Molecular characterization of *mlo*-based powdery mildew resistance and the role of heterotrimeric G-protein signaling in Arabidopsis defense

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Abbreviations

%	percent
°C	degrees Celsius
3’	downstream region (of a gene or sequence)
4MI3G	4-methoxyindol-3-ylmethylglucosinolate
5’	upstream region (of a gene or sequence)
APS	ammonium persulfate
A. thaliana	*Arabidopsis thaliana*
Avr	avirulence
Bgh	*Blumeria graminis* forma specialis *hordei*
bp	base pair(s)
C	carboxy-terminal
Ca^{2+}	calcium ions
CDPK	calcium-dependent protein kinase
CaM	calmodulin
CaMBD	calmodulin binding domain
CC	coiled-coil
cfu	colony forming unit
COI1	CORONATINE INSENSITIVE 1
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
CYP79B2/3	Cytochrome 79B family member 2/3
d	days
DAMP	danger-associated molecular patterns
DNA	deoxyribonucleic acid
dH_2O	de-ionized water
EDR1	ENHANCED DISEASE RESISTANCE 1
EIN2	ETHYLENE INSENSITIVE 2
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
dn-OPDA	10-oxo-dinor-phytodienoic acid
dNTP	deoxynucleosidetriphosphate
dpi	days post inoculation
dsRNAi	double stranded RNA interference
DTT	dithiothreitol
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EDTA	ethylenediaminetetraacetic acid
EFR	EF-Tu receptor
EMS	ethyl methanesulfonate
E. cichoracearum	*Erysiphe cichoracearum*
E. pisi	*Erysiphe pisi*
ET	ethylene
ETI	effector-triggered immunity
flg	flagellin
f. sp.	forma specialis
Fig.	Figure
FLS2	FLAGELLIN SENSING RECEPTOR 2
g	gram
g	gravity constant (9.81 ms-1)
G. orontii	*Golovinomyces orontii*
GPCR	G-protein coupled receptor

G-protein	guanine nucleotide-binding protein
GSL5	GLUCAN SYNTHASE-LIKE 5
h	hours
hpi	hours post inoculation
HR	hypersensitive response
HRP	horseradish peroxidase
Hv	*hordeum vulgare*
I3A	indol-3-ylmethylamine
I3G	indol-3-ylmethylglucosinolate
IG	indole glucosinolates
Ile	isoleucine
JA/JAs	jasmonic acid/jasmonates
kb	kilobase(s)
l	liter
LRR	leucine rich repeats
m	milli
M	molar (mol/l)
µ	micro
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated protein kinase
min	minutes
MLO	MILDEW RESISTANCE LOCUS O
mRNA	messenger ribonucleic acid
MTI	MAMP-triggered immunity
MW	molecular weight
n	nano
N	amino-terminal
OD	optical density
OPDA	12-oxo-phytodienoic acid
P	probability value
P	pico
p35S	35S promoter of CaMV
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphat buffered saline
PCR	polymerase chain reaction
PDF1.2	PLANT DEFENSIN 1.2
PEG	polyethylene glycol
PEN1-3	PENETRATION 1-3
pH	negative decimal logarithm of H^+ concentration
PMR4	POWDERY MILDEW RESISTANCE 4
PR1	PATHOGENESIS-RELATED 1
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	pathovar
R	resistance
RA	raphanusamic acid
RLK	receptor-like kinase
RLP	receptor-like protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RPW8	RESISTANCE TO POWDERY MILDEW 8

rpm	rounds per minute
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	seconds
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Taq	*Thermophilus aquaticus*
T-DNA	transfer DNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TM	transmembrane
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TRIS	tris-(hydroxymethyl)-aminomethane
Trp	tryptophane
U	unit
UV	ultraviolet
V	volt
vir	virulence
(v/v)	volume per volume
(w/v)	weight per volume
Ws-0	*Arabidopsis thaliana* ecotype Wassilewskajia-0
wt	wild-type
YFP	yellow fluorescent protein

Summary

Powdery mildew is a common fungal disease of monocotyledonous and dicotyledonous plant species. Successful pathogenesis by the biotrophic fungus depends on the presence of plant-specific MLO (MILDEW RESISTANCE LOCUS O) proteins, as mutations in particular *MLO* genes confer durable powdery mildew resistance in barley, tomato and Arabidopsis. In the absence of MLO, fungal spores fail to invade the host epidermal cell, resulting in an early termination of fungal pathogenesis.

MLO proteins define a family of heptahelical plasma membrane-localized proteins, reminiscent of G-protein coupled receptors (GPCRs) in metazoans that activate heterotrimeric G-protein signaling. A genetic approach was chosen in this study to assess the role of MLO proteins as putative plant GPCRs and results from these experiments demonstrate that powdery mildew susceptibility conferred by MLO is independent of the heterotrimeric G-protein complex. However, data from this analysis suggest a function of the heterotrimeric G-protein in basal defense mechanisms against powdery mildew fungi as well as in the integration of MAMP (microbe-associated molecular patterns) perception into downstream immune responses.

Metabolomic analysis performed in this study indicates that the adapted powdery mildew fungus, *Golovinomyces orontii,* is able to suppress the accumulation of the defense-relevant indolic glucosinolate, 4MI3G (4-methoxyindol-3-ylmethylglucosinolate) in Arabidopsis, thereby inhibiting the PEN2-dependent glucosinolate defense pathway. This inhibition requires functional MLO, suggesting that successful defense suppression either operates through the MLO protein or that it requires the formation of post-invasive fungal infection structures. Other data obtained here demonstrate that MLO proteins negatively regulate transcriptional activation of defense-related genes in response to powdery mildew challenge as well as upon MAMP treatment, implicating MLO functions in MAMP-triggered defense signaling.

