CURRENT REVIEW

***mlo*-Based Resistance: An Apparently Universal “Weapon” to Defeat Powdery Mildew Disease**

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Loss-of-function mutations of one or more of the appropriate *Mildew resistance locus o* (*Mlo*) genes are an apparently reliable “weapon” to protect plants from infection by powdery mildew fungi, as they confer durable broad-spectrum resistance. Originally detected as a natural mutation in an Ethiopian barley landrace, this so-called *mlo*-based resistance has been successfully employed in European barley agriculture for nearly four decades. More recently, *mlo*-mediated resistance was discovered to be inducible in virtually every plant species of economic or scientific relevance. By now, *mlo* resistance has been found (as natural mutants) or generated (by induced mutagenesis, gene silencing, and targeted or nontargeted gene knock-out) in a broad range of monocotyledonous and dicotyledonous plant species. Here, we review features of *mlo* resistance in barley, discuss approaches to identify the appropriate *Mlo* gene targets to induce *mlo*-based resistance, and consider the issue of pleiotropic effects often associated with *mlo*-mediated immunity, which can harm plant yield and quality. We portray *mlo*-based resistance as an apparently universal and effective weapon to defeat powdery mildew disease in a multitude of plant species.

In temperate climates, the common and widespread powdery mildew disease constitutes a constant and ubiquitous threat to agriculture and horticulture (Glawe 2008). Worldwide, around 10,000 plant species are host to more than 650 species of powdery mildew fungi (Takamatsu 2004, 2008). Among these are cereals and crops such as barley (*Hordeum vulgare*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*), pea (*Pisum sativum*), tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*); fruits like apple (*Malus domestica*), grapevine (*Vitis vinifera*), peach (*Prunus persica*), and strawberry (*Fragaria × ananassa*; *F. vesca*); the spice plant pepper (*Capsicum annuum*); ornamental plants such as petunia (*Petunia hybrida*) and rose (*Rosa hybrida*); and other economically relevant plants, for example, tobacco (*Nicotiana tabacum*). Powdery mildew outbreaks often result in severe harvest losses, for instance by reduction of grain yield and quality in barley and wheat (Dean et al. 2012). Quantitative and race-specific resistances usually provide either incomplete or short-lived protection against powdery mildew disease (Jørgensen and Wolfe 1994).

In the 1930s and 1940s, a game-changing type of powdery mildew resistance was discovered. Loss-of-function of the gene

Mildew resistance locus o (*Mlo*) in barley was found to confer recessively inherited broad-spectrum resistance against the ascomycete phytopathogen (Jørgensen 1992; Lyngkjær and Carver 2000). In contrast to prototypical resistance (*R*) gene-mediated race-specific resistance, which is typically inherited dominantly or semidominantly, *mlo*-based resistance is non-race specific and, thus, effective against the vast majority of powdery mildew isolates (Brown 2015; Lyngkjær and Carver 2000). Out of the more than 40 barley *mlo* mutant alleles known to date (Reinstädler et al. 2010), the natural *mlo*-11 (Piffanelli et al. 2004) and the induced *mlo*-9 (Schwarzbach 1967) allele are the two *mlo* mutations predominantly deployed in agriculture since the late 1970s and early 1980s. Now, more than half of the spring barley commercially grown in central Europe is largely immune to powdery mildew, due to the introgression of *mlo* resistance into a broad panel of barley varieties (Jørgensen 1992; McGrann et al. 2014). Although *mlo* has been recently introgressed into a few winter barley lineages, this resistance is currently not yet employed in commercially grown winter barley (Czembar et al. 2016).

The barley *Mlo* gene was cloned about 20 years ago and was, subsequently, found to code for a member of a family of integral membrane proteins (Büschges et al. 1997; Devoto et al. 2003). As experimentally shown, barley *Mlo* is a plasma membrane-localized seven-transmembrane domain protein with an extracellular amino terminus and an intracellular carboxy-tail (Devoto et al. 1999). However, little is known about the molecular function of the protein. The carboxy-terminal tail, which is predicted to be intrinsically disordered (Kusch et al. 2016), includes a domain that binds calmodulin and is required for full susceptibility to powdery mildew infection in barley (Kim et al. 2002). Moreover, the protein possesses four conserved cysteines in two of the extracellular domains, likely important for structural integrity (Elliott et al. 2005), and a number of conserved short peptide motifs scattered in the intra- and extracellular domains (Appiano et al. 2015a; Kusch et al. 2016). However, at present the biochemical activity of the protein remains mysterious (Acevedo-Garcia et al. 2014). Given that *Mlo* is coexpressed with genes involved in plant defense, it seems plausible that *Mlo* exerts a function in plant immunity (Humphry et al. 2010).

Characteristics of *mlo* resistance in barley.

The obligate biotrophic barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* proliferates on the host upon penetration of the cell wall and “intracellular” establishment of the haustorium, which is the primary site of contact between the fungus and the plant cell (O’Connell and Panstruga 2006). Once the interaction is established, secondary hyphae emerge that spread epiphytically on the plant surface (Fig. 1A) and later

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give rise to conidiophores that bear new conidiospores (Bélanger et al. 2002). Barley *mlo*-based resistance is characterized by an effective arrest of fungal pathogenesis at very early stages of infection. Usually, *B. graminis* f. sp. *hordei* spores attempt to penetrate epidermal host cells but fail, as formation of haustoria and secondary hyphae is not observed on *mlo* genotypes (Fig. 1B). Consequently, sporulation does not occur and the fungus cannot complete its asexual life cycle (Fig. 2). Subsidiary cells of stomatal complexes are the exception, because rare powdery mildew colonies and even sporulation can be found after successful colonization of this cell type. The extent of this effect varies between *mlo* mutants dependent on the genetic background but is, seemingly, not dependent on the type of *mlo* allele (Jørgensen and Mortensen 1977).

