THE FUNCTION OF MLO, A NEGATIVE REGULATOR OF DEFENCE, IS CONSERVED IN MONOCOT AND DICOT PLANTS

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ABBREVIATIONS

Avr	avirulence gene
Bgh	*Blumeria graminis* f.sp. *hordei*
Bgt	*Blumeria graminis* f.sp. *tritici*
CaM	calmodulin
CaMBD	calmodulin binding domain
CC	coiled-coil
Cub	C-terminus of ubiquitin
Flg	flagellin
FOA	5´-fluoroorotic acid
FRET	fluorescence resonance energy transfer
GPCR	G-protein coupled receptor
G-protein	heterotrimeric G-protein
GUS	β-glucoronidase
HIS3	histidine auxothropic marker gene
HR	hypersensitive response
JA	jasmonic acid
kD	kilodalton
LPS	lipopolysaccharide
LRR	leucin-rich repeat
MLO	mildew-resistance locus o
NBS	nucleotide binding site
NOD	nucleotide-binding oligomerization domain
Nub	N-terminus of ubiquitin
NuI/A/G	isoforms of Nub
PAMPs	pathogen-associated molecular pattern

PG	petidoglycan
PLV	Protein A-LexA-VP16
PR	pathogenesis-related
R	resistance gene
ROR2	required for *mlo* resistance 2
R-URA3	orotidine-5´-phosphate decarboxylase with an arginine in position 1
SA	salicylic acid
SNAP	soluble *N*-ethylmaleimide-sensitive-factor-association protein
SNARE	SNAP receptor
TIR	*drosophila* toll/human interleukine il-1 like receptor
TLR	Toll-like receptor
TM	transmembrane
TRP	tryptophan auxothropic marker gene
Ub	ubiquitin
UBPs	ubiquitin specific proteases
µm	micrometer

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SUMMARY

Host cell entry is a critical step during pathogenesis of many microbial pathogens including animal pathogenic bacteria and fungal parasites of plants. Some microbes exploit human chemokine G-protein coupled receptors (GPCRs) to enter host cells. Barley MLO, the prototype of a plant-specific protein family, exhibits a topology and subcellular localisation that is reminiscent of GPCRs. Mutations in barley *MLO* confer resistance against all known isolates of the powdery mildew fungus, *Blumeria gramis* f. sp. *hordei* (*Bgh*), suggesting that MLO function might be exploited for pathogenesis by the fungal parasite.

The cytosolic calcium sensor calmodulin was previously identified as a protein interacting with MLO *in vivo* and was shown to enhance *mlo*-mediated susceptibility *in planta*. To identify further proteins that physically interact with *A. thaliana* MLO isoforms, the yeast split-ubiquitin system was employed. This revealed calmodulin as a common interactor of MLO proteins and identified four additional candidate interactors. To unravel their potential function in defence modulation, barley homologues of the selected candidate genes were tested by transient expression in single barley epidermal cells. However, neither dsRNAi-mediated gene silencing nor overexpression revealed a significant effect on *Bgh* penetration success in either wild-type (*MLO*) or mutant (*mlo*) genotypes.

Recently, a gene required for *mlo*-mediated resistance (*ROR2*) in barley was found to encode a plasma membrane-resident syntaxin protein. In addition, a genetic screen to identify *A. thaliana* mutants enabling enhanced entry of the inappropriate *Bgh* fungus led to the identification of *PEN1*. The gene was shown to encode a syntaxin that is functionally homologous to barley ROR2. In this study, *A. thaliana* MLO proteins were demonstrated to interact with a subset of syntaxins in the yeast split-ubiquitin system.

To date, it was unclear whether durable broad-spectrum *mlo*-mediated resistance is a feature restricted to the monocot barley. In this study, *A. thaliana* insertion mutants of the candidate ortholog of barley *MLO*, *AtMLO2*, were isolated and found to be resistant against the powdery mildew fungus *Golovinomyces orontii*. In addition, *Atmlo2* mutants exhibit enhanced resistance to inappropriate powdery mildew fungi. In contrast, infection phenotypes to bacterial or oomycete pathogens appeared unaltered. These results indicate that MLO function is evolutionarily conserved in both monocot and dicot plant species. It is therefore possible that the ability of powdery mildew fungi to target specific MLO isoforms for entry into plant cells was invented at least 200 million years ago.

ZUSAMMENFASSUNG

Der Eintritt in die Wirtszelle stellt eine kritische Phase während der Pathogenese vieler mikrobieller Krankheitserreger inklusive tierpathogener Bakterien und pilzlicher Pathogene von Pflanzen dar. Einige Mikroben können humane Chemokinrezeptoren, die zur Klasse der G-Protein gekoppelten Rezeptoren gehören, ausnutzen um Eintritt in ihre jeweiligen Wirtszellen zu erlangen. Das MLO-Protein aus Gerste ist der Prototyp einer pflanzenspezifischen Proteinfamilie und weist eine Topologie und subzelluläre Lokalisation auf, die an G-Protein gekoppelte Rezeptoren erinnert. Mutationen im *Mlo*-Gen der Gerste vermitteln Resistenz gegen alle bekannten Isolate des Mehltaupilzes, *Blumeria graminis* f. sp. *hordei* (*Bgh*), was darauf hinweist, dass die Funktion des MLO-Proteins möglicherweise für die Pathogenese durch den pilzlichen Parasiten ausgenutzt wird.

In vorangegangenen Experimenten war der cytosolische Calcium-Sensor Calmodulin als ein Protein das *in vivo* mit MLO interagiert und *in planta* die MLO-vermittelte Suszeptibilität erhöht identifiziert worden. Um weitere Proteine zu ermitteln, die physisch mit *A. thaliana* MLO-Isoformen interagieren, wurde das Hefe „split-ubiquitin"-System angewendet. Dies führte zur Identifizierung von Calmodulin als gemeinsamer Interaktor von MLO-Proteinen und erbrachte zusätzlich vier neue Interaktor-Kandidaten. Um eine mögliche Funktion dieser Kandidaten in der Modulation von Abwehrreaktionen zu überprüfen wurden Gerste-Homologe der ausgewählten Kandidaten-Gene mittels transienter Expression in Einzelzellen der Blattepidermis der Gerste getestet. Jedoch führten weder dsRNAi-vermitteltes Gen „silencing" noch Überexpression zu signifikanten Veränderungen der *Bgh*-Penetrationseffizienz in Wildtyp- (*Mlo*) oder Mutanten-Genotypen (*mlo*).

Vor kurzem wurde gezeigt, dass ein Gen das für *mlo*-vermittelte Resistenz in Gerste notwendig ist (*Ror2*) ein Plasmamembran-lokalisiertes Syntaxin codiert. Zusätzlich wurde in einem genetischen screen zur Identifizierung von *A. thaliana*-Mutanten mit erhöhter Penetrationsrate gegenüber dem unpassenden *Bgh*-Mehltaupilz das *Pen1*-Gen gefunden, welches ein funktionales Homolog des Gerste

ROR2-Proteins codiert. In der vorliegenden Arbeit wurde gezeigt, dass *A. thaliana* MLO-Proteine mit einer Syntaxin-Untergruppe im Hefe „split-ubiquitin"-System interagieren können, was darauf hindeutet, dass diese Syntaxine eine weitere Klasse von generellen MLO-Interaktoren darstellen.

Bis heute war es unklar, ob dauerhafte *mlo*-vermittelte Breitspektrum-Resistenz nur in Gerste vorkommt. Im Rahmen dieser Arbeit wurde gezeigt, dass *Arabidopsis* Insertionsmutanten im nächstverwandten Homolog des *Mlo*-Gens aus Gerste, *AtMLO2*, resistent gegen den Mehltauerreger *Golovinomyces orontii* sind und eine erhöhte Penetrationsresistenz gegenüber unpassenden Mehltaupilzen aufweisen. Im Gegensatz hierzu erscheinen die Infektionsphänotypen der *Atmlo2*-Mutante gegenüber bakteriellen Pathogenen oder Oomyceten unverändert. Darüber hinaus zeigt die *Atmlo2*-Mutante ähnliche pleiotrope Effekte wie *mlo*-Mutanten der Gerste, nämlich spontane Callose-Depositionen und einen deregulierten Zelltod von Mesphyllzellen. Diese Resultate zeigen, dass die Funktion des MLO-Proteins in monokotylen und dikotylen Pflanzen evolutionär konserviert ist.

1. INTRODUCTION

Both plants and animals are continually exposed to pathogens and, as a result, have evolved defence mechanisms to recognise and defend themselves against a wide array of potential pathogens (Mysore et al., 2003). Recent studies have revealed similar features of pathogen virulence and host resistance in both plant and animal diseases (reviewed in Cohn et al., 2001; Staskawicz et al., 2001; Nürnberger and Brunner, 2002).

The immune system in animals comprises innate and acquired immunity, both of which act together to protect the host from pathogens (reviewed in Medzhitov and Janeway, 1997). The innate immune system recognises a broad spectrum of pathogens using a set of invariant receptors (reviewed in Underhill and Ozinsky, 2002). In contrast, the acquired immune system is based on receptors generated by somatic mechanisms during the embryonic development of each individual organism (Medzhitov and Janeway, 1997). Moreover, specialized cell types, which are part of a circulatory blood system, are the key players of the animal immune system.

In contrast, plants lack the ability to generate new resistance specificities and rely on preformed receptors to detect pathogens and trigger defence responses (reviewed in Holt et al., 2003). In addition, plants lack a circulating immune system, but can recognise pathogens and trigger defence responses at the level of each single cell (cell-autonomous resistance; Nürnberger et al., 2004).

1.1. PATHOGEN-ASSOCIATED MOLECULAR PATTERS (PAMPs)

In animals, pathogen-derived molecules, which bind to pattern recognition receptors and trigger the expression of immune response genes, are referred to as pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway, 2002;

Underhill and Ozinsky, 2002; Zasloff, 2002). PAMPs are unique to microorganisms (pathogenic or not) and are not produced by (potential) hosts. Moreover, PAMPs are usually molecular structures essential for the survival of the pathogen. These structures are not subject to variability as mutations affecting them are generally lethal for the microorganism (Medzhitov and Janeway, 1997; Medzhitov and Janeway, 2002; Underhill and Ozinsky, 2002). PAMPs that trigger innate immune responses in various animal organisms include lipopolysaccharides (LPS) of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, bacterial flagellin as well as glucans, chitins and proteins derived from fungal cell walls (Aderem and Ulevitch, 2000; Underhill and Ozinsky, 2002).

1.1.1. PAMP recognition in animals and plants

In animals, PAMPs are recognised by host molecules such as the Toll and Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins. TLRs are a family of membrane-bound receptors, whereas NOD proteins reside within the cytoplasm and detect microbial motifs in the host cell (reviewed in Athman and Philpott, 2004).

The prototype of Toll-like plasma membrane receptors was identified in the fruit fly *Drosophila melanogaster* as a regulator of dorsal-ventral polarity during embryogenesis and was shown to have a role in innate immunity (Stein et al., 1991; Lemaitre, 1996). The Toll receptor is characterised by the presence of an extracellular domain containing a leucine-rich repeat (LRR) domain and a cytoplasmic domain highly homologous to the mammalian interleukin1 (IL-1) receptor referred to as the Toll/IL-1 receptor (TIR; Akira, 2003; Takeda et al., 2003). The role of Toll signalling in innate immunity in *Drosophila* was initially revealed by antifungal responses to the *Aspergillus fumigatus* (Lemaitre, 1996). Adult flies carrying a mutation in the Toll gene failed to induce expression of the antifungal peptide drosomycin when infected with *A. fumigatus* (Lemaitre, 1996).

In humans, the Toll-like receptor (TLR) family is formed by 10 members that recognise different PAMPs (Athman and Philpott, 2004). For example, TLR2 recognises peptidoglycans of Gram-positive bacteria (Birchler et al., 2001), TLR4

LPS of Gram-negative bacteria (Biragyn et al., 2002) and TLR5 bacterial flagellin (Hayashi et al., 2001).

TLRs interact with PAMPs in the extracellular compartment or within specialized intracellular compartments such as the Golgi apparatus, whereas NOD1 and NOD2 respond to bacteria in the cytosol (Athman and Philpott, 2004). NOD1 and NOD2 are two molecules of a protein family involved in the intracellular sensing system and characterised by a nucleotide binding site (NBS) domain and leucine-rich repeats (LRR; reviewed in Athman and Philpott, 2004). NOD1, also called CARD4 (caspase-activating and recruitment domain-4), is composed of LRR repeats at its carboxyl terminus, one amino terminal CARD and a central NBS. Recently, the bacterial ligand sensed by NOD1 was identified to be a structure derived from peptidoglycan (PG) mainly found in Gram-negative bacteria (Chamaillard et al., 2003; Girardin et al., 2003). NOD2 (CARD 15) is similar to NOD1 except that it presents two amino terminal CARD domains (Ogura et al., 2001). Recent studies have indicated that PG-derived muramyl dipeptide (MDP) is the essential structure sensed by NOD2 (Girardin et al., 2003).

The response downstream of an individual TLR is dependent on the activated signalling pathway that appears to be determined by the adaptor molecules interacting with the different TLRs (reviewed in Akira and Takeda, 2004; Athman and Philpott, 2004). Many of the TLR-induced inflammatory responses are dependent on a common signalling pathway mediated by the adaptor molecule MyD88 (Akira and Takeda, 2004; Athman and Philpott, 2004).

Intriguingly, many PAMPs identified in animals have been found to act also as general elicitors of defence responses in several plant species (Boller, 1995; Nürnberger and Scheel, 2001; Nürnberger and Brunner, 2002). For example, various structural elements of LPS from Gram-negative bacteria induce plant defence reactions (Meyer et al., 2001; Newman et al., 2002). Recently, a small peptide (Pep-13) present within a cell wall transglutaminase of *Phytophtora* strains was shown to activate plant defence in parsley and potato (Nürnberger et al., 1994; Brunner et al., 2002). Moreover, a cold-shock-inducible RNA-binding protein (RNP-1) from various Gram-positive bacteria was identified as PAMP (Felix and

Boller, 2003). A well-studied example of a PAMP recognised by plant receptors is flagellin, a 55 kD monomer obtained from bacterial flagella (Felix et al., 1999). The N-terminal fragment of bacterial flagellin, flg22 (Felix et al., 1999), was used to screen an EMS-mutagenized population of *A. thaliana* for flagellin-insensitive plants (Gomez-Gomez et al., 1999). This screen provided two independent mutations, which mapped to a single gene (FLS2) encoding a putative transmembrane receptor kinase with an extracellular LRR domain and a receptor-like kinase (LRR-RLK). Strikingly, this protein shares a similar modular structure with *Drosophila* Toll and human TLR proteins (Gomez-Gomez et al., 1999; Underhill and Ozinsky, 2002), but a low amino acid sequence similarity (Gomez-Gomez et al., 1999; Hayashi et al., 2001). This data suggests that during evolution the same biochemical modules (LRR) were selected for PAMP recognition in the animal and plant lineages (Nürnberger et al., 2004). The absence of sequence similarity might further suggest that both proteins arose independently as a result of convergent evolution (Nürnberger et al., 2004). This view is further supported by the fact that both receptors apparently recognize different structures of flagellin (Felix et al., 1999; Hayashi et al., 2001). Treatment of plants with flg22 induces the expression of numerous defence-related genes and triggers resistance to pathogenic bacteria in wild-type plants, but not in plants carrying mutations in the flagellin receptor gene FLS2. This induced resistance seems to be independent of salicylic acid, jasmonic acid and ethylene signalling (Zipfel et al., 2004).

1.2. PLANT RESISTANCE (*R*) GENE-MEDIATED RESISTANCE

In plants, one of the most effective, and better studied, defence signal transduction cascades is mediated by resistance (R) proteins that are able to detect specific pathogen proteins encoded by *Avr* genes of particular pathogen races (Flor, 1971; Martin, 1999; Ellis et al., 2000). The most prevalent class of functionally defined *R* genes encode proteins that display a structural homology to the mammalian NOD proteins (Inohara and Nunez, 2003; Jones and Takemoto, 2004). R proteins are composed of an intracellular nucleotide-binding site (NBS) domain and a C-terminal leucine-rich repeat (LRR) domain (reviewed in Hammond-Kosack

and Parker, 2003). As N-terminal domain, NBS-LRR proteins possess either a putative coiled-coil (CC) domain or a region sharing homology with the cytoplasmic TIR domain of the Toll receptor (Hammond-Kosack and Parker, 2003; Jones and Takemoto, 2004). R proteins are thought to act as molecular antennae that register interactions between pathogen avirulence factors (Avr) and their host targets ("guard hypothesis"; Van Der Biezen and Jones, 1998). *R* gene-mediated resistance is commonly associated with rapid necrosis of plant cells at the site of invasion, the so-called hypersensitive response (HR), resulting in efficient containment of the pathogen (Van Der Biezen and Jones, 1998). Activation of HR triggers a systemic resistance response known as systemic acquired resistance (SAR). The SAR response includes accumulation of the signal molecule salicylic acid (SA) and expression of a set of pathogen-related (PR) proteins, some of which were demonstrated to have antimicrobial activity (Glazebrook, 2001).

Pathogen isolate-specific Avr proteins are considered as factors that contribute to host infection, although the biochemical function of most Avr proteins is still unknown (reviewed in Nürnberger et al., 2004). Some Avr proteins, such as AvrRPM1, AvrB and AvrPto generated by strains of the bacterial pathogen *P. syringae*, were shown to be targeted to the plasma membrane subsequent to injection into the plant cytosol (Nimchuk et al., 2000; Shan et al., 2000). In addition, pathogenic fungi secrete a number of Avr proteins, like Avr9 (*Cladosporium fulvum*; Lauge et al., 2000, AvrPita (*Magnaporthe grisea*; Jia et al., 2000) and AvrMla6 (*Blumeria graminis*; Halterman et al., 2001). However, direct interaction between R and Avr proteins was demonstrated only in few cases, namely Pita and AvrPita (Jia et al., 2000); RRS1-R and PopP2 (Deslandes et al., 2003) as well as PTO and AvrPto (Martin GB, 1993).

In barley, individual NB-LRR genes have been shown to encode the MLA proteins (MLA1, MLA6 and MLA12; Halterman et al., 2001; Zhou et al., 2001; Shen et al., 2003). A mutant screen for genes required for *Mla12* resistance led to the identification of the *Rar1* gene (Jørgensen, 1988). The susceptible *rar1* mutants are unable to mount an HR response (Freialdenhoven et al., 1994). Genetic studies have shown that many, but not all, *Mla* resistance alleles require wild-type *Rar1* to be

fully functional (Jørgensen, 1996). Recently, the *A. thaliana* orthologue of RAR1 was shown to be required for the function of a subset of race-specific pathogen resistance genes (Austin et al., 2002; Azevedo et al., 2002; Muskett et al., 2002). The genes affected included those for resistance to both fungal (*Peronospora parasitica*) and bacterial (*Pseudomonas syringae*) pathogens, and represented members of both the TIR and CC subclasses of NB-LRR resistance proteins (Muskett et al., 2002; Tornero et al., 2002). Like MyD88 in animals, RAR1 appears to be required by several R proteins to trigger resistance in different plant species

1.3. HUMAN CELL SURFACE RECEPTORS CORRUPTED FOR PATHOGENESIS

To enter host cells, pathogens can exploit the human defence system, for example by misuse of chemokine receptors (Pease and Murphy, 1998). Chemokines comprise a superfamily of structurally related secreted proteins of 8 to 10 kD molecular weight that regulate migration and activation of mammalian leukocytes (Baggiolini et al., 1997). Chemokines induce leukocyte chemotaxis by binding to specific 7-transmembrane domain G protein-coupled receptors (GPCR) on the cell surface (Pease and Murphy, 1998). GPCR proteins mediate activation of downstream pathways by heterotrimeric guanine-nucleotide-binding (G) proteins composed of α, β and γ subunits. The cytosolic domains of GPCRs are coupled to the Gα subunit and promote the exchange of GDP for GTP in the associated Gα subunit. GTP binding to the Gα subunit leads to the disruption of interaction between Gα and Gβγ, resulting in the separation of Gα from the Gβγ dimer. Then, Gα and/or Gβγ interact with effector molecules resulting in activation of downstream pathways (Morris and Malbon, 1999; Jones and Assmann, 2004).

The chemokine system has been implicated in the regulation of diverse biologic processes, including host defence, hematopoiesis, inflammation and development (Baggiolini et al., 1997). Direct genetic evidence for some of these roles has been reported (reviewed in Pease and Murphy, 1998). For example, individuals lacking either the CCR5 or the Duffy GPCR protein due to inherited mutations are

highly resistant to infection with HIV-1 and *Plasmodium vivax*, respectively (Miller et al., 1976; Samson et al., 1996). The glycoprotein gp120 of the HIV-1 envelope binds the chemokine receptor CCR5 using the CD4 antigen to enter target cells (Samson et al., 1996; Pease and Murphy, 1998). In the second example, *Plasmodium vivax*, a protozoan and a major cause of human malaria, uses the Duffy antigen receptor for chemokines to enter erythrocytes (Hadley and Peiper, 1997; Pease and Murphy, 1998). The connection of *P. vivax* to Duffy has many parallels to the CCR5 HIV-1 co-receptor, including exploitation of a chemokine receptor for cell entry, and existence of an inactivating mutation in a corresponding chemokine receptor gene that confers high-level resistance to infection by the corresponding pathogen (Pease and Murphy, 1998). Interestingly, mice and people with these defective genotypes appear normal, implying that the respective functions are either not used or are fully compensated under normal, unstressed conditions (Pease and Murphy, 1998).

The human genome contains at least 800 GPCR genes and 17 Gα genes (Pierce et al., 2002; Jones and Assmann, 2004). Interestingly, the *A. thaliana* genome encodes only one GPCR protein (GCR1; Josefsson, 1997; Jones and Assmann, 2004). Furthermore, only one canonical Gα gene has been found in the genomes of *Arabidopsis (GPA*1; MA et al., 1990), rice *(RGA*1; Ishikawa A, 1995) and other diploid angiosperms (Assmann, 2002). In plants, heterotrimeric G-proteins were found to be involved in seed germination, root growth and architecture, as well as shoot morphology (Jones and Assmann, 2004; Perfus-Barbeoch et al., 2004). To date, there are no reports on heterotrimeric G-protein-dependent pathogen signalling in *A. thaliana* (Perfus-Barbeoch et al., 2004). However, a rice mutant in Gα (*rga1*) showed a more severe infection phenotype when challenged with a virulent strain of bacterial blight, *Xantomonas oryzae* pv. *oryzae*, but a phenotype comparable to the wild-type when inoculated with the *M. grisea* rice blast fungus (Suharsono et al., 2002; Komatsu et al., 2004).

1.4. PUTATIVE COMPATIBILITY FACTORS IN PLANTS

In comparison to animals, little is known about plant host factors that are required for pathogenesis and that may serve as receptors for the entry of pathogenic microbes. The recessive inheritance of resistance to single or closely related pathogen species in plants might serve as an indicator for the existence of these molecules, known as "compatibility factors" or "docking molecules" (Panstruga and Schulze-Lefert, 2003). Genetic analysis of resistant lines in natural plant populations and of induced mutations revealed single recessive resistance loci in different plant species. For example, mutations in the barley *MLO* gene confer resistance against the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*; Büschges et al., 1997; see below). Resistance to powdery mildew fungi can also be due to mutations in the *Er-1* gene in pea (*Pisum sativus*; Tiwari et al., 1998); in the *Ol-2* gene in tomato (*Lycopersimun esculentum*; Ciccarese et al., 2000) and in the *PMR* genes in *A. thaliana* (Vogel and Somerville, 2000). A screen to identify *A. thaliana* genes required for susceptibility against the powdery mildew *Erysiphe cichoracearum* led to the identification of six independent loci (*pmr1* to *pmr6*). To date only two of these loci, *pmr4* and *pmr6* have been characterised. *PMR4* encodes a callose synthase (see below; Jacobs et al., 2003; Nishimura et al., 2003) whereas *PMR6* encodes a pectate lyase-like protein (Vogel et al., 2002). Resistance caused by mutation in *PMR6* was found to be independent of the well-characterised salicylic acid- or jasmonic acid-dependent pathways, supporting the idea that *PMR6* might act as a compatibility factor. Furthermore, this hypothesis was corroborated by the fact that *PMR6* mutants are resistant to the closely related powdery mildew species *Golovinomyces orontii*, but are fully susceptible to unrelated pathogens like virulent strains of either *Pseudomonas syringae* or *Peronospora parasitica* (Vogel and Somerville, 2000; Vogel et al., 2002). In contrast, induced *A. thaliana edr1* mutants confer resistance to both fungal and bacterial pathogens, suggesting that wild-type *EDR1* is unlikely to serve as a pathogen "docking molecule", but is possible involved further downstream as negative regulator in disease resistance signalling (Frye and Innes, 1998).

1.5. BROAD-SPECTRUM *mlo* RESISTANCE

Genetic analysis of barley resistance responses to the virulent powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*), revealed two major pathways: race-specific resistance triggered by single R proteins (Jørgensen, 1994) and broad-spectrum resistance mediated by mutant alleles of the *MLO* gene (Büschges et al., 1997).

Resistance mediated by recessive *mlo* alleles is effective against all known isolates of the fungal pathogen. However, *mlo* alleles are ineffective against other fungal diseases including barley leaf rust (*Puccinia striiformis*), stripe rust (*Puccinia hordei*), scald (*Rhynchosporium secalis*), and the fungus *Gaeumannomyces graminis* (Jørgensen, 1977). This data suggests that *MLO* might encode a pathogen-specific compatibility factor. Interestingly, *mlo* mutants show enhanced disease susceptibility to the hemibiotrophic rice blast fungus, *Magnaporthe grisea*, and to the necrotrophic fungus *Bipolaris sorokiniana* (Jarosch et al., 1999; Kumar et al., 2001). This shows that wild-type *MLO* influences sensitivity to more than one pathogen species in opposite directions (Panstruga and Schulze-Lefert, 2003). In analogy to the human chemokine receptors, *Bgh* might target MLO to corrupt a plant defence pathway (Panstruga and Schulze-Lefert, 2003). Overexpression of *MLO* in the *MLO* wild-type genotype results in supersusceptibility against *Bgh*, suggesting that the homozygous wild-type *MLO* gene under its own promoter is an incomplete suppressor of resistance (Kim et al., 2002a).

The development of *Bgh* on *mlo*-resistant plants is arrested at the penetration stage and is associated with the formation of a localised cell wall apposition (papilla) beneath the fungal appressorium (Jørgensen and Mortensen, 1977). The molecular organization of papillae is still poorly understood, but a major constituent is the carbohydrate polymer β-1,3 glucan (callose). This polymer was considered to be a physical barrier against pathogen invasion. Recently, it has been shown that a single glucan synthase-like isoform in *A. thaliana*, glucan synthase-

like5 (GSL5)/powdery mildew resistance4 (PMR4), is essential to synthesize papillary callose (Jacobs et al., 2003; Nishimura et al., 2003). Surprisingly, mutants in which the *GSL5/PMR4* gene is disrupted exhibit broad-spectrum enhanced disease resistance against virulent powdery mildew fungi, including *Erysiphe cruciferarum*, *Golovinomyces orontii* and the oomycete *Peronospora parasitica*, but not to the bacterial pathogen *Pseudomonas syringae*. This indicates a role for the wild-type gene in the fungal colonization of host plants rather than in disease resistance (Schulze-Lefert, 2004).

Most of the *mlo* mutants have been generated by radiation or chemical treatment from various domesticated barley lines and all of them display broad-spectrum resistance to all tested *Bgh* isolates (Jørgensen, 1992). At least one allele (*mlo*-11) arose spontaneously in Ethiopian land-races found in a region of high rainfall and high pathogen pressure (Jørgensen, 1992). Unlike all other characterized *mlo* alleles with mutations in the coding sequence, a complex repeat structure upstream of the *MLO* gene leads to drastically reduced MLO transcript levels (Piffanelli et al., 2004). This is reminiscent of the naturally occurring promoter mutation in the Duffy chemokine receptor mediating resistance against malaria (Pease and Murphy, 1998). It can be speculated that the *mlo*-11 allele conferred a selective advantage to barley populations in the Ethiopian highlands and was maintained during barley domestication (Piffanelli et al., 2004).

1.5.1. The MLO topology is reminiscent of GPCR proteins but MLO acts independently of heterotrimeric G-proteins

MLO genes have been identified in higher plants and bryophytes, but not in prokaryotes, yeast and animals (Devoto et al., 1999; Devoto et al., 2003). The barley wild-type *MLO* gene encodes a 7 transmembrane domain protein of ~60 kD (Fig. 1.1; Büschges et al., 1997; Devoto et al., 1999) that was shown to be localised in the plasma membrane (Devoto et al., 1999). In the *A. thaliana* genome, 15 *MLO* homologues were identified, whereas in rice (*Oryza sativa*) twelve *MLO* homologues were found indicating that *MLO* genes are present as medium-size

gene families (Devoto et al., 1999; Devoto et al., 2003). MLO family members exhibit only moderate amino acid sequence identity (around 35–45% identical residues among members) and possess two regions of extraordinary sequence divergence: the first extracellular loop and the carboxyl terminus (Devoto et al., 2003). MLO protein topology, subcellular localisation in the plasma membrane and sequence diversity among the family are reminiscent of the G-protein coupled receptors (GPCR). However, genetic evidence and pharmacological studies demonstrated that MLO functions independently of Gα suggesting that MLO might act differently from GPCRs (Kim et al., 2002a; Panstruga and Schulze-Lefert, 2003).

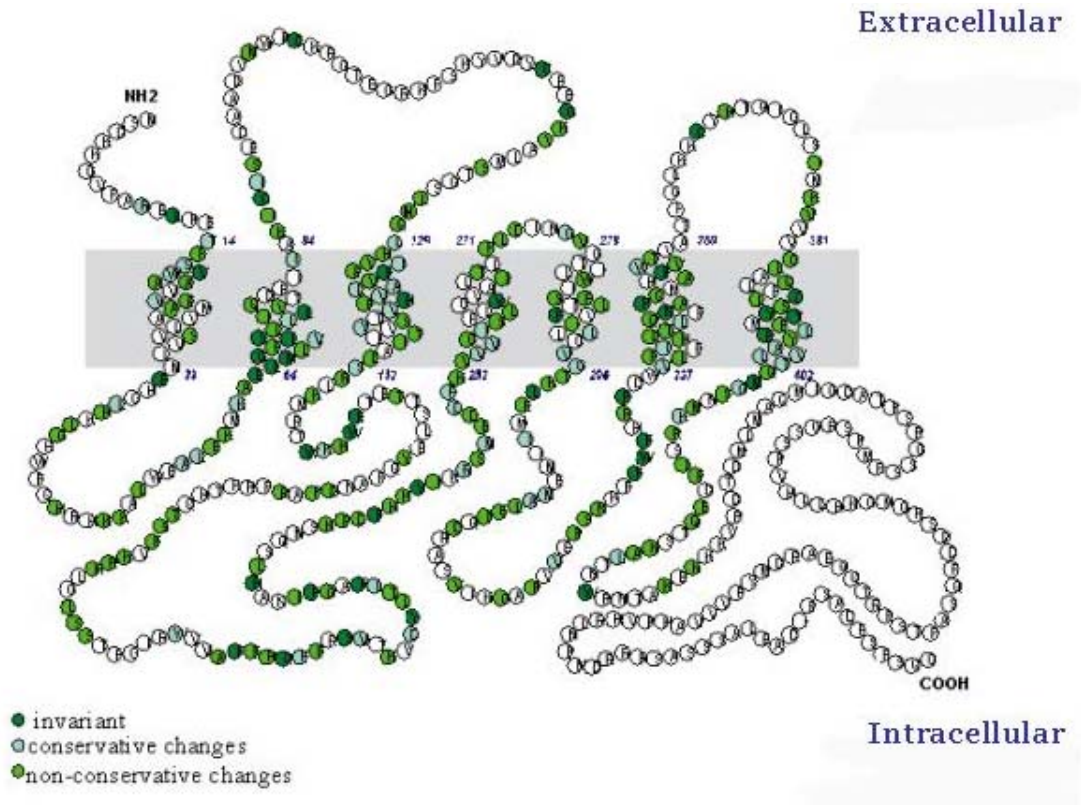

Fig 1.1

A common scaffold topology for the MLO family.

The graphic represents the membrane topology of the barley MLO protein. The plasma membrane lipid bilayer is represented by a grey horizontal bar. Circles with letters represent amino acids identified by the single-letter amino acid code. Levels of conservation of individual residues across MLO family members are indicated using a color-code: invariant residues, dark green; conservative changes in positions with at least 50% identical residues, light green; non-conservative changes in positions with at least 50% identical residues, green. Numbers indicate amino acid positions (Figure from Devoto et al., 1999).

Recently, an interaction between a rice MLO protein and calmodulin (CaM) was identified in a screen for novel calmodulin-interacting proteins (Kim et al. 2002b). CaM is a small (around 17 kD) protein highly conserved among eukaryotes and acts as a cytoplasmic calcium sensor. The CaM binding domain (CaMBD) of MLO is located in the proximal region of the carboxyl terminus and is structurally conserved among all family members (Kim et al., 2002a; Devoto et al., 2003), suggesting that CaM binding might be a general feature of MLO proteins (Panstruga and Schulze-Lefert, 2003). Moreover, barley MLO was demonstrated to bind CaM both *in vivo* and *in vitro* (Kim et al., 2002a). MLO variants carrying mutations in the CaMBD, that disrupt the interaction with CaM, can complement *mlo* resistance only partially, indicating that CaM binding activates the *MLO* susceptibility pathway (Kim et al., 2002a).

Several *mlo* resistance alleles result from either single amino nucleotide substitutions or small in-frame deletions in *MLO* (Büschges et al., 1997; Piffanelli et al., 2002). The majority of the encoded mutant proteins are unstable *in planta*, whereas three mutant variants, each affecting residues in cytoplasmic loops, accumulate in the plasma membrane like the wild-type protein (Piffanelli et al., 2002; Müller et al. in press). These mutations are at a distance from the CaMBD and compromise MLO activity to a greater extent than the CaMBD mutations. Thus, the substitutions in the cytoplasmic loops may affect interactions with additional, yet unidentified, factors (Panstruga and Schulze-Lefert, 2003).

1.5.2. Genes required for *mlo* resistance

In barley, re-mutagenesis of *mlo* resistant lines led to the identification of partially susceptible suppressor mutants. Genetic analysis revealed recessive mutations in two genes, named *ROR1* and *ROR2* (required for *mlo* resistance; Freialdenhoven et al., 1996). The existence of partially susceptible *ror* mutants in the presence of a *mlo* null mutation demonstrates that MLO is not an absolute requirement for powdery mildew pathogenesis (Panstruga and Schulze-Lefert, 2003). Furthermore, mutations of either *ROR1* or *ROR2* in the genetic background

of wild-type *MLO* plants result in supersusceptibility, indicating that *ROR* genes might act as positive regulatory components of a basal *Bgh* defence mechanism (Collins et al., 2003; Panstruga and Schulze-Lefert, 2003). It was proposed that MLO might act as docking receptor during pathogenesis and that mutations in *ROR* genes may open a bypass route for pathogenesis (Panstruga and Schulze-Lefert, 2003). The *ROR2* gene was recently isolated and was found to encode a syntaxin protein, localized in the plasma membrane (Collins et al., 2003). Syntaxins are member of the superfamily of SNARE proteins that are conserved in animals and plants and control intracellular vesicle trafficking (Bock et al., 2001; Sanderfoot and Raikhel, 2001; Collins et al., 2003).