Zusammenfassung

Mehltau ist eine weit verbreitete Pilzkrankheit von monokotyledonen und dikotyledonen Pflanzenarten. Die pflanzenspezifischen MLO (MILDEW RESISTANCE LOCUS O)-Proteine sind dabei unerlässlich für eine erfolgreiche Infektion durch den biotrophen Mehltaupilz, da Mutationen in bestimmten *MLO* Genen in Gerste, Tomate und Arabidopsis zur dauerhaften Mehltauresistenz führen. Durch Abwesenheit der MLO-Proteine wird der Eintritt der Pilzspore in die pflanzliche Epidermiszelle verhindert, wodurch es zum früher Erliegen der Mehltauinfektion kommt.

MLO-Proteine gehören zur Familie der heptahelikalen Transmembranproteine und ähneln damit G-Protein gekoppelten Rezeptoren (GPCR), welche durch Aktivierung von heterotrimeren G-Proteinen Signaltransduktionswege einleiten. Im Rahmen dieser Arbeit wurde ein genetischer Ansatz gewählt, um die potentielle Rolle von MLO-Proteinen als G-Protein gekoppelte Rezeptoren in Arabidopsis zu untersuchen. Dabei wurde gezeigt, dass die MLO-vermittelte Mehltauinfektion unabhängig von der Aktivität heterotrimerer G-Proteine ist. Vielmehr liefert die vorliegende Arbeit Hinweise für eine Funktion des heterotrimeren G-Protein Komplexes in der Pflanzenabwehr gegenüber Mehltaupilzen sowie in der Vermittlung von Immunantworten nach Erkennung von Mikroben-assoziierten molekularen Signaturen (MAMPs; microbe-associated molecular patterns).

Des Weiteren deuten Analysen von Sekundärmetaboliten in Arabidopsis darauf hin, dass der adaptierte Mehltaupilz, *Golovinomyces orontii*, dazu fähig ist, die Akkumulation des Abwehr-relevanten Indol-Glucosinolats, 4MI3G (4-Methoxyindol-3-ylmethylglucosinolat), zu unterdrücken und damit den PEN2-anhängigen Abwehrmechanismus zu inhibieren. Dieser Prozess ist abhängig vom funktionalen MLO-Protein und lässt daher vermuten, dass die effektive Unterdrückung pflanzlicher Abwehr durch den Mehltaupilz entweder durch die Manipulation des MLO-Proteins durch den Pilz erfolgt oder aber die Invasion des Pilzes in die Pflanzenzelle voraussetzt. Weitere Ergebnisse aus dieser Studie demonstrieren, dass MLO-Proteine die transkriptionelle Aktivierung von Abwehrgenen nach Inokulation mit dem Mehltaupilz sowie nach Behandlung mit MAMPs negativ regulieren. Letzteres weist auf die Funktion von MLO-Proteinen in MAMP-induzierten Abwehrmechanismen hin.

1. Introduction

Plants are permanently exposed to a wide range of pathogens including viruses, bacteria, fungi and oomycetes that have different infection strategies and life styles. While necrotrophic parasites feed on dead plant tissue, biotrophic pathogens entirely rely on the living plant and its metabolites. Nevertheless, plant disease represents the exception and most plants are immune to the majority of pathogenic microbes. This phenomenon is termed 'non-host resistance' and describes the immunity of an entire plant species against all variants of a non-adapted pathogen species (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005; Lipka et al., 2010). Non-host resistance defines the most robust and durable form of plant immunity and has been proposed to comprise successive layers including constitutive plant barriers as well as inducible reactions (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005; Lipka et al., 2010). The former constitutes physical and chemical barriers like a rigid cell wall and toxic compounds. If a pathogen successfully evades these constitutive plant defense barriers it faces the risk of recognition by plant membrane-localized pattern recognition receptors (PRRs), which sense highly conserved microbial molecules, the microbe-associated molecular patterns (MAMPs). Following MAMP perception a plethora of immune responses is initiated leading to MAMP-triggered immunity (MTI), which defines the first layer of plant innate immunity (Chisholm et al., 2006; Jones and Dangl, 2006; Bent and Mackey, 2007; da Cunha et al., 2007; Dodds and Rathjen, 2010). Successful pathogens evolved strategies to suppress MTI by delivering effector molecules into the plant cell enabling plant colonization. In turn plants possess resistance (R) proteins to recognize effectors, leading to effector-triggered immunity (ETI) defined as the second layer of plant immunity.

1.1 Microbe-associated molecular pattern-triggered immunity (MTI)

Like in animals, cell surface pattern recognition receptors (PRRs) initiate immune responses upon detection of microbe-associated molecular patterns (MAMPs). MAMPs represent highly conserved and indispensable microbial structures, including bacterial lipopolysacharides (LPS), flagellin, coldshock proteins and elongation factor Tu (EF-Tu); fungal chitin, ergosterol and xylanase as well as β-glucan, Pep-13 and heptaglucosides from oomycetes (Boller and Felix, 2009). PRRs consist of extracellular leucine-rich repeat (LRR) or peptidoglucan-bindig LysM domains and are largely grouped into receptor-like kinases (RLKs) and receptor-like proteins (RLPs) according to the presence or absence of an