Barley *mlo*-based prepenetration resistance is characterized by several distinctive features. Local cell-wall appositions (CWAs) that contain the β -glucan cell-wall polymer callose are found at attempted penetration sites of *B. graminis* f. sp. *hordei*, which is reminiscent of the response to nonhost pathogens (Skou 1982; Skou et al. 1984). The number and diameter of these CWAs, or papillae, is increased in *mlo* mutants, compared with wild-type barley (*Mlo* genotype), upon interaction with *B. graminis* f. sp. *hordei* (Stolzenburg et al. 1984). In addition, other defense-associated compounds overaccumulate at *B. graminis* f. sp. *hordei* infection sites in barley *mlo* genotypes, including the phenol conjugate *p*-coumaroyl-hydroxyagmatine (von Röpenack et al. 1998) and reactive oxygen species such as hydrogen peroxide (Hückelhoven et al. 2000; Piffanelli et al. 2002). These events coincide with a faster and more pronounced accumulation of defense-related transcripts in *mlo* null mutant plants (Zierold et al. 2005). Mesophyll cells were further described to undergo localized cell death below sites of attempted *B. graminis* f. sp. *hordei* penetration, which can result in macroscopically visible necrotic leaf spots (Piffanelli et al. 2002).

Requirements for *mlo* resistance in barley.

Deciphering the molecular basis of *mlo*-based resistance has been subject of several studies. Chemical mutagenesis has been employed to identify suppressor mutants of *mlo*-based resistance, thereby uncovering two genetic loci required for full resistance, *Required for mlo-specified resistance1* (*Ror1*) and *Ror2* (Freialdenhoven et al. 1996). Both *ror1* and *ror2* mutations partially suppress penetration resistance to powdery mildew in barley *mlo* null mutant plants and reinstate host cell entry rates of up to 20% (Freialdenhoven et al. 1996). The *Ror2* protein encodes a t-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein that is likely involved in pathogen-induced secretion of antimicrobial compounds at infection sites. *Ror2* is considered to be the ortholog of *Arabidopsis* PENETRATION1 (*PEN1*), which contributes to nonhost resistance against powdery mildews (Collins et al. 2003). The *Ror1* gene has not been cloned

yet, but its genomic location has been narrowed down to a 0.18-cM interval in the peri-centromeric region of the long arm of chromosome 1H (Acevedo-Garcia et al. 2013). *Ror1* and *Ror2* seem to partake in two separate pathways that add to *mlo*-based resistance (Collins et al. 2003). The two proteins modulate *mlo*-dependent hyperaccumulation of hydrogen peroxide upon *B. graminis* f. sp. *hordei* challenge (Hückelhoven et al. 2000; Piffanelli et al. 2002) and also contribute to basal resistance against the rice blast pathogen, *Magnaporthe grisea* (Jarosch et al. 2005).

In addition to the genetic approach, pharmacological assays were devised to uncover requirements for *mlo*-based resistance in former studies. The glucose analog 2-deoxy-D-glucose (DDG) as well as mannose act as partial suppressors of *mlo*-based resistance to *B. graminis* f. sp. *hordei*, resulting in up to 15% host cell entry in the case of mannose and more than 50% penetration by *B. graminis* f. sp. *hordei* in the case of DDG. Furthermore, autofluorescence and size of papillae are affected by these treatments, suggesting that synthesis and localized deposition of callose is necessary for full resistance to powdery mildew in barley *mlo* plants (Bayles et al. 1990; Lyngkjær et al. 1997). Treatment of mannose or DDG in combination with α -aminooxy- β -phenylpropionic acid (AOPP) enhances this effect and further increases *B. graminis* f. sp. *hordei* penetration rates, while AOPP alone is insufficient to affect *mlo*-based resistance. AOPP is an inhibitor of phenylalanine ammonia lyase, which catalyzes the first dedicated reaction in the synthesis of phenolics in plants (Lyngkjær et al. 1997). In addition, actin cytoskeleton function was discovered to be involved in *mlo*-based resistance. The actin cytoskeleton undergoes extensive reorganization upon powdery mildew attack, reorienting toward and accumulating at attempted host cell-entry sites. This reorganization occurs faster in *mlo* mutant cells, compared with *Mlo*-expressing cells, in response to *B. graminis* f. sp. *hordei* (Opalski et al. 2005). Treatment of barley leaves with the actin polymerization inhibitor cytochalasin E severely affects resistance to *B. graminis* f. sp. *hordei* in barley *mlo* plants, as does overexpression of actin depolymerizing factors (ADFs) (Miklis et al. 2007). ADFs increase the turnover of filamentous actin by depolymerization of globular monomeric actin from the polymer, thus negatively regulating actin polymerization (Carlier et al. 1997). Microtubule inhibitors (propyzamide, oryzalin), however, do not alter resistance to *B. graminis* f. sp. *hordei* in *mlo* mutant plants (Miklis et al. 2007).

Barley *Mlo* function is mechanistically linked to Ca^{2+} homeostasis. The ubiquitous Ca^{2+} sensor calmodulin binds to barley *Mlo* and this interplay is required for full susceptibility to *B. graminis* f. sp. *hordei* (Kim et al. 2002). Binding of calmodulin to *Mlo* increases during the *B. graminis* f. sp. *hordei* entry phase in planta (Bhat et al. 2005), while treatment with the Ca^{2+} chelator EGTA attenuates this interaction in vitro (Kim et al. 2002). Moreover, exogenous application of Ca^{2+} partially suppresses *mlo* resistance in barley coleoptiles (Bayles and Aist

Fig. 1. *mlo*-based resistance prevents host cell penetration. Barley leaves were inoculated with *Blumeria graminis* f. sp. *hordei* (isolate K1) and photographs were taken at 48 h postinoculation. Fungal structures were stained with Coomassie brilliant blue. **A**, The plant lines were the susceptible cultivar Ingrid and **B**, *mlo-3* in Ingrid background. sp = spore, ap = appressorium, ha = haustorium, and hy = hyphae. Scale bar = 30 μm .