Recent screens for *A. thaliana* mutants allowing increased entry by the inappropriate *Bgh* fungus led to the identification of the *PEN1* gene (Collins et al., 2003). Interestingly, *PEN1* encodes a syntaxin, which belongs to the clade of two syntaxins showing the highest sequence similarity to barley ROR2, demonstrating a conserved role for syntaxins and thus possibly vesicle transport in *Bgh* defence (Collins et al., 2003). Moreover, the conserved function of the barley ROR2 and *A. thaliana* PEN1 syntaxins provides a link between non-host and penetration (basal) resistance (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert, 2004).

1.5.3. *mlo* mutants display pleiotropic phenotypes

Another characteristic phenotype of barley *mlo* mutant plants is a deregulated cell death reaction that is under developmental control. Compared to wild-type plants, *mlo* plants grown under pathogen-free conditions exhibit early necrotic leaf spotting and chlorosis (Wolter M, 1993). Furthermore, *mlo* mutant plants show spontaneous callose deposition even in absence of pathogen challenge (Wolter M, 1993; Peterhänsel et al., 1997).

Mutations leading to cell death misregulation have also been described in other plant species (reviewed in Lorrain et al., 2003). These mutants are called lesion mimic, as their phenotype is reminiscent of the pathogen-inducible hypersensitive response (Lorrain et al., 2003). Some of these mutants have been isolated using different screens related to plant responses to pathogens. Mutations

resulting in constitutive expression of defence mechanisms in *A. thaliana*, as the *cpr* (constitutive expresser of *PR*; Bowling et al., 1997; Silva et al., 1999) mutations and the *cet* (constitutive expression of thionin; Hilpert et al., 2001) mutations, also cause spontaneous lesions. The *ssi4* (suppressor of salicylic acid insensitivity of *npr1*) mutation affects the salicylic acid-dependent pathway, displays spontaneous necrotic lesions and increased resistance to *P. parasitica* and *P. syringae* (Shirano et al., 2002). *SSI4* encodes a TIR-NBS-LRR protein and the mutation caused by a single amino acid substitution in the NBS domain leads to constitutively activated defence mechanisms (Shirano et al., 2002). It was suggested that some of the LMM mutants affect control mechanisms of plant defence. Apart from the onset of cell death in the absence of pathogens, accumulation of defence-related gene transcript has not been observed in *mlo* plants (Peterhänsel et al., 1997).

1.5.4. A potential parallel between human chemokine receptor and barley MLO function during pathogenesis

Striking parallels can be found between the role of chemokine co-receptors during HIV infection and MLO function in *Bgh* pathogenesis. In both cases, either absence of a host 7-TM protein or presence of a mutant variant of the host 7-TM protein results in disease resistance to the respective pathogen (reviewed in Panstruga and Schulze-Lefert, 2003). The 7-TM protein involved in HIV recognition is a polypeptide with a known function for the host, whereas the role of barley MLO in non-infected wild-type plants is still unclear, but might be related to leaf senescence. Resistance due to the absence of wild-type isoforms of either MLO or CCR5 appears to be restricted to a particular pathogen. Spontaneous polymorphisms in chemokine receptor genes as well as in *MLO* (*mlo*-11) might have led to disease-resistant populations, indicative of selective advantages of the respective polymorphisms. During pathogenesis, neither chemokine co-receptor nor MLO require signalling via heterotrimeric G-proteins (Panstruga and Schulze-Lefert, 2003).

It was proposed that MLO might serve as a docking molecule for *Bgh*, allowing invasive growth (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert and

Panstruga, 2003). However, this model does not explain the fact that most amino acids affected in barley *mlo* mutants are located in cytoplasmic domains of the protein (Büschges et al., 1997; Piffanelli et al., 2002) and also provides no explanation for the importance of CaM for MLO activity (Kim et al., 2002a). Thus, a dual function of MLO in both docking and defence suppression might be more plausible. The fungus may target MLO and corrupt or initiate MLO-dependent signalling, which can trigger defence suppression. It is possible that MLO is activated by *Bgh*-derived effectors, without docking, to initiate defence suppression (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert, 2004). Both models are corroborated by the fact that *mlo* resistance is effective only against a single pathogen species (*Bgh*), which might have evolved a specific mechanism to corrupt MLO for its own advantage (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert, 2004). Considering that absence of *MLO* affects pathogenesis of different pathogens in opposite directions, it can be proposed that MLO might modulate mutually inhibitory defence responses (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert, 2004). *Bgh*-derived molecules might compete with plant-derived MLO ligands to alter the balance of mutually inhibitory defence pathways to the advantage of the pathogen (Panstruga and Schulze-Lefert, 2003; Schulze-Lefert, 2004).

As mentioned above, the binding of calmodulin to MLO was demonstrated to modulate MLO activity in the pathogen defence responses in barley. However, the role of MLO in the pathway leading to *Bgh* defence/susceptibility is still unclear. Identification of further interactors of MLO could be instrumental to better understand the biological role of the MLO protein family. In this study, the split-ubiquitin system, an alternative yeast two-hybrid method, was employed to identify polypeptides that physically interact with MLO proteins. Respective prey library screens revealed four novel candidate MLO interactors. The split-ubiquitin system was further used to investigate the interaction between MLO proteins and syntaxins in a targeted manner. This uncovered a subset of syntaxins as common interactors of MLO proteins.

To investigate whether *mlo*-mediated penetration resistance represents a characteristic of monocot plant species, a reverse genetic approach in the dicot

model plant *A. thaliana* was employed. Insertion mutant lines in the three most sequence-related *AtMLO* homologues to barley MLO were analysed for their susceptibility against several pathogens, revealing AtMLO2 as the pivotal modulator of defence responses against powdery mildew fungi.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Antibiotics

Ampicillin (1000 x): 50 mg/mL in H_2O
Kanamycin (200 x): 50 mg/mL in H_2O
Rifampicin (1000 x): 100 mg/mL in ethanol
Stock solution stored at –20° C

2.1.2. Bacterial strains

E. coli strains:

DH5α

Genotype: F$^-$ *sup*E44 Δ*lac*U169 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

DB3.1

Genotype: F$^-$ *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*-5 λ$^-$ *leu mtl*-1

2.1.3. Yeast strain

All constructs were expressed in the *Saccharomyces cerevisiae* strain JD53 (Dohmen et al., 1995).

Genotype: *MAT*α *his3-*Δ*200 leu2-3, 112 lys2-801 trp1-*Δ*63 ura3-52*

2.1.4. Pathogens

2.1.4.1. *Barley powdery mildew Blumeria graminis f. sp. hordei (Bgh)*

The barley powdery mildew *Bgh* isolate K1 was maintained on seven-day-old barley seedlings I10, a near-isogenic line of Ingrid. Plants or detached leaves were kept at 20° C, 60% relative humidity, and 16 h light/8 h darkness after inoculation with *Bgh* conidio spores.

2.1.4.2. *Wheat powdery mildew Blumeria gramis f. sp. tritici (Bgt)*

The wheat powdery mildew isolate JIW2 was propagated on the susceptible wheat (*Triticum aestivum*) cultivar Cerco cultivated at 22°C 16 h light/8 h darkness, 60 % relative humidity (Elliott et al., 2002).

2.1.4.3. *Pea powdery mildew Erysiphe pisi*

The pea powdery mildew *E. pisi* isolate *Birmingham* was maintained on 3-week-old pea plants, cultivar Linga. Pea and inoculated *A. thaliana* plants were kept at 22° C, 70% humidity, 500 µEm²/s and 12h light/12 h darkness in a protected environment.

2.1.4.4. *A. thaliana powdery mildew Golovinomyces orontii*

The *A. thaliana* powdery mildew *G. orontii* was propagated on *A. thaliana* Col-0 plants cultivated at 20° C and 16 h light/ 8 h darkness, 80% humidity in a protected environment.

2.1.4.5. *Oomycete pathogen Peronospora parasitica*

P. parasitica isolates *Noco2* and *Cala2* were maintained by weekly subculturing on susceptible recipient plants as described previously (McDowell et al., 2000; Dangl et al., 1992).

2.1.4.6. Bacterial pathogen Pseudomonas syringae

All *P. syringae* strains were maintained in DMSO stock conserved at –80° C. The strains were streaked on fresh agar plates 2 days before usage.

2.1.5. Plant materials

All barley seedlings were grown at 20° C and 16 h light/8 h darkness in a protected environment.
I10: a near-isogenic line in Ingrid background containing wild type *MLO*, susceptible to *Bgh*.
Ingrid *mlo*-3: generated by seven backcrosses with cv. Ingrid, fully resistant to *Bgh*.

All *A. thaliana* plants used in this work are in the Col-0 genetic background. Except as noted, all *A. thaliana* plants were grown at 20° C and 10 h light/ 14 h darkness in a protected environment.

2.1.6. Vectors

pDONR 201	Invitrogen, Heidelberg
pMet GWY Cub R-URA3 Cyc1	single copy plasmid in yeast, used as bait vector in the Split-Ubiquitin system; modified as Gateway® (GWY) compatible (Deslandes et al., 2003; Wittke et al., 1999)
pCup NuI GWY Cyc1	single copy plasmid in yeast, used as prey vector in the Split-Ubiquitin system; modified as Gateway® (GWY) compatible (Deslandes et al., 2003; Wittke et al., 1999)

pUbi GUS	construct expressing the GUS reporter under control of the maize ubiquitin promoter.
pUAMBN	construct used for single-cell gene silencing experiments. The construct contains two Gateway® cassettes: one in sense and the other in antisense orientation, separated by a 1 Kb *HvMla1* intron and driven by a maize Ubiquitin promoter (M. Miklis et al. unpublished; Schweizer et al., 2000).
pMUG	construct expressing both the GUS reporter under control of maize ubiquitin promoter and the barley wild type *MLO* gene driven by a second maize ubiquitin promoter and followed by the *Agrobacterium* NOS terminator (S. Bieri).
pUbi GATE	construct used for transient overexpression of genes in barley epidermal cells by ballistic transformation. The vector is Gateway® compatible.
pEXSG GWY $^{CFP}/_{YFP}$	construct used for expression of proteins fused to the N-terminal part of either CFP (cyan fluorescent protein) or YFP (yellow fluorescent protein) under control of a 35S promoter. The vector is Gateway® compatible.
pENSG $^{CFP}/_{YFP}$ GWY	construct used for expression of protein fused to the C-terminal part of either CFP (cyan fluorescent protein) or YFP (yellow fluorescent

	protein) under control of a 35S promoter. The vector is Gateway® compatible.
pUbi $^{CFP}/_{YFP}$ GWY	construct used for expression of protein fused to the C-terminal part of either CFP (cyan fluorescent protein) or YFP (yellow fluorescent protein) under control of a maize ubiquitin promoter. The vector is Gateway® compatible.

2.1.7. Oligonucleotides

Listed below are primers used in the present study and synthesized by Invitrogen or Promega.

Name	Sequence 5´-3´
AtMLO3-B1	**GWYF**[1]- AC ATA ATG ACG GAT AAA GAA GAA
AtMLO3-B2	**GWYR**[2]- G CCT TTC AGT TTT CTC TTG
AtMLO5-B1	**GWYF**- AC ATA ATG GCT GGA GGA GGA GGT
AtMLO5-B2	**GWYR**-G GGG ACC GCT TAA GAG GTC
AtMLO13-B1	**GWYF**-AC ATA ATG GCA GAA GCA AGG TCT
AtMLO13-B2	**GWYR**-G AGG GTT TTC ACT TTG GAC
ADH	GTG AAC TTG CGG GGT TTT TCA GTA T
Nu start	TAG AAT TCC ATG GGG ATC CTG GCG GCC GCC ATG CAG ATT TTC GTC AAG
NuI	GAT TTT CGT CAA GAC TTT GAC CGG TA
Cyc1	TTT CGG TTA GAG CGG ATG TG
pYes	TAA GTC GAC ACG GAT TAG AAG CCG CCG A
Ura3-rev	CCT ACC ACC TCT TAG CCT TAG CAC AAG A
pMet	ATT CTA TTA CCC CCA TCC
AtMLO1-B1	**GWYF**-AC ATA ATG GGT CAC GGA GGA GAA
AtMLO1-B2	**GWYR**-G GTT GTT ATG ATC AGG TGT
AtMLO1-B2-HIS	**GWYR**-G GTG ATG GTG ATG GTC GAG
AtMLO4-B1	**GWYF**-AC ATA ATG ATG AAA GAA GGA AGG

AtMLO4-B2	**GWYR**-G AGT CCT CCT AAA CAA CTC
AtMLO6-B1	**GWYF**-AC ATA ATG GCG GAT CAA GTT AAA
AtMLO6-B2	**GWYR**-G TCG CTT AAA CGA AAA ATC
AtMLO10-B1	**GWYF**-AC ATA ATG GCC ACA AGA TGC TTT
AtMLO10-B2	**GWYR**-G GTC AAT ATC ATT AGC AGG
AtMLO1W191R-1	**CAA TGG AAG AAA CGA GAG GAT TCG ATC**
AtMLO1W191R-2	GAT CGA ATC CTC TCG TTT CTT CCA TTG
AtMLO1W363D-1	GAT GAG CAT TTC GAC TTC AGC AAA CCT
AtMLO1W363D-2	AGG TTT GCT GAA GTC GAA ATG CTC ATC
AtMLO1P367L-1	TGG TTC AGC AAA CTC CAA ATT GTT CTC
AtMLO1P367L-2	GAG AAC AAT TTG GAG TTT GCT GAA CCA
NuI	GAT TTT CGT CAA GAC TTT GAC CGG TA
Cyc1	TTT CGG TTA GAG CGG ATG TG
AtMLO12-B1	**GWYF**-ACA TAA TGG CAA TAA AAG AGC GA
AtMLO12-B2	**GWYR**-GCT TCT TGA ACG TAA ACT C
PPI-B1	**GWYF**-ACA TAA TGG GAA TGG AGG TCG TC
PPI-B2	**GWYR**-G GTT GCT GGT GCT CGC CGG
PPI-over-5	CAT CGG AAG CTT GTA GCC ACC ATG GGA ATG GAG GTC GTC
PPI-over-3	CGT CGC GGA TCC TCA CTC AAA TGA GCG
CYCL1-B1	**GWYF**-AC ATA ATG GCC AAC CCG AAG GTG
CYCL1-B2	**GWYR**-G GAG CTG GCC GCA GTC GGC
SQS-B1	**GWYF**-AC ATA ATG TGA CCA ATG CTT TGA
SQS-B2	**GWYR**-GCT TGC CAT ACA GCA CGC C
CYT1-B1	**GWYF**-AC ATA ATG G CCG GCG ACA AGA AG
CYT1-B2	**GWYR** -GCT CAG ATT TGC TGT AGT G
CYT2-B1	**GWYF**-ACA TA ATG T CGT CCT CCT CCT CC
CYT2-B2	**GWYR**-GGA CAG ACT CCG ACT TGG T
Nu-X	CAT TGG AAG TTG AAT CTT CCG
OsMLO-B1	**GWYF**-T AC ATA ATG GCA GGG GGA GGA GGG
OsMLO-B2	**GWYR**-G CCG TTG TAC ACT GAA GGA
PpMLO-B1	**GWYF**- T AC ATA ATG GCC GGG GGC GAA GAT
PpMLO-B2	**GWYR**- G TGA CTT GTT CTC TTC ATT
HvPPI-C	AGG CCC GAA GGT CAG TTC
HvPPI-D	CGG TAT CAG CTG GCT CAG
HvPPI-B2 stop	**GWYR**- G TCA GTT GCT GGT GCT CGC

HvPPI-C2	CAG CAG CCA GTA GCA CAA G
HvPPI-D2	CAA TTC GGT CAA TGA CTC G
HvCyclo#1 stop	**GWYR**- G CTA GAG CTG GCC GCA GTC
HvCytb5#2 stop	**GWYR**- G CTA GAC AGA CTC CGA CTT
18c2-AtMLO12-c2	TGA GTA CCA ATA TGC CA
18d2-AtMLO12-d2	CAG CCA AAG ATA TGA GTC CC
HvCytb5 #1 stop	**GWYR**- GCT ACT CAG ATT TGC TGT
HvSQSstop	**GWYR**- GTC ACT TGC CAT ACA GCA C
HvPPI-D	AGT CGT ACC GGT CAT GGG
AtMLO1G351E-1	GTA GCC ATT GAA GAA GAC TTA GTG GTG
AtMLO1G351E-2	CAC CAC TAA GTC TTC TTC AAT GGC TAC
AtMLO1-mlo10-1	GTC ACT CAT GTA CAT GCT TTT ATT
AtMLO1-mlo10-2	AAT AAA AGC ATG TAC ATG AGT GAC
AtMLO5-mlo10-1	TCA AGG TTT AGG CAT GAG ACA TCA
AtMLO5-mlo10-2	TGA TGT CTC ATG CCT AAA CCT TGA
AtMLO12-mlo10-1	GAG AGG TTC AGA AGA GAT ACA TCG
AtMLO12-mlo10-2	CGA TGT ATC TCT TCT GAA CCT CTC
AtMLO2-B1	**GWYF**-AC ATA ATG GCA GAT CAA GTA AAA
AtMLO2-B2	**GWYR**-G TTT CTT AAA AGA AAA ATC

[1] GWYF: forward attB1 primer Gateway® compatible

[2] GWYR: reverse attB2 primer Gateway® compatible

2.1.8. Enzymes

2.1.8.1. Restriction enzymes

Restriction enzymes were purchased from New England Biolabs (Schwalbach), Boehringer (Mannheim), GIBCO BRL, Pharmacia Biotech (Braunschweig), and Stratagene (Heidelberg) unless otherwise stated. 10 x buffers for restriction enzymes were companied with the enzymes and supplied by manufacturers.

2.1.8.2. Nucleic acid modifying enzymes

Standard PCR reactions were performed using homemade *Taq* DNA polymerase while for the cloning of PCR products, *Pfu* or Expand High Fidelity polymerase were used.
Modifying enzymes were listed below and purchased from various sources:

Taq-DNA Polymerase	Homemade
Pfu DNA Polymerase	Stratagene (Heidelberg)
Expand High Fidelity System	Roche (Mannheim)
T4 DNA ligase	Roche (Mannheim)
Klenow	
DNase I, from bovine pancrease	
RNase I, from bovine pancrease	
Superscript II RT	Invitrogen (Heidelberg)
GATEWAY ® -Technology	Invitrogen (Heidelberg)
BP-Clonase	Invitrogen (Heidelberg)
LR-Clonase	Invitrogen (Heidelberg)
Lysozyme	Roche (Mannheim)

2.1.9. Chemicals

Laboratory grade chemicals and reagents were purchased from Roth (Karlsruhe), Serva (Heidelberg), Boehringer (Mannheim), Merck (Darmstadt), Beckman (München), GIBCO BRL (Neu Isenburg) and Sigma (Deisenhofen) unless otherwise stated. Chemicals for yeast culture, transformation and agar plates were obtained from Sigma or Merck unless otherwise stated.
5´Fluoroorotic acid (FOA) was purchased either from BioTech Trade & Service (Germany) and stored at –20° C or from RPI (Research Product International Corp.) and stored at 4° C.

2.1.10. Media

Unless otherwise indicated all media were sterilized by autoclaving at 121°C for 20 minutes. Heat labile solutions were sterilized using filter sterilisation units prior to addition of autoclaved components.
For the addition of antibiotics and other heat liable components the solution or media were cooled down to 55°C.

<u>*E.coli* Media</u>

LB (Lauria Bertani) Broth	
tryptone peptone	1%
yeast extract	0.5%
NaCl	0.5%

Agar plates
1.5-2% agar was added to the above broth.

<u>Yeast media</u>

YEPD media (1 L)	
Difco peptone	20 g
Yeast extract	10 g
Glucose 50%	40 mL
Water	to 1 L

Dropout Media (1 L)

	-H[1]	-T[2]	-HT	-HTU	-U[3]
glucose (g)	20	20	20	20	20
Yeast Nitrogen Base (g)	6,7	6,7	6,7	6,7	6,7
Dropout mix (selective AA / g)	-H / 1,92	-T / 1,92	-HTUL / 1,4	-HTUL / 1,4	-U / 1,92
Ura (mg)	-	-	50	-	-
His (mg)	-	-	-	-	-
Trp (mg)	-	-	-	-	-
Lys (mg)	-	-	-	-	-
Leu (mg)	-	-	380	380	-
Agar (g)	15	15	15	15	15
Water	to 1 L	to 1 L	to 1 L	to 1 L	to 1 L

[1] H: histidine

[2] T: tryptophan

[3] U: uracile

Minimal Media (1 L)

	-H[1]	-T[2]	-HT	-HTU	-U[3]
glucose (g)	20	20	20	20	20
Yeast Nitrogen Base (g)	6,7	6,7	6,7	6,7	6,7
Ura (mg)	50	50	50	-	-
His (mg)	-	76	-	-	76
Trp (mg)	76	-	-	-	76
Lys (mg)	76	76	76	76	76
Leu (mg)	380	380	380	380	380
Agar (g)	15	15	15	15	15
Water	to 1 L	to 1 L	to 1 L	to 1 L	to 1 L

[1] H: histidine

[2] T: tryptophan

[3] U: uracile

FOA-containing Minimal Media (1 L):

1 g of FOA dissolved in 10mL of DMSO was added to the listed minimal media

<u>*P. syringae* media</u>

NGYA

Bacto proteose peptone	5 g
Yeast extract	3 g
Glycerol	20 mL
H_2O	to 1 L

Agar plates
1.5-2% agar was added to the above broth.

2.1.11. Buffers and solutions

General buffers and solutions
Sodium acetate, 3 M

$NaC_2H_3O_2 \cdot 3H_2O$	408 g
H_2O	1000 mL

Dissolve sodium acetate trihydrate in 800 mL H2O, adjust pH to 4.8, 5.0, or 5.2 (as desired) with 3 M acetic acid, add H2O to 1 L. Filter sterilize.

TE (Tris/EDTA) buffer

Tris/HCl (pH 8.0, 7.5)	10 mM
EDTA (pH 8,0)	1 mM
Tris/HCl	1 M
Tris-Base	121 g
H_2O	1000 mL

Dissolve 121 g Tris base in 800 mL, adjust to desired pH with concentrated HCl, adjust volume to 1 L with H_2O, filter sterilize if necessary, can be stored up to 6 months at 4° C or at room temperature.

EDTA (ethylenediaminetetraacetic acid)-stock (0.5 M, pH 8.0)

Na_2EDTA	186,1 g
H_2O	1000 mL

Dissolve 186.1 g Na_2EDTA in 700 mL water, adjust pH to 8.0 with 10 M NaOH (~50 mL; add slowly), add water up to 1 L. Filter sterilize.

SDS (sodium dodecyl sulfate or sodium lauryl sulfate) (20% w/v)

SDS	20 g
H_2O	100 mL

Slightly heat may be necessary to fully dissolve the powder

Ethidium bromide stock (10 mg/mL)

ethidium bromide	0.2 g
H_2O	20 mL

Stored at 4° C in dark bottle. Do not sterilize.

TAE (Tris/acetate/EDTA) buffer (10x)

Tris base	24.2 g
glacial acetic acid	5.71 mL
$Na_2EDTA{\cdot}2H_2O$	3.72 g
H_2O	to 1 L

2.2. METHODS

2.2.1. Nucleic acid manipulation

2.2.1.1. Polymerase chain reaction (PCR) amplification

PCR amplification buffer, 10x

200 mM Tris/HCl (pH 8.4)

500 mM KCl

25 mM $MgCl_2$

Stock solution is sterilized by autoclaving

Plasmid or genomic PCR *(Taq* polymerase)
Reaction mix

Components	Amount per reaction (50 µL)
Template DNA (genomic or plasmid)	20 ng
10x PCR amplification buffer	5 µL
10 mM dNTPs mix (dATP, dCTP, dGTP, dTTP)	1 µL
Forward oligo (50 pmol or 10 µM)	1 µL
Reverse oligo (50 pmol or 10 µM)	1 µL
Homemade TAQ DNA polymerase	1 µL
H_2O	To 50 µL

Thermal profile

Stage	Temperature (°C)	Time	N° of cycles
Initial denaturation	93	3 minutes	
Denaturation	93	30 seconds	25-35 x
Annealing	50-58	30 seconds	
Extension	72	1-2 minutes	

PCRs with other polymerases (e. g. *Pfu*), or Expand High Fidelity System were performed according to the manufacturer's protocol.

2.2.1.2. Restriction endonuclease digestion of DNA

All restriction digests were performed using the manufacturers recommended conditions. Typically, reactions were carried out in 1.5 mL eppendorf tubes using 1-2 Units of restriction enzyme per 10-20 µL of reaction volume. All digests were

carried out at the appropriate temperature in incubators with proper temperature for a minimum of 30 minutes.

2.2.2. DNA analysis

2.2.2.1. Plasmid DNA isolations

Plasmid DNA was isolated by alkaline lysis method (Birnboim and Doly, 1979). High quality DNA for single-cell transient gene expression assay or DNA sequencing was isolated using Qiagen or MACHEREY-NAGEL(MN) Mini-, Midi- or Maxi-prep kits.

2.2.2.2. Plant genomic DNA isolation

Genomic plant DNA was isolated using the Edwards method as described in Edwards et al. 1991.

2.2.2.3. Isolation of DNA fragments from agarose-gel

The Nucleospin Extract-Kit (MACHEREY-NAGEL) was used to extract DNA fragments from agarose-gels according to the manufacturer's protocol.

2.2.2.4. DNA sequencing

DNA sequences were determined by the Automatische DNA-Isolierung und Sequenzierung (ADIS-Unit) at the MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry (Sanger et al., 1997). PCR products were purified with the Nucleospin Extract-Kit (MACHEREY-NAGEL) or Qiagen Extract Kit, ensuring sufficient amount at appropriate concentration to be directly sequenced.

2.2.2.5. DNA sequence analysis

Sequencing data were analysed mainly using Clone Manager 6, version 6.00 and alignments made using ClustalW (www.ch.embnet.org).

2.2.2.6. Database searching

DNA sequence data were directly used for database searching using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/), or translated into polypeptide for motif similarity searching. Other used databases include TAIR (http://www.Arabidopsis.org/), TIGR (http://www.tigr.org) and the IPK Barley ESTs Database (http://pgrc.ipk-gatersleben.de/).

2.2.3. Transformation of *E. coli*

2.2.3.1. Preparation of electro- and heatshock-competent cells.

Electro-competent cells:10 mL of an overnight culture of *E. coli* strain DH5α was added to 1 L of LB broth and shaken at 37°C until the bacterial growth reached an OD= 0.5-0.6. The bacteria were pelleted at 5000 x g for 20 minutes at 4° C and the pellet gently resuspended in ice-cold sterile water. The cells were pelleted as before and again resuspended in ice-cold water. The process was repeated twice. Finally the cells were gently resuspended in a 1/100 volume of the initial culture in 10% sterile glycerol, pelleted once more and then resuspended in 5 mL 10% glycerol. 50 µL aliquots of cells were frozen in liquid nitrogen and stored at –80° C until use.

Heat-shock-competent cells: 100 mL of an overnight culture of *E. coli* strain DH5α was added to 1 L of LB broth and shaken at 37°C until the bacterial growth reached an OD= 0.2 The bacteria were pelleted at 5000 x g for 10 minutes at 4° C and gently resuspended in 250 mL ice-cold sterile 100 mM $MgCl_2$. The cells were incubated for 5 minutes on ice, then pelleted as before and again resuspended in 50 mL ice-cold

100 mM $MgCl_2$. The cells were incubated for 20 minutes on ice, then pelleted as before. Finally the cells were gently resuspended in 10 mL of a solution prepared with 85% 100 mM $CaCl_2$ and 15% glycerol. 50 µL aliquots of cells were stored at –80° C until use.

2.2.3.2. Transformation of electro- and heatshock-competent cells

Electro-transformation: 20 to 50 ng of salt-free ligated plasmid DNA (or ~1µL of ligated mix from 10 µL ligation reaction) was mixed with 50 µL of electro-competent cells, and transferred to a cold BioRad electroporation cuvette (1 mm electrode distance). The BioRad gene pulse apparatus was set to 25 µF capacitance, 1.7 kV voltage and the pulse controller to 200 ohms. The cells were pulsed once at the above settings for a few seconds, 500 µL of LB medium was immediately added to the cuvette and the cells were quickly resuspended and incubated at 37°C for 1 hour. A fraction (~150-300µL) of the transformation mixture was plated onto selection media plates.

Heat-shock transformation: 100-250 ng of plasmid DNA was mixed with 50 µL of heat-shock-competent cells in a 1.5 mL Eppendorf tube and incubated on ice for 20 minutes. Subsequently, the cells were transferred in a water bath at 42° C for 1 minute and incubated on ice for 1 minute. 1 mL of LB medium was added to the cells before incubating them for 1 hour at 37° C. A fraction (~150-300µL) of the transformation mixture was plated onto selection media plates.

2.2.4. The Split-Ubiquitin system

All constructs were expressed in the *Saccharomyces cerevisiae* strain JD53. The single-copy Cub-URA3 fusion vector (see above) was used as a Gateway destination vector (Invitrogen). Each *AtMLO* coding sequences were amplified by PCR and recombined into the bait vector by using the Gateway system. The primers used are listed in the table in Chapter 2.1.7.

2.2.4.1. High-efficiency transformation of yeast competent cells

The following protocol was modified from Gietz, R.D. and R.A. Woods, 2002

1. Start a 10 mL overnight culture at 30° C in YEPD or dropout medium lacking the selective amino acids, if the yeast strain already contains a plasmid
2. Start a new culture in 50 mL YEPD using 5 x 10^6 cells/mL (2.5 x 10^8 cells in total) and grow for 5-6 hours at 30 °C to have a final density of 2 x 10^7 cells/mL.
3. Centrifuge at 3500 rpm for 3 min.
4. Resuspend the cells in 25 mL of cold-sterile water and centrifuge again.
5. Resuspend the cells in 1 mL of 100 mM LiAc (freshly made from a 1M stock).
6. Centrifuge at 13000 rpm for 10 seconds.
7. Resuspend the cells in 500 µL of 100 mM LiAc.
8. For 1 transformation use 50 µL of cells and spin down (15 seconds, 13000 rpm). Remove the supernatant and add 1 µg of plasmid DNA. Vortex at low speed 2 seconds and, while vortexing, add 300 µL of Transformation Mix.
9. Incubate at 30 °C for 30 minutes.
10. Incubate at 42 °C for 60 minutes.
11. Centrifuge 6000 rpm, 10-15 second.
12. Eliminate supernatant. Add carefully 300-500 µL of sterile water. Gently resuspend the cells by inverting the tubes.
13. Plate a dilution of the transformed mixture on selective plate to estimate the number of transformants. Plate the rest of the transformed mixture on selection plates.

Transformation Mix (1 mL)

50% PEG 3350	680 µL
1 M LiAc	100 µL
1 mg/mLCarrier DNA	140 µL
H_2O	80 µL

2.2.4.2. Yeast colony PCR

A yeast colony was resuspended in 25 µL of 20 mM NaOH and 2 µL of the lysate was used as template in a PCR reaction as described in 2.2.1.1. The cycle number of the PCR reaction was increased to 40x.

2.2.4.3. Plasmid rescue

The following protocol was adapted from Liang and Richardson, 1992 and Uhrig et al., 1999.

1. Start an overnight culture in 5 mL of appropriate medium
2. Centrifuge the cells at 4000 rpm for 5 min at 20°C
3. Resuspend the cells in 1,9 mL 1 M Sorbitol
4. Add 100 µL 1 M KPO_i pH 7,5
5. Add a spatula tip of yeast lytic enzyme (16,500 U/g; ICN Cat.No. 150214)
6. Vortex to dissolve yeast lytic enzyme
7. Incubate the cells for 30 min at 37°C
8. Centrifuge the cells at 14000 rpm, for 5 min, at room temperature
9. Resuspend the cells in 500 µL P1-buffer (from Qiagen Plasmid Miniprep Kit or similar)
10. Add 500 µL P2-buffer and mix
11. Incubate the cells for 10 min at 37°C
12. Add 700 µL N3-buffer and mix
13. Distribute the suspension into two Eppendorf tubes, centrifuge for 10 min at 14000 rpm
14. Successively load supernatant onto one Qiagen Mini column
15. Perform a standard Qiagen mini column
16. Elute the DNA with 50 µL sterile H2O
17. Transform up to 15 µL by electroporation in an *E. coli* strain

2.2.4.4. Gap repair system

The gap repair system is based on the ability of yeast to repair DNA sequences carraing gaps *in vivo* by homologous recombination.
The yeast strain was transformed with a linearized vector containing a selectable marker and an autonomous replicating sequence together with a PCR product that spanned the gap in the vector. The homologous recombination between the vector and the PCR product occurring *in vivo* resulted in a particular plasmid containing the PCR product, which was efficiently propagated (Kostrub et al., 1998; Muhlrad et al., 1992).

2.2.5. Generation of NuI-PPI1 noTM fusion protein

The prey vector expressed by the clone 3F8, identified in the split-ubiquitin system using AtMLO3 as bait, was rescued as described in Chapter 2.2.4.3. The plasmid was linearised by enzyme restriction with SalI. Subsequently, the linearised vector was modified by Klenow fill-in and ligate.

2.2.6. PCR direct mutagenesis

To generate *AtMLO1* variant alleles, site-direct mutagenesis using PCR was performed. In the first step, wild-type AtMLO1 coding sequence was used as template to generate primary PCR products in two separate PCR reactions. Both primary PCR products contain the desired mutation as well as overlap regions that are attached to the beginning and end of the coding sequence. These overlaps are needed for production of the linear expression template in the second PCR. The mutation is introduced by primers containing a nucleotide exchange leading to an amino acid substitution. The external primers (Gateway compatible) are used to attach the overlap regions.

Allele	Primer Name	Sequence 5´-3´
Atmlo1-1	AtMlo1-B1	**GWYF**-AC ATA ATG GGT CAC GGA GGA GAA
	AtMlo1W191R-1	CAA TGG AAG AAA CGA GAG GAT TCG ATC
	AtMlo1W191R-2	GAT CGA ATC CTC TCG TTT CTT CCA TTG
	AtMlo1-B2	**GWYR**-G GTT GTT ATG ATC AGG TGT
Atmlo1-27	AtMlo1-B1	**GWYF**-AC ATA ATG GGT CAC GGA GGA GAA
	AtMlo1W363D-1	GAT GAG CAT TTC GAC TTC AGC AAA CCT
	AtMlo1W363D-2	AGG TTT GCT GAA GTC GAA ATG CTC ATC
	AtMlo1-B2	**GWYR**-G GTT GTT ATG ATC AGG TGT
Atmlo1-29	AtMlo1-B1	**GWYF**-AC ATA ATG GGT CAC GGA GGA GAA
	AtMlo1P367L-1	TGG TTC AGC AAA CTC CAA ATT GTT CTC
	AtMlo1P367L-2	GAG AAC AAT TTG GAG TTT GCT GAA CCA
	AtMlo1-B2	**GWYR**-G GTT GTT ATG ATC AGG TGT

Note: the underlined triplets indicate the position of the nucleotide exchange leading to amiono acid substitutions.