intracellular kinase domain (Boller and Felix, 2009). The best-characterized PRRs are the flagellin sensing receptor kinase FLS2 (Gómez-Gómez and Boller, 2000) and the elongation factor EF-Tu receptor EFR (Zipfel et al., 2006). The highly conserved flagellin and EF-Tu epitopes, flg22 and elf18, respectively are sufficient to trigger MAMP responses (Felix et al., 1999; Kunze et al., 2004). While FLS2 orthologues are present in tomato, tobacco and rice, EF-Tu perception is restricted to Brassicacea (Felix et al., 1999; Kunze et al., 2004; Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008). Upon flg22 binding, FLS2 rapidly forms a complex with the LRR-RLK BAK1 (BRI1-associated receptor kinase 1), which is required for full activation of subsequent defense responses (Chinchilla et al., 2007). Other known plant PRRs are the rice LRR-RLK XA21 (for a sulfated peptide from the *Xanthomonas* protein Ax21) (Lee et al., 2009), the LysM-RLP CeBiP from rice (for fungal chitin) (Kaku et al., 2006), the tomato LRR-RLPs LeEIX1/2 (for fungal xylanase EIX) (Ron and Avni, 2004), the LysM-RLK CERK1 from Arabidopsis (for fungal chitin) (Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010) and legume NFR1/5 (for rhizobial Nod factor) (Radutoiu et al., 2007). Plants also evolved receptors that sense endogenous molecules termed danger-associated molecular patterns (DAMPs). Classical DAMPs are plant cell wall fragments, the oligogalacturonides (OGs), that are released by microbial enzymes and are likely perceived by the wall-associated kinase 1 (WAK1) (D'Ovidio et al., 2004; Brutus et al., 2010). Recent studies identified the endogenous Arabidopsis elicitor Pep1 and its two LRR-RLKs PEPR1 and PEPR2 (Huffaker et al., 2006; Yamaguchi et al., 2006; Huffaker and Ryan, 2007; Krol et al., 2010; Yamaguchi et al., 2010). Pep1, which originates from its precursor PROPEP1, is generated in response to pathogens and their MAMPs. It activates immune responses as well as expression of its own gene and therefore has been proposed to act as a MAMP amplifier (Huffaker and Ryan, 2007).

Recognition of MAMPs and DAMPs by the respective PRRs triggers a common set of defense responses indicating that down-stream signaling events converge to a stereotypical response. Early MAMP responses that occur within minutes are ion fluxes across the plasma membrane like Ca^{2+} influx, generation of reactive oxygen species (ROS) and nitric oxide (NO), activation of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades as well as ethylene production (Schwessinger and Zipfel, 2008; Boller and Felix, 2009; Boudsocq et al., 2010). Moreover, accumulation of antimicrobial compounds and reprogramming of gene expression occur. Typical late responses, which develop over one to several days, comprise callose deposition and inhibition of seedling growth (Boller and Felix, 2009). To date, the contribution of the individual defense responses

to the establishment of robust immunity as well as the mechanisms leading to such diverse outputs are largely unknown.

1.2 Effector-triggered immunity (ETI)

Successful pathogens are able to suppress MTI through the delivery of effector molecules into the plant cell. Bacteria transfer effector proteins by a dedicated needle apparatus, the type III secretion system (TTSS), whereas effector delivery of fungal and oomycete parasites occurs via a specialized infection structure, the haustorium, which seems to involve secretory pathways (Cunnac et al., 2009; Panstruga and Dodds, 2009). The development of effector proteins by plant pathogens led to the evolution of plant resistance (R) proteins that specifically recognize effectors (gene-for-gene resistance) (Flor, 1971). In absence of a matching R protein the effector escapes the recognition by the plant, resulting in plant disease (compatible interaction), whereas presence of a cognate R protein leads to termination of pathogen growth (incompatible interaction) (Chisholm et al., 2006). The recognized effector is referred to as avirulence (Avr) protein. In contrast to non-host immunity, which acts at the species level, R gene-mediated resistance is cultivar-specific.

Most R proteins belong to the intracellular NB-LRR protein family with a nucleotide binding site (NB) and leucin-rich repeat (LRR) domains, that can be further divided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor (TIR) NB-LRR according to their N-terminus (Dangl and Jones, 2001). The best characterized members of the NB-LRR class include RPS2, RPM1 and RPS5 that confer resistance to *Pseudomonas syringae* pv. *tomato* bacteria carrying the corresponding effector.

Multiple *R* genes have been identified that encode transmembrane receptor-like kinase (RLK) or receptor-like proteins (RLP) with an extracellular LRR including the well-studied tomato RLPs of the Cf family (Jones et al., 1994; Bent and Mackey, 2007).

R proteins can recognize effectors either directly by physical interaction or indirectly by detecting effector-mediated modifications of plant targets. Direct effector recognition has been reported in few cases including the R/Avr protein interactors Pi-ta/AvrPita in rice, L/AvrL567 or M/AvrM in flax (Jia et al., 2000; Dodds et al., 2004; Dodds et al., 2006; Catanzariti et al., 2010; Dodds and Rathjen, 2010). Indirect effector recognition has been observed in a number of cases and is often referred to as the ‘guard’ model (Van Der Biezen and Jones, 1998; Dangl and Jones, 2001). This model postulates that R proteins guard a host protein (guardee), which is targeted and modified by pathogen effectors. One of the best-studied guardees is Arabidopsis RIN4 (RPM1 INTERACTING PROTEIN 4), which is

guarded by the two NB-LRR proteins RPM1 (RESISTANCE TO PSEUDOMONAS MACULICOLA 1) and RPS2 (RESISTANCE TO PSEUDOMONAS SYRINGAE 2) and is targeted by the sequence-unrelated *P. syringae* effectors, AvrRpm1, AvrB and AvrRpt2. RPM1-mediated disease resistance is activated by AvrRpm1- or AvrB-mediated phosphorylation of RIN4, whereas the protease effector AvrRpt2 cleaves RIN4 for degradation, which in turn activates RPS2-mediated resistance (Mackey et al., 2002; Axtell and Staskawicz, 2003; Mackey et al., 2003).

