings have to invade the host cell for establishment of a specialized intracellular feeding structure termed 'haustorium'. This requires penetration of the host cell wall as well as invagination of the host plasma membrane to accommodate the emerging fungal haustorium [5].

In barley, presence of the MLO protein is an absolute requirement for successful penetration of the host cell wall by the corresponding compatible powdery mildew species, *Blumeria graminis* f. sp. *hordei*. Barley genotypes lacking functional MLO, either due to natural genetic variation [6] or induced lesions in the *Mlo* gene [7,8], are resistant against all known isolates of the fungal pathogen. Owing to its broad-spectrum nature and its unusual durability, recessively inherited barley *mlo* resistance has been a success story in European spring barley agriculture for the last 25 years [9].

Is *mlo* disease resistance specific for powdery mildew?

As mentioned above, it is plausible that each pathogen species evolved its own specific means to suppress and overcome general or specialized host defence mechanisms. For example, the cowpea rust fungus, *Uromyces vignae*, seems to interfere with the formation of thin cytoplasmic plasma membrane-cell wall adhesion sites (also known as Hechtian strands) for host-cell invasion on its host plant, cowpea [10]. In contrast, formation of Hechtian strands was not decreased at attempted penetration sites of compatible or incompatible powdery mildew species, suggesting that an induced reduction in Hechtian strands at penetration sites might be a specific pathogenic strategy of the cowpea rust

Key words: *Arabidopsis*, barley, defence suppression, fungal pathogenesis, MLO, powdery mildew.

Abbreviations used: PAMP, pathogen-associated molecular pattern; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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fungus. Likewise, powdery mildew fungi appear to have specialized on MLO proteins as portals for host cell entry. Compared with *Mlo* wild-type plants, barley *mlo* mutants do not differ in the infection phenotype to a range of other phytopathogens, as for example barley leaf rust (*Puccinia striiformis*), stripe rust (*Puccinia hordei*), and the take-all fungus (*Gaeumannomyces graminis*) [11]. Interestingly, however, *mlo* mutants are more susceptible to the hemibiotrophic rice blast fungus, *Magnaporthe grisea*, and to the necrotrophic fungus *Bipolaris sorokiniana* [12,13]. In contrast, preliminary evidence for enhanced disease resistance of *mlo* genotypes to *Rhynchosporium secalis* (the causal agent of scald) was reported recently [14]. It remains to be examined in the future whether these differential infection phenotypes are a direct or indirect consequence of the lack of MLO function.

Why does *mlo* resistance not prevail in nature?

Wild barleys exhibit an enormous degree of genetic diversity (including significant polymorphism of resistance genes), rendering natural populations generally less sensitive to pathogen threat compared with genetically homogenous monocultures, nowadays typically employed in agriculture [15,16]. Thus, it appears that *mlo* resistance is not required in wild populations to keep spread of the powdery mildew pathogen in check. In addition, absence of functional barley MLO is intimately associated with a range of developmentally controlled pleiotropic effects, including the spontaneous deposition of callose-containing cell wall appositions (papillae) in pathogen-free grown, unchallenged plants as well as a premature onset of leaf senescence [8,17–19]. This impaired leaf physiology ultimately results in some reduced grain yield of *mlo* mutants compared with wild-type plants. Thus, the advantage of broad-spectrum powdery mildew resistance is entangled with a physiological penalty that initially hampered the use of *mlo* mutants in agriculture [17]. Finally, enhanced disease resistance of *mlo* mutants to a subset of hemibiotrophic and necrotrophic pathogen species (see above) might result in counter-selection of *mlo* mutant plants in natural environments. However, at least one *mlo* mutant arose naturally but was found at low frequencies in its habitat in the Ethiopian highlands [6]. This could either indicate that the recently emerged cultivation-associated polymorphism did not have sufficient time yet to spread more broadly or that *mlo* mutants may indeed have a competitive disadvantage in natural populations.