2.2.7. Single-cell transient gene expression assay in barley epidermal cells using particle bombardment

Overview

A reporter plasmid containing the *GUS* genes and the respective effector plasmids were mixed prior to the coating of particles (molar ratio 2:1, respectively, maximum of 5 µg DNA). The bombarded leaves were transferred onto 1% agar plates supplemented with 85 µM benzimidazol and incubated at 20° C for 15 h before high-density inoculation with *Bgh* spores. Leaves were stained for GUS activity and single leaf epidermal cells attacked by *Bgh* germLings were evaluated under the microscope at 48 h after spore inoculation. In dsRNAi single-cell silencing experiments, particles were co-coated with a construct encoding an intron-spliced dsRNAi construct according to Azevedo et al. 2002 (molar ratio 1:1:1, 5 µg total DNA). In the gene silencing experiments, the bombarded leaves were incubated at 20 °C for 96 h before high-density inoculation to allow turnover of preformed proteins of interest.

Particle Delivery System: Biolistic-PDS-1000/He *(BIO-RA*D)

Material preparations:

Plant material: one-week old barley plants grown in phytochamber under controlled conditions.

Sucrose and benzimidazol agar (1-1.5%) plates

Gold particles (0.9-1.0 μm): washed and coated with reporter and effector constructs

Spermidine solution (0.1 M)

$CaCl_2$ solution (2.5 M)

Ethanol (70% and pure)

glycerol (50% in water)

Particle bombardment:

Use rupture disc (900 psi), apply vacuum up to 27 inch, trigger shooting

Fungal inoculation

Dusting off high-density fresh *Bgh* conidio spores on bombarded leaves

GUS staining

Infiltration with GUS staining solution into bombarded leaves in

Falcon tubes, leave for at least 10 h at 37 °C

GUS destaining and fixing

Remove GUS staining solution and add in destaining solution

Use coomassie solution to stain fungal surface structures

Evaluation of haustorium index (%) under light microscopy.

Other materials required and recipes

Macrocarrier

Rupture disc

Gold particles

Hepta adapter (including browser, macrocarier holder, stopping screen holder)

Gus staining solution (1L)

1M Na_2HPO_4 57.7 mL

1M NaH_2PO_4 42.3 mL

0.5M Na_2EDTA 20.0 mL

$K_4Fe[CN]_6$ 2.112 g

$K_3Fe[CN]_6$ 1.646 g

Triton X-100 (v/v) 0.1%

methanol (v/v) 20%

X-gluc 1 g

X-gluc: 5-bromo-4-chloro-3-indoxyl-D-glucuronic acid, cyclohexylammonium salt, from Roth

Destaining solution:

stock solution

50% glycerol

25% lactic acid

H_2O

work solution

stock solution : ethanol (v/v) = 1 : 2

Coomassie solution

coomassie (w/v) 0.6%

coomassie: Serva Blue R, from Serva

ethanol

2.2.8. Selection of T-DNA insertion homozygous lines

Each *A. thaliana* T-DNA insertion lines used in this work was selected by PCR analysis. The primers employed are listed in the table below.

Allele	Name	Sequence 5´-3´
AtMLO2	Ara7	TGG AGC AAG ACG AGA GTC A

	Ara8	ATT TTG TTA TTA TGA CTT CAA GC
	2B2	GGT TAT TTC TTA TCC AAC TAG TAA TGT AC
	GarlicLb2	GCT TCC TAT TAT ATC TTC CCA AAT TAC CAA
AtMLO6	6C2	GTG AAA GGC ACA CCG CTA G
	6bw2	AGA TCT CCA AAC CTA GAA C
	GarlicLb2	GCT TCC TAT TAT ATC TTC CCA AAT TAC CAA
AtMLO12	18C2	TGA GTA CCA ATA TGC CA
	18d2	CAG CCA AAG ATA TGA GTC CC
	18C	CCT CGC CGA AAT TTA GCC ACC AAG
	Spm11	GGT GCA GCA AAA CCC ACA CTT TTA CTT C

2.2.9. Pathogen test

2.2.9.1. P. syringae

Pseudomonas syringae strains DC3000 and DC3000 *avrRpm1* were grown overnight at 28°C in NGYA medium containing the appropriate antibiotics (concentrations: rifampicin 50 µg/mL, kanamycin 50 µg/mL). Bacteria were pelleted, washed three times with 10 mM $MgCl_2$, resuspended, and diluted in 10 mM $MgCl_2$ to the desired concentration (for symptom development 5 x 10^6 cfu/mL, for bacterial growth 10^6 cfu/mL). The bacterial solutions were infiltrated from the abaxial side into a sample leaf using a 1 mL syringe without a needle. Control (mock) inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed by homogenizing discs originating from infiltrated areas of four different leaves in 1 mL of 10 mM $MgCl_2$ and plating appropriate dilutions on NGYA medium containing the appropriate antibiotics. Quantification of colony numbers was done after 1, 3 and 5 days.

2.2.9.2. P. parasitica

Two-week-old seedlings were sprayed with 4 x 10^4 *Peronospora* conidiospores/mL suspended in sterile distilled water. Phenotypes of the inoculated leaves were

quantified 7 days after inoculation by microscopic analysis using UV light excitation.

2.2.9.3. Powdery mildew

2.2.9.3.1. *E. pisi*

Four-week-old *A. thaliana* plants were inoculated with an adequate inoculation density and samples were collected for further evaluations after 7 days.

2.2.9.3.2. *G. orontii*

Four-week-old *A. thaliana* plants were inoculated with an adequate inoculation density and samples were collected for further evaluations after 2, 3 and 10 days.

2.2.9.3.3. *Bgh and Bgt*

Four-week-old *A. thaliana* plants were inoculated with an adequate inoculation density (~ 200 conidia/mm^2) and samples were collected for further analysis after 3 days.

2.2.10. Microscopic analysis

All *A. thaliana* samples were treated with either one or a combination of the following treatments (Adam and Somerville, 1996; Vogel and Somerville, 2000).

To visualize microscopic lesions (cell death), leaves were vacuum-infiltrated in a solution of phenol, lactic acid, glycerol, and water (1:1:1:1) plus 200 µg/mL trypan blue. The tubes with the samples were placed in a boiling water bath for 1 min. The leaves were destained in a chloral hydrate solution and examined under bright-field illumination.

To visualize callose, leaves were cleared in lactophenol solution (1 phenol:1 lactic acid: 2 glycerol: 1 H_2O) diluted 1:1 in ethanol. Cleared leaves were stained in aniline blue solution (0.067 M potassium phosphate buffer pH 5.8 containing 0.05% aniline blue) and examined for fluorescence.

To visualise epiphytic fungal structures, infected leaves were cleared in lactophenol solution (1 phenol:1 lactic acid: 2 glycerol: 1 H_2O) diluted 1:1 in ethanol. Cleared leaves were stained in a solution of 0.6% Coomassie Blue in ethanol, rinsed in water and mounted.

3. IDENTIFICATION OF MLO INTERACTORS USING THE SPLIT-UBIQUITIN SYSTEM

3.1. INTRODUCTION

Barley MLO is the prototype of a 7-transmembrane domain protein family unique to plants. Lack of the *MLO* wild-type allele in barley leads to broad-spectrum resistance against the barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei, Bgh*). Recently, it was shown that the C-terminal region of barley MLO protein binds calmodulin (CaM) and that the binding modulates MLO activity in the pathogen defence response (Kim et al., 2002a). Barley MLO proteins with a mutation in the calmodulin binding domain complement only partially the *mlo*-5 null mutant (Kim et al., 2002a). Moreover, several *mlo* mutant alleles with single nucleotide substitutions or small deletions, which do not affect the CaM binding domain, were identified. Some of them (*mlo*-10, *mlo*-27 and *mlo*-29) encode stable MLO proteins that confer full resistance to *Bgh* (Büschges et al., 1997; Piffanelli et al., 2002); Müller et al. in press). These findings suggest that MLO and CaM are not the only components of the *Bgh* defence/susceptibility pathway. Thus, at least one more protein with a significant role should be involved in the pathway via interaction with MLO. Identification of further MLO-interacting proteins could be instrumental to better understand the biological role of the MLO family and to dissect the defence/susceptibility pathway controlled by barley MLO.

3.2. THE YEAST SPLIT-UBIQUITIN SYSTEM

In the present work, the recently developed yeast split-ubiquitin system was employed to identify MLO interactors. The split-ubiquitin system is an alternative yeast two-hybrid assay using proteolytic stability as growth readout (Johnsson and

Varshavsky, 1994). Ubiquitin (Ub) is a ubiquitously conserved eukaryotic protein of 76 amino acids (Sharp and Li, 1987) that is usually transferred, as multi-Ub chain, to an internal lysine residue of a substrate as a signal for its degradation. However, in some cases a single ubiquitin is conjugated to the NH_2-terminal amino group of the substrate (Varshavsky et al., 2000; reviewed in Glickman and Ciechanover, 2002). The ubiquitin protein can be divided and expressed in two halves, a N-terminal part (Nub) and a C-terminal part (Cub). The split-ubiquitin system (Fig. 3.1) is based on the ability of Nub and Cub to re-assemble into a native-like ubiquitin in yeast cells when the two halves are separately linked to proteins that interact *in vivo*. Specific ubiquitin proteases (UBPs) recognize the reconstituted ubiquitin and cleave the reporter protein fused to Cub (Fig. 3.1 A). In this study, URA3 was used as reporter protein. The *Ura3* gene encodes orotidine-5'-phosphate decarboxylase, an enzyme required for biosynthesis of uracil. In addition, URA3 can also convert the non-toxic compound 5'-fluoroorotic acid (FOA) into a toxic compound, 5'-fluorouracil. The URA3 reporter protein used in the split-ubiquitin assay has an arginine in position 1 (R-URA3). The presence of an arginine as N-terminal residue of URA3 leads to a rapid degradation of the cleaved reporter protein by the enzymes of the so-called N-end rule (Varshavsky, 1996). When two interacting proteins are fused to Nub and Cub-R-URA3, respectively, and co-expressed in yeast cells, a native-like ubiquitin is formed and the R-URA3 is cleaved and degraded (Fig. 3.1 C). The rapid degradation of R-URA3 results in uracil auxotrophy and FOA-insensitivity of the yeast cells. Wild-type Nub possesses an isoleucine in position 13 (NuI) and has a high affinity for Cub. Replacement of I-13 by alanine (NuA) or glycine (NuG) decreases the affinity between Nub and Cub; in particular, NuG shows the lowest and NuI the highest affinity for Cub (Johnsson and Varshavsky, 1994; Stagljar et al., 1998; Dünnwald et al., 1999; Wittke et al., 1999).

3.2.1. MLO bait constructs used in the split-ubiquitin screening

The amino acid sequences of the MLO family members were aligned and the subsequent analysis showed that MLO proteins clustered in 4 different clades plus

an outgroup, *At*MLO3 (Fig 3.2; Devoto et al., 1999; Kim et al., 2002b); Devoto et al., 2003). Among the 15 *A. thaliana* members, proteins from each clade were selected: *At*MLO4 (clade I); *At*MLO1 (clade II); *At*MLO5 and *At*MLO10 (clade III); *At*MLO2 and *At*MLO12 (cladeIV) as well as the outgroup *At*MLO3. Full-size sequences of all selected *AtMLO* genes and of *HvMLO* were fused to the N-terminus of Cub-R-URA3 and used as bait construct in the split-ubiquitin system. The usage of baits belonging to distinct phylogenetic subgroups might facilitate the identification of common interactors of the MLO protein family.

3.2.2. *A. thaliana* prey libraries used in the screens

The split-ubiquitin system was previously employed to test the interaction between some MLO family members and calmodulin (Kim et al., 2002a). In particular, *Hv*MLO, *At*MLO1 and *At*MLO2 were used as baits. Barley CaM3, which is highly identical to each of the seven *A. thaliana* isoforms, was fused to the 3'-end of all the three Nub isoforms (NuI, NuA and NuG). Although no interaction between *Hv*MLO and *Hv*CaM3 was detected, both *At*MLO1 and *At*MLO2 interacted with *Hv*CaM3 when fused to NuI (Kim et al., 2002a). In addition, both *A. thaliana* MLO proteins interacted with neither NuA-HvCaM3 nor NuG-HvCaM3 (R. Panstruga, personal communication). Based on this data, fusion of the cDNA library to NuI was expected to identify more clones than fusion to either NuA or NuG. However, it has to be considered that using a NuI-cDNA library might lead to the identification of more "false positives" than using a NuA- or a NuG-cDNA library.

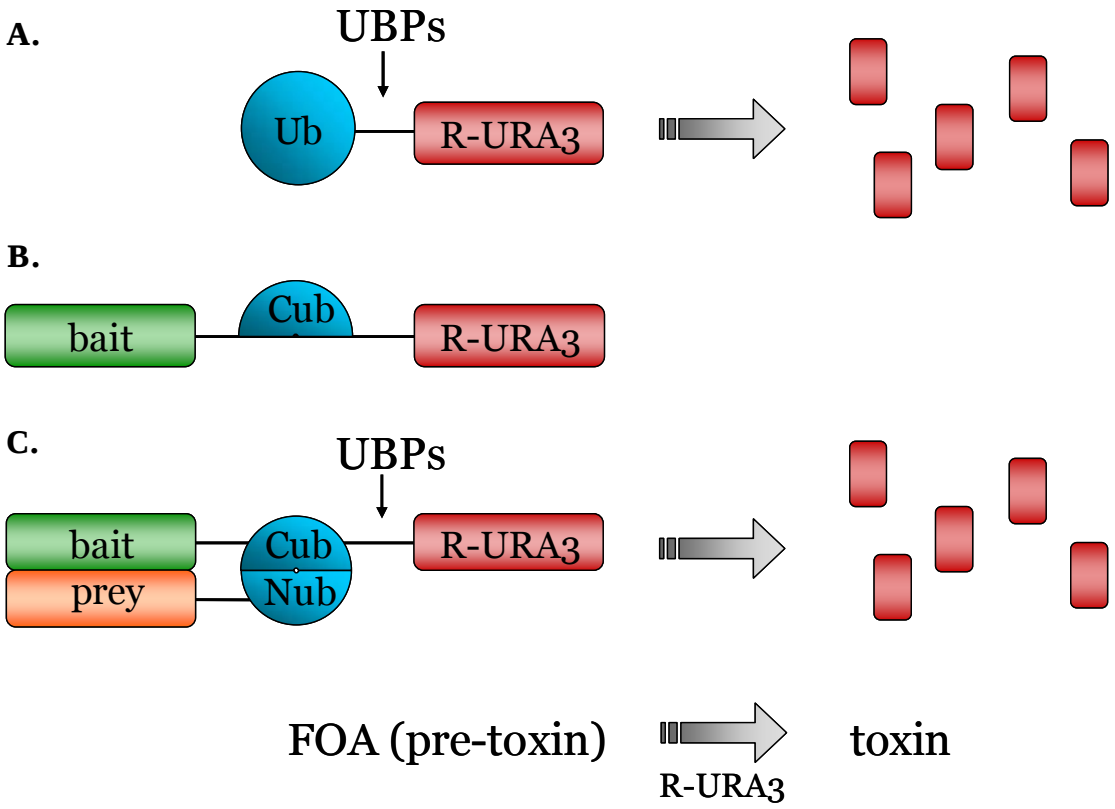

Fig 3.1

Schematic representation of the yeast split-ubiquitin two-hybrid system.

A. A fusion protein of ubiquitin (blue) and URA3 with an arginine as first amino acid (R-URA3, red) is cleaved by ubiquitin-specific proteases (UBPs) and free R-URA3 becomes rapidly degraded. **B.** When only the C-terminal part of ubiquitin (Cub, blue) is fused between a bait protein (green) and R-URA3, no cleavage occurs. **C.** A prey protein (orange) is fused to the N-terminal half of ubiquitin. If the bait interacts with the prey, an ubiquitin-like molecule is formed and the fusion is cleaved. The free R-URA3 is rapidly degraded, resulting in uracil auxotrophy and FOA resistance of the respective yeast strain.

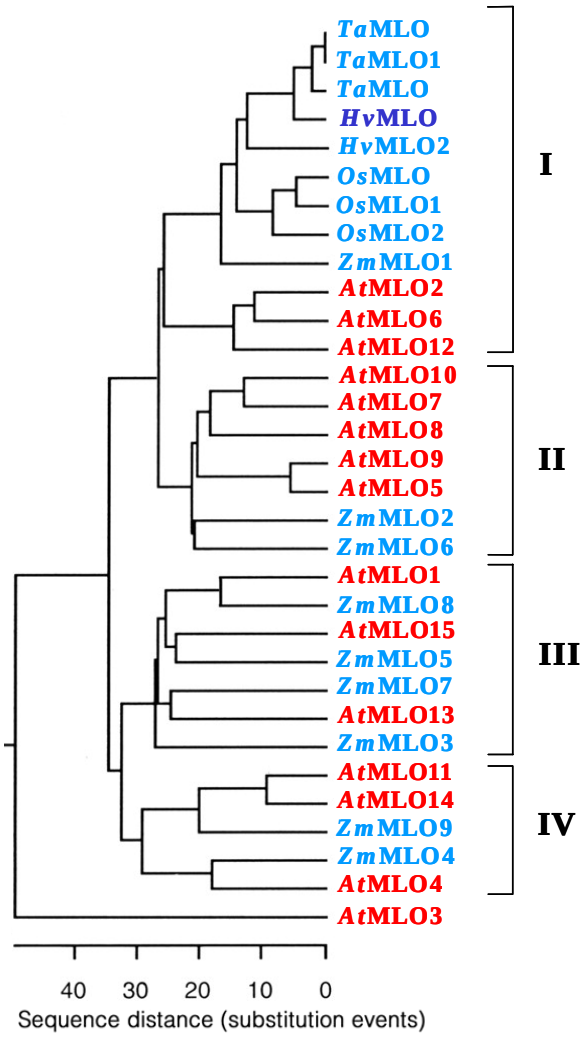

Fig 3.2

Phylogenetic tree of MLO proteins.

Amino acid sequences of 32 MLO-like proteins were aligned (Figure adopted from Kim et al., 2002b. The phylogenetic analysis shows that the plant MLO family is divided into four major clades plus one outgroup (*At*MLO3). MLO family members share approximately 35-45% identity at the amino acid level. Barley MLO is indicated in dark blue. The fifteen *A. thaliana* homologues are indicated in red.

Two different cDNA libraries from *A. thaliana* were used for screening. One library was created using mRNA from *A. thaliana* flower tissue at different developmental stages and represents around 1.5 million independent clones with an average insert size of 1.2 kb (L. Deslandes and I. Somssich, unpublished). The other cDNA library was generated using mRNA from various plant tissues, including flowers, and consists of 400.000 independent clones of large insert size (around 1.2 kb) and 700.000 independent clones of smaller insert size (around 0.6 kb) (I. Ottenschläger and K. Palme, unpublished).

3.2.3. *At*MLO proteins interact with *Hv*CaM but not with *Hv*Gα

All *At*MLO bait constructs generated were first tested for the interaction with either *Hv*CaM or *Hv*Gα. As mentioned before, calmodulin was found to interact with *Hv*MLO, *At*MLO1 and *At*MLO2 *in vivo* Kim, 2002 #20}. Apart from *At*MLO3, all *At*MLO baits (*At*MLO1, *At*MLO2, *At*MLO4, *At*MLO5, *At*MLO10 or *At*MLO12) were able to grow on FOA-containing medium when co-transformed with NuI-*Hv*CaM.

Since MLO protein structure is reminiscent of the animal G-protein coupled receptors, MLO proteins were suggested to potentially interact with Gα protein, one of the three polypeptides (Gα, Gβ, Gγ) that form the hetero-trimeric G-protein complex associated with proto-typical G-protein coupled receptors. Plants generally contain only one gene encoding a heterotrimeric G-protein α-subunit, which was demonstrated not to be required for *Hv*MLO function (Kim et al., 2002). No growth was detected when NuI-*Hv*Gα was transformed together with any of the *At*MLO bait constructs. Due to reproducibility of these results, yeast cells co-expressing NuI-*Hv*CaM and either *At*MLO1-Cub-R-URA3 or *At*MLO5-Cub-R-URA3 were used as positive control in all split-ubiquitin experiments; yeast cells co-transformed with NuI-*Hv*Gα and either *At*MLO1-Cub-R-URA3 or *At*MLO5-Cub-R-URA3 were employed as negative control.

3.2.4. Library screens and confirmation of candidate genes

As a first step to perform a yeast split-ubiquitin screen, the yeast strain JD53 was transformed with an *AtMLO* bait construct. As a selectable yeast marker, the bait vector contains the *HIS3* gene that catalyses an essential step in histidine biosynthesis. The presence of HIS3 results in histidine-prototrophy of JD53 yeast cells carrying the bait construct. Transformants were selected for presence of the bait construct on dropout medium lacking histidine. In order to examine bait fusion protein stability, the yeast strain expressing the bait construct was streaked on dropout medium lacking both histidine and uracil. If the fusion protein MLO-Cub-R-URA3 is functional and stable, the yeast cells are able to grow on this selective medium.

The yeast strain containing a stable bait fusion protein was subsequently transformed with the cDNA library by the lithium acetate transformation method. As a selectable yeast marker, the cDNA library vector (prey construct) contains the *TRP* gene required for tryptophan biosynthesis.

Each of the *At*MLO1, *At*MLO2 and *Hv*MLO bait constructs was used in four independent library screens, whereas each of the other bait constructs (*At*MLO3, *At*MLO4, *At*MLO5, and *At*MLO10) was employed in a single library screen. The *At*MLO12 bait construct was not used in a library screen, but employed for testing interactions with candidate proteins in a targeted manner (see below). Thus, a total of 16 independent library screens was performed.

To determine the transformation efficiency, an aliquot of the transformed yeast cells was plated on dropout medium lacking both histidine and tryptophan. On average, 2-5 x 10^5 transformants were obtained from each transformation. Thus, around 3 x 10^6 to 8 x 10^6 transformants were analysed in total. Transformants were selected on minimal medium lacking both histidine and tryptophan and additionally containing FOA. The appearance of colonies on the selective plate was monitored during the following 5 to 10 days. All transformants that appeared on selective agar plates were re-streaked on a fresh minimal medium agar plate containing FOA to confirm the growth phenotype (Fig. 3.3). Among all re-streaked transformants, around 20% were confirmed with respect to the FOA growth

phenotype. Growth of yeast strains carrying both the bait and the prey construct on FOA-containing medium indicates interaction of the two proteins. Yeast colony PCR reactions were performed to amplify the cDNA present in the prey construct. The PCR products were purified and sequenced.
As the split-ubiquitin system was never before used to screen a plant cDNA library, information about common "false positives" was not available. It should be stressed that the system employed can detect close proximity of or transient interaction between two proteins inside the yeast cell. Furthermore, plant proteins expressed in yeast cells can be localised in different compartments than in plant cells. In order to reduce the number of putative "false positive" clones the yeast strain containing the bait vector was re-transformed with both the linearised prey vector and the PCR product of interest. Yeast cells are able to homologously recombine the vector and the PCR product *in vivo* resulting in a prey plasmid containing the PCR product (gap repair system; Muhlrad et al., 1992). Among all candidates re-tested, 93 were confirmed upon re-transformation. Only prey constructs verified upon independent transformation were chosen for recovery of the prey constructs. Among these clones, those showing growth on FOA-containing medium with more than one bait constructs were selected for further analysis (Fig. 3.4).

It is noteworthy that not all tested bait constructs were equally well suited for the split-ubiquitin assay. The bait clones differed both in the number of originally identified prey clones and in the ratio of initially identified to subsequently confirmed prey constructs. For example, more than 250 clones were identified in independent screens using *At*MLO1 as bait construct (Fig. 3.5), whereas no clones were identified in multiple independent screens using *Hv*MLO as bait construct. However, only a small percentage of the clones identified with *At*MLO1 was confirmed upon re-transformation in a yeast strain containing the same bait construct. A similar ratio of confirmed to initially identified clones was obtained using the *At*MLO4 bait construct, while the ratio of confirmed candidates upon re-transformation to clones identified

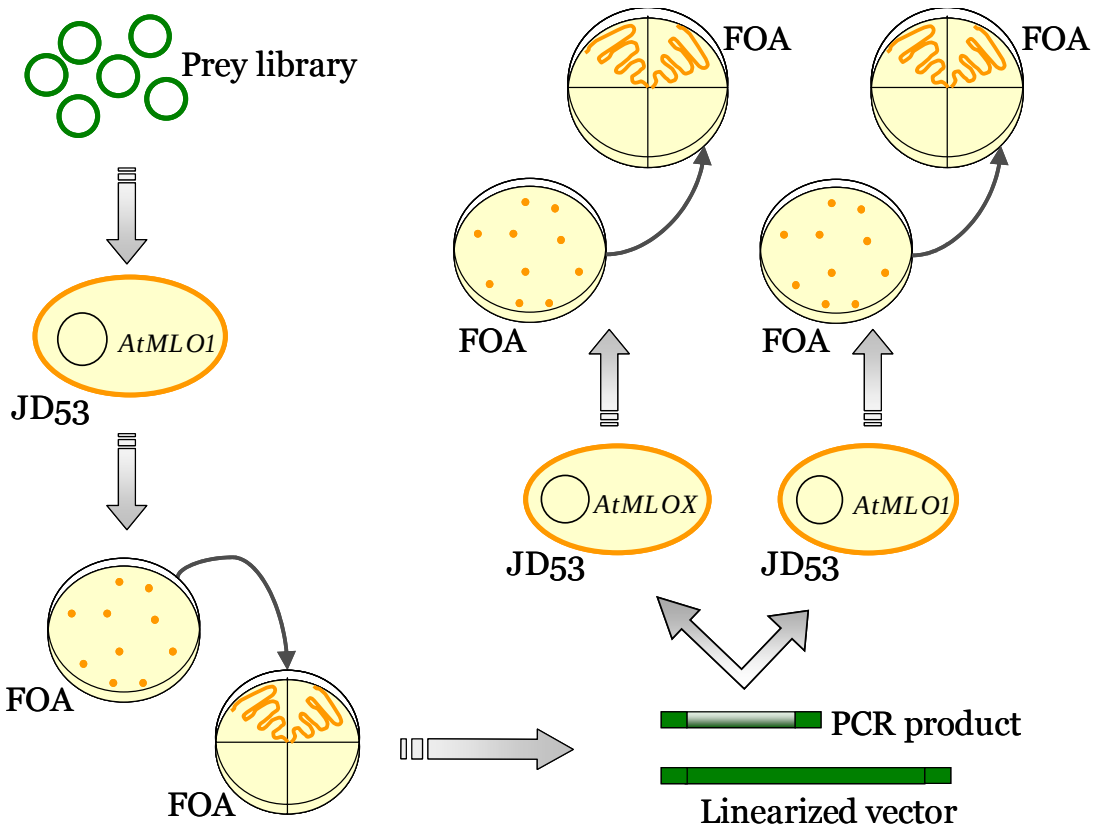

Fig 3.3

Flow chart depicting the general steps of the split-ubiquitin screening procedure.

A prey library is transformed into a yeast strain that contains a bait construct (here: *At*MLO1). Transformants are selected for their ability to grow on FOA-containing agar plates. Colonies that are able to grow on FOA medium are re-streaked on a fresh FOA plate to confirm the phenotype. The cDNA of the candidate prey clone is amplified by yeast colony PCR. The PCR product plus the linearised prey vector are transformed into a yeast strain containing either the same bait construct (here: *At*MLO1) or another bait construct (*At*MLOX). Transformants are tested for their ability to grow on the selective media.

in the screens was higher using *At*MLO5 or *At*MLO10 bait constructs. Particularly low rates of confirmed clones were obtained employing *At*MLO3 or *At*MLO2 as bait constructs. The former bait construct allowed the identification of around 40 clones, but only 3 of them were confirmed upon re-transformation. None of the 81 clones identified in the performed screens using *At*MLO2 was re-confirmed.

A list of all sequenced candidates is included in the Supplementary Data section (Table SD.1). The majority of the listed clones were confirmed upon re-transformation before sequencing to reduce the number of putative "false positives". None of the clones listed in the table possesses a stop codon in the translated sequence. On average, only proteins not larger than 300 to 400 amino acids were full-length.

Among all identified clones, four were chosen for further analysis. The selected clones have been isolated in independent screens using either all bait constructs (*At*PPI1); all but *At*MLO3 (*At*CYT b5); using *At*MLO1, *At*MLO5 or *At*MLO10 as bait constructs (*At*CYP1), or using *At*MLO1 or *At*MLO5 (*At*SQS). Moreover, two proteins with unknown function (At1g17080 and At1g62480) were identified independently using either *At*MLO1 or *At*MLO5 as bait constructs, but were not selected for further analysis (see below, Chapter 3.5).

3.3. CANDIDATE GENES IDENTIFIED USING THE SPLIT-UBIQUITIN SYSTEM

3.3.1. Cyclophilin

Cyclophilins possess peptidyl-prolyl *cis-trans* isomerase (rotamase) activity that catalyses the rotation of the amino bond between a proline residue and the preceding amino acid from a *cis* to a *trans* conformation (Fisher G, 1985). The *A. thaliana* genome contains 29 cyclophilin genes, the largest cyclophilin family identified in any organism to date

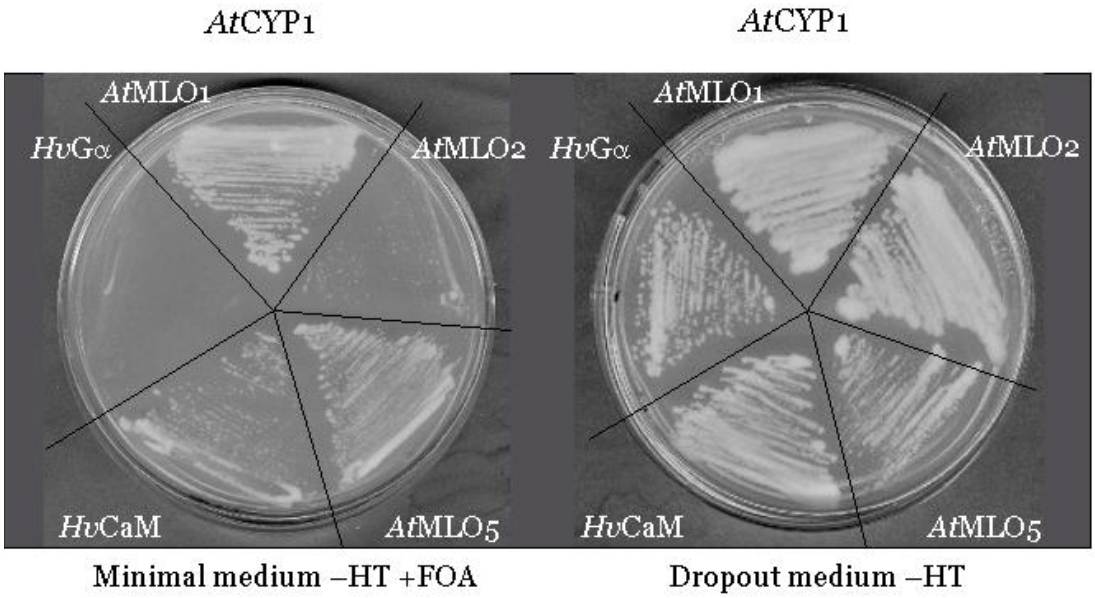

Fig 3.4

Interaction of *At*MLO proteins and *At*CYP1.

Yeast cells expressing the prey construct pNuI-*At*CYP1 together with one of the bait fusion proteins *At*MLO1-Cub-R-URA3 (*At*MLO1), *At*MLO2-Cub-R-URA3 (*At*MLO2) or *At*MLO5-Cub-R-URA3 (*At*MLO5) were streaked on medium lacking histidine and tryptophan (dropout medium –HT, on the right) or on selective medium lacking histidine and tryptophan plus additionally containing FOA (minimal medium –HT +FOA, on the left). Interaction was revealed by growth on the FOA-containing medium. Yeast cells expressing the prey vector pCup-NuI-HvCaM and the bait vector pMet-AtMLO5-Cub-URA3 were used as positive control; yeast cells expressing the prey vector pCup-NuI-HvGα and the bait vector pMet-AtMLO5-Cub-URA3 were used as negative control.

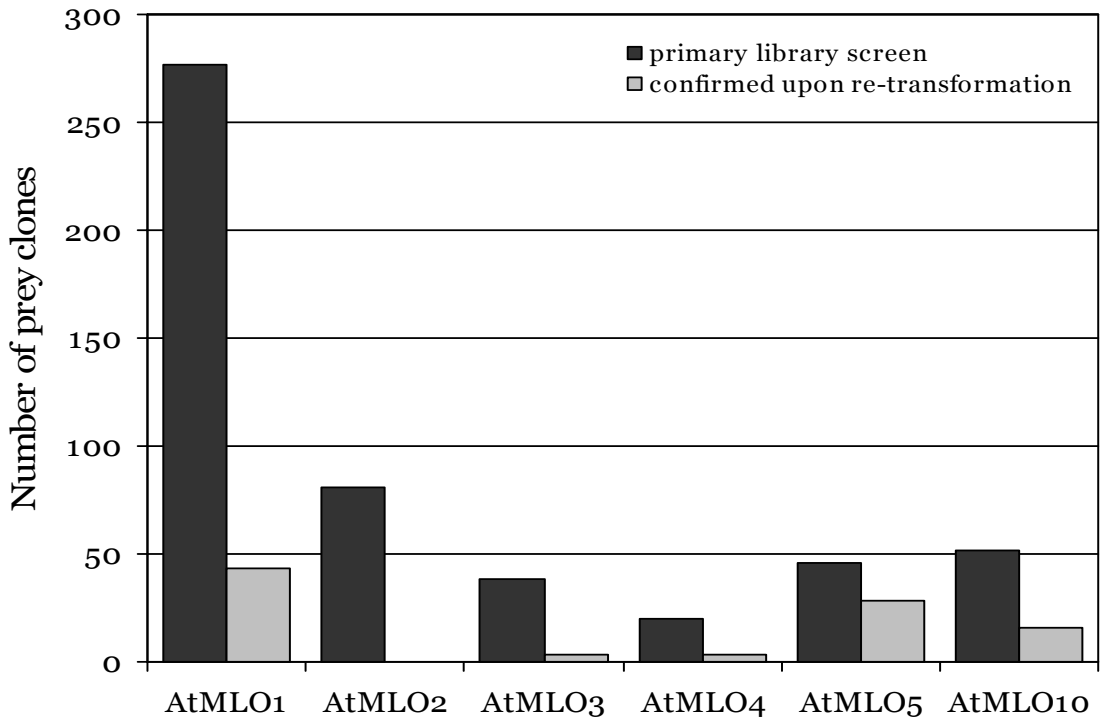

Fig 3.5

The ratio between initially identified and subsequently confirmed clones varies from bait to bait.

Black columns indicate the number of clones identified in the screens using a particular *At*MLO bait; grey columns indicate the number of these clones that were confirmed upon re-transformation using the same *At*MLO bait construct.

(Romano et al., 2004). Recently, it was shown that *A. thaliana* cyclophilins can be grouped in three phylogenetic clusters. Five isoforms of the same clade, *At*ROC1, *At*ROC2, *At*ROC3, *At*ROC5 (*At*CYP1) and *At*ROC6, share an amino acid identity of around 75-90%, have all a similar molecular weight (~20 kD) and are all predicted to be located in the cytosol (Chou and Gasser, 1997; Romano et al., 2004).