The signal transduction pathway downstream of activated plant NB-LRR receptors is poorly understood. However, EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) is required for signaling of TIR-NB-LRRs, whereas NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE 1) mediates signaling of CC-NB-LRR proteins (Parker et al., 1996; Aarts et al., 1998; Peart et al., 2002; Hu et al., 2005; Wiermer et al., 2005). EDS1 and its interacting partners PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG101 (SENESCENCE ASSOCIATED GENE 101) are important activators of salicylic acid signaling (1.3) and play therefore also a central role in basal resistance (Feys et al. 2001, 2005). Forward genetic screens revealed a requirement of HSP90 (HEAT SHOCK PROTEIN 90), RAR1 (REQUIRED FOR MLA12 RESISTANCE) and SGT1 (SUPPRESSOR OF G2 ALLELE OF SKP1) for function of some NB-LRR proteins (Austin et al., 2002; Azevedo et al., 2002; Muskett et al., 2002; Tornero et al., 2002; Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004).

ETI-induced defense responses show significant overlap with those of MTI (Nimchuk et al., 2003; Nürnberger et al., 2004). However, effector recognition by R proteins results in a stronger and faster immune response that is usually associated with localized programmed death of the attacked cell, the hypersensitive response (HR) (Greenberg and Yao, 2004).

1.3 Phytohormones and plant defense

Plant defense against pathogenic microbes involves regulation by the phytohormones salicylic acid (SA), jasmonic acid (JA), its conjugates termed jasmonates (JAs), and ethylene (ET) (Glazebrook, 2005; Pieterse et al., 2009). A cross-talk between the different hormone signaling pathways allows the plant to fine-tune the induction of defense responses against different pathogens. It is commonly accepted that SA mostly regulates defense against pathogens with a biotrophic lifestyle, whereas defense responses activated by JA and ET signaling rather act against necrotrophic pathogens (Glazebrook, 2005). Mainly antagonistic interactions have been reported for JA and SA, whereas JA and ET often act synergistically

(Glazebrook, 2005; Memelink, 2009). In turn pathogens evolved strategies to manipulate the plant hormone signaling network for their own benefit. For example, several strains of the hemibiotrophic bacterium *P. syringae* produce coronatine, a functional JA mimic, which inhibits SA defense by inducing the JA signaling pathway (Bender et al., 1999). Although the role of SA in resistance against biotrophs and JA in defense against necrotrophs has been demonstrated in many plant-pathogen interactions, the situation is far more complex and plenty of exceptions to this rule exist (Glazebrook, 2005; Pieterse et al., 2009).

SA is synthesized in chloroplasts from chorismate by the isochorismate synthase SID2 (SA INDUCTION-DEFICIENT 2) (Wildermuth et al., 2001; Lu, 2009). SA-induced cellular redox changes lead to monomerization of NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) the key component in SA signaling (Mou et al., 2003; Dong, 2004). NPR1 monomers are translocated from the cytoplasm to the nucleus and modulate the expression of SA-dependent genes including the *PR* (*PATHOGENESIS-RELATED*) genes (Zhang et al., 1999; Tada et al., 2008). EDS1 and its interactors PAD4 and SAG101 are also important players in SA signaling (Feys et al., 2001; Feys et al., 2005; Wiermer et al., 2005).

JA and its conjugates are lipid-derived compounds synthesized in chloroplasts (Wasternack and Kombrink, 2009). JA signaling operates through the F-box COI1 (CORONATINE INSENSITIVE 1) SCF (Skip/Cullin/F box) E3 ubiquitin ligase complex. The SCF^{COI1} complex promotes proteasomal degradation of JAZ (jasmonate-ZIM-domain protein) repressors of transcription factors such as MYC2, thereby activating the expression of JA-responsive genes including the *PDF1.2* (*PLANT DEFENSIN 1.2*) genes (Xie et al., 1998; Dombrecht et al., 2007; Chung et al., 2009).

ET is sensed by a family of endoplasmic reticulum (ER)-associated ET receptors (ETRs) that initiate a regulatory cascade leading to stabilization and activation of transcription factors, such as EIN3, and expression of ethylene-responsive genes including *PDF1.2* (Schaller and Bleecker, 1995; Chao et al., 1997; Hua and Meyerowitz, 1998; Hua et al., 1998; Sakai et al., 1998; Hall et al., 2000; Zhu and Guo, 2008). The membrane protein EIN2 (ETHYLENE INSENSITIVE 2) is a central element of the ethylene signaling pathway, but its biochemical function remains elusive (Alonso et al., 1999).

1.4 Pathogenesis of powdery mildew fungi

Powdery mildew parasites are Ascomycete fungi that cause disease of about 10,000 distinct plant species resulting in severe agricultural yield losses (Takamatsu, 2004; Micali et al., 2008). They are obligate biotrophic phytopathogens that entirely rely on living host tissue and

exclusively infect epidermal cells in order to reproduce (Figure 1.1) (Plotnikova et al., 1998; Eichmann and Hückelhoven, 2008; Micali et al., 2008). Within a few hours after the landing of a conidiospore on the plant leaf surface a germ tube is formed and differentiates into the appressorium, which is used by the fungus to penetrate into the plant epidermal cell. Penetration attempts are typically associated with host cell wall appositions termed papillae, which contain callose (β-1,3-glucan), pectins and phenolics and are formed locally at infection sites (Hardham et al., 2007; Hückelhoven, 2007). Successful host cell invasion results in the development of the specialized fungal infection structure, the haustorium, which is thought to allow nutrient uptake from the plant as well as the delivery of effectors into the host cell to manipulate plant defense (Ellis et al., 2006; Panstruga and Dodds, 2009). Subsequently, the fungus spreads on the plant leaf by epiphytic growth of secondary hyphae. Several days after infection conidiophores are formed and conidiospores are released to initiate new asexual infection cycles.