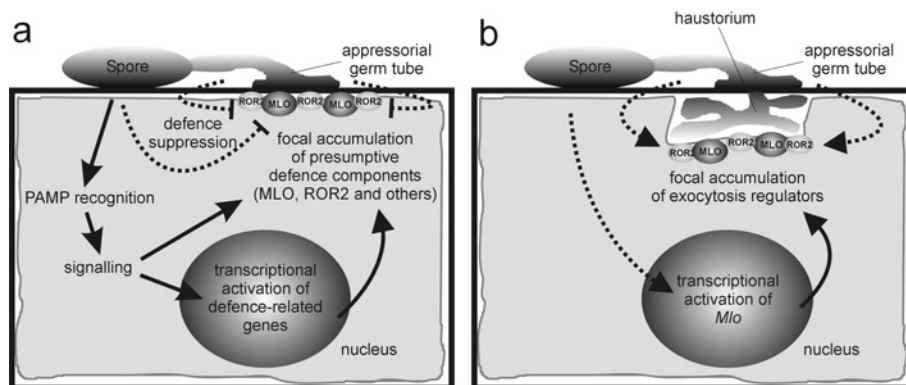
From genes to biochemistry: what might the MLO protein do?

Barley MLO is an integral plasma membrane-localized protein, possessing seven hydrophobic membrane-spanning helices [20]. The cytoplasmic C-terminus harbours an amphiphilic α -helix that serves as a calmodulin binding do-

main. Calcium-dependent calmodulin binding to this peptide domain was previously shown both *in vitro* and *in vivo* and contributes about half of the susceptibility-conferring activity of MLO [21]. Preliminary evidence suggests that MLO may form homooligomers and requires interplay of cytoplasmic domains for functionality [22]. Recently, a gene *Ror2* (*required for mlo resistance 2*) that, when mutated, suppresses *mlo*-mediated resistance was found to encode a plasma membrane-resident syntaxin. Syntaxins belong to the superfamily of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins representing key mediators of membrane fusion events during vesicle trafficking in yeast and animal cells [23]. Lack of wild-type ROR2 partially compromises penetration resistance in *mlo* genotypes, suggesting that syntaxin activity is required for effective *mlo* resistance [24,25]. As shown by transient expression of fluorophore-tagged protein variants in barley epidermal cells, both MLO and ROR2 focally accumulate at sites of attempted fungal cell-wall penetration, whereas a range of control proteins did not exhibit this specific subcellular accumulation pattern. Thus, it appears that MLO, ROR2 and potentially additional proteins form a novel pathogen-triggered micro-domain at biotic stress sites [26]. *In planta* fluorescence resonance energy transfer analysis revealed that, at these subcellular regions, interaction between MLO and the cytoplasmic calcium sensor calmodulin transiently increases during successful fungal host cell invasion [26]. Moreover, fluorescence resonance energy transfer experiments and studies using the yeast split-ubiquitin method [26] uncovered direct physical interaction between MLO and ROR2. The intensity of this interaction is drastically lowered between a subset of single amino acid substitution *mlo* mutant proteins and wild-type ROR2 as well as between wild-type MLO and the barley variant encoded by the barley *ror2* mutant, characterized by a 31 amino acid in-frame deletion in the central region of the syntaxin protein [25,26]. In conclusion, MLO might modulate SNARE protein-dependent and vesicle transport-associated processes at the plasma membrane, i.e. exocytosis or homotypic vesicle fusion events [23,25,28]. Cargo delivered by vesicles may include regulatory proteins and toxic metabolites (to defeat the microbial intruder at the site of attempted cell-wall penetration; Figure 1a) or plasma membrane material (to mediate net growth of the host plasma membrane for accommodation of the 'intracellular' fungal haustorium; Figure 1b). Current genetic data, however, strongly favour the first scenario, since the second model is difficult to reconcile with the fact that *mlo/ror2* double mutants exhibit partially restored susceptibility. Alternatively, for the regulation of membrane fusion events, MLO and the ROR2 syntaxin may form a novel biochemical complex of yet unknown function. Future analysis of biological processes governed by distinct MLO isoforms will help to elucidate the exact biochemical role of MLO proteins. Preliminary evidence suggests, for example, that *AtMlo5* and *AtMlo9* that are specifically expressed in pollen play a role during pollen tube germination (S. Noir and R. Panstruga, unpublished work).