1987) and overexpression of the Ca^{2+} -dependent protein kinase *HvCDPK3* or the junction domain of *HvCDPK4* in barley *mlo* cells partially increases the *B. graminis* f. sp. *hordei* entry rate (Freymark et al. 2007). Taken together, cellular Ca^{2+} levels seem to modulate both *Mlo* function and *mlo*-based resistance.

Programmed cell death is an important factor of plant defense. BAX INHIBITOR 1 (BI-1) is a negative regulator of cytochrome c release-related stress-induced cell death in mammalian and plant cells. Barley *BI-1* expression is responsive to *B. graminis* f. sp. *hordei* infection, and local overexpression of *BI-1* in barley *mlo* cells reconstitutes susceptibility to *B. graminis* f. sp. *hordei* almost to wild-type levels, suggesting that regulation of cell death is another crucial aspect of *mlo*-based resistance (Hückelhoven

et al. 2003). Further, *BI-1* silencing increases resistance to powdery mildew in otherwise susceptible barley *Mlo* plants (Eichmann et al. 2010) and, at least in *Arabidopsis*, BI-1 was found to interact with calmodulin and requires intact Ca^{2+} -dependent ATPases for cell death-inhibiting activity (Ihara-Ohori et al. 2007). Thus, BI-1 is a common element of cell death regulation and a modulator of penetration resistance.

Taken together, the combination of genetic and pharmacological assays suggests that multiple molecular processes and pathways collectively contribute to *mlo*-based resistance in barley. These include exocytosis mediated by the syntaxin Ror2, actin cytoskeleton function, callose biosynthesis and deposition, Ca^{2+} -related pathways, as well as regulation of cell death.

Fig. 2. Examples for *mlo* resistance in various plant species. The plant species were inoculated with the respective adapted powdery mildew species. Upper panel, susceptible wild-type or *Mlo* overexpression. Lower panel, powdery mildew-resistant line based on *mlo* loss-of-function. Species and the respective *Mlo/mlo* variant are indicated above each picture. Photographs were contributed by Y. Bai and S. Pessina (apple, grapevine), J. Berg (cucumber), Z. Zheng (pepper), M. Appiano (tomato), H. Cheng (melon), Z. Qiu and M. Bao (rose), K. Gruner (wheat), C. Consonni (*Arabidopsis*), and M. Arai (tobacco).

Modulation of barley *mlo*-based resistance by abiotic and biotic factors.

While *mlo*-based resistance is typically very stable in the field, several abiotic factors have been found to modulate this type of plant defense. A number of studies identified drought stress followed by sudden relief of this state (by watering) as a modulator of resistance, since several *mlo*-resistant barley cultivars experiencing this condition displayed moderate susceptibility to powdery mildew (Baker et al. 1998, 2000; Newton and Young 1996). Further, heat treatment (36°C for 30 to 120 min) was discovered to partially break immunity of barley *mlo* leaf segments to powdery mildew. However, the weak, heat-induced susceptibility of *mlo* plants is temporary and resistance recovers to levels of unstressed leaves after 13 h (Schwarzbach 2001).

Some reports describe the occurrence of barley powdery mildew isolates that partially overcome *mlo*-based resistance. The natural Japanese isolate Race1 exhibits limited virulence on barley *mlo* null mutant plants, with colony numbers reaching up to 13% of a susceptible wild-type cultivar (Lyngkjær et al. 1995). In addition, field isolates with temporarily increased *mlo* virulence have been collected in the United Kingdom and Czech Republic, resulting in colony numbers of up to 16% of a susceptible *Mlo* genotype. However, this partially enhanced virulence was lost in subsequent generations (Schwarzbach et al. 2002). In contrast to these naturally occurring cases of partial *mlo* virulence, the *B. graminis* f. sp. *hordei* isolate HL-3/HL-5 was selected toward enhanced virulence on *mlo* plants from the Czech isolate GE3 (Schwarzbach 1967, 1979). HL-3/HL-5 displayed a penetration rate of up to 18% on *mlo* null mutant plants but did not present increased aggressiveness on the respective susceptible (*Mlo* genotype) parental cultivar (Lyngkjær and Østergård 1998). Noticeably, *mlo*-virulent *B. graminis* f. sp. *hordei* strains do not appear to be prevalent or dominant in nature or agricultural settings, even though partially overcoming *mlo*-based resistance is, in principle, possible, as evidenced by the above-mentioned instances. Perhaps the combination of *mlo*-based resistance, isolate-specific resistance genes, and fungicides proves too high a barrier for *B. graminis* f. sp. *hordei* to adapt to. Alternatively, partially *mlo*-virulent *B. graminis* f. sp. *hordei* isolates may suffer from selective disadvantages in other respects.