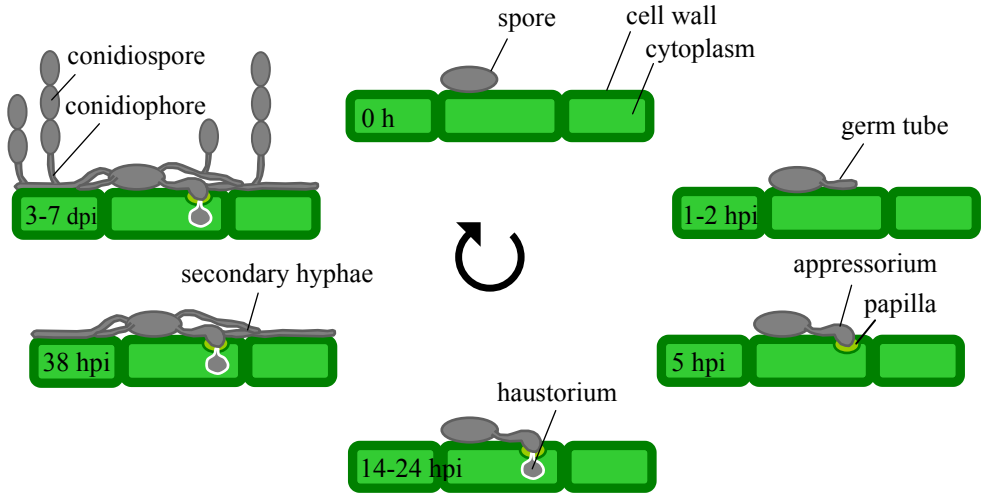

Figure 1.1. Schematic illustration of the asexual life cycle and pathogenesis of the Arabidopsis powdery mildew fungus. The scheme represents the chronological order of events after the landing of a spore on the leaf surface. Within 1–2 hours post-inoculation (hpi) a germ tube is formed, which differentiates into an appressorium for host cell penetration. Penetration attempts are typically associated with the formation of papillae. At about 14–24 hpi, sporelings that successfully penetrated the host cell wall establish a haustorium within the epidermal cell enabling epiphytic growth of secondary hyphae. At 3–7 days post-inoculation (dpi) conidiophores with new conidiospores are formed.

The powdery mildew species *Erysiphe cruciferarum* (Koch and Slusarenko, 1990), *Golovinomyces cichoracearum* (Adam and Somerville, 1996), *Golovinomyces orontii* (Plotnikova et al., 1998) as well as the tomato powdery mildew fungus *Oidium neolycopersici* (Whipps et al., 1998; Ellis and Turner, 2001; Jones et al., 2001; Xiao et al., 2001; Bai et al., 2008; Göllner et al., 2008) are able to successfully reproduce on Arabidopsis plants.

The recent genome sequencing of the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) represents a milestone in the powdery mildew research and provides a powerful tool for investigation and understanding of these biotrophic pathogens (Spanu et al., 2010). Further genome sequencing of the pea and Arabidopsis powdery mildew fungi *Erysiphe pisi* and *G. orontii*, respectively, is currently ongoing. Interestingly, *Bgh* genome analysis revealed a massive retrotransposon proliferation and genome-size expansion. Moreover, several genes encoding enzymes for primary and secondary metabolism as well as transporters are missing in the *Bgh* genome, possibly reflecting the cause or consequence of the biotrophic powdery mildew life style.

1.5 Arabidopsis powdery mildew resistance

In many plant species resistance to powdery mildew fungi is conferred by dominantly or semi-dominantly inherited resistance (*R*) genes, such as those located at the polymorphic barley *Mla* locus (Jørgensen, 1994), the tomato *Ol* locus (Huang et al., 2000; Bai et al., 2005) or the *Run1* gene in grapevine (Donald et al., 2002; Barker et al., 2005), which provide isolate-specific powdery mildew immunity. In contrast, no true *R* gene-mediated isolate-specific resistance against powdery mildew fungi has been reported in Arabidopsis (Göllner et al., 2008). Instead Arabidopsis possesses a unique type of dominantly inherited *R* gene, termed *RPW8* (*RESISTANCE TO POWDERY MILEW 8*), which confers broad-spectrum powdery mildew resistance in several Arabidopsis accessions (Xiao et al., 2001). Examination of the sequence polymorphism of 32 Arabidopsis accessions at the *RPW8* locus revealed that this locus defines a key player in resistance to powdery mildew disease in Arabidopsis (Xiao et al., 2004). RPW8 proteins are atypical R proteins that contain an N-terminal transmembrane domain and a CC domain, but lack the typical NB site and LRR motifs characteristic for most R proteins (Wang et al., 2007). Interestingly, RPW8 resistance shares characteristics with *R* gene-mediated immunity. Both types of resistance are dependent on SA, require EDS1 and result in HR-like cell death (Xiao et al., 2001; Xiao et al., 2003; Xiao et al., 2005). Intriguingly, recent studies have revealed that *RPW8*-mediated broad-spectrum powdery mildew resistance involves specific targeting of RPW8 to the haustorial complex of the invading pathogen (Wang et al., 2009).

In a forward genetic screen for Arabidopsis mutants that displayed enhanced powdery mildew (*G. cichoracearum*) resistance in the absence of constitutive *PR1* gene expression three recessive mutants, termed *enhanced disease resistance* (*edr*) *1*, *edr2* and *edr3*, were isolated (Frye and Innes, 1998; Frye et al., 2001; Tang and Innes, 2002; Tang et al., 2005a; Tang et al.,

2005b; Tang et al., 2006). Powdery mildew development in all *edr* mutants was arrested after successful host cell penetration but before conidiophore formation. This late-onset of resistance was dependent on SA and accompanied by accelerated cell death leading to large necrotic lesions. The *EDR1* gene encodes a CTR1-like protein kinase, *EDR2* encodes a mitochondrial protein with a pleckstrin homology (PH) domain and a steroidogenic acute regulatory protein-related lipidtransfer (START) motif and *EDR3* encodes a dynamin-like protein with an N-terminal GTPase domain and a C-terminal GTPase effector region (Frye and Innes, 1998; Tang et al., 2005a; Tang et al., 2005b; Tang et al., 2006).