In three independent screens performed with the split-ubiquitin system, full-length *At*CYP1 was identified using either *At*MLO1, *At*MLO5 or *At*MLO10 fused to the Cub-R-URA3 reporter protein as bait construct. The identified *At*CYP1 clone was tested in a targeted manner with the other MLO bait constructs (*At*MLO2, *At*MLO3, *At*MLO4, *At*MLO12). Growth on FOA-containing medium was observed with all bait constructs, except *At*MLO3.

To investigate the specificity of these interactions, homologues of *At*CYP1 were tested in the split-ubiquitin system. The full-length cDNAs of the *At*CYP1 homologues (*At*ROC1, *At*ROC2, *At*ROC3 and *At*ROC6) were fused to the C-terminus of NuI and transformed into yeast cells containing either *At*MLO1-Cub-R-URA3 or *At*MLO2-Cub-R-URA3 fusion proteins. Yeast cells carrying both plasmid constructs were tested for their ability to grow on FOA-containing medium. Yeast cell co-expressing *At*MLO1 and *At*ROC6 displayed a strong growth phenotype, whereas *At*MLO1-expressing cell transformed with *At*ROC1, *At*ROC2 or *At*ROC3 exhibited a weak growth phenotype. Moreover, a clear interaction was detected between *At*MLO2 and either *At*ROC1 or *At*ROC3, whereas a weak interaction was observed between *At*MLO2 and *At*ROC6. No growth of yeast cells co-expressing *At*MLO2 and *At*ROC2 was monitored.

A table summarizing the interaction phenotypes in the split-ubiquitin system is reported in the Supplementary Data section (Table SD.2)

3.3.2. Proton Pump Interactor

The *A. thaliana* protein pump interactor1 (*At*PPI1) acts as a stimulator of the plasma membrane H^+-ATPase activity *in vitro* (Morandini et al., 2002). The *A. thaliana AtPPI1* gene encodes a polypeptide consisting of 612 amino acids that does not show significant similarity to any known protein. Analysis of the amino acid

sequence suggests the presence of a transmembrane domain in the distal region of the C-terminus.

In split-ubiquitin screens, different clones encoding various lengths of the C-terminus of *At*PPI1 were identified independently with all *At*MLO baits (Table SD.1 and data not shown). Due to the presence of a putative transmembrane domain in the C-teminus of *At*PPI1, it is conceivable that *At*PPI1 and *At*MLO proteins co-localise in yeast cells. To reduce the possibility of a "false positive" interaction due to co-localisation, a construct carrying the PPI1 C-terminus without the predicted TM domain was generated. One of the identified clones was modified by restriction digest in order to introduce a stop codon just upstream of the presumptive TM domain (Fig. 3.6). Yeast cells were co-transformed with the modified prey construct and different bait constructs. The truncated form of *At*PPI1 was still able to interact with several of the tested baits (*At*MLO1, *At*MLO2, *At*MLO3 or *At*MLO10) but not with *At*MLO5.

Furthermore, the full-length coding sequence of *At*PPI was fused to the 3´-terminus of NuI and analysed for interaction with *At*MLO proteins in the split-ubiquitin system. As reported in Table SD.2, no growth on FOA-containing medium was detected when NuI-AtPPI full-length was co-expressed with any of the bait constructs.

3.3.3. Cytochrome b5

Cytochrome b5 (CYT b5) is a component of the stearyl-CoA desaturase system and acts as an electron transfer intermediate between reductase and oxidative enzyme (Schenkman and Jansson, 2003). The *A. thaliana* genome contains six cytochrome b5 genes that share an identity of 40-70% at the amino acid level.

A.

561 588

wt: EKPIRNRTRGRGPETIPRAILKRKKSTNYWVYAAPAALVVLLLLVLGYYYVL*

no TM: EKPIRNRTRGRGPETIPRAILKRKKSID*

B.

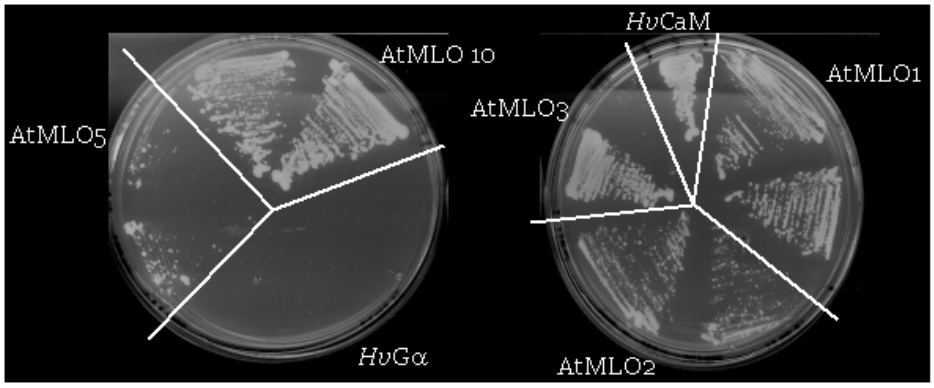

Fig 3.6

Interaction between *At*MLO proteins and *At*PPI1 variants.

A. Representation of the last 50 amino acids of *At*PPI1. The first line shows the wild-type situation with the putative transmembrane (TM) domain indicated in red. The second line corresponds to the mutant form of *AtPPI1* (*Atppi1*-no TM) where a stop codon, indicated by the red asterisk, prevents the translation of the transmembrane region. Two amino acids substituted in the mutant, due to the cloning procedure, are indicated in pink letters. **B.** Yeast cells expressing the prey vector carrying the mutant form of *AtPPI1* (pCup-NuI-AtPPI-noTM) together with various *At*MLO proteins fused to Cub-R-URA3 were grown on selective agar plates lacking histidine and tryptophan and containing FOA. Interaction was revealed by growth on the selective medium. Yeast cells expressing the wild type prey vector pCup-NuI-AtPPI and the bait vector pMet-AtMLO1-Cub-URA3 were used as positive control; yeast cells expressing the prey vector pCup-NuI-HvGα and the bait vector pMet-AtMLO1-Cub-URA3 were used as negative control.

In independent screens, four different isoforms of cytochrome b5 were identified using *At*MLO1, *At*MLO4, *At*MLO5 and *At*MLO10 as bait constructs. Two identified isoforms were full-length (At5g48810 and At2g32720) whereas the others (At5g53560 and At2g46650) represent only the C-terminal region of the CYT b5 proteins (Table SD.1 and data not shown).

3.3.4. Squalene synthase

Squalene synthase, also known as farnesyl-diphosphate farnesyl transferase, is an ER membrane enzyme that converts two molecules of farnesyl-diphosphate (FPP) into squalene (Brown and Goldstein, 1980).

Performing split-ubiquitin screens, *At*SQS was identified three times independently, twice using *At*MLO1 and once using *At*MLO5 as bait constructs (Table SD.1). The identified clones represent the C-terminal region of the protein and were tested in a targeted manner for the interaction with other *At*MLO baits (*At*MLO2, *At*MLO3, *At*MLO4, *At*MLO10 or *At*MLO12; Table SD.2). Growth on FOA-containing medium was detected when *At*SQS was co-transformed with *At*MLO1, *At*MLO2, *At*ML5, *At*MLO10 or *At*MLO12; no growth was observed when *At*SQS was transformed together with *At*MLO3 or *At*MLO4.

3.4. INTERACTION BETWEEN PREY CLONES AND *Atmlo1* MUTANT ALLELES

As mentioned above, few barley *mlo* mutant alleles are known to encode a stable protein variant. One of them (*mlo*-L420R/W423R) has a double amino acid exchange in the CaM-binding domain that disrupts the interaction with CaM (Kim et al. 2002b). Two other *mlo* mutant alleles contain nucleotide substitutions that lead to a single amino acid exchange in the third intracellular loop of the protein: *mlo*-27 (G318E) and *mlo*-29 (P334L) (Piffanelli et al., 2002). Interaction between *At*MLO proteins and the candidate interactors might be disrupted by one of these mutations. As *At*MLO1 was a suitable bait for the split-ubiquitin system, it was

chosen for generating a set of mutants mirroring the barley *mlo* mutants. The *Atmlo1* mutant alleles were created by PCR-based site directed mutagenesis. Both *Atmlo1*-27 and *Atmlo1*-29 show a single amino acid substitution, G351E and P367L respectively; *Atmlo1*-LWRR is characterized by two amino acid exchanges, L453R and W456R.

The three *At*MLO1 mutant alleles were fused to the 5´end of Cub-R-URA3 and tested with a sub-set of candidate interactors in the split-ubiquitin system. As reported in table SD.2, the *At*MLO1 mutants behaved very similarly to the wild-type *At*MLO1 bait construct. In accordance with previous data (Kim et al., 2002a), strains carrying *Atmlo1*-LWRR and *Hv*CaM did not grow on FOA-containing media, indicating that no interaction occurred between the two proteins. In contrast, *At*MLO1-27 and *At*MLO1-29 were still able to interact with *Hv*CaM, suggesting that amino acid substitutions in the third cytoplasmic loop of *At*MLO1 do not interfere with CaM binding at a distance. Furthermore, no differences were observed in the interaction between the *At*MLO1 mutants and the candidate proteins compared to the wild-type *At*MLO1 protein.

3.5. TESTING THE POTENTIAL BIOLOGICAL RELEVANCE OF THE CANDIDATE INTERACTORS BY TRANSIENT SINGLE CELL GENE EXPRESSION TECHNOLOGY

The split-ubiquitin system allowed the identification of putative *At*MLO-interactors, but it is necessary to perform other assays to verify the interaction. One approach to investigate the biological meaning of the interaction between MLO and the candidates *in planta* is to test their relevance in the context of the barley-powdery mildew interaction. To evaluate the effect of candidate gene silencing or overexpression in this plant-microbe interaction, the single cell transient gene expression technology was employed (reviewed in Panstruga, 2004). This method is based on the delivery of DNA constructs in single barley epidermal cells by particle bombardment and subsequent inoculation of the samples with powdery mildew spores. Application of this technique depends on few conditions. First, the outcome of the interaction between barley and the fungus appears to be determined in a cell-

autonomous manner. Thus, it is possible to evaluate the impact of either gene silencing or overexpression by quantification of the pathogen success in single transformed cells. Second, the powdery mildew fungus attacks exclusively epidermal cells and it develops in a highly synchronous manner. These characteristics allow an easy evaluation of infection success by microscopy of stained challenged leaves.

The amino acid sequences of the identified *A. thaliana* candidate MLO-interacting proteins were used for BLAST analysis against a comprehensive barley EST database (http://pgrc.ipk-gatersleben.de) and the most closely related barley sequences were selected for further analysis (Fig. 3.7). The barley homologues of the *A. thaliana* proteins identified in the split-ubiquitin system were cloned into appropriate vectors and tested in single cell gene silencing and overexpression experiments. Barley homologues of the *A. thaliana* "unknown proteins" identified in the screens were not found. Thus, these proteins were not further analysed. It has to be mentioned that besides *Hv*SQS, all barley EST sequences identified encode full-length proteins. BLAST searches of the *At*SQS protein sequence led to the identification of an EST sequence encoding only the C-terminus (~ 35% of the full-size *A. thaliana* sequence) of the corresponding barley protein.

3.5.1. Transient single cell gene silencing of the candidate genes

Gene silencing can be triggered by the expression of inverted repeat DNA constructs (Panstruga, 2004). The barley sequences were cloned as inverted repeats separated by an intron into plasmid pUAMBN (M. Miklis, P. Schulze-Lefert and R. Panstruga, unpublished data), and ballistically co-transformed

A.

```
AtCYP1     1MSNPRVFFDMSLSGTPIGRIEMELFADTTPNTAENFRALCTGEKGMGKLGKPLHFKGSIF
HvCyp1     1MANPKVFFDMTVGGAPAGRIVMELYKDAVPRTVENFRALCTGEKGVGKSGKPLHYKGSSF

AtCYP1    61HRVIPGFMCQGGDFTAKNGTGGESIYGAKFKDENFIKKHTGAGILSMANSGPNTNGSQFF
HvCyp1    61HRVIPDFMCQGGDFTRGNGTGGESIYGEKFADEKFVHKHTKPGILSMANAGPNTNGSQFF

AtCYP1   121ICTDKTSWLDGKHVVFGQVVKGLDVVKAIEKVGSDSGKTSKVVTITDCGQLS
HvCyp1   121ICTVPCNWLDGKHVVFGEVVEGMDVVKNIEKVGSRSGICSKQVVIADCGQL-
```

B.

```
AtCYTb5      1 MGGDG---KVFTLSEVSQHSSAKDCWIVIDGKVYDVTKFLDDHPGGDEVILTSTGKDATD
HvCytb5-1    1 MAGDK---KVFGFEEVARHNVTKDCWLVIAGKVYDVTSFMDEHPGGDEVLLAVTGKDATS
HvCytb5-2    1 MSSSSSTTKVFTLEEVAKHASKDDCWLVIAGKVYNVTKFLDDHPGGDDVLLSSTAKDATD

AtCYTb5     58 DFEDVGHSSTAKAMLDEYYVGDIDTATVPVKAKFVPPTSTKAVATQDKSSDFVIKLLQFL
HvCytb5-1   58 DFEDIGHSDSAREMMEKYHIGEIDASTIPAKRTFVPPQ--QGSHVQAKDNDILIKILQFL
HvCytb5-2   61 DFEDVGHSTTARAMMDEYYVGEIDATTIPTKVKYMPPK--QPHYNQDKTPEFIIKILQFL

AtCYTb5    118 VPLLILGLAFGIRYYTKTKAPSS
HvCytb5-1  116 VPIFILGLAFGIRHYSKSE----
HvCytb5-2  119 VPLAILGLAVAVRIYTKSESV--
```

C.

```
AtPPI1   1 MGVEVVNSGGFEVAPAPFEGKPEKNCKLDQGKGDDAPINFGSVGELPKN---AEENNNKV
HvPPI    1 MGMEVV---GAEAAPAQVKVADEEVALFQDKESQATARAREEAAVFGSDNGKAAANAPNG

AtPPI1  58 VNSDAPKNAAEEWPVAKQIHSFYLVKYRSYADPKIKAKLDLADKELEKLNKARTGVLDKL
HvPPI   58 SDMAPPKDAADEWPEPKQTYTFYFVKVRSFEDPKLRAKLEQAEKDFQNKIQARSKIIEAI

AtPPI1 118 RAKRAERSELFDLLDPLKSERKGFNTMFDEKRKEMEPLQQALGKLRS-NDGGSARGPAIC
HvPPI  118 KAKKTERAAVLAELRPLSAENRQYNEAFNEKLEEMKPFRNRLGKFRDENNAMRAESAGLC

AtPPI1 177 SSEEELNSMIYSYQYRIQHESIPLTEEKQILKEIRLLEGTRDKVIANAAMRAKIKESMGQ
HvPPI  178 SSLEELEHEIKRLNHRISHESISLDEEKRLIKEIKTLEKTRPKVSSNAAKRAKMQDTVVE

AtPPI1 237 KDDIQGQVKLMGAGLDGVKKERQAISARINELSEKLKATKDEITVLENELKTVSEKRDKA
HvPPI  238 RDAIQDQVKIIGDGIDGVKKERQAVRSKIKVLDDEMKVVDGEIALLQEDLNAATARKDKA

AtPPI1 297 YSNIHDLRRQRDETNSEYYQNRTVLNKARDLAAQKNISELEALANAEVEKFISLWCSKKN
HvPPI  298 YESLTELRKLRDLANASFHQNRIVLNKARDYSSRNEVEELQELHKTEVEKFMTQWCSSKT

AtPPI1 357 FREDYEKRILQSLDSRQLSRDGRMRNPDEKPLIAPEAAPSKATPSETEVVPKAKAKPQPK
HvPPI  358 FREDYEKRILTSLNGRQLTRDGRMRNPDEKPIFIETQQPVAQEP--VPLKAPLKQAKEAA

AtPPI1 417 EEPVSAPKPDATVAQNTEKAKDAVKVKNVADDDDDEVYGLGKPQKEEKP--VDAATAKEM
HvPPI  416 APQVVAPK-EEPLAKASAKS---AKVKASVDADDD-AYEAEPPKEKPKPKEVDVAKLKEI

AtPPI1 475 RKQEEIAKAKQAMERKKKLAEKAAAKAAIRAQKEAEKKEKKEQEKKAKKKTGGNTETETE
HvPPI  471 KRQEEIEKNKLALERKKKQAEKQAAKAAARAQKEAEKKLKKE-EKKTKKKTEPADTDEPT

AtPPI1 535 EVPEASEEEIEAPVQEEKP----QKEKVFKEKPIRNRTRGRGPETIPRAILKRKKSTNYW
HvPPI  530 DSDTKSDEAAEAQAEDEFTPTTLNKEQKQNVRPRNVVTKTKAP--LPKAILKRKKAQSYR

AtPPI1 591 VYAAPAALV------VLLLLVLGYYYVL-------
HvPPI  588 SWATPAVVISAVAVLVALLAALGYYQYYRPASTSN
```

Fig 3.7

Amino acid sequence alignment of *A. thaliana* prey proteins and the closest identified barley homologues.

A. Amino acid sequence alignment of *A. thaliana* cyclophlin (*At*CYP1, AGI number At4g34870) and its closest barley homologue (*Hv*Cyp, GenBank accession number BE196287). Residues identical to those in the *A. thaliana* protein are boxed in black, conservative changes are boxed in grey and gaps are shown as hyphens. **B.** Amino acid sequence alignment of *A. thaliana* cytochrome b5 (*At*CYTb5, AGI number At5g48810) and its closest barley homologues (*Hv*Cytb5-1, GenBank accession number BE216802; *Hv*Cytb5-2, GenBank accession number BE421668). Residues marked like in A. **C.** Amino acid sequence alignment of *A. thaliana* protein pump interactor PPI (*At*PPI1, AGI number At4g27500) and its closest barley homologue (*Hv*PPI, GenBank accession number BQ467418). Residues marked like in A. All sequences were aligned using ClustalW (www.ch.embnet.org).

with a GUS reporter construct into epidermal cells of detached barley leaves.

Subsequently, these were incubated for 96 h to allow the turn-over of pre-existing endogenous target proteins prior to inoculation with powdery mildew conidiospores (Kim et al., 2002a). 48 h after pathogen challenge, leaves were stained for GUS activity and subsequently evaluated for pathogen penetration efficiency. Transient single cell gene silencing experiments were performed in both *MLO* (susceptible) and *mlo* (resistant) barley genotypes.

The susceptible wild-type barley cultivar Ingrid *MLO* was co-bombarded with the GUS reporter construct and either an individual or a pool of all gene silencing constructs. The same barley line bombarded with the GUS reporter construct plus the empty silencing vector served as negative control and resulted in a successful fungal penetration rate of about 10%. This relatively low penetration success is due to an increased resistance of detached barley leaves incubated for an extended time period (96 h) before being challenged with the fungus. Reasons for this increased resistance are still unclear, but might be related to the stress of prolonged *in vitro* incubation of the detached leaves. Consistent with previous findings that *Mlo* overexpression results in supersusceptibility (Kim et al. 2002b), an increased successful penetration rate of around 60% was observed when cultivar Ingrid was co-bombarded with a vector overexpressing wild-type MLO (positive control). Almost no variation in the penetration rate compared to the negative control was observed when silencing of the candidate genes was performed. A moderate increase in successful penetration rate (to about 20% absolute penetration success) was observed when all silencing constructs were co-bombarded (Table SD.3; Table SD.4 and Fig. 3.8).

In another set of experiments, the silencing constructs were co-bombarded with a bifunctional construct expressing both the GUS reporter and wild-type *MLO*. As mentioned above, simultaneous overexpression of *MLO* renders cell supersusceptible (Kim et al. 2002b) and should thus allow a better visualisation of a (potential) decrease in successful penetration. Barley cells bombarded with the bifunctional GUS-MLO overexpressing construct alone showed a successful penetration rate of around 60%, consistent with the results obtained in the previous

experiment (see above). No significant variation was observed when this construct was co-bombarded with the gene silencing constructs (Table SD.5; Table SD.6 and Fig. 3.9).

The resistant barley line BC Ingrid *mlo*-3 was co-bombarded with the GUS reporter plasmid and the dsRNAi silencing constructs. The barley line used in this experiment is fully resistant to *Bgh*, as consistently confirmed by the low penetration rate of about 2% in the control samples (transformed with the GUS reporter plasmid only). A similar penetration rate was detected in the barley cells when silencing of the candidate genes was performed (Table SD.7; Table SD.8 and Fig. 3.10).

3.5.2. Transient single cell overexpression of the candidate genes

Transient gene expression technology was also employed to test the effect of overexpression of the candidate genes. As in the previous experiments, both susceptible and resistent barley genotypes were used, but detached leaves were inoculated 6-16 hours after bombardment as no turnover of pre-existing proteins was necessary.

The susceptible wild-type cultivar Ingrid *MLO* was co-bombarded with a GUS reporter construct and the individual overexpressor constructs. As outlined above, detached *MLO* wild-type leaves are more susceptible to *Bgh* when inoculated few hours after bombardment (6 to 16 h) than after 96 h. Due to this reason, an experiment involving *MLO* overexpression was not necessary. No significant difference was observed upon overexpression of either single genes or a pool of all candidate genes (Table SD.9; Table SD.10; Fig. 3.11).

A similar experiment was performed using the resistant barley line Ingrid *mlo*-3. As in the previous experiments, candidate gene overexpression did not alter resistance to the fungus in the *mlo*-3 genotype (Table SD.9; Table SD.10).

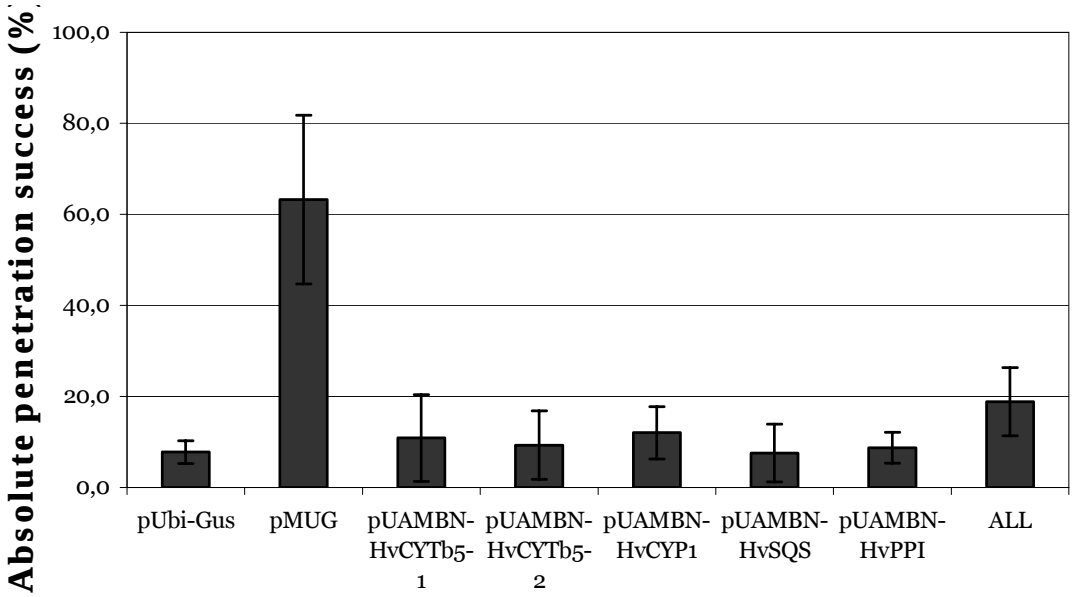

Fig 3.8

Single cell gene silencing of barley candidate genes in wild-type *MLO* cells.

The susceptible wild-type barley line Ingrid *MLO* was bombarded with the vectors listed in Table SD.3. Leaves were inoculated with *Bgh* 96 hours after bombardment and stained for GUS activity 48 hours after inoculation. As positive control, the same barley line was bombarded with a construct overexpressing *MLO* (pMUG). Columns represent the mean and the standard deviation of three independent experiments.

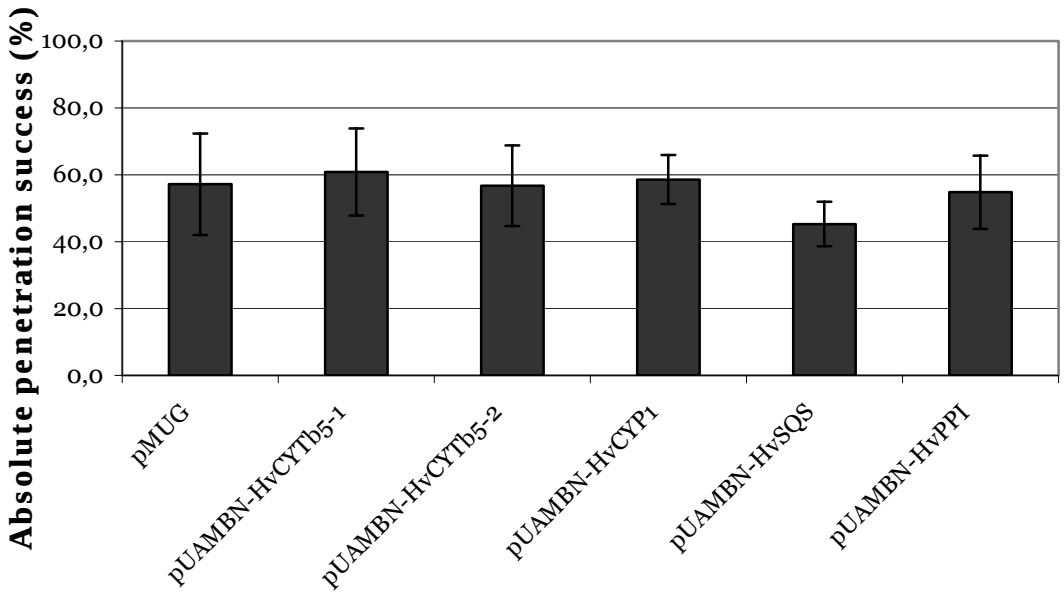

Fig 3.9

Single cell gene silencing of barley candidate genes in wild-type *MLO* cells overexpressing *MLO*.

The susceptible wild-type barley line Ingrid *MLO* was bombarded with the vectors listed in Table SD.5. Leaves were inoculated with *Bgh* 96 hours after bombardment and stained for GUS activity 48 hours after inoculation. Columns represent the mean and the standard deviation of four independent experiments.

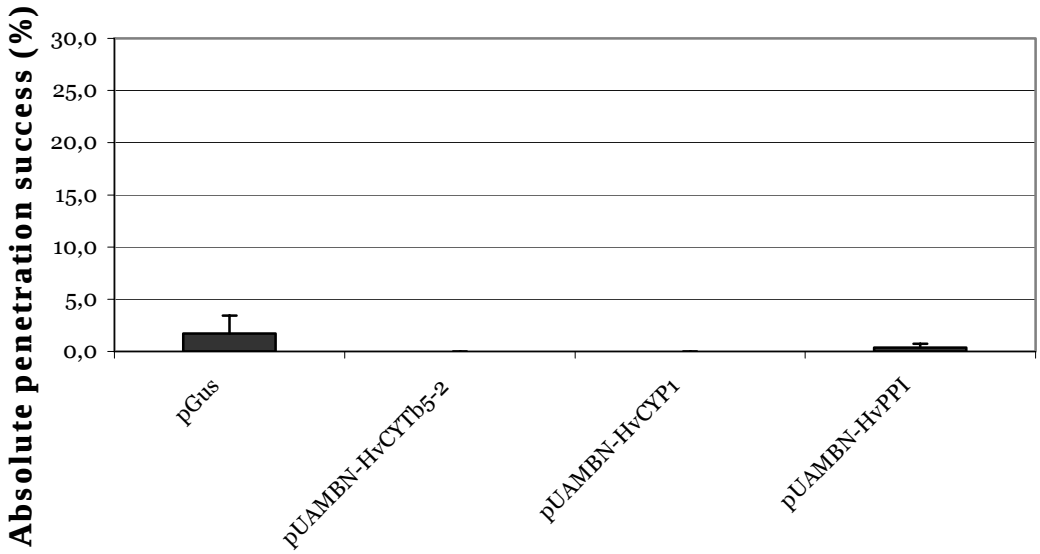

Fig 3.10

Single cell gene silencing of barley candidate genes in mutant *mlo*-3 cells.

Resistant mutant barley line Ingrid *mlo*-3 was bombarded with the vectors listed in Table SD.7. Relative penetration success and standard deviations were calculated based on the experiments listed in Table SD.7. Columns represent the mean and the standard deviation of three independent experiments.

3.6. INTERACTION OF BARLEY HOMOLOGUES WITH *A. thaliana* BAITS IN THE SPLIT-UBIQUITIN SYSTEM

Besides transient single cell gene expression/silencing, barley homologues of the *A. thaliana* candidate genes were also analysed in the split-ubiquitin system. The cDNA sequences of the barley homologues were cloned into the prey vector in order to test a potential interaction with the *At*MLO baits. The growth phenotype on FOA-containing media revealed that *Hv*CYT b5 can interact with all tested *At*MLO baits, except *At*MLO4. In contrast, when NuI-*Hv*CYP1 or NuI-*Hv*SQS were co-expressed with any of the bait constructs, no growth was observed. Using full length NuI-*Hv*PPI as prey, growth was observed when co-expressed with either *At*MLO1, *At*MLO5 or *At*MLO10; no growth on FOA-containing medium was observed with *At*MLO2, *At*MLO3, *At*MLO4 or *At*MLO12 bait constructs.

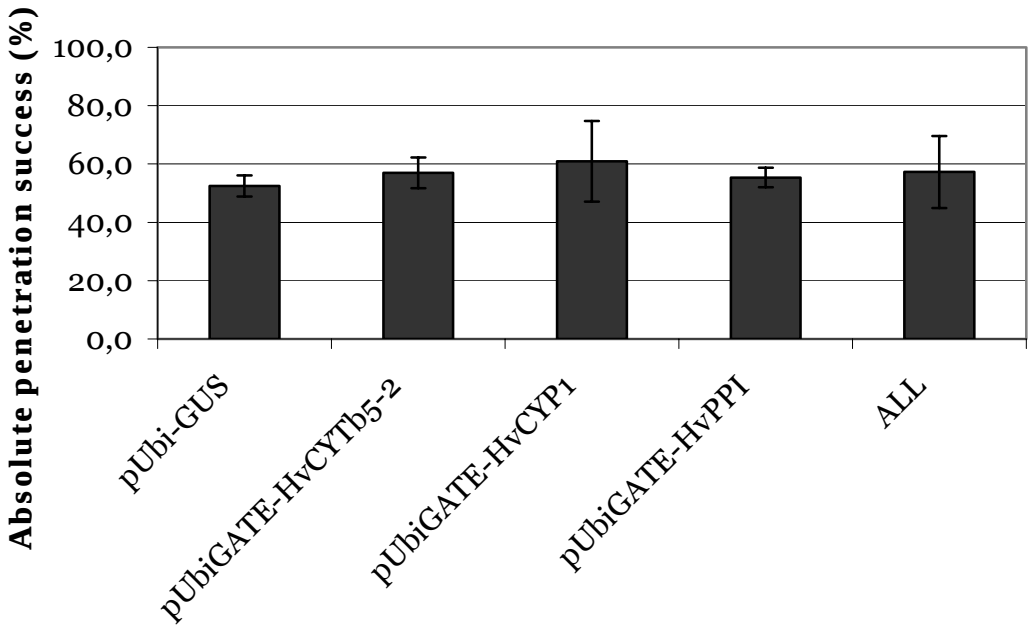

Fig 3.11

Single cell overexpression of barley candidate genes in wild-type *MLO* cells.

The susceptible wild-type barley line Ingrid *MLO* was bombarded with the vectors listed in Table SD.9. Percentage penetration success and standard deviations were calculated based on the experiments listed in Table SD.9.

4. DISCUSSION

4.1. THE SPLIT-UBIQUITIN SYSTEM

Several subdomains of barley MLO were tested as baits in previous studies using a classical yeast two-hybrid assay, but identification of putative interactors was unsuccessful (R. Panstruga, P. Piffanelli, personal communication). Thus, a recently developed alternative yeast two-hybrid system was employed.

4.3.1. Advantages and disadvantages of the split-ubiquitin system

The split-ubiquitin system provides important advantages compared to the traditional yeast two-hybrid assay. The readout based on proteolytic stability renders the system suitable not only for soluble proteins or for subdomains of transmembrane proteins but also for full-size polytopic membrane proteins, provided that Nub and Cub are fused to cytosolic domains of the proteins. Screening with full-length integral membrane proteins offers the opportunity to identify protein-protein interactions as they occur in their natural settings. In addition, the system may enable the identification of protein-protein interactions that require multiple bait or prey peptide domains to occur.

A major disadvantage of the split-ubiquitin system is the high number of false positives that arise in the screens (this study; Thaminy et al., 2003). This feature can be due to different independent factors. Mutations in the bait promoter may lead to silencing of the fusion construct resulting in FOA resistance that is independent of the bait-prey interaction. Equivalent effects might be caused by mutations affecting the URA3 gene resulting in an inactive form of the URA3 protein or by mutations in the bait that result in a premature stop codon. Furthermore, a high expression level of a NuI-prey fusion might lead to incorrect

compartmentalization of the protein, resulting in unspecific interaction with the bait construct. To reduce the number of false positives, re-transformation of yeast cells with both the prey and bait constructs has been shown to be useful. Additionally, in this study, only candidates interacting with several sequence-diversified bait isoforms were selected for further analysis.

4.3.2. Characteristics of bait and prey fusion proteins

Despite the fact that *Hv*CaM was demonstrated to bind *Hv*MLO both *in vitro* and *in vivo* (Kim et al., 2002a), no interaction between the two proteins was detected in the split-ubiquitin system. In the same assay, *Hv*CaM was found to interact with either *At*MLO1 or *At*MLO2 baits. Thus, *A. thaliana* MLO proteins were selected to be used as baits in the split-ubiquitin screens. Furthermore, overlap of candidates identified independently with different *At*MLO baits facilitated the identification of common candidate interactors of the MLO family. The bait constructs investigated were not equally well suited for the split-ubiquitin system. Some of them (*At*MLO1, *At*MLO3, *At*MLO4, *At*MLO5 and *At*MLO10) led to the identification of several candidates while no interactors were isolated using *At*MLO2 or *Hv*MLO bait constructs (see Chapter 3.2.4). This difference might be due to the particular conformation of the *At*MLO2 or *Hv*MLO bait fusion proteins.

Recently, the split-ubiquitin system was employed to investigate homo- and hetero-interactions between *A. thaliana* K^+ channels (Obrdlik et al., 2004) and to identify interactors of either a human endoplasmic reticulum-associated protein (Wang et al., 2004) or of a human G-protein coupled receptor (Thaminy et al., 2003). In all referred cases, NuG, the Nub isoform characterised by the lowest affinity to Cub, was employed. To examine the specificity of the interaction, the human G-protein coupled receptor ErbB3 was co-expressed with a non-interacting protein fused to either NuG or NuI. Interestingly, no interaction was observed when the bait construct was co-transformed with the NuG fusion protein, while NuI fusion led to activation of the reporter system (Thaminy et al., 2003). This unspecific interaction might occur because NuI has the tendency to associate with

Cub independently of an additional protein-protein interaction (Johnsson and Varshavsky, 1994; Stagljar et al., 1998; Thaminy et al., 2003). Furthermore, unspecific interaction with the NuI fusion protein might be due to the multicopy prey vector used in this study possibly resulting in extraordinary high expression levels of the prey protein (Thaminy et al., 2003).