Pleiotropic phenotypes of barley *mlo* mutants.

mlo-based resistance is unique, as it confers durable broad-spectrum resistance specifically to powdery mildews but,

Fig. 3. Scheme illustrating the proposed *Mlo*-dependent anticorrelation of powdery mildew susceptibility and the extent of pleiotropic phenotypes. The relative level of susceptibility to powdery mildew is shown on the left y axis, the relative level of pleiotropic phenotypes is given on the right y axis. The hypothesized anticorrelation between these traits is depicted as being dependent on the plant genotype (*Mlo/mlo*), which is indicated on the x axis. Please note that, in this scheme, the correlations are shown to be linear, which is not necessarily the case (as symbolized by the dashed lines). This simplified scheme also does not take into account the developmental stage of the plant, which is an additional determinant of the extent of the pleiotropic phenotypes.

seemingly, not to most other pathogens. The only exception reported so far is the hemibiotrophic oomycete *Phytophthora pal-mivora*, to which barley *mlo* plants are more resistant in younger leaf sections but not in older leaf areas or roots (Le Fevre et al. 2016). However, defense against some nonbiotrophic pathogens is affected in barley *mlo* plants. Barley *mlo* mutants were reported to be more susceptible to the hemibiotrophic rice blast fungus *M. grisea*, which is associated with increased leaf chlorosis upon infection and is independent from the *Ror* genes (Jarosch et al. 1999). The mutant was also found to be more sensitive to *Bipolaris sorokiniana* toxin-containing culture filtrates (Kumar et al. 2001) and to allow increased proliferation of the necrotrophic fungus *Fusarium graminearum* in barley spikes (Jansen et al. 2005). In addition, barley *mlo* plants show increased susceptibility to *Ramularia* leaf spot caused by *Ramularia collo-cygni* in a *Ror1*- and *Ror2*- as well as growth condition-dependent manner (McGrann et al. 2014; Pinnschmidt and Sindberg 2009). In contrast to these reports, a recent study determined that there seems to be no difference in colonization by *Fusarium* spp. and *R. collo-cygni* between *mlo* and wild-type (*Mlo*) barley plants under field conditions (Hofer et al. 2015). Collectively, these data suggest a trade-off between powdery mildew resistance and susceptibility to nonbiotrophic pathogens. Nevertheless, this trade-off might depend on the particular field or laboratory conditions.

Barley *mlo* mutants exhibit additional pleiotropic phenotypes that can impact harvest yields. A microscopically visible feature of *mlo* mutants is the spontaneous formation of CWAs, detectable as discrete callose-containing deposits in aging leaves in the absence of any pathogen or other obvious stress condition (Wolter et al. 1993). This situation is reminiscent of a deregulated defense program (lesion-mimic mutant [Walbot et al. 1983]). Likely as a further consequence of endogenously deregulated physiology, *mlo* mutants display early leaf senescence, which is characterized by spontaneous mesophyll cell death and premature chlorophyll decay, resulting in macroscopically visible leaf chlorosis and necrosis (Peterhänsel et al. 1997; Piffanelli et al. 2002; Wolter et al. 1993). This effect has also been reported in field trials in Scotland and Ireland. In the latter studies, barley *mlo* mutants showed consistently increased occurrence of abiotic leaf spots, emphasizing the potential problem of these pleiotropic effects for barley cultivation (Makepeace et al. 2007).

Interestingly, the recently described *mlo*-11 (*cnv2*) allele present in an Ethiopian landrace (Eth295), which exhibits partially relieved powdery mildew resistance, seemingly does not suffer from the early senescence phenotype (Ge et al. 2016). Similar observations have been made with induced barley *mlo* mutants (*mlo*-12 and *mlo*-28) that show a weak resistance phenotype (Hentrich 1979; Piffanelli et al. 2002). Notably, *mlo*-based resistance is effective at all developmental stages, while pleiotropic effects seem to be only apparent in mature or older (i.e., flowering or senescing) plants. Based on these data, an anticorrelation seems to exist between the level of powdery mildew susceptibility and the extent of premature leaf senescence in *mlo* plants (Fig. 3).

Discovery of *mlo* resistance in other plant systems.

Resistance conferred by loss-of-function *mlo* mutants was long believed to represent a unique feature of monocotyledonous barley. Approximately a decade ago, however, it was found that *mlo*-based resistance is also effective in the dicotyledonous model plant *Arabidopsis thaliana* (Table 1) (Consonni et al. 2006). Since then, *mlo*-based resistance has been described in many additional plant species (Fig. 2; Table 1; Box 1). These instances comprise naturally occurring resistant accessions of tomato and pea (Bai et al. 2008; Humphry et al. 2011) or induced *mlo* resistance in wheat (Acevedo-Garcia et al. 2017; Wang et al. 2014). Thus, *mlo*-based resistance emerges as a powerful and apparently

universal genetic resource against powdery mildew disease in a wide range of angiosperms.

***Arabidopsis* and barley reveal conserved features of *mlo*-based resistance.**

Arabidopsis AtMLO2, *AtMLO6*, and *AtMLO12* (please note that, in plant species other than barley, the nomenclature can either be *Mlo* or *MLO*; we use *Mlo* as the generic version throughout this article) were identified as co-orthologs of barley *Mlo*. Transgenic transfer-DNA (T-DNA) insertion lines and chemically induced mutants of *MLO2* (syn. *POWDERY MILDEW RESISTANCE2 [PMR2]* [Vogel and Somerville 2000]) revealed that the three *MLO* genes unequally add to the interaction with powdery mildew; *AtMLO2* is the major and *AtMLO6* and *AtMLO12* are minor contributors to powdery mildew susceptibility. Accordingly, the *Arabidopsis mlo2* mutant displays macroscopically visible resistance against powdery mildew but microscopically retains partial susceptibility concerning host cell penetration and the establishment of fungal microcolonies. The *mlo2 mlo6* double mutant exhibits increased resistance and the *mlo2 mlo6 mlo12* triple mutant is fully immune, resembling the phenotype of barley *mlo* plants (Consonni et al. 2006). Of the three single mutants, only *mlo2* shows increased resistance to *Golovinomyces cichoracearum* and *G. orontii*, as infections are macroscopically symptom-less (Consonni et al. 2006). Similar to barley *mlo* plants, *Atmlo2* lines display early senescence and chlorosis in aging rosette leaves as well as spontaneous callose deposition in unchallenged leaves.