In an independent screen for *powdery mildew resistant* (*pmr*) Arabidopsis mutants, in tolal six *pmr* mutants, *pmr1* to *pmr6*, with enhanced resistance to the virulent powdery mildew fungus *G. cichoracearum* were identified and to date four of them were cloned (Vogel and Somerville, 2000; Vogel et al., 2002; Jacobs et al., 2003; Nishimura et al., 2003; Vogel et al., 2004; Consonni et al., 2006).

The *PMR2* gene was found to be allelic to *MLO2* (*MILDEW RESISTANCE LOCUS O 2*), which encodes one of 15 Arabidopsis members of a heptahelical plasma membrane-localized protein family that show high sequence similarity to barley *Hv*MLO, the founder of this plant-unique protein family (see 1.5.3) (Büschges et al., 1997; Consonni et al., 2006).

PMR4, also termed GLUCAN SYNTHASE-LIKE5 (GSL5), was identified as a wound- and pathogen associated callose synthase (Jacobs et al., 2003; Nishimura et al., 2003). Interestingly, *pmr4* mutants were described to constitutively hyperaccumulate SA and mutations in components of the SA pathway suppressed *pmr4*-mediated resistance (Nishimura et al., 2003). Moreover, *pmr4* plants exhibit enhanced resistance to *Hyaloperonospora parasitica*, indicating that enhanced immunity in this mutant is likely the result of constitutive SA-dependent defense activation that is effective against a broad range of pathogens (Jacobs et al., 2003).

The *pmr5* and *pmr6* mutants exhibited altered cell wall compositions indicating a putative function in the maintenance of cell wall integrity. *PMR6* encodes a pectate lyase-like protein while *PMR5* codes for an unknown protein likely targeted to the ER (Vogel et al., 2002; Vogel et al., 2004). Double mutant analysis suggests that PMR5 and PMR6 likely control parallel defense pathways that are independent of SA, ET and JA signaling. Furthermore, *pmr5* and *pmr6* are fully susceptible to pathogens unrelated to powdery mildew fungi, indicating that both proteins may be true powdery mildew specific compatibility factors.

1.5.1 The role of phytohormones in Arabidopsis powdery mildew defense

First evidence for an involvement of SA signaling in powdery mildew resistance came from early analysis of plants bearing *pad4*, *eds5* or *npr1* mutations, which supported enhanced growth of the adapted powdery mildew fungus *G. orontii* (Reuber et al., 1998). All three components are linked to SA accumulation or signaling (Lu, 2009). In accordance with a role of SA in powdery mildew immunity, mutants with hyperactive SA-dependent defense responses, such as *pmr4* or *edr1*, exhibited enhanced resistance against these fungal pathogens (Frye et al., 2001; Nishimura et al., 2003). Infection with *G. cichoracearum* did not induce JA/ET-dependent *PDF1.2* gene expression, a marker gene of JA/ET-signaling (Zimmerli et al., 2004). Interestingly, ectopic activation of the JA/ET signaling pathway by treatment of Arabidopsis plants with exogenous JA or ethanol reduced the infection of *G. cichoracearum* (Zimmerli et al., 2004). Similarly, a mutation in the cellulose synthase CEV1, which results in constitutive activation of the JA/ET singnaling pathway, led to enhanced resistance against *G. orontii* and *G. cichoracearum* (Ellis and Turner, 2001; Ellis et al., 2002a; Ellis et al., 2002b). Together, these findings suggest that JA/ET-dependent responses are not induced by compatible powdery mildew fungi, but if they are triggered artificially, they are effective. In contrast, the non-adapted powdery mildew fungus *Bgh* induced *PDF1.2* gene expression, indicating that non-adapted powdery mildew pathogens are able to trigger the JA/ET defense pathway (Zimmerli et al., 2004). Therefore, it has been concluded that adapted powdery mildew fungi, such as *G. orotnii*, either fail to elicit JA/ET defense or are able to actively suppress it (Zimmerli et al., 2004).

1.5.2 Arabidopsis powdery mildew non-host resistance

Non-host resistance is the most effective and durable form of plant immunity (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005; Lipka et al., 2010). Arabidopsis non-host resistance to non-adapted powdery mildew fungi comprises two layers: pre-invasion defense that controls fungal penetration at the cell periphery and post-invasion defense, which can be associated with a cell death response (Collins et al., 2003; Lipka et al., 2005). Three key components designed PENETRATION1 (PEN1), PEN2 and PEN3 of pre-invasion resistance were identified in a genetic screen for Arabidopsis mutants with enhanced entry success of the non-adapted powdery mildew fungi *Bgh* and *E. pisi* (Collins et al., 2003; Lipka and Panstruga, 2005; Stein et al., 2006). Genetic analysis suggests that PEN1 and PEN2 act in

independent entry control pathways, whereas PEN2 cooperates with PEN3 in the same pathway (Lipka et al., 2005; Stein et al., 2006).

PEN1 encodes a plasma membrane-associated syntaxin with a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) domain, which forms a pathogen-induced ternary SNARE complex with the SNARE protein SNAP33 (Synaptosomal-associated protein 33) and the endomembrane-anchored VAMP721 or VAMP722 (vesicle associated membrane protein 721/722) (Kwon et al., 2008). In eukaryotes, ternary SNARE complexes are key players in vesicle-associated membrane fusion and secretion processes (Lipka et al., 2007). Therefore, it is thought that the PEN1-SNAP33-VAMP721/722 complex mediates pathogen-induced vesicle-dependent secretion at the plasma membrane. Moreover, PEN1 was found to focally accumulate in papillae at sites of attempted fungal penetration, assuming that PEN1-mediated secretion possibly delivers anti-microbial as well as cell wall-reinforcing cargo (Assaad et al., 2004; Bhat et al., 2005; Kwon et al., 2008; Meyer et al., 2009).