Previous experiments showed that interaction between *Hv*CaM and *At*MLO1 was only detectable when *Hv*CaM was fused to the NuI isoform. No interaction was observed when *Hv*CaM was fused to either the NuA or NuG isoform (R. Panstruga, personal communication). Thus, in this study, NuI, the wild-type Nub isoform, was preferred to NuA or NuG as a fusion partner for the prey library, despite its higher affinity to Cub. Moreover, candidates isolated in the screens were fused to either NuA or NuG and co-expressed with any of the *At*MLO bait constructs. Interaction was detected exclusively between *At*MLO1 and either NuA-*At*PPI1 or NuG-*At*PPI1 (data not shown).

Collectively, data obtained by others and in this study indicate that none of the Nub isoforms is generally better suited than others. The optimal constellation may depend on the reporter system used (see below) and/or the individual bait/prey interaction pair.

4.3.3. Different reporter systems can be employed in the split-ubiquitin system

In the present study, readout of the split-ubiquitin system was based on the degradation of the reporter protein URA3. The URA3 protein is required for biosynthesis of uracil and, additionally, can degrade FOA into a toxic compound. High toxicity of the FOA-derived product enables a strict selection of yeast cells in which protein-protein interaction has led to complete degradation of the R-URA3. Previously, the URA3 reporter protein was successfully employed to study interactions of *Saccharomyces cerevisiae* proteins (Wittke et al., 1999; Laser et al., 2000; Pätzold and Lehming, 2001).

Besides URA3, other reporter systems have been employed. In particular, the artificial transcription factor protein A-LexA-VP16 (PLV) was used (Stagljar et al.,

1998; Thaminy et al., 2003; Obrdlik et al., 2004; Wang et al., 2004). Reconstitution of a native-like ubiquitin protein leads to cleavage of PLV that activates both *lacZ* and *HIS3* reporter genes. The PLV reporter system was improved in order to minimize the background arising from non-specific release of PLV. To reduce the expression level of the bait, the bait construct was integrated into the yeast chromosome (Wang et al., 2004). Moreover, to inhibit the basic leakage of the histidine reporter gene, a competitive inhibitor (3-aminotriazole) of an enzyme involved in histidine biosynthesis was employed (Wang et al., 2004).

To date, the PLV-based split-ubiquitin system was successfully employed in prey library screens for protein-protein interactions, subsequently confirmed by co-immunoprecipitation (Thaminy et al., 2003; Wang et al., 2004), while URA3 was mainly used for testing interactions of known proteins in a targeted manner (Dünnwald et al., 1999; Wittke et al., 1999; Laser et al., 2000). In the present study, a URA3-based split-ubiquitin prey library screen was performed for the first time, but interaction between MLO protein and the candidates has still to be confirmed by different experimental approaches.

4.3.4. MLO-CaM interaction is conserved between monocots and dicots

Based on biocomputational analysis, putative calmodulin binding domains (CaMBD) were previously identified in the C-terminus of all investigated MLO isoforms, suggesting that CaM binding might be a conserved feature of MLO proteins (Kim et al., 2002a). Surprisingly, *At*CaM was not identified in any performed screen. However, the barley NuI-*Hv*CaM prey construct was identified several times independently in the same screen when a contamination by this vector occurred accidentally (data not shown). Moreover, a NuI-*At*CaM prey construct, identified by using an unrelated bait (a WRKY transcription factor; L. Deslandes, personal communication), was tested in a targeted manner with several *At*MLO bait constructs. No growth on FOA-containing medium was detected. Sequencing of the clone revealed that in addition to the full-size coding sequence partial sequence of the *At*CaM 5´UTR was fused to NuI resulting in an in-frame insertion of a linker sequence, consisting of five arginine residues, between NuI and the CaM protein.

The presence of this linker might cause a conformational change in the NuI-*At*CaM fusion protein, rendering the protein potentially unable to bind to *At*MLO CaMBDs in the split-ubiquitin system. Similarly, in a recent publication, a protein previously identified in a classical two-hybrid screen as interactor of the human ErbB3 G-protein coupled receptor, was not recovered in a split-ubiquitin screen (Thaminy et al., 2003). These results indicate that identification of candidates might be influenced by particular prey fusion protein conformations or by the distance between NuI and Cub during bait-prey interactions.

*Hv*CaM was tested in a targeted manner with all available *At*MLO bait constructs. With the exception of the *At*MLO3-Cub-R-URA3 fusion protein, interaction between NuI-*Hv*CaM and any of the *At*MLO bait constructs was detected by growth of the yeast cells co-expressing the two vectors on FOA-containing medium (see Chapter 3.2.3). For the first time, it was shown that the MLO-CaM interaction is conserved among almost all MLO family members. This data indicate that the interaction between CaM and MLO proteins is conserved in both monocots and dicots and among isoforms within the same plant species suggesting that this interaction plays an important role for MLO function.

In addition, all available *At*MLO bait constructs were tested for interaction with *Hv*Gα. It was previously demonstrated that *Hv*Gα does not contribute to the defence-modulating function of barley MLO (Kim et al., 2002). In accordance with this finding, no interaction between *Hv*Gα and any of the *At*MLO baits was detected using the split-ubiquitin system. This outcome further corroborates the notion that MLO acts independently of Gα proteins.

4.2. TRANSIENT SINGLE CELL GENE EXPRESSION TECHNOLOGY TO ANALYSE MLO-CANDIDATE INTERACTIONS *in vivo*

Like for the classical yeast two-hybrid method, interactions identified in the split-ubiquitin system have to be confirmed by an independent experimental procedure. Co-immunoprecipitation can be problematic for integral membrane

proteins especially when the expression level is low as in the case of MLO proteins. As an alternative, fluorescence resonance energy transfer (FRET) experiments may be used to confirm the interaction *in planta*. Furthermore, in the case of MLO, transient single cell gene expression can be employed to assess the impact of each candidate gene on the outcome of the barley-powdery mildew interaction.

Since its development for the analysis of cereal-powdery mildew interactions (Nielsen et al., 1999; Schweizer et al., 1999), several laboratories have extensively used the transient single cell gene expression technology (reviewed in Panstruga, 2004). This system is extremely powerful for rapid analysis of candidate genes and allows testing several genes simultaneously (Panstruga, 2004).

According to the findings of this study, none of the candidate genes identified in the yeast split-ubiquitin screens seems to have an impact on the barley-powdery mildew interaction (see Chapter 3.5). It has to be considered that silencing experiments require several hours of pre-incubation after particle bombardment and before pathogen challenge to allow turnover of pre-existing proteins. The actually required period is not known and can vary from protein to protein. Thus, the 96 hours pre-incubation period used in this study might have been too short to observe any altered disease phenotype compared to the control. An extended pre-incubation period is not possible because of the accelerated senescence processes of detached leaves. However, transient single cell silencing of all candidate genes simultaneously revealed a small increase of susceptibility in the *MLO* wild-type genotype (Chapter 3.5.1). This result might indicate an additive effect of some of the candidate genes in the resistance pathway against the powdery mildew fungus. Moreover, MLO might also act in pathways that give no readout in the barley-*Bgh* interaction. Thus, it is possible that the candidate proteins interact with MLO in other pathways.

Finally, it is possible that not the appropriate barley gene was used for the overexpression/gene silencing experiments. Though the most closely related barley genes were selected from the barley EST database, it is conceivable that the isoforms that interact with MLO and possibly interfere with MLO function during barley-powdery mildew interactions are missing in the current EST collections.

4.3. PUTATIVE MLO-INTERACTORS IDENTIFIED USING THE SPLIT-UBIQUITIN SYSTEM

4.3.1. Cyclophilin

The first cyclophilin (CYP A) protein was identified as a specific target of the immunosuppressant cyclosporin A (CsA) in mammalian T-cells (Handschumacher et al., 1984). Cyclophilins are ubiquitous proteins characterised by a peptidyl-prolyl *cis-trans* isomerase activity and are present in all subcellular compartments. The high degree of conservation found in CYP amino acid sequences of distantly related organisms suggests an important conserved cellular function for cyclophilins (Trandinh et al., 1992; Chou and Gasser, 1997). In yeast, it was found that cyclophilins are not essential for growth, but their presence can be critical for survival after heat shock (Mclaughlin et al., 1992; Sykes et al., 1993). Expression of plant cyclophilins was reported to be induced by several biotic and abiotic stresses, including salt stress, heat and cold shock, salicylic acid, wounding and fungal infection (Godoy et al., 2000; Kong et al., 2001). Recently, it was demonstrated that a cyclophilin of the phytopathogenic fungus *Magnaporthe grisea* is required for the formation of infection structures (Viaud et al., 2002). The *M. grisea* cyclophilin gene (Cyp) putatively encodes a cytosolic form of cyclophilin. Absence of the CYP1-encoded cyclophilin in *Magnaporthe* resulted in a lower rate of plant infection and affected the function of appressoria, which did not penetrate efficiently the plant cuticle. Furthermore, two cyclophilin genes were identified in the human pathogenic fungus *Cryptococcus neoformans* and it was shown that double mutants are severely attenuated in virulence (Wang et al., 2001; Viaud et al., 2002).

Recently, phylogenetic analysis of the *A. thaliana* cyclophilin protein family revealed the presence of 29 cyclophilin isoforms that can be grouped in three major clusters. (Romano et al., 2004). *A. thaliana* cyclophilin proteins exhibit a molecular weight range of 20 to 100 kD. Five cytosolic isoforms (CYP1, ROC1, ROC2, ROC3 and ROC6) of clade I share the same molecular weight (~20 kD) and a high identity at the amino acid level (75-90%). Phylogenetic analysis indicates that *At*ROC3

(At2g16600) and *At*CYP1 (At4g34870), as well as *At*ROC1 (At4g38740) and *At*ROC6 (At2g21130), possess a considerable sequence identity at both the DNA and the protein level. These pairs are likely to have resulted from a recent local duplication event of a common ancestor gene resulting in a tandemly-oriented gene pair that underwent a subsequent segmental duplication event (Chou and Gasser, 1997; Romano et al., 2004). Thus, the high sequence similarity due to recent gene duplication and a putative functional redundancy of the *A. thaliana* cyclophilin proteins might explain why *At*MLO proteins did not exhibit specificity for a particular cyclophilin isoform. However, the four *A. thaliana* cyclophilins that share the highest similarity (ROC1, 3, 6 and CYP1) interact with both *At*MLO1 and *At*MLO2 in the split-ubiquitin system, whereas the most distantly related protein (*At*ROC2; At3g56070) shows a faint binding to *At*MLO1 and no interaction with *At*MLO2. It would be interesting to examine the specificity of the interaction between MLO proteins and this cyclophilin group by testing less related cytosolic cyclophilins belonging both to the same and other clades.

4.3.2. Proton Pump Interactor

Previously, the C-terminal autoinhibitory domain of *A. thaliana* plasma membrane H^+-ATPase ("proton pump" *At*AHA1) was used as bait in a classical yeast two-hybrid screen. The N-terminal part of an uncharacterised gene was identified as interactor of the proton pump and was named proton pump interactor1 (PPI1; Morandini et al., 2002). It was demonstrated that H^+-ATPase activity was stimulated *in vitro* by binding of *At*PPI1 (Morandini et al., 2002). Though *At*PPI1 is rich in charged residues, particularly in the C-terminus, the last 24 amino acids contain no charged or polar residues, suggesting the presence of a TM domain possibly tail-anchoring the protein to a cellular membrane. Recent data indicate both a plasma membrane and a Golgi localisation of the protein (P. Morandini, personal communication). In plants, plasma membrane H^+-ATPase is known to be involved in many different processes such as pH homeostasis, solute import and export (Palmgren, 2001) as well as plant defence (Zhou et al., 2000). It was suggested that inhibition of H^+-ATPase activity could be exploited by pathogens to

overcome plant resistance (Kato et al., 1993; Schaller and Öcking, 1999). Furthermore, it was demonstrated that the PM proton pump is activated in barley epidermal cells penetrated by an avirulent powdery mildew isolate (Zhou et al., 2000).

As *At*PPI1 appears to be both an activator of the H^+-ATPase and an MLO-interactor, it is tempting to speculate that it can act as a scaffold between its interactors suggesting that the interaction between *At*PPI1 and *At*MLO might be required for the activation of the PM proton pump by *At*PPI1. In contrast, MLO could act as a competitor of H^+ ATPase activity by sequestering PPI1.

The split-ubiquitin data obtained in this study indicate that the presence of the presumptive TM domain of *At*PPI1 is not required for the interaction with the *At*MLO baits, except *At*MLO5. This result suggests that the interaction between *At*MLO proteins and *At*PPI1 is most likely not simply due to a co-localisation of the proteins in the same yeast cell compartment. In contrast, yeast cells co-expressing the full-length sequence of *At*PPI1 fused to NuI and any of the MLO baits were not able to interact. The *At*PPI1 gene encodes a rather large polypeptide (predicted molecular weight of 68.8 kD; Morandini et al., 2002). Thus, absence of interaction between *At*MLO bait constructs and full-length *At*PPI1 might be either due to the distance between NuI and Cub or due to the structural conformation of the NuI fusion protein.

4.3.3. Cytochrome b5

Cytochrome b5 is a small heme protein associated with the ER membrane in animals, plants and yeast cells (Mitoma and Ito, 1992; Zhao et al., 2003). The large cytoplasmic N-terminus is a hydrophobic region containing the heme domain and participating in electro-transferring functions. CYT b5 is tail-anchored to the membrane by its C-terminal hydrophobic transmembrane domain (Hanlon et al., 2000; Zhao et al., 2003). Two different conformations were proposed for the membrane-binding domain: a single membrane-spanning helix and a hairpin-type structure spanning only half of the lipid bilayer (Visser et al., 1975). To date, it is

unclear which conformation the CYT b5 tail adopts *in vitro* or *in vivo* (Vergeres and Waskell, 1995; Hanlon et al., 2000). Furthermore, it was also proposed that a common mechanism for targeting of CYT b5 operates in mammalian, plant and yeast cells (Zhao et al., 2003). Cytochrome b5 is known to form a heterodimeric complex with cytochrome P450 in the presence of monooxygenase substrates (Jansson and Schenkman, 1996). Cytochrome P450 monooxygenases are a group of heme-containing proteins that catalyse oxidative reactions (Chapple, 1998). In plants, they play important roles in the biosynthesis of cell wall constituents (lignin), signal molecules (salicylic acid) and antimicrobial plant defence compounds (phytoalexins; Godiard et al., 1998).

Identification of four out of six *A. thaliana* isoforms in the split-ubiquitin screens might indicate a non-specific interaction between MLO and CYT b5. However, isoform specific interactions might be possible due to localisation in different tissues and/or different expression patterns in plant cells. As little is known about the function of both CYT b5 and MLO, it is very difficult to speculate about a potential role for the CYT b5-MLO interaction. It is not possible to exclude that the interaction between MLO and CYT b5 is due to their proximity in yeast cells rather than to a real interaction of biological significance.

Further analysis of the *in planta* interaction is currently in progress using the fluorescence resonance energy transfer (FRET) assay. Results of these experiments might elucidate whether the interaction between CYT b5 and MLO occurs in plant cells.

4.3.4. Squalene synthase

Squalene synthase catalyses the first pathway-specific reaction of the sterol branch of the isoprenoid pathway and is thought to be a key enzyme of sterol biosynthesis (Brown and Goldstein, 1980). The C-terminus of the protein is likely to contain a transmembrane domain, which is predicted to anchor the protein to the ER membrane. The catalytic site resides in the N-terminal region of the protein and it was found to be located on the cytoplasmic face of the ER (Robinson et al., 1993). Challenging of tobacco cell cultures with a fungal elicitor (elicitin of *Phytphthora*

parasitica) leads to suppression of sterol biosynthesis. This effect was reported to be correlated with a dramatic decrease in SQS enzyme activity (Devarenne et al., 2002).

As for Cyt b5, little is known about the regulation of the SQS gene and enzymatic activity of the protein. Thus, a convincing hypothesis concerning a putative role for SQS-MLO interaction cannot be proposed. Furthermore, it cannot be excluded that SQS and MLO might localise in the same compartment in yeast cells but not in plant cells.

Further investigation of the *in planta* interaction is currently in progress using the FRET assay. Results of these experiments might elucidate whether the interaction between SQS and MLO occurs in plant cells.

4.4. *A. thaliana* INSERTION MUTANT LINES

As reported in this study (see Chapter 5), *Atmlo2* T-DNA insertion lines display resistance to the *A. thaliana* powdery mildew fungus, *Golovinomyces orontii*. Thus, *A. thaliana* T-DNA insertion mutants in any of the candidate interactor genes (Table 4.1) might be used as an alternative to transient gene silencing in barley. *A. thaliana* single insertion lines could be investigated for altered infection phenotypes against *G. orontii*. Moreover, double mutant lines, such as *Atmlo2/cyp1* and *Atmlo2/ppi1*, could be analysed for restored susceptibility against the powdery mildew fungus.

However, the effect of disruption of a single *A. thaliana* gene, encoding a putative MLO-interactor protein, might be covered by functional redundancy among family members. To bypass this problem, more than one family member gene should be mutated. Thus, *A. thaliana* double and triple insertion mutant lines should be generated. Alternatively, double strand RNA interference (dsRNAi) technology could be employed to silence simultaneously several sequence-related genes in stably transformed *A. thaliana* lines (Jacobs et al., 2003).

Table 4.1
Compilation of *AtCYP1*, *AtPPI1*, *AtCYT b5* and *AtSQS* T-DNA insertion lines.

Gene	Designation (location of insertion)	
AtCYP1 (At4g34870)	GABI_0578C07 [1]	(Exon)
AtPPI1 (At4g27500)	SAIL_0713_E06.b.1a[1]	(Promoter)
	SALK_009375	(5´UTR)
	SALK_058333	(Exon 6)
	SALK_040701	(Exon 6)
	SALK_058008	(Exon 6)
	SAIL_0361_F09.b.1a[1]	(Last exon)
	GABI_0383A11	(3´UTR)
	SALK_116049	(3´UTR)
AtCYT b5 (At5g48810)	SALK_045010	(3´UTR)
	GABI_0328H06	(Exon 2)
	GABI_0688G10	(5´UTR)
	SALK_012962	(5´UTR)
	GABI_0570D04	(5´UTR)
AtSQS (At4g34640)	SALK_077057	(5´UTR)
	SAIL_1284_H07	(Intron 1)
	SALK_034266	(Exon 6)
	SALK_087515	(Exon 6)
	SALK_034431	(Exon 6)
	SAIL_390_E02	(Intron 11)
	GABI_0152C02	(3´UTR)
	GABI_0399G06	(3´UTR)

[1] *A. thaliana* T-DNA insertion lines already crossed with *Atmlo2* insertion lines

5. AN *A. thaliana mlo2* INSERTION MUTANT LINE PHENOCOPIES THE BARLEY *mlo* POWDERY MILDEW RESISTANCE PHENOTYPE

5.1. INTRODUCTION

Recessive mutations in the barley *MLO* gene confer durable, broad-spectrum resistance to all isolates of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). The resistance due to *mlo* mutant alleles occurs at the penetration stage and is associated with deposition of a localised cell wall apposition (papilla) at the attempted penetration site. Barley *mlo* mutant plants display also some pleiotropic phenotypes, such as spontaneous deposition of callose, a major component of papillae, in pathogen-free growth conditions. Furthermore, uninoculated *mlo* mutant plants exhibit spontaneous mesophyll cell death that leads to premature leaf senescence (Wolter M, 1993; Peterhänsel et al., 1997; Piffanelli et al., 2002). Barley MLO homologues were identified in both monocotyledous and dicotyledonous plant species (Devoto et al., 1999; Kim et al., 2002b; Devoto et al., 2003). However, to date, barley MLO is the only family member with a defined function.

To better understand the role of MLO in defence/susceptibility and cell death, functional characterisation of other family member proteins will be instrumental.

5.2. GENERATION OF *AtMLO* INSERTION LINES

In the *A. thaliana* genome 15 MLO homologues were identified (Devoto et al., 1999; Kim et al., 2002b; Devoto et al., 2003). Among these, a group of three genes, *At*MLO2, *At*MLO6 and *At*MLO12, showing the highest sequence similarity to *Hv*MLO, was chosen for further characterisation.

Consistent with publicly available microarray data (http://affy.arabidopsis.info/narrays/experimentbrowse.pl; http://www.arabidopsis.org/tools/bulk/microarray/index.jsp), microarray experiments performed on *A. thaliana* plants inoculated with avirulent strains of *Pseudomonas syringae* (M. Bartsch and J. Parker, unpublished) indicate that the three *A. thaliana* genes considered are transcriptionally highly upregulated after pathogen treatment (data not shown). Taken together, the high sequence homology to barley MLO and the corresponding mRNA upregulation suggested that *At*MLO2, *At*MLO6 and *At*MLO12 might be involved in defence responses upon pathogen challenge.

A. thaliana T-DNA or transposon insertion lines in Col-0 background for each of the three genes were obtained from the SAIL (Syngenta Biotechnology) and SLAT (The Sainsbury Laboratory, Norwich, U.K.) populations respectively (Table 5.1). Insertions in these lines are located in exon 6 (*AtMLO2*), exon 13 (*AtMLO6*) and intron 7 (*AtMLO12*), and are predicted to represent null mutants. One homozygous insertion line for each gene was selected by PCR and further analysed. A schematic representation of the genomic structure of the *Atmlo* insertion lines used in this study is shown in Fig. 5.1. These lines are referred to as *Atmlo2* (Garlic_0878H12), *Atmlo6* (Garlic_0523D09) and *Atmlo12* (SLAT_24-21).

Due to the close phylogenetic relationship, a (partial) functional redundancy among the three family member proteins was expected. To test this hypothesis, double and triple insertion lines were generated. Homozygous double mutant insertion lines are referred to as *Atmlo2/mlo6* (♀*Atmlo6* crossed with ♂*Atmlo2*), *Atmlo2/mlo12* (♀*Atmlo12* crossed with ♂*Atmlo2*) and *Atmlo6/mlo12* (♀*Atmlo6* crossed with ♂*Atmlo12*). The homozygous triple mutant insertion line is referred to as *Atmlo2/mlo6/mlo12* (♀*Atml2/mlo12* crossed with ♂*Atmlo6/mlo12*).

Table 5.1
Compilation of *AtMLO2*, *AtMLO6* and *AtMLO12* insertion lines.

Allele	Designation (ecotype, location of insertion)	
*At*mlo*2*-5	Garlic_0878H12	(Col-0; exon 7)
*At*mlo*2*-6	SALK_050191	(Col-0; last exon, 14)
*At*mlo*2*-7	SALK_079850	(Col-0; exon 10)
*At*mlo*6*-1	CSH sp2	(Ws-0; intron 2)
*At*mlo*6*-2	Garlic_0523D09	(Col-0; last exon, 14)
*At*mlo*6*-4	Garlic_0506C09	(Col-0; exon 11)
*At*mlo*12*-1	SLAT 24-21	(Col-0; intron 7)
*At*mlo*12*-3	SALK_004420	(Col-0; intron 5)
*At*mlo*12*-4	Garlic_0573C10	(Col-0; intron 1)
*At*mlo*12*-5	Garlic_0050_C10	(Col-0; intron 7)

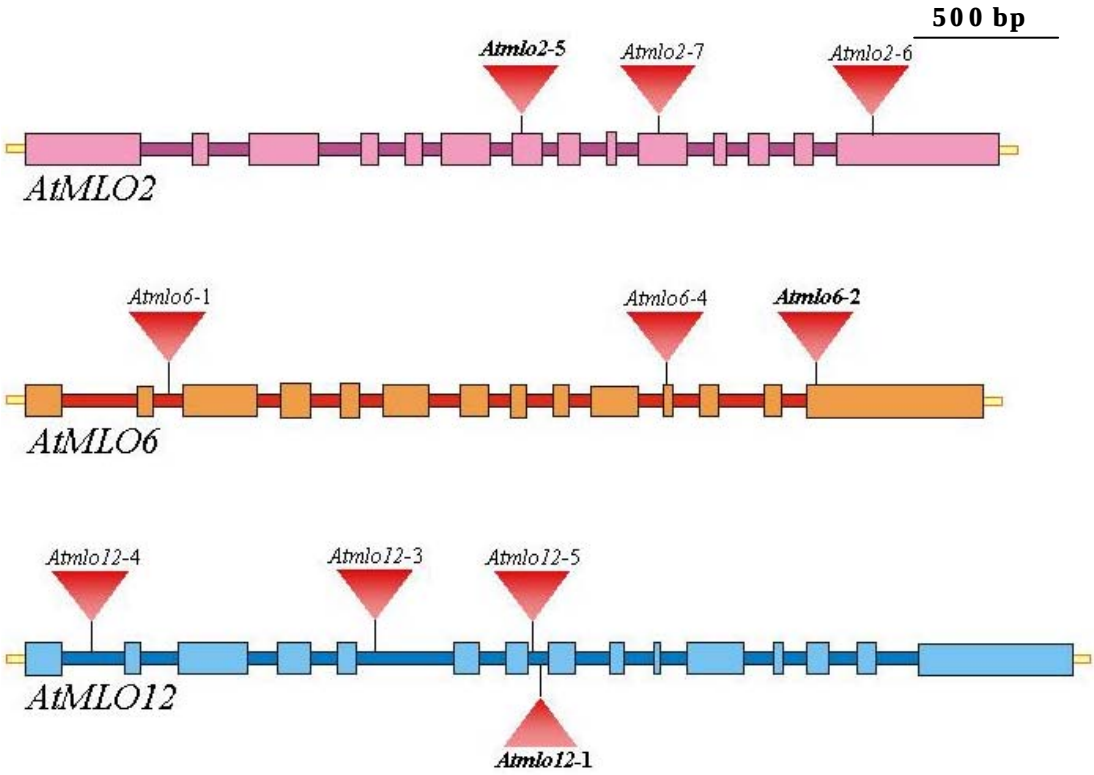

Fig. 5.1

Schematic representation of *A. thaliana mlo* insertion lines.

The figure shows a schematic representation of the *AtMLO2*, *AtMLO6* and *AtMLO12* genomic structure. Exons are represented by large rectangles (pink, orange and light blue for the three genes, respectively) and introns by small ones (dark pink, red and blue). Positions of the insertions are indicated by red triangles. Bold letters indicate the alleles that have been used in the present study.

AtMLO INSERTION LINES CHALLENGED WITH THE BACTERIAL PATHOGEN *Pseudomonas syringae*

The transcriptional upregulation of the *A. thaliana* genes *AtMLO2, AtMLO6* and *AtMLO12* after *P. syringae* challenge might indicate an involvement of the three genes in the defence pathway against this pathogen. *P. syringae* is a Gram-negative, plant pathogenic bacterium that infects plant leaves by entering through stomatal cells. In this study, both a virulent (*Pst* DC3000; Whalen et al., 1991) and a near-isogenic avirulent strain (*Pst* DC3000/*avrRpm1*; Bent, 1992) were employed. Inoculation of wild-type and insertion mutant lines (single, double and triple mutant lines) was performed by infiltration of bacterial suspensions into the abaxial leaf surface using a syringe without needle.

The macroscopic disease symptoms of *Pst* DC3000/*avrRpm1* inoculated plants were monitored during the subsequent 5 days. When inoculated with the avirulent strain, wild-type Col-0 leaves collapse within few hours and lesions appear as a dry collapsed zone surrounded by healthy tissue as a consequence of the hypersensitive response characteristic for the RPM1/avrRPM1 interaction. *Atmlo* mutant lines showed an infection phenotype comparable to the wild-type Col-0 plants.

After inoculation with a bacterial suspension of virulent *Pst* DC3000, wild-type Col-0 plants and all insertion lines exhibited a chlorotic leaf phenotype characteristic of a compatible interaction. To determine if similar macroscopic symptom development was reflected by comparable bacterial growth in *mlo* mutant and wild-type plants, bacterial growth tests were performed. Leaf discs were collected at 0, 1, 3 and 5 days post inoculation and bacterial growth was monitored by dilution plating of ground leaf disk tissue. The growth curve for any of the insertion lines was comparable to the Col-0 wild-type (data not shown). Collectively, this data indicates that insertions in *AtMLO2*, *AtMLO6* and *AtMLO12* do not significantly interfere with compatible or incompatible *A. thaliana*/*P. syringae* interactions.

5.3. *AtMLO* INSERTION LINES CHALLENGED WITH THE OOMYCETE PATHOGEN *Peronospora parasitica*

A. thaliana mlo mutant lines were also challenged with the obligate biotrophic oomycete *Peronospora parasitica*. This pathogen usually affects young seedling and leaves causing a destructive disease known as downy mildew. Both a virulent (Noco2; Parker et al., 1997) and an avirulent (Cala2; Holub et al., 1994) strain were used to inoculate two-week-old seedlings of wild-type Col-0 and *mlo* single, double and triple mutant lines.

At the macroscopic level, *A. thaliana* wild-type Col-0 plants inoculated with *P. parasitica* Noco2 showed yellowish, necrotic leaves and massive sporulation of the pathogen 5-7 days post inoculation. All mutant insertion lines displayed a phenotype comparable to the wild-type plants.

Col-0 plants inoculated with *P. parasitica* Cala2 developed an HR at the attempted penetration site, visible as whole cell fluorescence under UV light excitation. Neither macroscopic nor microscopic differences between the Col-0 control and any of the *mlo* mutant lines were observed upon Cala2 inoculation (data not shown).

These data suggest that the tested *mlo* mutants do neither affect compatible nor incompatible *A. thaliana*/*P. parasitica* interactions to a significant extent.

5.4. *AtMLO* INSERTION LINES CHALLENGED WITH POWDERY MILDEW FUNGI

Considering that mutations in the barley *MLO* gene confer resistance to the *Bgh* powdery mildew fungus, *A. thaliana MLO* insertion lines were inoculated with conidiospores of various powdery mildew pathogens. In particular, interactions with *Golovinomyces orontii*, virulent on *A. thaliana*, as well as with the inappropriate barley (*Bgh*), wheat (*Blumeria graminis* f. sp. *tritici*, *Bgt*) and pea (*Erysiphe pisi*) powdery mildew fungi were investigated.

5.4.1. *Atmlo2* plants are resistant to the host pathogen, *G. orontii*

A. thaliana wild-type Col-0 is a host for *G. orontii* (Plotnikova, 1998), which can successfully complete its life cycle on this ecotype. Massive sporulation of *G. orontii* is macroscopically visible on *A. thaliana* Col-0 plants at ~10 days after inoculation.

Rosette leaves of four-week-old *A. thaliana* wild-type Col-0 and *mlo* mutant plants were inoculated with *G. orontii* conidiospores. As expected, a clear susceptible phenotype of Col-0 was detected (Fig. 5.2). Susceptibility comparable to Col-0 was also observed for *MLO* single mutant lines *Atmlo6*, *Atmlo12* and the double mutant line *Atmlo6/mlo12*. In contrast, the *MLO* single insertion line *Atmlo2*, the double mutant lines *Atmlo2/mlo6*, *Atmlo2/mlo12*, and the triple mutant line *Atmlo2/mlo6/mlo12* were resistant to the pathogen (Fig. 5.3).

To exclude the possibility that the resistant phenotype to *G. orontii* was due to a second independent insertion in the genome of the tested *Atmlo2* insertion line, two further independent *Atmlo2* insertion lines were tested. Both homozygous mutant lines *Atmlo2*-6 (SALK_050191) and *Atmlo2*-7 (SALK_079850) were resistant to *G. orontii* (data not shown).

In addition, the plant-pathogen interaction was investigated in all *Atmlo* mutants at the microscopic level. In the *Atmlo2* single and double insertion lines, a decreased successful penetration was observed. In the triple mutant, the penetration attempts of mildew spores failed coincident with appressorium formation (data not shown). Consequently, the inability of powdery mildew spores to switch from surface to invasive growth resulted in

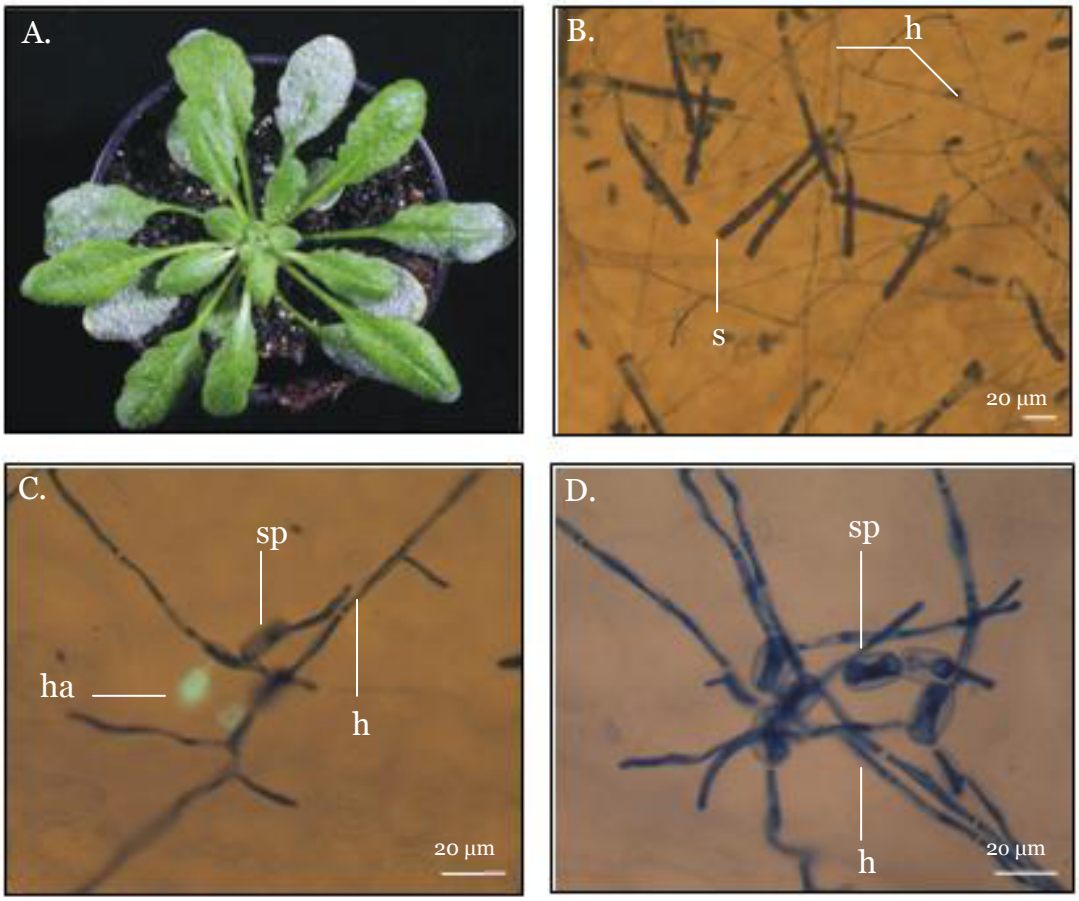

Fig. 5.2

***A. thaliana* wild-type Col-0 infected with *G. orontii*.**

A. 5-week-old *A. thaliana* MLO wild-type plant 10 days after inoculation with *G. orontii*. **B.** Secondary hyphal growth (h) and sporulation (s) of *G. orontii* on an *A. thaliana* leaf 10 days after inoculation. **C.** Callose-encapsulated spherical haustorium (ha) differentiated by the pathogen after successful penetration of an epidermal cell. **D.** *G. orontii* spores (sp) at higher magnification. Leaves were collected 10 days (B.) and 72 hours after inoculation (C. and D.) with the pathogen and stained with aniline blue for callose detection and Coomassie blue for highlighting epiphytic fungal structures.