Thanks to the availability of abundant genetic resources, extensive analyses have been performed to unravel the molecular basis of *mlo* resistance in *Arabidopsis* (Acevedo-Garcia et al. 2014; Kuhn et al. 2016). Similar to the *ror2* mutation in barley, the *Arabidopsis pen1* mutation partially suppresses resistance of *mlo2*. In addition, *PEN2* (a glycosyl hydrolase) and *PEN3* (an ABC transporter), both involved in nonhost resistance against powdery mildews, are required for *mlo2*-based resistance (Consonni et al. 2006). The *mlo2 mlo6 mlo12* mutant also hyperaccumulates salicylic acid (SA), but mutants affected in SA, jasmonic acid, or ethylene synthesis do not compromise *mlo2*-based resistance (Consonni et al. 2006). Further, *mlo2*

mutants show an age-dependent increase in steady-state levels of the phytoalexin camalexin and indolic glucosinolates. Accordingly, mutations in *CYP79B2/CYP79B3*, encoding two redundant monooxygenases required for the synthesis of these tryptophan-based compounds, restore susceptibility to *G. orontii* in the *mlo2* genotype (Consonni et al. 2010). In addition, *POWDERY MILDEW RESISTANCE4 (PMR4)/GLUCAN SYNTHASE-LIKE5* has an effect on *mlo2* mutants. *mlo2 pmr4* mutants do not exhibit spontaneous callose deposition (as opposed to the *mlo2* mutant) but resistance to powdery mildew is unaltered (Consonni et al. 2010). Taken together, these findings reveal that the essential characteristics of *mlo*-based resistance, such as prepenetration resistance and early leaf senescence, are conserved among monocots and dicots. Interestingly, however, *mlo* mutants in some plant species, such as apple, melon, pea, tobacco, and tomato, do not seem to suffer from undesired pleiotropic phenotypes (Table 1) (Bai et al. 2008; Cheng et al. 2015; Fujimura et al. 2016; Humphry et al. 2011; Pessina et al. 2016a). Whether these are species-specific or simply condition-dependent differences still needs to be explored.

Sources of *mlo* mutants.

Naturally occurring plant accessions exhibiting *mlo*-based resistance are an obvious source for *mlo* mutants and, in the case of barley and pea, are widely employed for breeding and agriculture. Ethiopian landraces of barley were the first known example of such natural *mlo* mutants. This mutant (designated *mlo-11*) carries a complex repeat array consisting of multiple partial copies of the *Mlo* gene directly upstream of the genuine *Mlo* gene copy, causing accumulation of aberrant transcripts and a massive reduction of the authentic *Mlo* transcript (Piffanelli et al. 2004). Interestingly, the landrace Eth295, a recently discovered derivative of the *mlo-11* mutant, is an example of a natural variant showing partial *mlo*-based resistance but not its usually associated pleiotropic phenotypes (Ge et al. 2016). In addition to barley, natural *mlo* mutants of a different type (e.g., single nucleotide polymorphisms, small insertions or deletions [indels] and transposon insertions) have been found in cucumber (Berg et al. 2015), melon (Cheng et al. 2015), pea (Humphry et al. 2011; Pavan et al. 2011; Sun et al. 2016a and b),

Table 1. Functionally validated examples of *mlo*-based powdery mildew resistance (in chronological order)

Plant species	<i>Mlo</i> gene	Type ^a	Pleiotropic phenotypes ^b	Reference
<i>Hordeum vulgare</i> (barley)	<i>Mlo</i>	Natural, chemical, radiation	CWAs, premature senescence	Jørgensen 1992 Wolter et al. 1993
<i>Arabidopsis thaliana</i> (thale cress)	<i>AtMLO2</i> <i>AtMLO6</i> <i>AtMLO12</i>	T-DNA, chemical	CWAs, premature senescence	Consonni et al. 2006
<i>Solanum lycopersicum</i> (tomato)	<i>SlMLO1</i>	Natural	None	Bai et al. 2008
<i>Pisum sativum</i> (pea)	<i>PsMLO1</i>	Natural, chemical	None	Humphry et al. 2011 Pavan et al. 2011 Sun et al. 2016a Sun et al. 2016b
<i>Fragaria vesca</i> (strawberry)	Unknown ^c	RNAi	Unknown	Jiwan et al. 2013
<i>Capsicum annuum</i> (pepper)	<i>CaMLO2</i>	RNAi	Reduced plant size	Zheng et al. 2013
<i>Triticum aestivum</i> (bread wheat)	<i>TaMlo1</i>	TALEN, TILLING	Premature senescence	Wang et al. 2014 Acevedo-Garcia et al. 2017
<i>Cucumis sativus</i> (cucumber)	<i>CsMLO5</i>	Natural	Unknown	Berg et al. 2015 Nie et al. 2015
<i>Rosa hybrida</i> (rose)	<i>RhMLO1</i>	RNAi	Unknown	Qiu et al. 2015b
<i>Nicotiana tabacum</i> (tobacco)	<i>NtMLO1</i>	Natural	None	Fujimura et al. 2016
<i>Cucumis melo</i> (melon)	<i>CmMLO2</i>	Natural	None	Cheng et al. 2015
<i>Vitis vinifera</i> (grapevine)	<i>VvMLO6</i> , <i>VvMLO7</i>	RNAi	Unknown	Pessina et al. 2016b
<i>Malus domestica</i> (apple)	<i>MdMLO19</i>	RNAi	None	Pessina et al. 2016a

^a T-DNA = transfer-DNA, RNAi = RNA interference, TALEN = transcription activator-like effector nucleases, and TILLING = targeting induced lesions in genomes.

^b CWA = cell-wall appositions. None indicates none observed in the tested growth regimes and unknown indicates the aspect was not mentioned in the respective publications.

^c Unknown = aspect not mentioned in the respective publications.

tobacco (Fujimura et al. 2016), and tomato (Bai et al. 2008). These examples illustrate that naturally occurring landraces and accessions can be a rich source for *mlo* mutants.