PEN2 encodes an atypical myrosinase associated with peroxisomes, which was shown to participate in the metabolism of a group of tryptophan (Trp)-derived secondary metabolites, the indole glucosinolates, to release potential antimicrobial products (Lipka et al., 2005; Bednarek et al., 2009). Specifically, the 4-methoxyindol-3-ylmethylglucosinolate (4MI3G) is supposed to be the biologically relevant PEN2 substrate leading to formation of yet unknown toxic compounds that are important for the restriction of fungal growth (Supplementary Figure 6.1) (Bednarek et al., 2009). PEN2-dependent hydrolysis of 4MI3G is also required for PMR4/GSL5-mediated callose accumulation after flg22 treatment (Clay et al., 2009). Interestingly, phylogenetic analysis suggests that the *PEN2* gene is an evolutionarily recent invention of Arabidopsis (Xu et al., 2004).

PEN3 encodes the pleiotropic drug resistance 8 (PDR8) ATP binding cassette (ABC) transporter (Stein et al., 2006). The absence of PEN3 results in hyperactivation of the SA-signaling pathway, which is suppressed in *pen2 pen3* double mutants, indicating that accumulation of toxic PEN2-derived products results in the hyperactivation of the SA-signaling in *pen3* mutant plants. Thus, it has been proposed that PEN2 and PEN3 act in the same defense pathway, where PEN3 transports toxic PEN2 hydrolysis products across the plasma membrane to restrict fungal growth (Lipka et al., 2005; Stein et al., 2006).

In contrast to PEN1, the PEN2/PEN3-dependent pathway contributes to defense against a broad range of pathogens with different life-styles (Lipka et al., 2005; Hiruma et al., 2010; Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010).

Although *pen* single mutants exhibit impaired non-host resistance at the cell periphery resulting in enhanced entry rates of non-adapted powdery mildew fungi, they mount effective post-invasion immunity, which is accompanied by a HR-like cell death response ultimately terminating fungal colonization (Lipka et al., 2005). Analysis with various mutant combinations revealed a requirement of EDS1, PAD4 and SAG101 for post-invasion defense (Lipka et al., 2005). Dramatic infection phenotypes were observed on *pen2 pad4 sag101* triple mutants in which *Bgh* was capable to complete its life cycle and growth and reproduction of *E. pisi* was macroscopically detectable (Lipka et al., 2005). Thus, removal of three genes renders Arabidopsis a fully susceptible plant for non-adapted powdery mildew fungi.

1.5.3 Broad-spectrum powdery mildew *mlo* resistance

In the genetic screen for Arabidopsis *pmr* mutants (see 1.5), the *PMR2* gene was isolated and later identified to be allelic to *MLO2* (*MILDEW RESISTANCE LOCUS O 2*) (Vogel and Somerville, 2000; Consonni et al., 2006). Arabidopsis MLO2 is one of 15 members of the seven transmembrane (7TM) domain MLO protein family (Devoto et al., 2003). Recessively inherited loss-of-function mutations of the founder of this plant unique gene family, barley *MLO*, confer durable and broad-spectrum resistance against all isolates of the compatible powdery mildew fungus *Bgh* (Jørgensen, 1992; Büschges et al., 1997). In the absence of MLO, fungal spores fail to penetrate the host epidermal cell, thus rendering the plant resistant to the pathogen. Because successful powdery mildew pathogenesis requires the presence of MLO, this protein is considered to be a negative modulator of powdery mildew defense (Panstruga and Schulze-Lefert, 2003; Panstruga, 2005). The phenomenon of *mlo*-based resistance was long considered as a unique type of plant immunity restricted to barley (Jørgensen, 1992; Jørgensen, 1994). However, broad-spectrum immunity against powdery mildew fungi based on loss-of-function *mlo* alleles has recently also been shown in the dicot plant species Arabidopsis and tomato (*Solanum lycopersicum*) (Consonni et al., 2006; Bai et al., 2008). These findings imply a conserved requirement of MLO proteins in pathogenesis to distantly related powdery mildew fungi in dicot and monocot plants. Mutations in the Arabidopsis *MLO2* gene and its closest homologs, *MLO6* and *MLO12* were found to be required for full powdery mildew resistance, while mutations in *MLO2* alone confer only partial resistance to the compatible pathogens *G. orontii* and *G. cichoracearum* (Consonni et al., 2006). Arrest of fungal penetration in *mlo* mutant plants is linked with localized cell wall remodelling leading to the formation of callose-containing papillae (Jørgensen and Mortensen, 1977; Skou, 1982, , 1985; Bayles et al., 1990; Wolter et al., 1993; Thordal-

Christensen et al., 1997). The fact that presence of MLO is absolutely required by powdery mildew fungi to successfully infect the host plant suggests that these pathogens may abuse MLO function(s) to suppress host defense (Panstruga, 2005).
Interestingly, barley and Arabidopsis *mlo* mutants exhibited enhanced susceptibility to fungal pathogens with a hemibiotrophic and necrotrophic lifestyle, indicating that MLO proteins influence pathogenesis of fungi with diverse lifestyles (Kumar et al., 2001; Jarosch et al., 2005; Consonni et al., 2006).