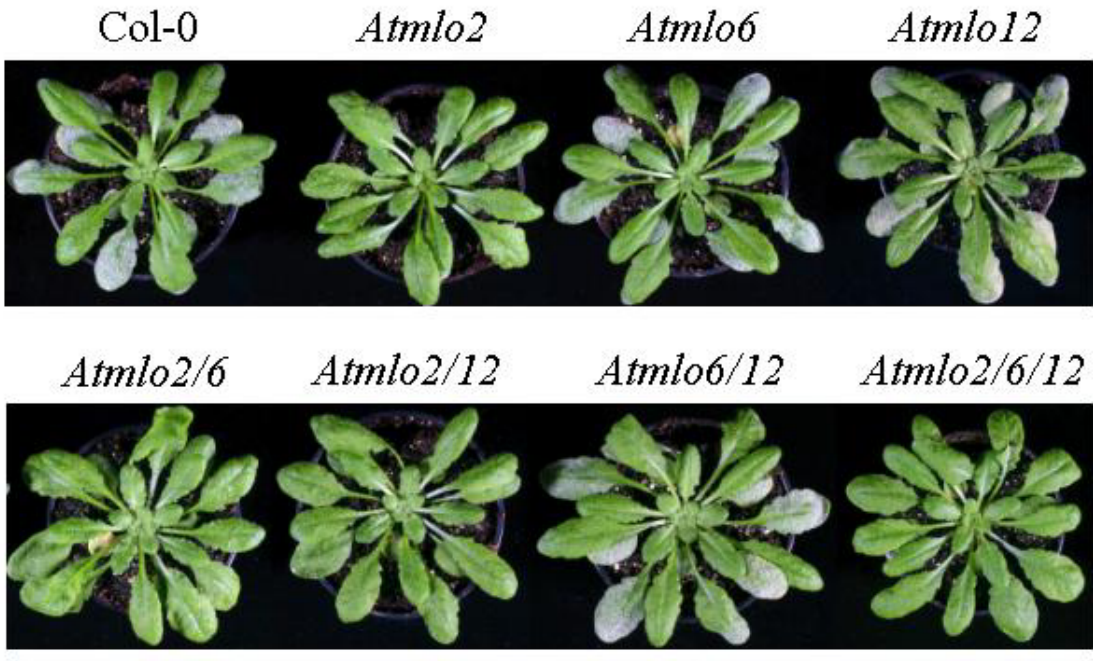

Fig. 5.3

Infection phenotypes of *A. thaliana MLO* insertion lines inoculated with *G. orontii*.

4-week-old *A. thaliana MLO* wild-type (Col-0) and *mlo* insertion lines (single mutant lines *Atmlo2*; *Atmlo6* and *Atmlo12*; double insertion lines *Atmlo2/mlo6*, *Atmlo2/mlo6* and *Atmlo6/mlo12*, indicated as *Atmlo2/6, Atmlo2/12* and *Atmlo6/12* respectively; triple insertion line *Atmlo2/mlo6/mlo12*, indicated as *Atmlo2/6/12*) were inoculated with *G. orontii*. The picture shows the infection phenotypes 10 days post inoculation.

absence of haustoria, hyphal growth and sporulation. Phenotype of the *mlo* triple insertion line is reminiscent of the penetration resistance in barley *mlo* mutants (Wolter M, 1993).

5.5.2. *Atmlo* mutants are more resistant to the inappropriate pathogens *Bgh* and *Bgt*

A. thaliana inappropriate pathogens are not able to complete their reproductive cycle on *A. thaliana* leaves. After landing on an *A. thaliana* leaf, *Bgh* or *Bgt* conidiospores form an appressorium and attempt to penetrate through the cell wall into the epidermal cell. In wild-type *A. thaliana*, about 70-80% of the conidia are stopped in association with the formation of a papilla. A proportion of 20-30% of the penetration attempts is successful and the spores can establish a haustorium and in rare cases initiate some secondary hyphal growth. Plant epidermal cells successfully penetrated can undergo a cell death reaction (reminiscent of the hypersensitive response) visible as whole cell autofluorescence (Fig. 5.4).

Rosette leaves of 4-week-old *A. thaliana* plants were inoculated with *Bgh* conidia. Leaf samples were collected 3 days post inoculation, stained with aniline blue and analysed under UV light excitation to evaluate the penetration rate. A sub-set of *Atmlo* insertion lines exhibited an altered successful penetration rate in comparison to wild-type Col-0. In particular, fungal spores showed a lower penetration success on the *Atmlo2* single mutant line as well as on the *Atmlo2/mlo6* and *Atmlo2/mlo12* double mutant lines compared to wild-type plants. Moreover, successful fungal penetration was essentially undetectable on the *Atmlo2/mlo6/mlo12* triple mutant line (Table SD.11; Fig. 5.5).

Comparable results were obtained with *Bgt* inoculated plants (data not shown).

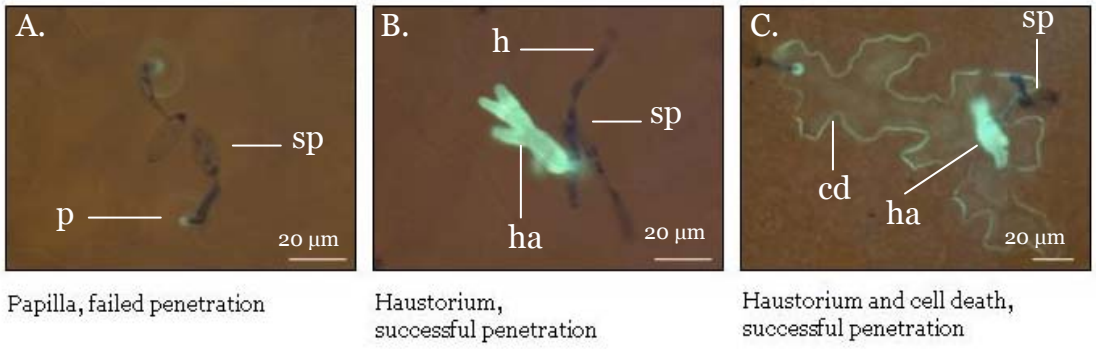

Fig. 5.4

Interaction sites on *A. thaliana* Col-0 after inoculation with barley powdery mildew (*Bgh*) conidiospores.

A. Papilla (p) formation coincident with failed penetration of a *Bgh* sporeling (sp) on a wild-type *MLO A. thaliana* epidermal cell. **B.** Callose-enclosed multidigitate haustorium (ha) formed by *Bgh* in a wild-type *MLO A. thaliana* epidermal cell after successful cell wall penetration. h, secondary hyphal growth. **C.** Callose-enclosed multidigitate haustorium formed by *Bgh* after successful cell wall penetration and subsequent whole cell callose deposition (indicative of cell death, cd). Leaves were collected 3 days after inoculation with *Bgh* and stained with aniline blue for callose detection and Coomassie blue for highlighting epiphytic fungal structures.

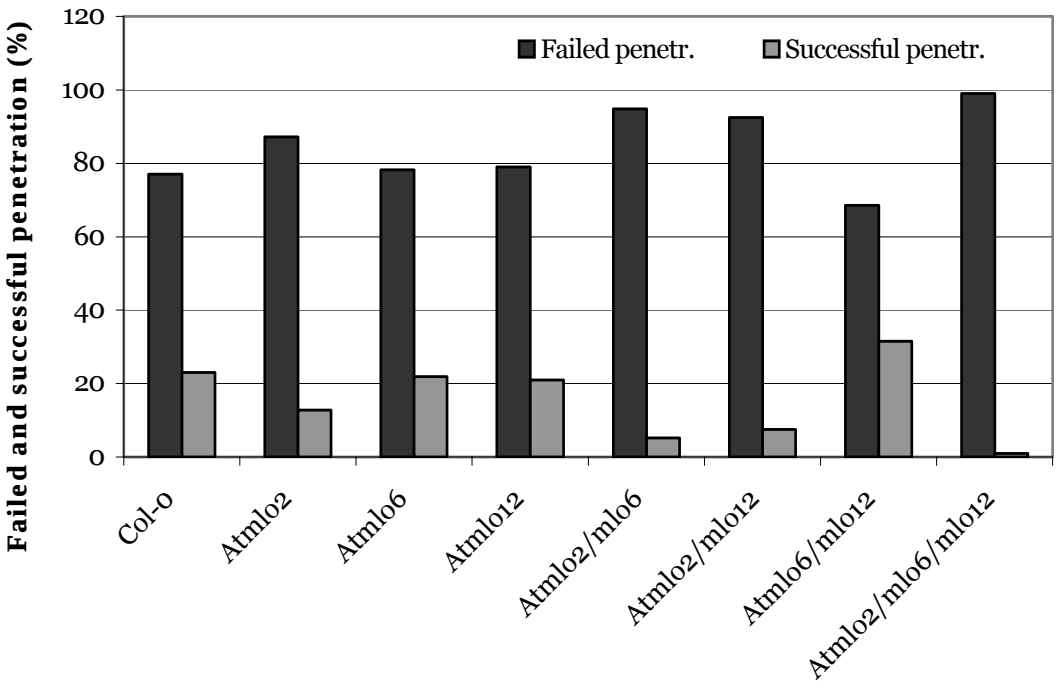

Fig 5.5

Quantitative analysis of *A. thaliana* plants inoculated with *Bgh*.

Rosette leaves of 4-week-old *A. thaliana* wild-type Col-0 and *mlo* insertion lines were inoculated with *Bgh* conidiospores. Samples were collected 3 days post inoculation. The graphic represents the percentage of successful penetration events. The data reported in the graphic represent the average of the two independent experiments shown in Table SD.11.

5.5.3. *Atmlo2* is more resistant to the non-host pathogen *E.pisi*

In contrast to the grass powdery mildew fungi *Bgh* or *Bgt*, *E. pisi* can regularly initiate some secondary hyphal growth also on the non-host plant *A. thaliana*, although it cannot complete its asexual life cycle and differentiate sporangiophores. As the haustorium established by *E. pisi* is small and difficult to visualise (Fig. 5.6) evaluation of secondary hyphal growth on *A.*
thaliana leaves inoculated with *E. pisi* was taken as a measure of successful host cell penetration. Additionally, percentage of cell death, displayed as whole cell autofluorescence upon aniline blue staining, was assessed.

Rosette leaves of 4-week-old *A. thaliana* plants were inoculated with *E. pisi* conidia. Leaf samples were collected 7 days post inoculation, stained with aniline blue and analysed under UV light excitation. Differences in successful penetration by *E. pisi*, indicated by presence of secondary hyphal growth, were detected between Col-0 and *Atmlo* mutant plants. Consistent with the experiments performed with *Bgh* and *Bgt*, a lower rate of secondary hyphal growth was observed in the *Atmlo2/mlo6* and *Atmlo2/mlo12* double mutant lines in comparison to Col-0, whereas a minor effect was observed in the *Atmlo2* mutant line. The same mutant lines exhibited also a lower proportion of cell death compared to wild-type plants. Moreover, neither secondary hyphal growth nor any cell death was detected in the *Atmlo2/mlo6/mlo12* triple mutant line (Table SD.12; Table SD.13; Fig. 5.7: Fig. 5.8).

5.6. *Atmlo2* PLANTS SHOW SIMILAR DEVELOPMENTALLY CONTROLLED PLEIOTROPIC PHENOTYPES AS BARLEY *mlo* MUTANTS

A. thaliana mlo insertion lines were investigated for pleiotropic phenotypes that characterise barley *mlo* mutant plants. *A. thaliana* wild-type Col-0 and *mlo* insertion lines were grown in powdery mildew-free conditions. Leaf samples were collected at different time points (6-8 weeks after sowing)

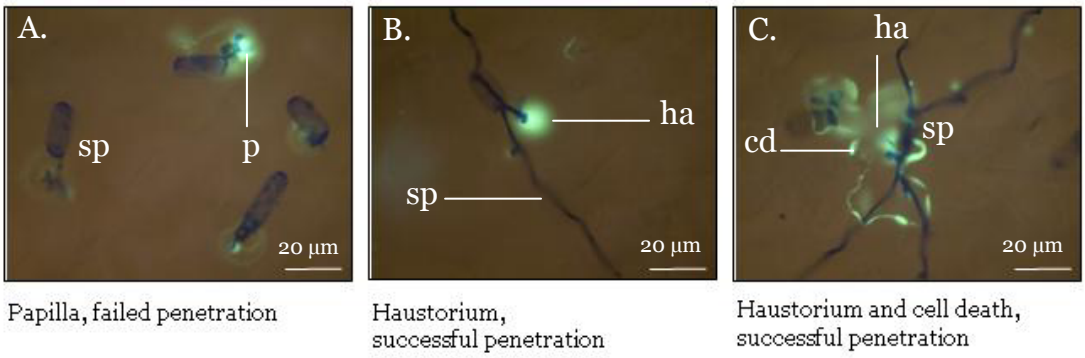

Fig. 5.6

Interaction sites on *A. thaliana* Col-0 after inoculation with pea powdery mildew (*E. pisi*) conidiospores.

A. Papilla (p) formation coincident with failed penetration of a spore (sp) of *E. pisi* on a wild-type *MLO A. thaliana* epidermal cell. **B.** Spherical haustorium (ha) formed by *E. pisi* in a wild-type *MLO A. thaliana* after successful cell wall penetration. h, secodary hyphal growth. **C.** Whole cell callose deposition (indicative of cell death, cd) of an *A. thaliana* epidermal cell after successful penetration by an *E. pisi* sporeling. Leaves were collected 7 days after inoculation with *E. pisi* and stained with aniline blue for callose detection and Coomassie blue for highlighting epiphytic fungal structures.

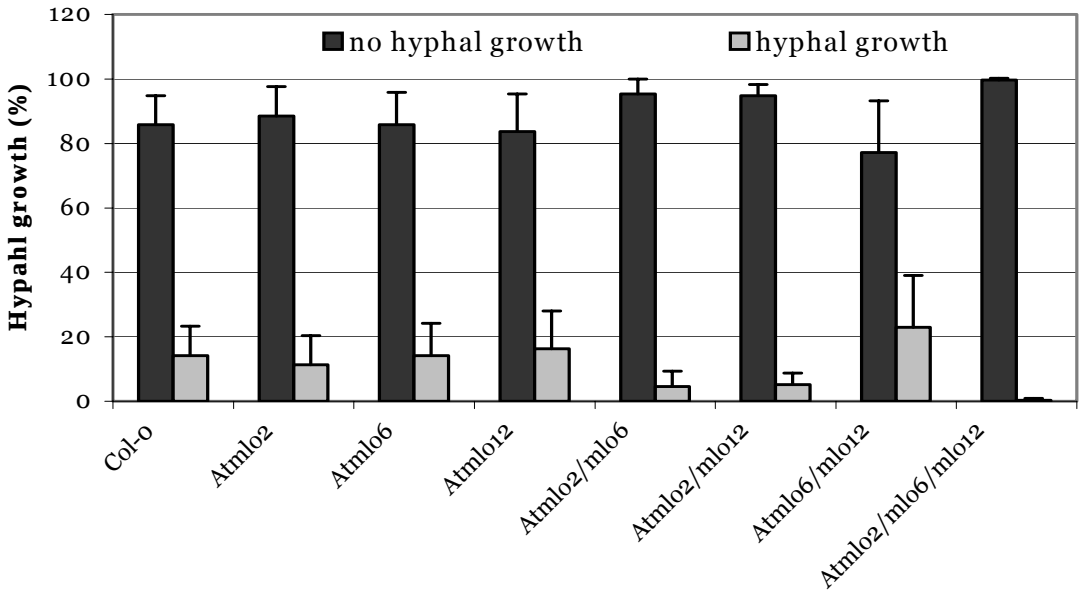

Fig. 5.7

Quantitative analysis of hyphal growth *A. thaliana mlo* mutant plants inoculated with *E. pisi*.

Rosette leaves of 4-week-old *A. thaliana* wild-type Col-0 and *mlo* insertion lines were inoculated with *E. pisi*. Samples were collected 7 days post inoculation. The graphic represents the percentage of fungal secondary hyphal growth subsequently to a successful penetration attempt. The data reported in the graphic represent the average and the standard deviation of the three independent experiments shown in Table SD.12.

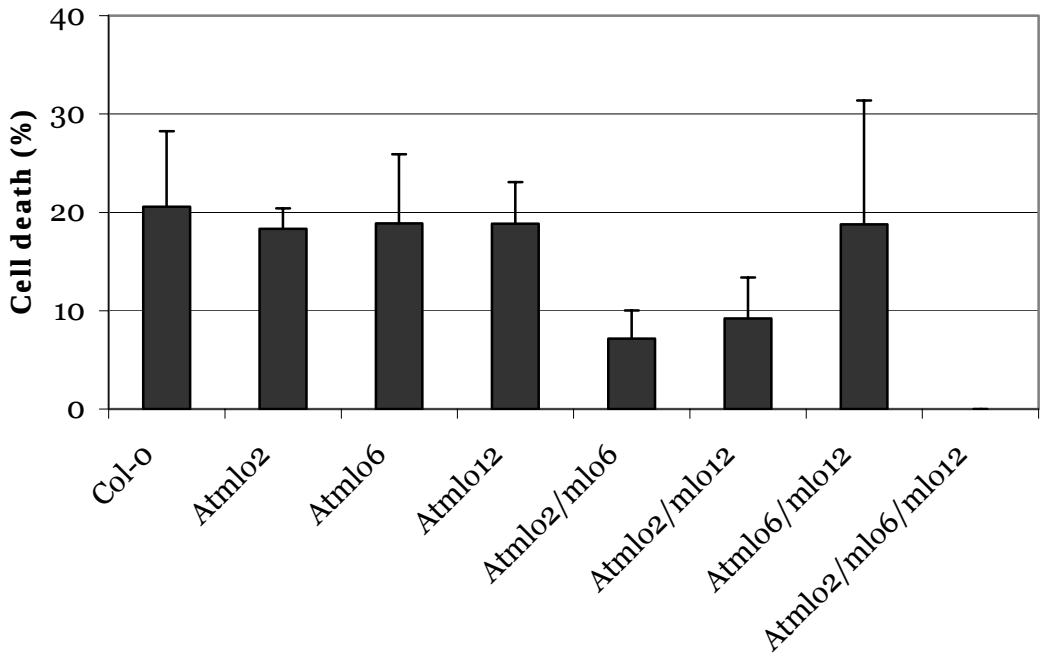

Fig. 5.8

Quantitative analysis of cell death in *A. thaliana mlo* mutant plants inoculated with *E. pisi* conidiospores.

Rosette leaves of 4-week-old *A. thaliana* wild-type Col-0 and *mlo* insertion lines were inoculated with *E. pisi*. Samples were collected 7 days post inoculation. The graphic represents the percentage of whole cell callose deposition (indicative of cell death) subsequent to a successful penetration attempt. The percentage of cell death is irrespective of the average hyphal growth reported in Fig 5.7. The data reported in the graphic represent the average and the standard deviation of the three independent experiments shown in Table SD.12. Asterisk indicates $p<0.05$ (Student´s t-test) compared to wild-type Col-0.

and stained with either aniline blue to visualise spontaneous callose deposition or trypan blue to highlight spontaneous cell death.

5.6.1. *Atmlo2* mutants exhibit spontaneous callose deposition

Wild-type Col-0 plants showed hardly any spontaneous callose deposition in the cells of rosette leaves neither at 6- nor at 7-weeks after sowing. A similar phenotype was observed in the *Atmlo6* and *Atmlo12* mutant lines. In contrast, 6-week-old *Atmlo2* mutant plants displayed some spontaneous callose deposition that became massive in 7-week-old leaf samples. Comparable results were obtained in the *Atmlo2/mlo6* and *Atml2/mlo12* double mutant lines and in the *Atmlo2/mlo6/mlo12* triple mutant line. The *Atmlo6/mlo12* double mutant line developed a moderate callose deposition only 7 weeks after sowing (Fig. 5.9). Similar results were observed in two independent repetitions of this experiment.

5.6.2. *Atmlo2* mutants display spontaneous mesophyll cell death

Wild-type Col-0 plants showed no cell death at any time point examined (6, 7 and 8 weeks after sowing). Exiguous cell death was observed in the *Atmlo6* and *Atmlo12* single mutant lines and in the *Atmlo6/mlo12* double mutant line at a late time point (8 week after sowing). Moreover, *Atmlo2* and the *Atmlo2/mlo6* and *Atmlo2/mlo12* double mutant lines exhibited some cell death already at 7 weeks after sowing which increased in 8-week-old samples. Finally, the *Atmlo2/mlo6/mlo12* triple mutant showed a slightly reduced cell death in comparison to the *Atmlo2* mutant lines (Fig. 5.10). In all cases, cell death was restricted to mesophyll tissue and was not observed in the epidermal cell layer. Similar results were detected in an independent repetition of this experiment.

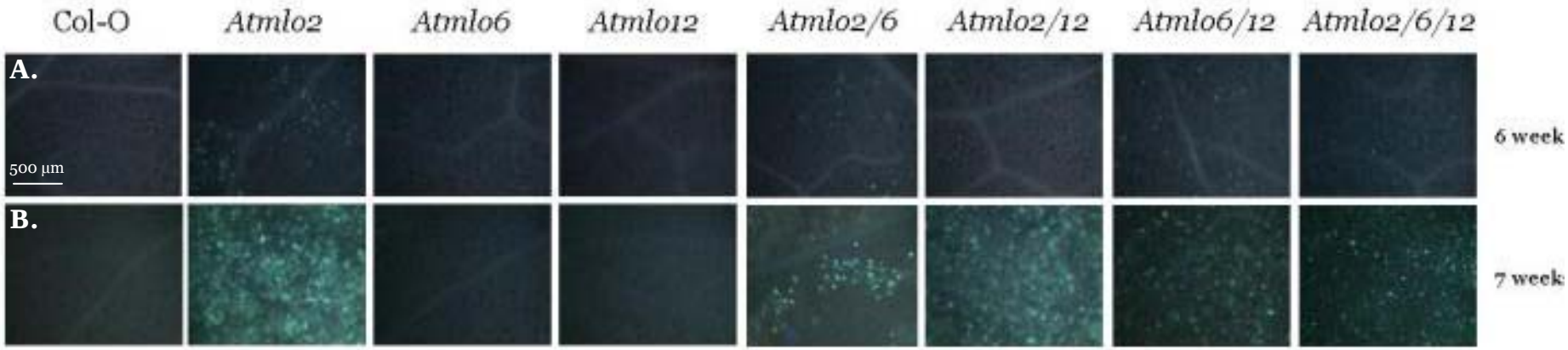

Fig. 5.9

Histochemical analysis of spontaneous callose deposition in *A. thaliana mlo* mutant leaves.

Panel A. and panel B. show spontaneous callose deposition in the epidermal cells of 6- and 7-week-old *A. thaliana* plants, respectively. One leaf of each genotype indicated was collected at the indicated time point from plants grown under powdery mildew-free conditions. Leaves were stained with aniline blue and analysed under UV fluorescence for callose detection. The experiment was repeated twice with similar results.

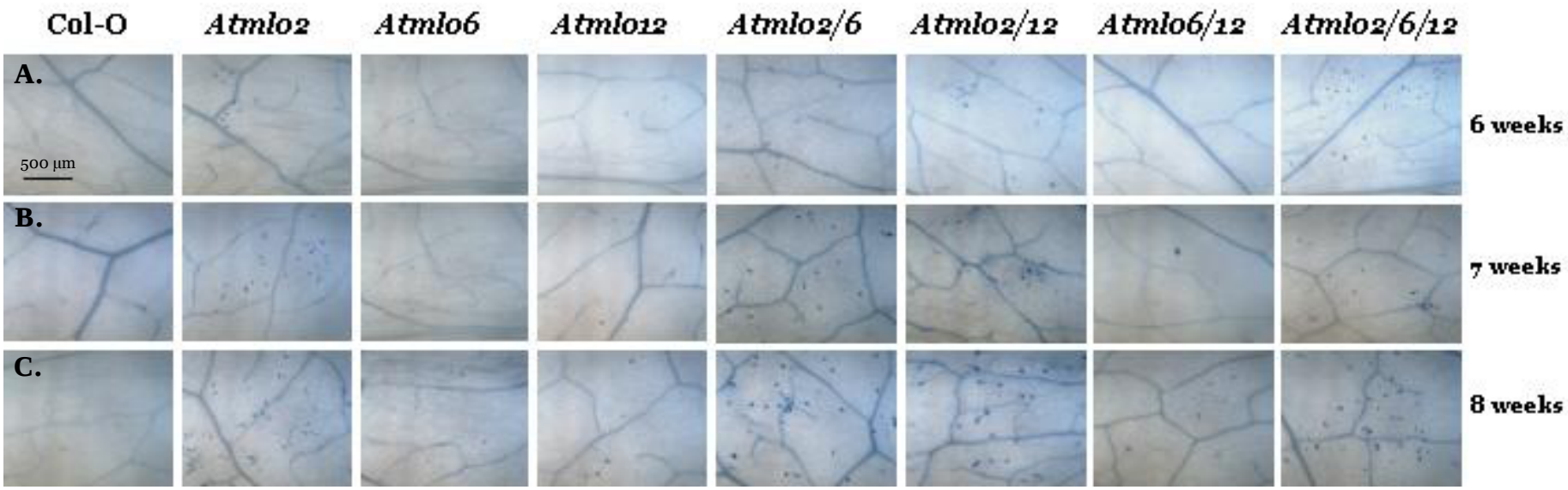

Fig. 5.10

Histochemical analysis of spontaneous mesophyll cell death in *A. thaliana mlo* mutant leaves.

Panel A., B. and C. show spontaneous cell death in the mesophyll layer of 6-, 7- and 8-week-old *A. thaliana* plants, respectively. One leaf of each genotype was collected at the indicated time point from plants grown under powdery mildew-free conditions. Leaves were stained with trypan blue and analysed by brightfield microscopy for cell death. The experiment was repeated once with similar results.

6. DISCUSSION

6.1. *At*MLO2 IS THE FUNCTIONAL HOMOLOGUE OF BARLEY MLO

Until now, barley MLO was the only member of the MLO protein family with an identified function (Büschges et al., 1997). Recessive *mlo* mutant alleles confer resistance that is effective against all tested isolates of *Bgh*. Interestingly, a wheat ortholog (TaMLO-B1) of barley MLO has been shown to complement the barley *mlo* mutant (Elliott et al., 2002). Despite this and although MLO homologues were found in all analysed plant species, it was unclear whether *mlo* resistance represents a phenomenon restricted to monocot plants. In this study, *AtMLO2*, one of the three highly sequence-related *A. thaliana* homologues of *HvMLO*, was found to be required for susceptibility against the powdery mildew fungus, *G. orontii*. Mutants in the two other close *A. thaliana* homologues of *HvMLO* (*AtMLO6* and *AtMLO12*) showed unaltered powdery mildew susceptibility.

The main characteristic of barley *mlo* resistance is the failure of the fungal infection process during penetration through the epidermal cell wall (Jørgensen, 1992; Büschges et al., 1997). In addition, *mlo* mutant plants grown under pathogen-free conditions exhibit developmentally controlled spontaneous callose deposition and early leaf senescence associated with cell death of mesophyll but not epidermal cells (Wolter, 1993; Peterhänsel et al., 1997). Recent data indicate that the barley *MLO* wild-type gene is upregulated upon biotic or abiotic stress stimuli (Piffanelli et al., 2002). The amount of wild-type *MLO* transcript in the leaf increased shortly after barley powdery mildew inoculation and after challenge with either the inappropriate wheat powdery mildew fungus (*Bgt*) or the hemibiotrophic fungus *M. grisea*. In addition, leaf wounding and treatment with the herbicide paraquat led to upregulation of *MLO* expression (Piffanelli et al., 2002).

A. thaliana mlo2, *mlo6* and *mlo12* single, double and triple mutant plants inoculated with *G. orontii* were microscopically analysed for fungal hyphal growth, an indirect indicator of penetration success. Preliminary data indicate that penetration is reduced, but not completely abolished in the *Atmlo2* single mutant line (see Chapter 5.5.1). Furthermore, the *Atmlo2-mlo6* double mutant line exhibits a more severe reduction of hyphal growth that is entirely abolished in the *Atmlo2-mlo6-mlo12* triple mutant (data not shown). Like barley *mlo* mutant plants, also *Atmlo2* single, double and triple mutant lines display pleiotropic phenotypes when grown under pathogen-free conditions. Rosette leaves of 6-week-old plants exhibit spontaneous callose deposition, which is extensived in older plants (7-week-old), indicating that this process is under developmental control (see Chapter 5.6.1). The mesophyll cell death leading to early leaf senescence in barley *mlo* plants appears to be less severe in *A. thaliana mlo* mutants (see Chapter 5.6.2).

Accumulating evidence suggests that leaf senescence and defence response share common components. Several senescence-associated genes (*SAG*) were reported to accumulate in *A. thaliana* leaves in response to challenge with pathogens. In particular, mRNA levels of *AtSAG25* were shown to increase after plant inoculation with a virulent strain of *P. syringae* (Kiedrowski et al., 1992; Quirino et al., 1999). In addition, *AtSAG101* was found to play an important role in leaf senescence (He and Gan, 2002) and to be involved in defence responses to both virulent and avirulent strains of *P. parasitica* (M. Wiermer and J. Parker, unpublished). Analysis of microarray expression data revealed that the three closest *A. thaliana* homologues of *HvMLO* (*AtMLO*2, *AtMLO*6 and *AtMLO*12) are upregulated upon different abiotic stress stimuli such as heat and cold treatments, drought or salt stresses and upon challenge with oomycete pathogens (*Phytophthora infestans*) or general elicitors like lipopolysaccharide (LPS) and a peptide of flagellin (flg22; NASCArray database). Moreover, an increase in mRNA levels of the three genes was observed upon inoculation with avirulent strains of *P. syringae* (M. Bartsch and J. Parker, unpublished results). These data indicate that *AtMLO*2, *AtMLO*6 and *AtMLO*12 are responsive to various abiotic and biotic stress stimuli like *HvMLO*.

Taken together, *AtMLO2* and *HvMLO* exhibit a comparable gene expression pattern upon biotic and abiotic stress stimuli, a similar resistance phenotype and analogous developmentally controlled pleiotropic phenotypes, indicating that *AtMLO2* is the functional homologue (ortholog) of *HvMLO*. However, the data discussed above, together with the results of *mlo2*-mediated non-host resistance (see Chapter 6.2), suggest that *Atmlo*2 might be the major component of resistance against powdery mildew in *A. thaliana*, but not the only one. Additional mutations in both *AtMLO*6 and *AtMLO12* genes contribute to a certain degree to confer full resistance against powdery mildew fungi indicating that functions of the three genes are partially redundant.

It is interesting to note that barley *mlo* mutations not only confer resistance against *Bgh*, but also enhance susceptibility to the hemibiotrophic fungus *M. grisea* and to the necrotrophic fungus *B. sorokiniana* (Jarosch et al., 1999; Kumar et al., 2001). These results indicate that absence of *MLO* affects pathogenesis of some parasites in opposite directions. Considering the similarities between barley *mlo* mutants and *A. thaliana mlo* mutants, it would be interesting to investigate *A. thaliana MLO* single, double and triple mutants for enhanced susceptibility against various pathogens. Currently, *Atmlo* mutants are analysed for their susceptibility against necrotrophic (*Plectospherella* and *Alternaria alternata*) and hemibiotrophic (*Colletotrichum higginsianum*, *C. destructivum*; *M. grisea*; *Phytophtora infestans*) fungal pathogens.

In conclusion, *mlo*-mediated resistance appears to be conserved among monocot and dicot plant species. A single *MLO* gene is involved in susceptibility/resistance against powdery mildew in both barley and A. *thaliana*. However, microscopic analysis of *A. thaliana mlo* double and triple insertion lines inoculated with *G. orontii* revealed full penetration resistance only in the *Atmlo2/mlo6/mlo12* triple mutant. This data may indicate a low level of redundancy of the three *A. thaliana MLO* genes. Furthermore, these results suggest that powdery mildew fungi might use the same pathogenic mechanisms to

infect/penetrate both monocots and dicots, implicating that *mlo* resistance could be likely engineered in any higher plant species, such as tomato, wine and wheat.

6.1.1. Pathogen-related genes conserved in monocots and dicots

Similar to *MLO* and to *HvROR2/AtPEN1* (discussed Chapter 6.2.1), other genes with a defence-related role are functionally conserved between monocots and dicots. Like *MLO*, *RAR1* was first isolated in barley as a gene required for resistance signalling triggered by multiple *R* genes, including *HvMLA6* and *HvMLA12* (Freialdenhoven et al., 1994). The *A. thaliana* homologue, *AtRAR1*, was subsequently shown to be required by *R* gene-mediated resistance against avirulent strains of both *P. syringae* and *P. parasitica* (Azevedo et al., 2002; Muskett et al., 2002). Moreover, *At*RAR1 was shown to interact with the *Sgt1* yeast homologues *At*SGT1a and *At*SGT1b in *A. thaliana* (Azevedo et al., 2002). In addition, also barley RAR1 can interact with *At*SGT1a and *At*SGT1b, indicating that interaction between RAR1 and SGT1 is conserved in both monocots and dicots and even functions across species. Recently, *Hv*SGT1 was demonstrated to be required for *R* gene-mediated resistance in barley (Azevedo et al., 2002). In contrast, homologues of the *A. thaliana RPW8* genes, comprising a new class of *R* genes, were only found in members within the *Brassicaceae* plant family (Xiao et al., 2004).

6.2. *At*MLO2 IS ALSO INVOLVED IN NON-HOST RESISTANCE

6.2.1. Non-host resistance

Non-host resistance is defined as immunity of an entire plant species to all genetic variants of a specific pathogen and is the most common form of disease resistance exhibited by plants (Heath, 2000).

Non-host resistance appears to be formed by several overlapping mechanisms including preformed barriers and induced defence responses (reviewed in Heath, 2000; Thordal-Christensen, 2003; Jones and Takemoto, 2004). Individual genes contributing to non-host resistance have been identified by mutational analysis in

A. thaliana. For example, *AtNHO1* encodes a glycerol kinase and is required for resistance against inappropriate isolates of *Botrytis cinerea* and *P. syringae* pathogens (Lu et al., 2001; Kang et al., 2003; reviewed in Mysore and Ryu, 2004). In most cases, non-host resistance against fungal pathogens is associated with the host cell penetration process. *A. thaliana* penetration (*pen*) mutants were identified by screening for mutants that showed increased penetration of the inappropriate fungal pathogen *Bgh* (Collins et al., 2003). Mutations in *PEN1* reduce the ability of the plant to arrest penetration attempts of *Bgh* conidia in comparison to the wild-type. *PEN1* encodes a syntaxin protein that appears to play a key role in papilla-related vesicle trafficking at the plasma membrane (Collins et al., 2003). Syntaxins are members of the SNARE family of proteins that mediate membrane-fusion events. An independent screen for genes required for *mlo* resistance in barley identified two mutants (*ror1* and *ror2*) with enhanced penetration of *Bgh* in *mlo* mutants (Freialdenhoven et al., 1996). Interestingly, *ROR2* is the functional homologue of *AtPEN1* gene (Collins et al., 2003). These results provide a mechanistic link between non-host and basal penetration resistance (Collins et al., 2003; Schulze-Lefert, 2004).