Figure 1 | Model of assumed MLO corruption by the powdery mildew fungus in barley

The black rectangle represents a barley leaf epidermal cell attacked by the appressorial germ tube of a fungal sporeling and the grey line inside the box depicts the plasma membrane. **(a)** PAMP recognition and subsequent signal transduction leads to activation of the innate immunity response, including transcriptional up-regulation of defence-related genes and focal accumulation of respective gene products at the biotic stress site. MLO modulates an ROR2-dependent defence mechanism at the cell periphery. The fungus interferes with plasma membrane-localized MLO for defence suppression (broken lines). **(b)** The fungal pathogen stimulates transcriptional up-regulation and post-translational focal accumulation of MLO/ROR2 at attempted penetration sites (broken lines). Enhanced focal exocytosis of plasma membrane material might be required for the accommodation of the fungal haustorium.



Defence sabotage: how might the fungus manipulate MLO during pathogenesis?

It is now common belief that microbial intruders in animals and plants are recognized early during attempted invasion by the presence and exposure of PAMPs to corresponding host immune receptors [1]. Thus, it is plausible to assume that powdery mildew fungi also trigger a set of biochemical activities characteristic of the innate immunity response, typically comprising altered ion fluxes, the generation of reactive oxygen species, a mitogen-activated protein kinase signalling cascade, and transcriptional activation of defence-related genes [1,29]. It is conceivable that the approx. 10-fold transcriptional up-regulation of *Mlo* [8] and recruitment of MLO and ROR2 to biotic stress sites are integral parts of these complex defence activities. Depending on whether MLO may contribute to the delivery of toxic cargo or haustorium accommodation (see above), the fungal pathogen might either aim at dampening or stimulating MLO activity during pathogenesis (Figure 1). This could be accomplished by direct manipulation of MLO, e.g. by altering post-translational modifications like phosphorylation through fungal effector proteins. Alternatively, peptide effectors or small compounds might directly impinge on protein–protein interactions, e.g. between MLO and ROR2. The situation could be even more complex since powdery mildews might have evolved means to subvert a mechanism that originally evolved for plant defence. In tomato (*Lycopersicon esculentum*), for example, the tomato leaf spot fungus, *Septoria lycopersici*, detoxifies the steroidal phytoalexin α -tomatine by tomatinase, an extracellular enzyme that hydrolyses glucose from the secreted antifungal compound. The cleavage product of this enzymatic reaction, β ₂-tomatine, has significantly less antifungal capacity and, in addition, serves a role in the suppression of induced defence

responses [30]. In this manner, the pathogen undermines a preformed defence barrier and specifically exploits it for defence suppression in its host plant, tomato. If a similar situation holds true for the barley–powdery mildew interaction, then the parasite might even stimulate exocytosis, in fact thought to be an integral part of a general pathogen–defence pathway.

Unique or not: are powdery mildew resistant *mlo* mutants confined to barley?

During the last 50 years, most plant pathologists and breeders have envisaged broad-spectrum *mlo* resistance as an oddity of the monocot species barley. However, results recently obtained in our laboratory indicate that broad-spectrum disease resistance against powdery mildews can be induced by knocking out a particular form of the 15 *Arabidopsis Mlo* genes. T-DNA (transferred DNA) insertions in *AtMlo2* confer enhanced resistance against all three powdery mildew species known to successfully colonize *Arabidopsis thaliana* (C. Consonni, R. Panstruga, M. Humphry, S. Somerville, unpublished results). This finding indicates that the capability of powdery mildew fungi to exploit MLO proteins for pathogenesis is ancient and was evolutionarily conserved for more than approx. 270 million years, the time when monocot and dicot plants were thought to have diverged from each other [31]. In other words, this means that the pathogenicity mechanism of angiosperm powdery mildew fungi had already evolved long before dinosaurs began to dominate terrestrial life [32]. In addition, this result corroborates the fact that there exists strong isoform-specificity among *Mlo* paralogues, since only a loss of particular MLO proteins in both *Arabidopsis* and barley conditions mildew resistance. It will be interesting to elucidate whether this isoform-specificity is the result

of a particular protein feature or, alternatively, the indirect consequence of distinct gene expression patterns, as recently exemplarily demonstrated for members of the plant syntaxin protein family [33].

I thank Volker Lipka for helpful comments on the manuscript. Research in my laboratory is funded by grants of the Max-Planck Society as well as grant PA861/1 'signal transduction in *mlo* resistance' of the Deutsche Forschungsgemeinschaft (DFG).

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Received 8 October 2004