Another widely explored source of *mlo* mutants is chemical- or radiation-based mutagenesis. The plant genotype of choice is either exposed to a chemical mutagen, e.g., ethyl methanesulfonate (EMS) or sodium azide (NaN₃), or a physical mutagen such as gamma radiation, and then, the respective offspring is selected for the desired powdery mildew resistance phenotype. Many of the known barley mutant alleles have been generated by radiation or EMS/NaN₃ mutagenesis, resulting in point mutations or small indels (Jørgensen 1992; Reinstädler et al. 2010). Some *Arabidopsis mlo2 (pmr2)* mutant lines have been generated by EMS mutagenesis as well (Consonni et al. 2006; Vogel and Somerville 2000). Combining induced mutagenesis with high-throughput genotyping for mutations in a given *Mlo* gene by a method called TILLING (targeting induced lesions in genomes) represents a fast, powerful, and nontransgenic procedure for the detection of *mlo* mutants even in complex genomes. For example, simultaneous mutation of the wheat homeologs of *TaMlo1* (*A1*, *B1*, *D1*) has been achieved by this method, producing several mutant combinations that display increased powdery mildew resistance (Acevedo-Garcia et al. 2017).

Another frequently used approach is gene silencing of candidate *Mlo* genes in the species of interest. This method takes advantage of the plant-endogenous RNA silencing machinery by providing small double-stranded RNA target sequences of the respective *Mlo* gene. In tomato, transient virus-induced gene silencing (VIGS) was one of the methods employed to show that *SlMlo1* is the gene responsible for powdery mildew susceptibility (Bai et al. 2008). A weak contribution of *SlMlo5* and *SlMlo8* to powdery mildew susceptibility was suggested in a later study using RNA interference (RNAi) in a susceptible tomato cultivar (Zheng et al. 2016). A VIGS-based approach was also utilized in pepper to silence *CaMLO1* and *CaMLO2* in the context of the interaction with *Leveillula taurica*, thereby enhancing resistance to this endophytic powdery mildew pathogen (Zheng et al. 2013). VIGS and other RNAi approaches were further followed in wheat (Várallyay et al. 2012) and several members of the family Rosaceae, e.g., apple, grapevine, rose, and strawberry (Jiwan et al. 2013; Pessina et al. 2016b; Pessina et al. 2016a; Qiu et al. 2015b). Most of these transgenic approaches were, however, limited to the validation of candidate *Mlo* genes that contribute to susceptibility to powdery mildew.

Recent advances in precision genome editing now allow targeted mutagenesis of a specific locus in plants. The respective

Box 1. Species with documented *mlo*-based powdery mildew resistance

Monocots

Barley (*Hordeum vulgare*)

- Archetype of *mlo*-based resistance (Jørgensen 1992).
- More than 40 mutant alleles known (Reinstädler et al. 2010).
- Most extensively studied *Mlo* protein, with experimental work focusing on interaction partners (Kim et al. 2002), protein stability (Elliott et al. 2005), structure-function analysis (Elliott et al. 2002, 2005; Reinstädler et al. 2010), and in planta protein dynamics (Bhat et al. 2005).

Wheat (*Triticum aestivum*)

- Three near-identical orthologs of barley *Mlo*: *TaMlo-A1*, *TaMlo-B1*, and *TaMlo-D1* (Elliott et al. 2002).
- Completely resistant TALEN/Cas9-derived triple mutants (Wang et al. 2014) and partially resistant TILLING-based mutants (Acevedo-Garcia et al. 2017).

Dicots

Apple (*Malus domestica*)

- Twenty-one *MLO* genes, four of which sort into phylogenetic clade V (Pessina et al. 2014).
- Two of these, *MdMLO11* and *MdMLO19*, and the clade VII member *MdMLO18* transcriptionally induced upon challenge with *Podosphaera leucotricha* (Pessina et al. 2014).
- Knockdown of *MdMLO19* but not of *MdMLO11* reduces symptoms by 75% (Pessina et al. 2016a).

Arabidopsis (*Arabidopsis thaliana*)

- *AtMLO2*, *AtMLO6*, and *AtMLO12* co-orthologs of barley *Mlo* (Consonni et al. 2006).
- First example of a species where mutations in multiple *Mlo* genes cooperatively contribute to full resistance (unequal genetic redundancy [Consonni et al. 2006]).
- Availability of ample genetic resources enables analysis of the molecular basis of *mlo* resistance (Consonni et al. 2006, 2010).

Cucumber (*Cucumis sativus*)

- Three clade V *MLO* genes closely related to *AtMLO2* (Schouten et al. 2014).
- One of these, *CsMLO13*, transcriptionally induced upon powdery mildew attack (Schouten et al. 2014).
- Transgenic expression of *CsMLO5* in the tomato *Slmlo1* mutant restores susceptibility to *Oidium neolycopersici* (Berg et al. 2015).
- Four natural *Cucumis sativus* mutant alleles of *CsMLO5* confer resistance to *Podosphaera xanthii* (Berg et al. 2015; Nie et al. 2015).

Grapevine (*Vitis vinifera*)

- Four *MLO* orthologs of *AtMLO2*, three of which are transcriptionally induced upon *Erysiphe necator* infection (Feechan et al. 2008; Winterhagen et al. 2008).
- Transgenic expression of *VvMLO11* and *VvMLO13* in the *Arabidopsis mlo2 mlo6 mlo12* triple mutant confers partial susceptibility to *Erysiphe cichoracearum* (Feechan et al. 2013).
- Contradictory, RNAi-based knockdown of *VvMLO6* and *VvMLO7* but not *VvMLO11* and *VvMLO13* results in increased resistance to *Erysiphe necator* (Pessina et al. 2016b).