6.2.2. *mlo* mutants are fully resistant to inappropriate powdery mildew fungi

Barley *mlo* mutant plants display full penetration resistance against *Bgh* and the inappropriate *Bgt* fungus (Peterhänsel et al., 1997). Moreover, overexpression of barley *MLO* in the wild-type *MLO* background revealed not only higher susceptibility against *Bgh*, but also higher successful penetration of the inappropriate *Bgt* fungus (Elliott et al., 2002). These results suggest that MLO plays a role not only in basal defence, but also in non-host resistance.

Atmlo2 mutant plants were found to be also slightly more resistant to inappropriate powdery mildew pathogens like *E. pisi*, *Bgh* and *Bgt*, while *Atmlo6* and *Atmlo12* showed penetration rates comparable to the wild-type. Moreover, the *Atmlo2/mlo6/mlo12* triple insertion line is fully resistant to the inappropriate powdery mildew pathogens, indicating a low level of functional redundancy of

MLO2, MLO6 and MLO12 proteins (see Chapters 5.5.2 and 5.5.3). This result suggests that penetration resistance mediated by *mlo* genes and non-host resistance might share some signalling components of the disease resistance pathway.

Analysis of disease symptoms of *Atmlo2/pen1* double mutants will reveal whether PEN1 is required in *mlo*-mediated resistance against powdery mildew in *A. thaliana*, like ROR2 is required in *mlo*-mediated resistance in barley.

6.3. *pmr2* IS ALLELIC TO *mlo2*

In the last years, six loci required for susceptibility to powdery mildew have been identified in mutagenic screens of *A. thaliana* (*pmr1-pmr6*; Vogel and Somerville, 2000). Interestingly, the recessive mutant *pmr2* was previously mapped to chromosome 1 in a region where also *AtMLO2* is located (Vogel and Somerville, 2000). Direct DNA sequencing of five *pmr2* alleles revealed point mutations in the *AtMLO2* coding sequence that result in either single amino acid substitutions or in one case in a mutation in an intron splice site (M. Humpry and S. Somerville, personal communication). Thus, it is likely that *AtPMR2* is *AtMLO2*.

In contrast to *cpr* mutants that constitutively express marker genes of both the SA- and JA/ethylene-dependent pathways (Clarke et al., 2000); see below), these genes were not found to be constitutively expressed in *Atmlo2/pmr2* plants (Vogel and Somerville, 2000). These results suggest that MLO2 might act in a defence pathway independent of SA and JA/ethylene. Investigation of crosses of *Atmlo2* with known *A. thaliana* disease resistance signalling mutants will allow a comprehensive genetic analysis of the signalling requirements of *mlo*-mediated resistance.

The signal transduction mechanisms controlling defence activation in *A. thaliana* are formed by interacting pathways that are dependent on the signalling molecules salicylic acid (SA) or jasmonic acid (JA) and ethylene (reviewed in Pieterse and van Loon, 1999; Glazebrook, 2001; Shah, 2003; Pieterse and Van Loon, 2004).

SA levels increase in *A. thaliana* at infection sites during compatible and incompatible interactions (Ryals et al., 1996). Evidence for the key role of SA in

pathogen defence came from the analysis of *A. thaliana* plants expressing the bacterial salicylate hydroxylase gene (*NahG*), which inactivates SA by converting it to cathecol (Gaffney et al., 1993; reviewed in Shah, 2003). Several genes play an important role in the SA-dependent pathway. For example, mutations in *AtEDS1* and *AtPAD4* genes strongly reduce SA accumulation at the infection site suggesting that both genes act upstream of SA (Zhou et al., 1998; Feys et al., 2001). A key element in controlling SA-mediated gene expression changes is NPR1 that appears to operate downstream of SA accumulation (Dempsey et al., 1999; Pieterse and Van Loon, 2004). Furthermore, translocation of NPR1 in the nucleus and subsequent interaction with the transcription factor TGA is required for expression of the pathogen-related gene *PR1*.

Recent studies revealed SA-independent disease resistance mechanisms in *A. thaliana* mediated by JA and ethylene (reviewed in Dong, 1998; Pieterse and van Loon, 1999; Pieterse and Van Loon, 2004). Some defence responses, such as expression of thionin (THI2.1; Epple et al., 1995) and defensin (PDF1.2; Penninckx et al., 1996), are controlled by the JA/Ethylene-dependent pathway (Pieterse and van Loon, 1999; Glazebrook, 2001; Pieterse and Van Loon, 2004). *PDF1*.2 gene expression is blocked in the ET-insensitive mutant *ein2* and the JA-insensitive mutant *coi1* (Penninckx et al., 1996) demonstrating that the signalling pathway involved in *PDF1*.2 induction requires components of the ET and JA response (Pieterse and van Loon, 1999; Glazebrook, 2001; Pieterse and Van Loon, 2004).

To unravel whether *mlo2*-mediated resistance requires components of the JA/ethylene- or the SA-dependent pathway double mutant lines *Atmlo2*/*ein2* and *Atmlo2*/*jar1*; *Atmlo2*/*npr1*, *Atmlo2*/*pad4*, *Atmlo2*/*eds1* or *Atmlo2*/NahG will be analysed for disease symptoms upon powdery mildew inoculation. Analyses to identify homozygous double mutant lines are currently in progress.

7. INTERACTION OF MLO PROTEINS WITH SYNTAXINS

7.1. INTRODUCTION

To investigate the molecular basis of *mlo*-mediated resistance in barley, a mutational approach in *mlo* mutant plants was performed. In the genetic screen, two genes required for full *mlo* resistance against *Bgh* were identified, *ROR1* and *ROR2* (Freialdenhoven et al., 1996). *HvROR2* encodes a plasma membrane-resident syntaxin (t-SNARE; Collins et al., 2003). Syntaxins are conserved in eukaryotes and are known to play a central role in vesicle trafficking in yeast and animal cells (Bonifacino and Glick, 2004).

In *A. thaliana*, a screen for mutants that exhibit increased penetration by the inappropriate *Bgh* fungus led to the identification of the *pen1* mutant (Collins et al., 2003). Intriguingly, *AtPEN1* encodes for one of the 24 syntaxins of *A. thaliana*, *At*SYP121. Furthermore, *At*PEN1 is the closest homologue of *Hv*ROR2 in *A. thaliana* (Fig. 7.1) and can complement the enhanced penetration phenotype of *ror2* mutant plants (i.e. it can restore full resistance in *mlo ror2* genotypes; Collins et al., 2003). This data indicates that the dicot PEN1 and the monocot ROR2 syntaxin proteins are functional homologues. Furthermore, the results suggest a molecular link between non-host and basal penetration resistance. It is conceivable that exocytosis is the common theme between the two kinds of resistance.

Barley MLO fused to the yellow fluorescent reporter protein (YFP) resides in the plasma membrane and, upon pathogen challenge, accumulates at attempted fungal penetration sites. A similar focal accumulation pattern upon pathogen treatment was also found for barley ROR2 fused to YFP (Fig. 7.2; R. Bhat et al. in press; Schulze-Lefert, 2004).

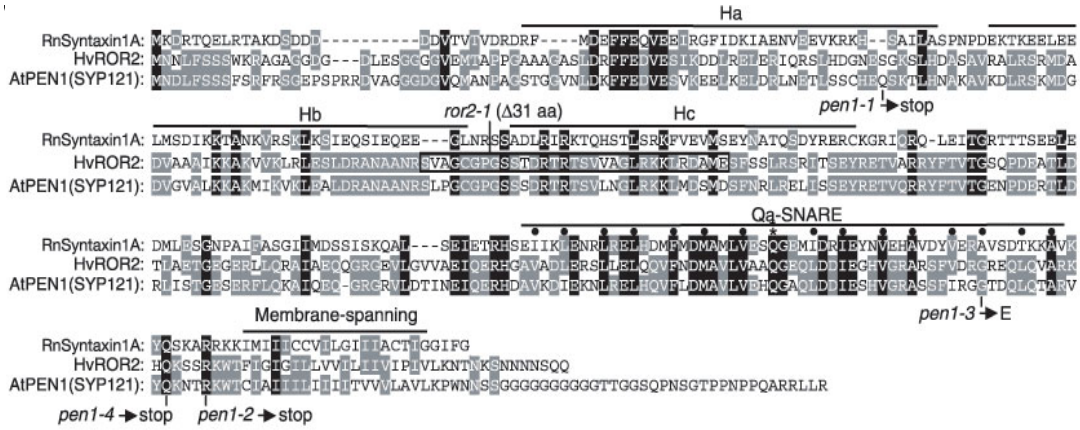

Fig. 7.1

Amino acid sequence alignment of *Hv*ROR2 and *At*PEN1 (SYP121).

The homologous syntaxins from rat (first line), barley (ROR2, second line) and from *A. thaliana* (PEN1/SYP121, third line) are aligned. Identical residues are boxed in black, conserved residues boxed in grey and gaps are indicated by hyphens. Black lines show location of the transmembrane domain, the Qa-type SNARE domain and the Ha, Hb and Hc helices. Mutational events identified in the *A. thaliana* syntaxin PEN1 and barley ROR2 (Collins et al., 2003) are indicated in the red rectangles. (Figure from Collins et al., 2003).

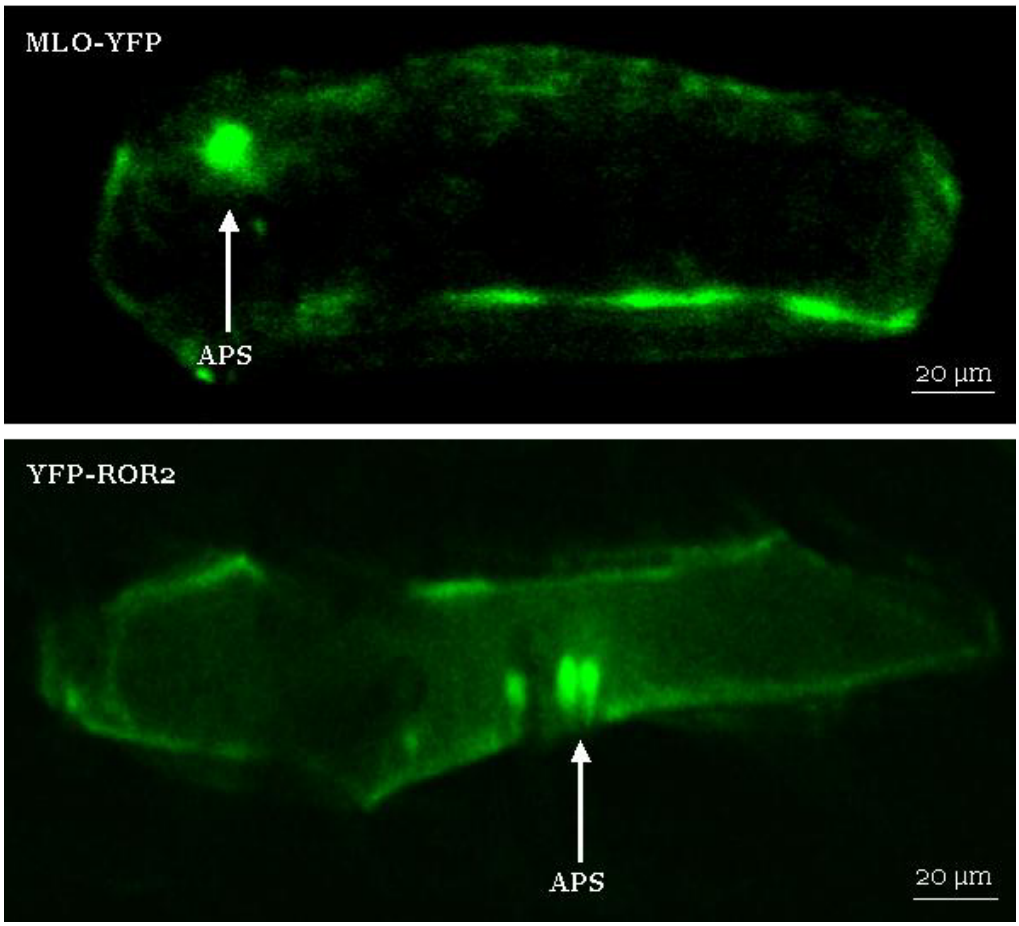

Fig. 7.2

Focal accumulation of *Hv*MLO-YFP and YFP-*Hv*ROR2 in a pathogen-challenged barley epidermal cell.

Barley leaves were bombarded with a construct containing either *MLO*-YFP or YFP-ROR2 and subsequently inoculated with barley powdery mildew (*Bgh*) conidiospores. Focal accumulation of the fusion proteins at the attempted penetration site (APS) is indicated by the red arrows (Figure kindly provided by R. Bhat).

The implication of both MLO and ROR2 in the same genetic pathway conditioning penetration resistance, as well as the temporal and spatial similarities in focal protein accumulation upon pathogen challenge prompted us to test a potential direct interaction between MLO proteins and syntaxins in the split-ubiquitin system.

7.2. INTERACTION BETWEEN MLO PROTEINS AND SYNTAXINS IN THE SPLIT-UBIQUITIN ASSAY

7.2.1. *A. thaliana* MLO proteins interact with *At*PEN1

Since, barley *MLO* is not suitable for split-ubiquitin studies, the interaction between *At*MLO proteins and PEN1 (the *A. thaliana* ortholog of barley ROR2) or ROR2, respectively, was analysed.

All available bait constructs (*At*MLO1, *At*MLO2, *At*MLO3, *At*MLO4, *At*MLO5, *At*MLO10 and *At*MLO12) were tested for interaction with either *At*PEN1 or *Hv*ROR2 fused to the C-terminus of NuI. Growth on FOA-containing medium of yeast cells co-expressing the prey and the bait vectors revealed interaction between NuI-*At*PEN1 and of the *At*MLO bait constructs (Fig. 7.3). In contrast, severely reduced growth (*At*MLO1, *At*MLO5 or *At*MLO10) or no growth at all (*At*MLO2, *At*MLO3, *At*MLO4 or *At*MLO12) was observed when NuI-*Hv*ROR2 was co-expressed with the bait constructs in yeast cells (Fig 7.4 and data not shown; Table SD.2).

In addition, a range of *Atpen1* and *Hvror2* mutant alleles were tested. The barley ROR2 mutant identified in the original genetic screen (Freialdenhoven et al., 1996) is characterised by a deletion of 93 nucleotides within the *Ror2* coding sequence leading to an in-frame deletion of 31 amino acids in the central region of the syntaxin (Fig. 7.1; Collins et al., 2003). Deletion of the corresponding region was engineered in the PEN1 prey construct (*At*PEN1Δ31). The *Atpen1*-3 mutant allele encodes a PEN1 variant

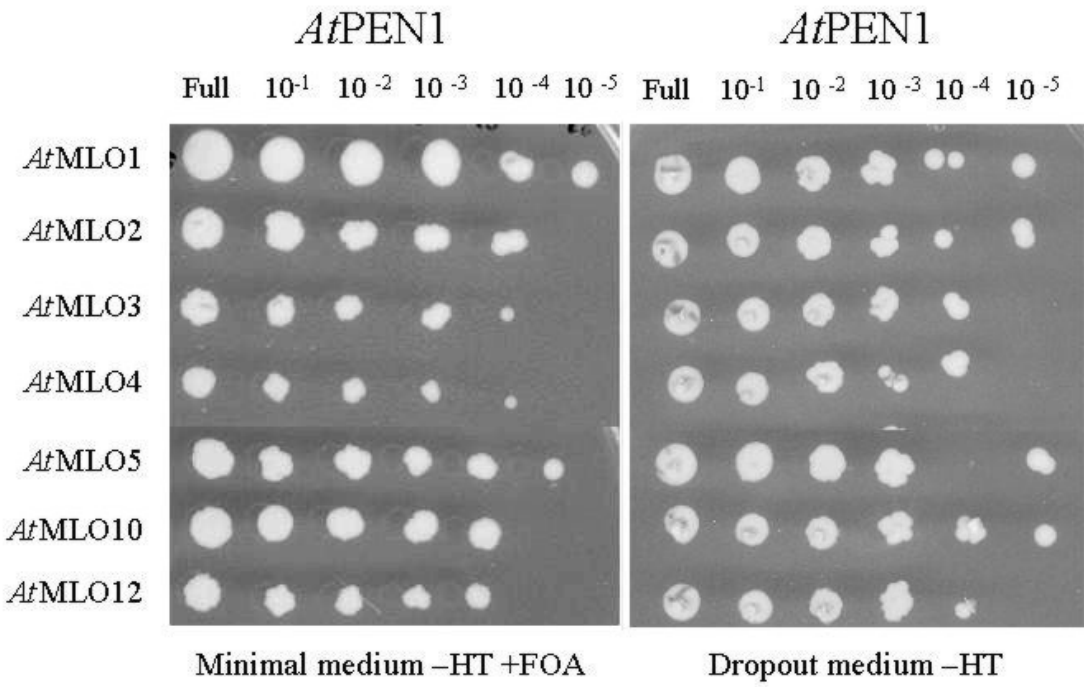

Fig. 7.3

***At*PEN1 syntaxin interacts with different *At*MLO proteins *in vivo*.** Ten-fold serial dilutions of cells co-expressing NuI-*At*PEN1 and various *At*MLO proteins fused to Cub-R-URA3 were grown on medium lacking histidine and tryptophan (dropout medium –HT, on the right) or on selective agar plates lacking histidine, tryptophan and containing additionally FOA (minimal medium –HT +FOA, on the left). Interaction between bait and prey proteins was revealed by growth on the FOA-containing medium.

with a single amino acid substitution (G269E; Collins et al., 2003), affecting a glycine highly conserved among the *A. thaliana* syntaxins. Yeast cells expressing NuI-PEN1-3 together with any of the bait constructs exhibited growth on FOA-containing medium comparable to wild-type *At*PEN1. In contrast, yeast cells co-expression of NuI-PEN1Δ31 and any of the bait constructs resulted in severely reduced growth on FOA-containing medium (Table SD.2; Fig. 7.4). Likewise, no interaction was detected when the *Hv*ROR2 mutant allele (ROR2Δ31) was co-expressed with any of the *At*MLO bait constructs (data not shown; Table SD.2).

Supporting the split-ubiquitin data, fluorescence resonance energy transfer (FRET) experiments demonstrated an *in planta* interaction between *Hv*MLO, fused to YFP, and *Hv*ROR2 fused to CFP (cyan fluorescent protein). Similar to the results obtained using the split-ubiquitin system, no interaction was detected when MLO-YFP was co-expressed with *Hv*ROR2Δ31-CFP (R. Bhat, R. Panstruga and P. Schulze-Lefert, unpublished).

7.2.2. *A. thaliana* MLO proteins can interact with different syntaxins in the split-ubiquitin system

The *A. thaliana* syntaxin family comprises 24 isoforms that can be divided in eight major clades (Fig. 7.5; Sanderfoot et al., 2000). PEN1 belongs to the clade named SYP1 that is further divided in 3 subgroups, SYP11, SYP12 and SYP13 (Sanderfoot et al., 2000). To test the specificity of the interaction between *At*PEN1 and *At*MLO proteins, other syntaxin family members were examined. One member of each SYP1 subgroups, *At*SYP111, also known as KNOLLE (Lukowitz et al., 1996), *At*SYP122 and *At*SYP132 was selected for analysis in the split-ubiquitin system. The full-length sequence of each gene was fused to the C-terminus of NuI in the prey vector. Yeast cells co-expressing either NuI-*At*SYP122 or NuI-*At*SYP132 and any of the MLO bait constructs were able to grow on FOA-containing medium. Interestingly, no

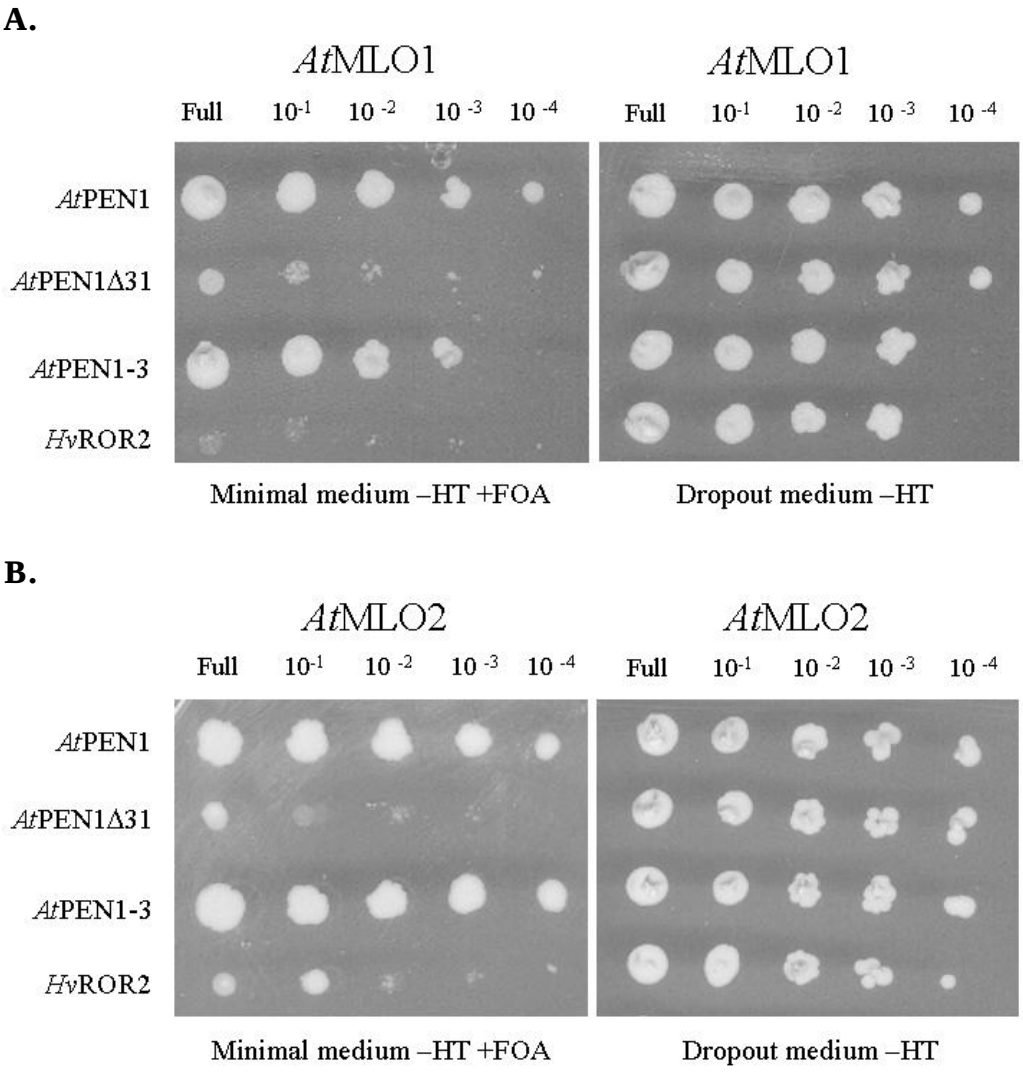

Fig. 7.4

***At*MLO proteins interact with syntaxin *At*PEN1 *in vivo*.**

Ten-fold serial dilutions of yeast cells expressing either *At*MLO1-Cub-URA3 (panel A.) or *At*MLO2-Cub-URA3 (panel B.) fusion proteins were transformed with constructs encoding the depicted proteins fused to Nub. Transformants were grown on agar plates lacking histidine and tryptophan (dropout medium –HT, right side) or on selective media lacking histidine and tryptophan and containing additionally FOA (minimal media –HT +FOA, left side). Interaction between bait and prey proteins was revealed by growth on the FOA plate.

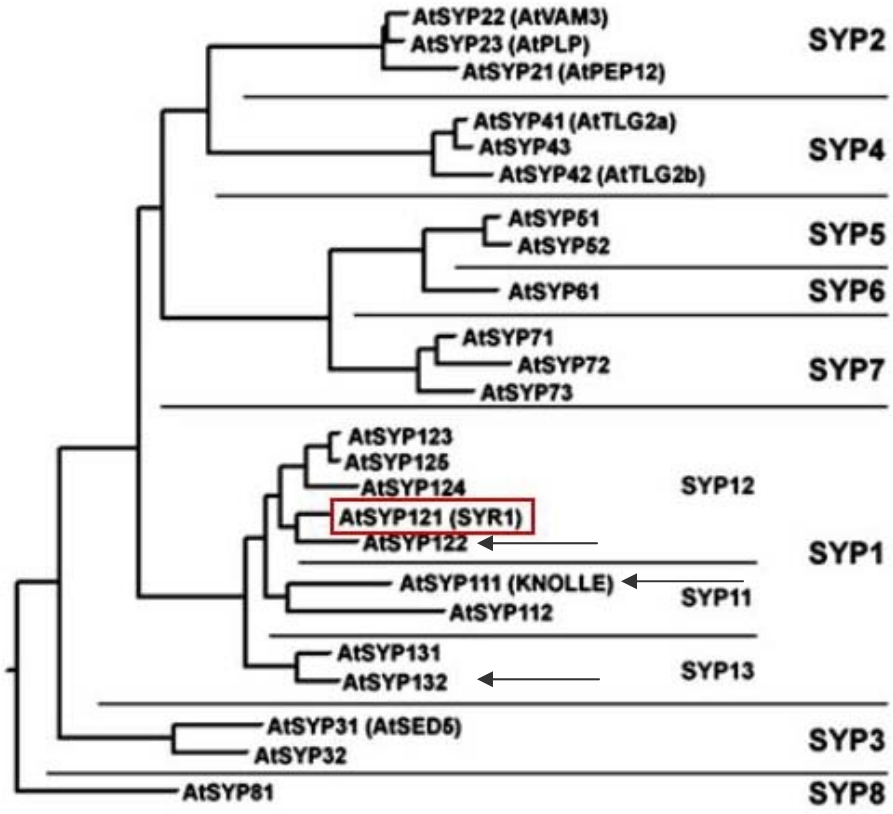

Fig. 7.5

Phylogenetic relationship of *A. thaliana* syntaxins.

The phylogenetic tree reveals the presence of eight major clades (SYP1–8) of *A. thaliana* syntaxins. For those *A. thaliana* syntaxins previously published, the prior name is given in parenthesis. *A. thaliana* syntaxin PEN1/SYP121 is indicated by the rectangle. The arrows indicate the various homologous syntaxins of *At*SYP121 that have been tested in this study for interaction with *At*MLO proteins using the split-ubiquitin system (for further details, see text). (Figure from Sanderfoot et al., 2000).

growth was detected when NuI-*At*SYP111 was expressed together with any of the *At*MLO bait constructs (Fig. 7.6 and data not shown; Table SD.2).

7.2.3. *A. thaliana mlo1* mutant variants interact with *At*PEN1 in the split-ubiquitin system

Barley ROR2 and *A. thaliana* syntaxins were also examined for the interaction with *At*MLO1 mutant variants. In detail, the *Atmlo1* double mutant in the CaM binding domain, *Atmlo1*-LWRR, or the mutants in the third intracellular loop, *Atmlo1*-27 and *Atmlo1*-29 (described above, see Chapter 3) were co-expressed with the prey constructs in yeast cells. Growth on FOA-containing medium comparable to the wild-type *At*MLO1 was observed for each mutant (Table SD.2).

7.2.4. *A. thaliana* MLO proteins do not interact with SNAP proteins in the split-ubiquitin system

The SNAP25 (SNARE-associated proteins) class of SNARE proteins was first described in the mammal neuron as a component of the synaptic membrane SNARE-complex (Oyler et al. 1992). Recent data indicate that SNAP proteins might be required for *Bgh* resistance in barley (Collins et al., 2003). Moreover, it was found that the barley SNAP25-homologue, designated as *Hv*SNAP34, forms complexes with *Hv*ROR2 in yeast cells (Collins et al., 2003). The *A. thaliana* genome encodes three SNAP25-like proteins, *At*SNAP29, *At*SNAP30 and *At*SNAP33 (Sanderfoot et al., 2000). Full-length sequences of these genes were fused to the C-terminus of NuI and co-expressed with any of the bait constructs in yeast cells. Interaction between *A. thaliana* SNAP and MLO proteins was not detected in the split-ubiquitin assay (Table SD.2).

To test the possibility that presence of the *At*PEN1 syntaxin is required for the interaction, a vector carrying *At*PEN1 under control of a galactose-

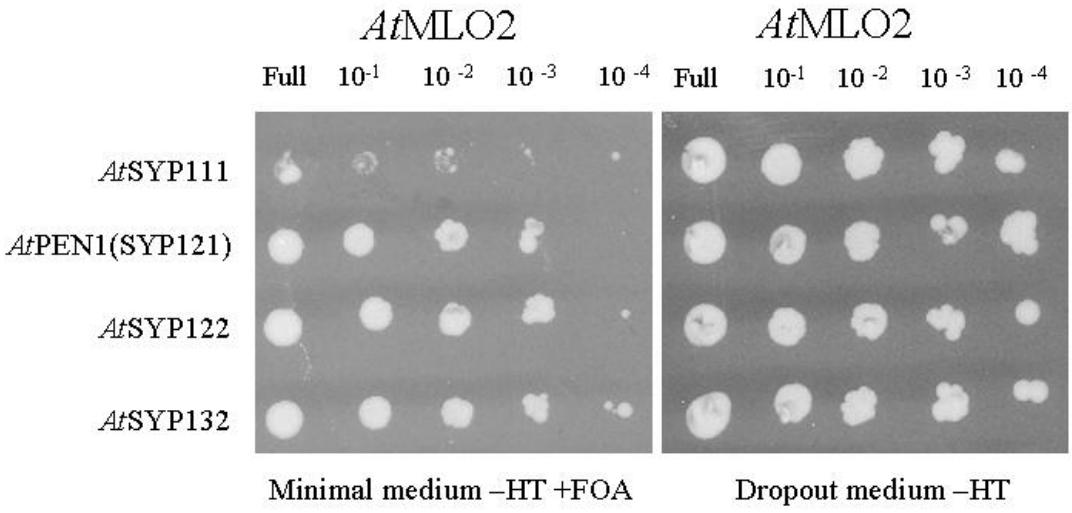

Fig. 7.6

_At_MLO2 interacts with different _A. thaliana_ syntaxins _in vivo_.

Ten-fold serial dilutions of cells co-expressing *At*MLO2-Cub-URA3 fusion proteins and various syntaxin isoforms fused to NuI were grown on medium lacking histidine and tryptophan (dropout medium –HT, on the right) or on selective agar plates lacking histidine, tryptophan and containing additionally FOA (minimal medium –HT +FOA, on the left). Interaction between bait and prey proteins was revealed by growth on the FOA-containing medium.

inducible promoter was constructed. Yeast cells co-expressing any of the *At*MLO bait constructs, *At*PEN1 and NuI-*At*SNAP vectors were tested for their growth on galactose medium containing FOA. Presence of *At*PEN1 had no effect on the yeast cells growth: no growth on FOA-containing medium was revealed (data not shown; Table SD.2).

8. DISCUSSION

It was recently reported that barley *ROR2* and *A. thaliana PEN1* encode orthologous syntaxins (Collins et al., 2003). In barley, it was shown that mutations in *ROR2* confer partial susceptibility against *Bgh* in the *mlo* mutant background (Freialdenhoven et al., 1996). In *A. thaliana*, *pen1* mutants display an increased cell wall penetration by the inappropriate pathogen *Bgh*. Conservation of a specific syntaxin function in both monocots and dicots indicates that basal penetration resistance mediated by *mlo* and non-host resistance against *Bgh* share at least one molecular component. Moreover, single cell expression of either *Hv*MLO or *Hv*ROR2 fused to YFP demonstrated that the two proteins focally accumulate at attempted fungal penetration sites upon pathogen challenge (R. Bhat, R. Panstruga and P. Schulze-Lefert, in press; Schulze-Lefert, 2004).

8.1. MLO PROTEINS INTERACT WITH *Hv*ROR2 AND *At*PEN1

The N-terminus of syntaxins is composed of three α-helical (Ha, Hb, Hc) regions that are capable to bind intramolecularly to the Qa-SNARE domain. This interaction generates a so-called “closed” conformation of the syntaxin protein resulting in inhibition of interactions with other SNARE proteins and in particular preventing the formation of a binary complex with SNAP25 proteins (Lerman et al., 2000; Munson et al., 2000; Misura et al., 2001).

In this study, binding of *A. thaliana* MLO proteins to PEN1 was detected in the yeast split-ubiquitin system (see Chapter 7.2.1). This interaction was disrupted by the deletion of 31 amino acids in the *At*PEN1 Hb/Hc domains, but not by a substitution of a highly conserved amino acid in the Qa-SNARE domain (see Chapter 7.2.1). Additionally, analysis of interaction between *Hv*MLO and *Hv*ROR2 was performed in living barley cells by fluorescence resonance energy transfer

(FRET; R. Bhat, R. Panstruga, P. Schulze-Lefert, unpublished). Transient expression in single epidermal cells of both *Hv*MLO fused to YFP and *Hv*ROR2 fused to CFP resulted in a strong FRET signal indicating interaction of the two proteins *in planta*. These results indicate that interaction between MLO and syntaxins (ROR2/PEN1) is conserved not only among MLO proteins within the same species (i.e. *A. thaliana*), but also between monocots and dicots (barley and *A. thaliana*). Like interaction with CaM, binding of syntaxins appears to be a common feature of MLO proteins.

Moreover, no FRET signal was detected when *Hv*MLO fused to YFP was co-expressed with the *Hv*ROR2-CFP mutant protein lacking 31 amino acids in the Hb/Hc domains (*Hv*ROR2Δ31). Deletion in the central region of syntaxin proteins might lead to a constitutively "open" state resulting in loss of interaction with *At*MLO proteins. Alternatively, *At*MLO might recognise and bind the Hb/Hc region of *At*PEN1 and deletions in this domain could affect the interaction between the two proteins. To examine whether the "open" state conformation of PEN1/ROR2 leads to loss of interaction with MLO, the effect of single amino acid replacements in the syntaxin protein leading to a constitutive "open" conformation will be investigated (S. Pajonk, R. Panstruga and P. Schulze-Lefert, unpublished).

8.2. *At*MLO PROTEINS INTERACT WITH A SUB-SET OF SYNTAXIN ISOFORMS IN THE SPLIT-UBIQUITIN SYSTEM

To test the specificity of the interaction between *At*MLO proteins and *At*PEN1, different *A. thaliana* syntaxin family members were assayed in the split-ubiquitin system. In addition to *At*PEN1/*At*SYP121, *At*MLO proteins can interact with *At*SYP122, a member of the subgroup of *At*PEN1 (see Chapter 7.2.2; Sanderfoot et al., 2000). Recently, it was reported that double mutants in *AtPEN1/SYP122* exhibit a severe dwarf phenotype suggesting an overlapping function between the two proteins (Assaad et al., 2004). Moreover, *At*MLO proteins interact with *At*SYP132 that belongs to a different subgroup within the clade of *At*PEN1 (see Chapter 7.2.2; Sanderfoot et al., 2000). In contrast, the syntaxin *At*SYP111 (also known as

KNOLLE) cannot interact with any *At*MLO protein tested in the split-ubiquitin assay (see Chapter 7.2.2). Mutants in *At*SYP111 were identified as seedling lethal gene and the gene was found to play a crucial role in the formation of the phragmoplast during cytokinesis (Lukowitz et al., 1996; Lauber et al., 1997). Thus, interaction between *At*MLO proteins and syntaxins appears to be specific for a particular subset of syntaxins. To test this hypothesis further, syntaxins belonging to different clades or subgroups will be investigated for their interaction with *At*MLO proteins.