(Continued on next page)

methods enable specific binding of one or more nucleases to a genomic sequence of interest, which can induce single- or double-strand breaks. Subsequent error-prone DNA repair on the basis of nonhomologous end-joining can then result in the formation of indel mutations at a specific genomic locus. Transcription activator-like effector nucleases (TALENs), originating from the plant-pathogenic bacterial genus *Xanthomonas* (Boch et al. 2009), have been used in wheat for simultaneous mutagenesis of the three *TaMlo1* homeologs. The knock-out of *TaMlo-A1*, *TaMlo-B1*, and *TaMlo-D1* by short indels induced full powdery mildew resistance (Wang et al. 2014). Additionally, in this study, a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 construct was employed to target *TaMlo-A1*. CRISPR-Cas9 is an alternative genome editing tool that consists of CRISPR single guide RNA, including the approximately 20-bp target sequence, or protospacer, and the nuclease Cas9 from *Streptococcus pyogenes*. The sgRNA directs Cas9 to the DNA target sequence (Jinek et al. 2012).

In sum, these examples nicely illustrate that *mlo* mutants can be retrieved from many sources and via different experimental approaches, depending on the specific demands, including the selection of natural mutants, induced mutagenesis, knockdown by gene silencing, and state-of-the-art genome modification methods.

Predicting the appropriate *Mlo* target.

In order to target one or more of the correct *Mlo* genes for induction of powdery mildew resistance, suitable candidates

have to be identified. Since *Mlo* genes are typically present as medium-sized families with 10 to 25 members per plant species (Kusch et al. 2016), the majority of these not involved in biotic stress functions, this task is not trivial. Generally, successful approaches include i) analysis of sequence similarity and phylogenetic relationship of candidate *Mlo* genes to described *Mlo* genes with a role in powdery mildew susceptibility, ii) evaluation of candidate *Mlo* transcript profiles upon powdery mildew infection, and iii) functional assessment based on bioassays.

The basic phylogenetic relationships within the *Mlo* protein family have been well-resolved. *Mlo* proteins involved in powdery mildew susceptibility belong to clade IV in monocots and clade V in dicots (Acevedo-Garcia et al. 2014; Kusch et al. 2016). Based on this knowledge, the identification of suitable *Mlo* genes by whole-genome surveys followed by phylogenetic classification has become common practice. These analyses also include searches for conserved peptide motifs, especially the C-terminally located tetra-peptide [DE]FSF seemingly always present in *Mlo* proteins involved in powdery mildew susceptibility (Panstruga 2005).

Often, several candidate *Mlo* genes are identified based on phylogeny and sequence analysis. The next step could be to analyze the expression pattern of the candidate *Mlo* genes by one or a combination of quantitative reverse transcription-polymerase chain reaction, microarray analysis, or whole genome shotgun RNA sequencing in the relevant tissues and upon powdery mildew attack. This approach is based on the previous

Box 1. (Continued from preceding page)

Melon (*Cucumis melo*)

- Sixteen melon *MLO* proteins, three of which classified as clade V (Iovieno et al. 2015).
- *CmMLO2*, *CmMLO3* and *CmMLO13* upregulated upon *Podosphaera xanthii* infection (Howlader et al. 2016).
- A natural mutant variant of *CmMLO5* confers resistance to *Podosphaera xanthii* (Cheng et al. 2015).

Pea (*Pisum sativum*)

- Six natural *Psmlo1* (*er1*) alleles and one induced mutant described that confer resistance to *Erysiphe pisi* (Humphry et al. 2011; Pavan et al. 2011; Sun et al. 2015, 2016a and b).
- Some of these extensively used in breeding without knowing the identity of the underlying gene (Rubiales and Fondevilla 2012).

Pepper (*Capsicum annuum*)

- Silencing of *CaMLO1* and *CaMLO2*, two genes with high sequence similarity to tomato *SlMlo1*, increases resistance to *Leveillula taurica* but also results in reduced plant size (Zheng et al. 2013).
- Silencing of *CaMLO2* also enhances resistance to the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (Kim and Hwang 2012).

Rose (*Rosa hybrida*)

- Four *MLO* genes with close phylogenetic relationship to *AtMLO2* and *SlMlo1* (Kaufmann et al. 2012).
- Two of these, *RhMLO1* and *RhMLO2*, transcriptionally induced upon infection with *Podosphaera pannosa* (Qiu et al. 2015a).
- Silencing of *RhMLO1* results in enhanced resistance to *P. pannosa* (Qiu et al. 2015b).

Strawberry (*Fragaria* spp.)

- Three clade V *MLO*s identified (Miao et al. 2016; Pessina et al. 2014).
- Expression of a peach (*Prunus persica*) clade V *MLO* antisense fragment in *Fragaria* × *ananassa* garden strawberry results in increased resistance to *Podosphaera macularis* (Jiwan et al. 2013).

Tobacco (*Nicotiana tabacum*)

- Five clade V *MLO* genes identified (Appiano et al. 2015b).
- Overexpression of one of these, *NtMLO1*, in the tomato *Slmlo1* mutant results in partial susceptibility to *Oidium neolycopersici* (Appiano et al. 2015b).
- Japanese tobacco cultivars, including Kokubu, exhibit resistance based on mutations affecting *NtMLO1* and *NtMLO2* (Fujimura et al. 2016).
- Transgenic tobacco cv. Kokubu expressing *NtMLO1* or *NtMLO2* display increased susceptibility (Fujimura et al. 2016).

Tomato (*Solanum lycopersicum*)

- The natural tomato *ol-2* mutant is defective in *SlMlo1* and shows resistance to *Oidium neolycopersici* (Bai et al. 2008) and the endophytic powdery mildew pathogen *Leveillula taurica* (Zheng et al. 2013).
- A later study identified three additional clade V *SlMlo* genes, and silencing of these suggests a minor contribution of *SlMlo5* and *SlMlo8* to susceptibility (Zheng et al. 2016).

notion that transcript levels of the *Mlo* genes that confer susceptibility to powdery mildew typically increase following pathogen challenge (Feechan et al. 2008; Howlader et al. 2016; Humphry et al. 2010; Pessina et al. 2014; Piffanelli et al. 2002; Qiu et al. 2015a; Schouten et al. 2014; Winterhagen et al. 2008; Zheng et al. 2013). Thus, responsiveness to biotic stress at the transcriptional level can be considered a further criterion to narrow down one or more appropriate *Mlo* target genes.