8.3. *At*MLO PROTEINS DO NOT INTERACT WITH *At*SNAP PROTEINS IN THE SPLIT-UBIQUITIN SYSTEM

Recent experiments identified another factor required for *Bgh* penetration resistance in barley (Collins et al., 2003). The barley SNAP25 homologue, *Hv*SNAP34, was required for full resistance against *Bgh* and was shown to interact with *Hv*ROR2 in the yeast two-hybrid system (Collins et al., 2003). Interactions between *A. thaliana* SNAP25 homologues (*At*SNAP29, *At*SNAP30 and *At*SNAP33) and *At*MLO proteins were analysed in the split-ubiquitin system. As no interactions were observed (see Chapter 7.2.4), binding of *At*MLO and *At*SNAP proteins might require the presence of *At*PEN1. A three-hybrid system was used to test this hypothesis. However, even in the presence of *At*PEN1 no interactions between *At*MLO and *At*SNAP proteins were detected. Thus, it is still unclear whether *At*MLO and *At*SNAP proteins interact *in vivo*. The requirement of different factors, which are not present in yeast, or localisation of the proteins in different compartments in yeast cells cannot be ruled out.

8.4. A POTENTIAL ROLE FOR MLO PROTEINS AS REGULATOR OF EXOCYTOSIS

SNARE proteins were found to play a role in various biological processes in plant cells. A screen for *A. thaliana* mutants impaired in the shoot gravitropic response led to the identification of *sgr3* and *zig* mutants, encoding the syntaxin

SYP22 and the VAMP VTI11 respectively (reviewed in Surpin and Raikhel, 2004). Mutants in these genes are deficient in the endodermis-specific gravitropic response and have vacuole defects. The *zig/vti11* mutant presents a typical "zig-zag" morphology of the inflorescence meristem, whereas *sgr3/syp22* mutants have abnormal vacuole morphology in both the endodermal and cortex cell layer (Surpin and Raikhel, 2004).

Mutations affecting the syntaxin SYP111, also known as KNOLLE, result in malformation of the epidermal embryonic cell layer due to abnormal cell divisions and enlargements. The *KNOLLE* mutation appears to disrupt the normal pattern of embryogenesis by altering the plane of cell division (phragmoplast), the rate of cell division and cell shape (Lukowitz et al., 1996). A similar, although weaker, phenotype is displayed by the *KEULE* mutant. KEULE encodes a member of the Sec1 family, proteins that regulate vesicle docking and fusion by interacting with syntaxins (see above). Interestingly, KNOLLE and KEULE proteins were found to interact *in vivo* and form a cytokinesis-specific complex together with the SNAP25-homologue AtSNAP33, which is found primarily at the plasma membrane and cell plate of dividing cells, where it co-localises with KNOLLE (Heese et al., 2001; Surpin and Raikhel, 2004).

Based on the data discussed above, two possible scenarios for MLO function can be proposed. The first hypothesis is that MLO might act as a regulator of exocytosis via interaction with syntaxins. Thus, plants might have evolved a novel regulatory component of exocytosis. The second possibility is that MLO and syntaxins (plus possibly further components) form a complex of still unknown functions.

In mammals and yeast, many factors are already known as SNARE-complex regulators (reviewed in Gerst, 2003). SNARE-complex regulation in the early part of the secretory pathway might restrict random SNARE pairing upon protein translocation into the ER. Furthermore, regulation can prevent *trans*-SNARE association between related SNAREs before they reach their appropriate compartments. Some regulatory factors (like SM; see below) ensure that only related SNAREs form functional *trans*-SNARE complexes, while other factors, such

as synaptophysin, may restrict the activity of SNAREs distal to the site of fusion (Gerst, 2003). It has been shown that syntaxins in the closed conformation interact preferentially with members of the Sec1/Munc18 (SM) family. The SM family is a highly conserved group of large membrane-associated proteins (Dulubova et al., 1999; Misura et al., 2000; reviewed in Gerst, 2003). SM family members were found to positively regulate the formation of SNARE complexes, however, their precise function is still unclear (Bryant and James, 2001; Peng and Gallwitz, 2002); reviewed in Gerst, 2003). Other regulators of SNARE complex are the synaptotagmin membrane proteins that can bind Ca^{2+}. They were found in higher eukaryotes, including plants (Craxton, 2004), and were proposed to act as calcium sensor modulating stimulus-coupled secretion (Chapman, 2002). In addition, complexins are neuronal-specific proteins implicated in regulated exocytic events (Marz and Hanson, 2002). Complexins were suggested to promote oligomerization of the SNARE complexes, by associating one of the two SNAP25 helices with the syntaxin of an adjacent SNARE complex.

In addition to positive regulators, also some negative regulators of SNARE-complexes have been identified. For example, the integral membrane proteins synaptophysin and synaptoporin were found to be major components of synaptic vesicles via interaction with VAMP proteins thereby preventing v-SNAREs from entering into SNARE complexes (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995).

Considering the results discussed above, it is tempting to speculate that the powdery mildew fungus evolved a mechanism to exploit the MLO/syntaxin complex for pathogenesis. MLO might dynamically interact with the Hb/Hc region of ROR2/PEN1 resulting in a “closed” conformation of the syntaxin protein and inhibiting the binding of ROR2/PEN1 with other SNARE proteins. The pathogen might target MLO, either directly or indirectly, causing a permanent arrest of the syntaxin in a “closed” conformation. Then, ROR2/PEN1 cannot interact with other SNARE proteins to direct vesicle transport towards the plasma membrane. Due to loss of interaction between MLO and ROR2/PEN1, syntaxin proteins might be present in the cell in a constitutive “open” conformation in *mlo* mutants. Thus,

vesicle transport towards the attempt penetration sites might be either more efficient or faster consequently resulting in increased resistance against the pathogen. If this scenario was true, then one would also expect that a ROR2-matching v-SNARE contributes to pathogen resistance. Further experimentation will be necessary to test this hypothesis and to identify the molecular composition of the cargo transported in the presumed plasma membrane-targeted vesicles.

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10. SUPPLEMENTARY DATA

Table SD.1
Prey clones identified using the split-ubiquitin system.

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
Fatty acid metabolism	At*MLO1*	1A14[8]	Cytochrome b5	Lipid, fatty-acid and isoprenoid biosynthesis	1	At5g53560
	*At*MLO5	5F10[8]	Cytochrome b5	Lipid, fatty-acid and isoprenoid biosynthesis	1	At5g48810
	*At*MLO10	XF4[8]	Cytochrome b5	Lipid, fatty-acid and isoprenoid biosynthesis	1	At5g48810
	*At*MLO10	XF8[8]	Putative cytochrome b5	Lipid, fatty-acid and isoprenoid biosynthesis	1	At2g46650
	*At*MLO4	4F1[8]	Putative cytochrome b5	Lipid, fatty-acid and isoprenoid biosynthesis	1	At2g32720
	*At*MLO5	5F21	Cytochrome b6	Lipid, fatty-acid and isoprenoid biosynthesis	2	At5g48811
	*At*MLO1	13FC[7]	Putative Acyl coa binding protein		?	At1g31812
	*At*MLO1	27FC[7,8]	Squalene syntetase	Secondary metabolism; biosynthesis of derivatives of homoisopentenyl pyrophosphate	1	At4g34640
	*At*MLO1	5F[7, 8]	Squalene syntetase	Secondary metabolism; biosynthesis of derivatives of homoisopentenyl pyrophosphate	1	At4g34640
	*At*MLO5	5F7[8]	Squalene syntetase	Secondary metabolism; biosynthesis of derivatives of homoisopentenyl pyrophosphate	1	At4g34640
	*At*MLO1	44FC[7]	Putative lipid transfer protein		Yes	At1g48750
	*At*MLO1	1A21	Putative glycine-rich protein			At2g05510
	*At*MLO1	1B24	Putative lipase	Lipid, fatty-acid and isoprenoid metabolism	No	At1g52760
	*At*MLO1	1B26	3-oxoacyl-[acyl-carrier-protein] synthase I precursor (beta-ketoacyl-acp synthase I) (KAS I)			At5g46290

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
	*At*MLO10	XF16	Unknown	Lipase	2	At2g20920
	*At*MLO5	5F20	Lipid transfer protein	Cellular transport and transport mechanism	1	At3g18280
Chaperones	*At*MLO1	1FC[8]	Peptidylprolyl isomerase (cyclophilin)	Protein destination; protein folding and stabilization	No	At4g34870
	*At*MLO5	5F24[8]	Peptidylprolyl isomerase (cyclophilin)	Protein destination; protein folding and stabilization	No	At4g34870
	*At*MLO10	XF21[8]	Peptidylprolyl isomerase (cyclophilin)	Protein destination; protein folding and stabilization	No	At4g34870
	*At*MLO1	1FC1	Unknown protein; similarity to known protein: bundle sheath defective protein 2 - Zea mays,		No	At3g47650
	*At*MLO1	1FC2	Putative heat-shock protein hsp70	Involved in protein folding, some of them in transport of proteins across membranes.	No	At3g09440
Vesicle transport	*At*MLO1	33FC[7]	Synaptobrevin (V-SNARE) SEC22	Putative vesicle transport protein	1	At1g11890
	*At*MLO1	1B19	Putative synaptobrevin	Vesicular transport	1	At2g33120
	*At*MLO5	5F9	Putative synaptobrevin	Vesicular transport	1	At2g33120
	*At*MLO5	5F13	Putative synaptobrevin	Vesicular transport	1	At2g33120
	*At*MLO5	5F33	Putative synaptobrevin	Vesicular transport	1	At2g33120
	*At*MLO1	1FC8	TLG1 - member of the syntaxin family of t-snares	Protein targeting, sorting and translocation	2	At1g79590
	*At*MLO1	1A26	Putative cis-Golgi SNARE protein	Protein targeting, sorting and translocation	Yes	At2g45200
proton pump interactor	*At*MLO1	1A11[8]	Proton pump interactor-	Plasmamembrane H+ atpase pump activator	1	At4g27500
	*At*MLO5	5F16[8]	Proton pump interactor-	Plasmamembrane H+ atpase pump activator	1	At4g27500

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
	*At*MLO10	XF15[8]	Proton pump interactor	Plasmamembrane H+ atpase pump activator	1	At4g27500
	*At*MLO3	3F8[8]	Proton pump interactor	Plasmamembrane H+ atpase pump activator	1	At4g27500
	*At*MLO4	4F5[8]	Proton pump interactor	Plasmamembrane H+ atpase pump activator	1	At4g27500
photosynthesis	*At*MLO1	48FC[7]	Photosystem II reaction center		1	At2g30570
	*At*MLO10	XF7	Photosystem II polypeptide,		1	At1g79040
	*At*MLO10	XF9	Photosystem II polypeptide,		1	At1g79040
	*At*MLO10	XF11	Photosystem II polypeptide,		1	At1g79040
	*At*MLO1	1A35	Hypothetical protein; similar to photosystemii polipep		1	At1g79040
	*At*MLO1	1FC19	Ribulose bisphosphate carboxylase,		No	At1g67090
	*At*MLO10	XF17	Ribulose-bisphosphate carboxylase		No	At1g67090
	*At*MLO5	5F44	Ribulose-bisphosphate carboxylase		No	At1g67090
Ca^{2+}-binding protein	*At*MLO1	2A2[7]	Ser/thr kinase or cam6 unclassified protein; high homology with calmoduline		No	At1g12310
detoxification	*At*MLO1	41FC[7]	Metallothionein-like protein		No	At3g09390
	*At*MLO1	1FC9	Selenium-binding protein like	Cell rescue, defense, cell death and ageing: detoxificaton	No	At4g14030
others	*At*MLO1	1A16[7]	Germin like protein	Hexameric glycoproteins oxalate oxidases or superoxide dismutase structural proteins or receptors		At1g72610
	*At*MLO1	FC5[7]	Cold-regulated protein cor15a		No	At2g42540

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
			precursor			
	*At*MLO1	1FC12	Putative elongation factor 1B alpha-subunit	Protein synthesis translation	No	At5g19510
	*At*MLO1	1A18	NAM / CUC2 - like protein		No	At4g35580
	*At*MLO1	1A29	Unknown protein/ squamosa promoter binding protein-like 12		1	At2g47080
	*At*MLO5	5F1	Unknown: similarity to glucose 6 phosphate/phosphate translocator-like protein	C-compound and carbohydrate transporters	6	At1g06890
	*At*MLO5	5F4	Putative protein phosphatase	Cellular communication/signal transduction	4	At5g10480
	*At*MLO5	5F5	Putative GTP binding protein, rab7 - like		No	At3g18820
	*At*MLO5	5F18	GTP-binding protein, ara-5	Ras-related small GTP-binding protein RAB1c	No	At1g02130
	*At*MLO5	5F6	Similarity to known protein: dihydrokaempferol 4-reductase	Biosynthesis of Phenylpropanoids	No	At4g33360
	*At*MLO5	5F8	Hypothetical: putative cold-regulated protein	Stress response	No	At1g20450
	*At*MLO5	5F14	Tubulin beta-7 chain	Cytoskeleton	No	At2g29550
	*At*MLO10	XF19	Tubulin beta-4 chain	Cytoskeleton	No	At5g44340
	*At*MLO5	5F19	Putative RING zinc finger protein	Unspecified signal transduction	2	At2g23780
	*At*MLO3	3F15	Putative RING zinc finger protein	Unspecified signal transduction	2	At2g23780
	*At*MLO10	XF12	Cysteine	Proteolytic degradation	1	At3g12490

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
			proteinase inhibitor			
	*At*MLO10	XF13	Putative caltractin calcium-dependent protein kinase - like	Unspecified signal transduction	No	At2g46600
	*At*MLO10	XF23	Bax inhibitor-1 like / / putative glutamate-/aspartate-binding peptide	Protein binding unspecified signal transduction	7	At5g47120
	*At*MLO5	5F32	Outer membrane lipoprotein	Biogenesis of plasma membrane	No	At5g58070
	*At*MLO5	5F45	Outer membrane lipoprotein	Biogenesis of plasma membrane	No	At5g58070
	*At*MLO5	5F37	Synaptic glycoprotein SC2-like	Lipid, fatty-acid and isoprenoid biosynthesis	6	At3g55360
unknown/ hypothetical protein	*At*MLO1	1B27	Unknown protein	Tonb-dependent receptor protein? (couple the cytoplasmic membrane proton motive force to active transport of iron-siderophore complexes and vitamin B(12) across the outer membrane)	Yes	At1g27350
	*At*MLO1	1A13	Unknown protein		Yes	At1g09920
	*At*MLO3	3F9	Hypothetical		No	At1g12300
	*At*MLO1	37FC[7]	Putative hydroxyproline-rich glycoprotein	Unclassified protein	No	*At*1g13930
	*At*MLO5	5F3[8]	Unknown		2	At1g17080
	*At*MLO5	5F38[8]	Unknown		2	At1g17080
	*At*MLO1	1A4[8]	Unknown		2	At1g17080
	*At*MLO1	1B22	Hypothetical protein	Similarity to hypothetical S. Pombe protein S.cerevisiae (DNA double-strand break (DSB) repair)	Yes	At1g18720
	*At*MLO1	18FC[7]	Hypothetical protein,	Unclassified protein: cellular transport and	Yes	At1g48750

Class[1]	Bait[2]	Clone[3]	Homology[4]	Characteristic		AGI number
				Activity[5]	TM domain[6]	
			similarity to lipid transfer - like protein	transport mechanisms		
	*At*MLO1	1A10	Hypothetical protein		Yes	At1g50740
	*At*MLO1	1B2	Hypothetical protein; Rubredoxin signature	Small electron-transfer prokaryotic proteins	Yes	At1g54500
	*At*MLO5	5F22[8]	Unknown		No	At1g62480
	*At*MLO1	51FC[7, 8]	Unknown protein	Unclassified protein	No	At1g62480
	*At*MLO1	1FC5[8]	Unknown protein		No	At1g62480
	*At*MLO1	1FC3	Unknown protein			At1g68875
	*At*MLO1	1A13	Unknown protein		No	At2g41010
	*At*MLO5	5F23	Putative	No similarity to proteins, but EST matches	4	At3g11395
	*At*MLO1	1B15	Unknown protein		Yes	At3g28050
	*At*MLO5	5F27	Hypothetical		1	At3g45160
	*At*MLO5	5F36	Expressed			At3g50685
	*At*MLO1	1A36	Hypothetical protein			At3g57280
	*At*MLO5	5F26	Hypothetical		No	At4g04200
	*At*MLO1	8FC[7]	Putative protein, similarity to known protein: LEA protein	Late embryogenesis-abundant protein	No	At4g13560
	*At*MLO4	4F7	Putative		No	At5g05060
	*At*MLO10	XF5	Putative		1	At5g12390
	*At*MLO10	XF22	Putative protein		No	At5g27860
	*At*MLO1	1F[7]	Unknown protein	Unclassified protein	Yes	At5g45420
	*At*MLO10	XF6	Sodium-dicarboxylate cotransporter-like		11	At5g47560
	*At*MLO1	1A8[7]	Unknown protein		Yes	At5g51010

[1] Identified clones were classified according to their putative function.
[2] *At*MLO bait used in the split-ubiquitin screen.

[3] Classification name of the clone identified in the split-ubiquitin screen.
[4] Based on blast analysis against *A. thaliana* genome using TAIR homepage.
[5] (Putative) function assigned to the candidate according to TAIR information.
[6] Indicate the number of (putative) TM present in the candidate protein. (Yes) indicates the presence of TM but not a defined number; (no) indicates that no TM domains were identified; (?) indicates that no information was available.
[7] Clone that was not re-tested for growth on FOA-containing media before sequencing.
[8] Clone was chosen for further analysis.

Table SD.2
Summary of interaction phenotypes in the split-ubiquitin system.

	*At*MLO1	*At*MLO2	*At*MLO3	*At*MLO4	*At*MLO5	*At*MLO10	*At*MLO12	*At*MLO1-LWRR[9]	*At*MLO1-29[10]	*At*MLO1-27[11]
*Hv*CaM	+	+	-	+	+	+	+	-	+	+
*Hv*Gα	-	-	-	-	-	+	-	-	-	-
*At*CYTb5[1]	+[8]	+	+	+[8]	+[8]	+[8]	+	+	+	+
*At*CYP1	+[8]	+	-	+	+[8]	+[8]	+	+	+	+/-
*At*PPI1	+[8]	+	+[8]	+[8]	+[8]	+[8]	+	+	+	+
*At*SQS	+[8]	+	-	-	+[8]	+	+	+	+	+
*At*PPI full length	-	-	-	-	-	-	-	NT[12]	NT	NT
*Hv*CYTb5-2	+	+	+	+/-	+	+	+	NT	NT	NT
*Hv*CYP1	-	-	-	-	-	-	-	NT	NT	NT
*Hv*PPI	+/-	-	-	-	+/-	+	-	NT	NT	NT
*Hv*SQS	-	-	-	-	-	-	-	NT	NT	NT
*At*SYP121	+	+/-	+	+	+	+	+	+	+	+/-
*At*SYP121 Δ31[2]	+/-	-	-	-	+/-	+/-	-	-	-	-
*At*SYP121 1-3[3]	+	+/-	+	+	+	+	+	+	+	+/-
*Hv*ROR2	+/-	-	-	-	+/-	+/-	-	-	+/-	-
*Hv*ROR2Δ31[2]	+/-	+/-	-	-	+/-	+	-	-	+/-	+/-
*At*SYP111 (Knolle)	-	-	-	-	-	-	-	NT	-	-
*At*SYP122	+	+	+/-	-	+	+	+/-	NT	+	+/-
*At*SYP132	+	+	+	-	+	+	+/-	NT	+	+
*At*SNAP29	-	-	-	-	-	+/-	+	NT	NT	NT
*At*SNAP30	-	-	-	-	-	+/-	+	NT	NT	NT
*At*SNAP33	-	-	-	-	-	-	-	NT	NT	NT
*At*ROC3[4]	+/-	+	NT	NT	NT	NT	NT	NT	NT	NT
*At*ROC1[5]	+/-	+	NT	NT	NT	NT	NT	NT	NT	NT
*At*ROC6[6]	+	+/-	NT	NT	NT	NT	NT	NT	NT	NT
*At*ROC2[7]	+/-	-	NT	NT	NT	NT	NT	NT	NT	NT

(+) indicates growth on FOA-containing medium; (-) indicates no growth; (+/-) indicates a reduced growth; grey stripped area indicates that no data are available for the specific experiment.

[1] AtCYT b5 isoform tested is At5g48810.
[2] Deletion of 31 amino acids in *Hv*ROR2 (119 to 150) or in *At*PEN1 (122 to 153).
[3] Single amino acid substitution (G269E).
[4] *A. thaliana* cyclophilin isoform At2g16600.
[5] *A. thaliana* cyclophilin isoform At4g38740.
[6] *A. thaliana* cyclophilin isoform At2g21230.
[7] *A. thaliana* cyclophilin isoform At4g34870.
[8] Clone identified in a screening
[9] AtMLO1 mutant allele that has two amino acid exchanges in the CaMBD (L453R; W456R).
[10] AtMLO1 mutant allele that has an amino acid exchange in the third intracellular loop (G351E).
[11] AtMLO1 mutant allele that has an amino acid exchange in the third intracellular loop (P367L).
[12] NT: not tested

Table SD.3
Single cell gene silencing of barley candidate genes in wild-type *MLO* cells.

Transformed vector(s)		1st experiment				2nd experiment				3rd experiment			
		Non-penetr. Cells[1]	Penetr. Cells[2]	Tot. cells[3]	% Penetr.[4]	Non-penetr. Cells[1]	Penetr. Cells[2]	Tot. cells[3]	% Penetr.[4]	Non-penetr. Cells[1]	Penetr. Cells[2]	Tot. cells[3]	% Penetr.[4]
pUbi-Gus[5]	pUAMBN[7]	64	5	69	7,2	17	2	19	10,5	101	6	107	5,6
pMUG[6]		25	129	154	83,8	46	64	110	58,2	35	32	67	47,8
pUbi-Gus	pUAMBN-HvCYTb5-1	74	20	94	21,3	84	8	92	8,7	75	2	77	2,6
pUbi-Gus	pUAMBN-HvCYTb5-2	140	30	170	17,6	39	3	42	7,1	63	2	65	3,1
pUbi-Gus	pUAMBN-HvCYP1	86	15	101	14,9	96	18	114	15,8	70	4	74	5,4
pUbi-Gus	pUAMBN-HvSQS	124	21	145	14,5	105	7	112	6,3	100	2	102	2,0
pUbi-Gus	pUAMBN-HvPPI	135	15	150	10,0	102	13	115	11,3	97	5	102	4,9
pUbi-Gus	ALL	NT[8]	NT	NT	NT	91	29	120	24,2	70	11	81	13,6

[1] Number of non-penetrated cells: fungal spore failed to penetrate into the epidermal cell.
[2] Number of penetrated cells: fungal spore succeeded in penetrating into the epidermal cell and established a haustorium.
[3] Total number of evaluated cells.
[4] Percentage of penetrated cells.
[5] GUS reporter construct.
[6] Bifunctional construct expressing both GUS reporter and wild-type MLO.
[7] Gene silencing vector.
[8] NT: not tested.

Table SD.4
Average data of single cell gene silencing of barley candidate genes in wild-type *MLO* cells.

Transformed vector(s)		% Penetr.[1]	St. Dev.[2]
pUbi-Gus[3]	pUAMBN[5]	7,8	2,5
pMUG[4]		63,2	18,5
pUbi-Gus	pUAMBN-HvCYTb5-1	10,9	9,5
pUbi-Gus	pUAMBN-HvCYTb5-2	9,3	7,5
pUbi-Gus	pUAMBN-HvCYP1	12,0	5,7
pUbi-Gus	pUAMBN-HvSQS	7,6	6,4
pUbi-Gus	pUAMBN-HvPPI	8,7	3,4
pUbi-Gus	ALL	18,9	7,5

[1] Percentage of penetrated cells calculated upon the three independent experiments reported in Table SD.3.
[2] Standard deviation calculated on the three experiments listed in Table SD.3.
[3] GUS reporter construct
[4] Bifunctional construct expressing both GUS reporter and wild-type MLO
[5] Gene silencing vector

Table SD.5
Single cell gene silencing of barley candidate genes in wild-type *MLO* cells overexpressing *MLO*.

Transformed vector(s)		1st experiment				2nd experiment				3rd experiment				4th experiment			
		Non-penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non-penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non-penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non-penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]
pMUG[5]		47	57	104	54,8	63	64	127	50,4	23	87	110	79,1	76	61	137	44,5
pMUG	pUAMBN[6]-HvCYTb5-1	35	82	117	70,1	NT[7]	NT	NT	NT	NT	NT	NT	NT	29	31	60	51,7
pMUG	pUAMBN-HvCYTb5-2	43	59	102	57,8	42	67	109	61,5	24	51	75	68,0	77	51	128	39,8
pMUG	pUAMBN-HvCYP1	59	64	123	52,0	45	86	131	65,6	67	74	141	52,5	50	90	140	64,3
pMUG	pUAMBN-HvSQS	45	45	90	50,0	NT	NT	NT	NT	NT	NT	NT	NT	60	41	101	40,6
pMUG	pUAMBN-HvPPI	60	60	120	50,0	76	118	194	60,8	51	101	152	66,4	47	34	81	42,0

[1] Number of non-penetrated cells: fungal spore failed to penetrate into the epidermal cell.
[2] Number of penetrated cells: fungal spore succeeded in penetrating into the epidermal cell and established a haustorium.
[3] Total number of evaluated cells.
[4] Percentage of penetrated cells.
[5] Bifunctional construct expressing both GUS reporter and wild-type MLO.
[6] Gene silencing vector.
[7] NT: not tested

Table SD.6
Average data of single cell gene silencing of barley candidate genes in wild-type *MLO* cells overexpressing *MLO*.

Transformed vector(s)		% Penetr.[1]	St. Dev.[2]
pMUG[3]	pMUG	57,2	15,2
pMUG	pUAMBN[4]-HvCYTb5-1	60,9	13,0
pMUG	pUAMBN-HvCYTb5-2	56,8	12,1
pMUG	pUAMBN-HvCYP1	58,6	7,4
pMUG	pUAMBN-HvSQS	45,3	6,7
pMUG	pUAMBN-HvPPI	54,8	10,9

[1] Percentage of penetrated cells calculated upon the three independent experiments reported in Table SD.5.
[2] Standard deviation calculated on the three experiments listed in Table SD.5.
[3] Bifunctional construct expressing both GUS reporter and wild-type MLO
[4] Gene silencing vector

Table SD.7
Single cell gene silencing of barley candidate genes in mutant *mlo*-3 cells.

Transformed vector(s)		1st experiment				2nd experiment				3rd experiment			
		Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]
pUbi-Gus[5]		103	0	103	0,0	23	1	24	4,2	102	1	103	1,0
pUbi-Gus	pUAMBN[6]-HvCYTb5-2	120	0	120	0,0	40	0	40	0,0	71	0	71	0,0
pUbi-Gus	pUAMBN-HvCYP1	91	0	91	0,0	40	0	40	0,0	80	0	80	0,0
pUbi-Gus	pUAMBN-HvPPI	67	0	67	0,0	20	0	20	0,0	90	1	91	1,1

[1] Number of non-penetrated cells: fungal spore failed to penetrate into the epidermal cell.
[2] Number of penetrated cells: fungal spore succeeded in penetrating into the epidermal cell and established a haustorium.
[3] Total number of evaluated cells.
[4] Percentage of penetrated cells.
[5] GUS reporter construct
[6] Gene silencing vector

Table SD.8
Average data of single cell gene silencing of barley candidate genes in mutant *mlo*-3 cells.

Transformed vector(s)		% Penetr.[1]	St. Dev.[2]
pUbi-Gus[3]		1,7	2,2
pUbi-Gus	pUAMBN[4]-HvCYTb5-2	0,0	0,0
pUbi-Gus	pUAMBN-HvCYP1	0,0	0,0
pUbi-Gus	pUAMBN-HvPPI	0,4	0,6

[1] Percentage of penetrated cells calculated upon the three independent experiments reported in Table SD.7.
[2] Standard deviation calculated on the three experiments listed in Table SD.7.
[3] GUS reporter construct
[4] Gene silencing vector

Table SD.9
Single cell overexpression of barley candidate genes in wild-type *MLO* or mutant *mlo*-3 cells.

Genotype	Transformed vector(s)		1st experiment				2nd experiment				3rd experiment			
			Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]	Non penet. Cells[1]	Penet. Cells[2]	Tot. cells[3]	% Penet.[4]
MLO	pUbi-Gus[5]		43	56	99	56,6	90	95	185	51,4	56	55	111	49,5
	pUbi-Gus	pUbiGATE[6]-HvCYTb5-2	12	20	32	62,5	51	66	117	56,4	82	89	171	52,0
	pUbi-Gus	pUbiGATE-HcvCYP1	60	49	109	45,0	39	86	125	68,8	35	78	113	69,0
	pUbi-Gus	pUbiGATE-HvPPI	44	64	108	59,3	14	16	30	53,3	53	61	114	53,5
	pUbi-Gus	ALL	18	34	52	65,4	37	28	65	43,1	40	69	109	63,3
mlo-3	pUbi-Gus		97	0	97	0,0	107	0	107	0,0	NT[7]	NT	NT	NT
	pUbi-Gus	pUbiGATE-HvCYTb5-2	80	0	80	0,0	10	0	10	0,0	NT	NT	NT	NT
	pUbi-Gus	pUbiGATE-HcvCYP1	62	2	64	3,1	64	1	65	1,5	NT	NT	NT	NT
	pUbi-Gus	pUbiGATE-HvPPI	107	0	107	0,0	77	0	77	0,0	NT	NT	NT	NT
	pUbi-Gus	ALL	49	0	49	0,0	94	0	94	0,0	NT	NT	NT	NT

[1] Number of non-penetrated cells: fungal spore failed to penetrate into the epidermal cell.
[2] Number of penetrated cells: fungal spore succeeded in penetrating into the epidermal cell and established a haustorium.
[3] Total number of evaluated cells.
[4] Percentage of penetrated cells.
[5] GUS reporter construct.
[6] Gene overexpression vector.
[7] NT: not tested

Table SD.10
Average data of single cell overexpression of barley candidate genes in wild-type *MLO* or mutant *mlo*-3 cells.

Genotype	Transformed vector(s)		% Penetr.[1]	St. Dev.[2]
MLO	pUbi-Gus[3]		52,5	3,6
	pUbi-Gus	pUbiGATE[4]- HvCYTb5-2	57,0	5,3
	pUbi-Gus	PUbiGATE-HvCYP1	60,9	13,8
	pUbi-Gus	PUbiGATE-HvPPI	55,4	3,4
	pUbi-Gus	ALL	57,3	12,3
mlo-3	pUbi-Gus		0,0	0
	pUbi-Gus	pUbiGATE- HvCYTb5-2	0,0	0
	pUbi-Gus	PUbiGATE-HvCYP1	2,3	1,1
	pUbi-Gus	PUbiGATE-HvPPI	0,0	0
	pUbi-Gus	ALL	0,0	0

[1] Percentage of penetrated cells calculated upon the three independent experiments reported in Table SD.9.
[2] Standard deviation calculated on the three experiments listed in Table SD.9.
[3] GUS reporter construct
[4] Gene overexpression vector

Table SD.11
Quantitative analysis of *Blumeria graminis* f. sp. *hordei* (*Bgh*) penetration efficiency on *A. thaliana MLO* insertion lines.

	1st experiment		2nd experiment	
Genotype	Failed penetr.[1]	Successful penetr.[2]	Successful penetr.	Failed penetr.
Col-0	77	23	77	23
Atmlo2	80	20	94	6
Atmlo6	76	24	80	20
Atmlo12	75	25	83	17
Atmlo2/mlo6	98	2	92	8
Atmlo2/mlo12	91	9	94	6
Atmlo6/mlo12	60	40	77	23
Atmlo2/mlo6/mlo12	99	1	99	1

[1] Percentage of spores that did not succeed in penetrating into an epidermal cell.
[2] Percentage of spores that succeeded in successfully penetrating into an epidermal cell and in establishing an haustorium. The number includes also cells that showed cell death subsequent of penetration.

Table SD.12
Quantitative analysis of secondary hyphal growth of *Erysiphe pisi* on *A. thaliana mlo* mutant lines

	1st experiment			2nd experiment			3rd experiment		
Genotype	No hyphal growth [1]	Hyphal growth[2]	Cell death[3]	No hyphal growth	Hyphal growth	Cell death	No hyphal growth	Hyphal growth	Cell death
Col-0	77	23	25	95	5	25	85	15	12
Atmlo2	80	20	19	98	2	20	88	12	16
Atmlo6	76	24	27	96	4	15	85	15	15
Atmlo12	75	25	22	97	3	14	79	21	21
Atmlo2/mlo6	98	2	4	98	2	8	90	10	10
Atmlo2/mlo12	91	9	7	98	2	14	95	5	7
Atmlo6/mlo12	60	40	33	92	8	9	79	21	14
Atmlo2/mlo6/mlo12	99	1	0	100	0	0	100	0	0

[1] Percentage of spores that did not succeed either in penetrating into an epidermal cell or in establishing an haustorium in a successfully penetrated cell.
[2] Percentage of spores that did succeed in successfully penetrating into an epidermal cell and in establishing an haustorium, indicated by the presence of secondary hyphal growth.
[3] Percentage of cells that present whole cell callose deposition (indicative of cell death) subsequent to a successful penetration attempt. This number is irrespective of secondary hyphal growth.

Table SD.13
Average data of the quantitative analysis of secondary hyphal growth of *Erysiphe pisi* on *A. thaliana mlo* mutant plants.

	Average[4]			St. Dev.[5]		
Genotype	% No hyphal growth [1]	% Hyphal growth[2]	% Cell death[3]	% No hyphal growth [1]	% Hyphal growth[2]	% Cell death[3]
Col-0	86	14	21	9,0	9,0	7,7
Atmlo2	89	11	18	9,0	9,0	2,1
Atmlo6	86	14	19	10,0	10,0	7,0
Atmlo12	84	16	19	11,7	11,7	4,3
Atmlo2/mlo6	95	5	7	4,6	4,6	2,8
Atmlo2/mlo12	95	5	9	3,5	3,5	4,1
Atmlo6/mlo12	77	23	19	16,1	16,1	12,6
Atmlo2/mlo6/mlo12	100	0	0	0,6	0,6	0,0

[1] Percentage of spores that did not succeed either in penetrating into an epidermal cell or in establishing an haustorium in a successfully penetrated cell.
[2] Percentage of spores that did succeed in successfully penetrating into an epidermal cell and in establishing an haustorium, indicated by the presence of secondary hyphal growth.
[3] Percentage of cells that present whole cell callose deposition (indicated as cell death) subsequently a successful penetration attempt. This number is irrespective of secondary hyphal growth.
[4] Average of the three independent experiments presented in Table SD.12.
[5] Standard deviation of the three independent experiments presented in Table SD.12.

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Köln, 14 Dezember 2004

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