For functional validation, three main methodologies have been described in the literature: i) silencing of one or more *Mlo* candidate genes via VIGS or RNAi in the plant species of interest, ii) overexpression of one or more *Mlo* candidate genes in a *mlo* mutant variant of this plant, or iii) overexpression of one or more *Mlo* candidate genes in a heterologous plant system with well-defined *mlo* resistance, i.e., *Arabidopsis mlo2 mlo6 mlo12*, tomato *Slmlo1*, or barley *mlo*. In each case, the effect of *Mlo* silencing or overexpression is assessed by scoring the powdery mildew infection success micro- or macroscopically or both. The mentioned approaches have been efficiently used in case of many plant species, including wheat (Acevedo-Garcia et al. 2017; Elliott et al. 2002; Várallyay et al. 2012), grapevine (Feechan et al. 2008, 2013; Pessina et al. 2016b; Winterhagen et al. 2008), rose (Kaufmann et al. 2012; Qiu et al. 2015a and b), pepper (Zheng et al. 2013), and apple (Pessina et al. 2014, 2016a), demonstrating the feasibility of this integrated strategy to identify one or more appropriate *Mlo* gene targets.

Concluding remarks.

In this essay, we portray *mlo*-based resistance as a powerful and durable “weapon” against powdery mildew infection, not only in barley but in a wide variety of crop, fruit, and other plant species, including both dicots and monocots. To our knowledge, *mlo* resistance seems to be a universal phenomenon, as we do not know of any example where loss-of-function of one or more of the appropriate clade IV and clade V *Mlo* genes failed to confer powdery mildew resistance. Thus, presence of one or more respective *Mlo* proteins seems to be a general prerequisite for successful powdery mildew colonization. There are, however, plant species (e.g., rice and maize) that harbor seemingly adequate clade IV *Mlo* genes but are not host to powdery mildews. Hence, other factors such as additional plant or pathogen components or climatic conditions, or both, also appear to be crucial for powdery mildew pathogenesis. *Mlo* proteins are, therefore, necessary but not sufficient to mediate colonization of a given plant species by powdery mildew fungi.

In the meantime, *mlo* resistance is widely used in plant breeding in several plant species, including barley, pea, and tomato. The tools are now available to predict one or more adequate *Mlo* targets and to test their function by a variety of assays. Modern precision gene editing technologies emerge as the methods of choice to induce *Mlo* knock-outs in a targeted manner. However, there is a still ongoing debate whether such approaches are to be considered transgenic or not (Huang et al. 2016). For the time being, TILLING remains an attractive and undoubtedly nontransgenic alternative to select suitable *mlo* mutants (Acevedo-Garcia et al. 2017).

Yet, many aspects regarding the molecular basis of *mlo* resistance remain unanswered to date. Due to its requirement for successful plant colonization, *MLO* is often referred to as a susceptibility factor for powdery mildews (van Schie and Takken 2014). On the other hand, coexpression data suggest an authentic function for *MLO* proteins in plant immunity and, thus, rather a potential role as a negative regulator of plant defense (Humphry et al. 2010). The latter would be consistent with the fact that *mlo* mutant plants show resemblance to mild lesion-mimic mutants (Walbot et al. 1983). Recently, many lesion-mimic mutants have

been shown to be suppressed by second-site mutations in genes coding for nucleotide binding site-leucine-rich repeat-type resistance proteins or pivotal defense signaling components, such as *PAD4*. It has been concluded that, in these instances, constitutive immunity and the lesion-mimic phenotype are the result of the inappropriate activation of resistance proteins due to the lack of the matching “guardee”. In such a scenario, *MLO* would represent the guardee that is guarded by a yet-unknown resistance protein, which would be constitutively active in the absence of *MLO*. However, the fact that *mlo*-mediated immunity and resistance gene-based immunity have differential genetic requirements in barley (Freialdenhoven et al. 1994, 1996) argues against such a scenario. Furthermore, resistance conditioned by the *Arabidopsis mlo2* mutant is not suppressed by mutations in *PAD4*, which is a necessary signaling component acting downstream of resistance proteins with an N-terminal toll interleukin 1 receptor domain (Consonni et al. 2006). Finally, resistance of *mlo* mutants is specific for powdery mildews, which is also inconsistent with such a setting. The exact role of *MLO* during fungal invasion thus remains unresolved for the time being.

Further key questions to be addressed by future research comprise the following:

- Is *mlo* resistance indeed a universal phenomenon in angiosperm plants?
- What are the defense pathways involved (what arrests fungal pathogenesis)?
- Why is *mlo* resistance so durable?
- Why do *mlo* mutants of some plant species show pleiotropic phenotypes, while *mlo* mutants of other plant species do (seemingly) not?
- Can we uncouple resistance from pleiotropic phenotypes, e.g., by mutation of particular regulatory genes?
- Why do some powdery mildew isolates have the capacity to overcome *mlo*-based resistance?
- What is the biochemical activity of the *Mlo* protein?
- Why is the presence of the *Mlo* protein required for successful powdery mildew pathogenesis?

Solving these issues is an important task for the coming years, as a deep understanding of the *Mlo* protein, the molecular mechanism of immunity, and the relationship between *mlo*-based resistance and potential pleiotropic phenotypes will help in designing the most efficient lines for defeating powdery mildew and for improving harvest yield and quality in the future.

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