1.5.3.1 Requirements for *mlo*-mediated powdery mildew resistance

Arabidopsis *mlo*-based powdery mildew resistance requires functional PEN1- and PEN2/PEN3 defense pathways (see 1.5.2), as mutations in one of the three *PEN* genes in the *mlo2* mutant background restore penetration rates of the respective powdery mildew fungus to wild-type like levels (Consonni et al., 2006). The barley orthologue of the PEN1 syntaxin, HvROR2, as well as a SNAP25 homolog, HvSNAP34, are required for *mlo* resistance in the crop plant barley, indicating that vesicle-mediated secretion is a common feature of *mlo* immunity in monocots and dicots (Freialdenhoven et al., 1996; Collins et al., 2003). Contrary to *mlo2*/*pen1* mutants, *mlo2*/*pen2* and *mlo2*/*pen3* double mutants not only display restored powdery mildew penetration rates but also wild-type like conidiation of the fungus, suggesting a role for PEN2/PEN3 in both, pre- and post-invasion defense, in the context of *mlo*-based immunity (Consonni et al., 2006). Besides the requirement of PEN2-hydrolyzed Trp-derived indolic compounds also the Trp-derived phytoalexin camalexin partially contributes to *mlo2*-mediated powdery mildew resistance (Consonni et al., 2010). Consistent with the role of Trp-derived indolic metabolites in *mlo2*-based resistance, triple mutants defective in MLO2 and the cytochrome P450 monooxygenases CYP79B2 and CYP79B3, which convert Trp into the precursor of most known Trp-derived metabolites, restored wild-type like susceptibility to the compatible powdery mildew fungus *G. orontii* (Supplementary Figure 6.1) (Consonni et al., 2010).
Arabidopsis mutant analysis revealed that *mlo*-mediated powdery mildew resistance acts independently of the SA-, JA- and ET- defense pathways (Consonni et al., 2006). In contrast, disruption of the actin cytoskeleton using pharmacological inhibitors as well as the ectopic expression of an *ADF* (*ACTIN-DEPOLYMERIZING FACTOR*) gene in barley epidermal cells revealed a requirement for a functional actin cytoskeleton in *mlo*-mediated resistance (Miklis et al., 2007). Plant cells attacked by fungal pathogens respond with rapid cellular rearrangement involving reorganization of the actin cytoskeleton, mobilization of organelles

and focal deposition of antimicrobials and cell wall material towards the fungal infection site (Schmelzer, 2002; Takemoto and Hardham, 2004; Lipka and Panstruga, 2005; Takemoto et al., 2006; Hardham et al., 2007). Perturbed actin cytoskeleton function also impeded defense against a range of fungal non-host pathogens (Kobayashi et al., 1997; Yun et al., 2003; Shimada et al., 2006; Miklis et al., 2007). Therefore, it is conceivable that a functional actin cytoskeleton is a general requirement for antifungal defense.

An *in vitro* screen for novel calmodulin (CaM) interacting proteins revealed the interaction between a rice MLO homolog and the calcium sensor calmodulin (Kim et al., 2002a). Likewise, interactions of CaM with the barley as well as several Arabidopsis MLOs were confirmed by yeast split-ubiquitin assays and FRET (fluorescence resonance energy transfer) microscopy, suggesting that CaM binding is a general feature of MLO proteins (Kim et al., 2002b; Bhat et al., 2005). Consistently, a calmodulin-binding domain (CaMBD), located in the cytoplasmic C-terminus of MLO, is conserved throughout the MLO protein family (Kim et al., 2002a; Kim et al., 2002b). Barley MLO variants carrying mutations in the CaMBD, which disrupt the interaction with CaM, only partially complemented *mlo* resistance, suggesting that CaM is an activator of MLO function (Kim et al., 2002b).

Transient expression studies in single barley leaf epidermal cells revealed that overexpression of the potential barley apoptosis suppressor, Bax inhibitor 1 (BI-1), as well as overexpression of a constitutive active variant of a barley calcium-dependent protein kinase (CDPK) isoform partially compromises *mlo*-mediated resistance to the adapted barley powdery mildew fungus *Bgh* (Hückelhoven et al., 2003; Freymark et al., 2007). Furthermore, in a barley genetic suppressor screen for components required for *mlo* resistance (*ror*), two mutants were isolated that exhibit partially disabled *mlo* resistance (Freialdenhoven et al., 1996). While the *ROR2* gene was cloned and shown to encode a homolog of the Arabidopsis PEN1 syntaxin, the identity of *ROR1* remains elusive (Collins et al., 2003).

1.5.3.2 Powdery mildew *mlo* resistance is reminiscent of non-host immunity

Interestingly, comparison of histological and phytopathological characteristics as well as conserved genetic requirements reveals common features of powdery mildew non-host resistance and *mlo*-mediated immunity. Therefore, it has been hypothesized by Humphry and colleagues (2006) that powdery mildew non-host resistance and *mlo*-mediated immunity are mechanistically related or possibly identical. In summary, both types of resistance confer durable and broad-spectrum immunity and mainly terminate powdery mildew pathogenesis at the stage of plant cell invasion, an early time point of fungal infection. Abortion of pathogen

penetration in both cases is associated with the formation of papillae. Moreover, *mlo*-based powdery mildew resistance and non-host penetration immunity depend on a functional actin cytosekeleton. Arabidopsis non-host resistance to the non-adapted barley and pea powdery mildew fungi, *Bgh* and *E. pisi*, respectively, requires the PEN1-dependent secretory defense pathway and the formation of PEN2-hydrolyzed antifugal glucosinolate products as well as PEN3, which has been implicated in the transport of these toxic compounds. Similarly, *mlo*-mediated resistance to the adapted-powdery mildew fungi *G. orontii* and *G. cichoracearum* also depends on functional PEN1- and PEN2/PEN3-defense mechanisms. Taken together, lack of MLO, the key compatibility factor for successful powdery mildew pathogenesis, appears to convert the compatible interaction between the adapted powdery mildew fungus and its host plant into a non-host interaction. Therefore, it seems that the pathogen encounters the same plant defense barriers on *mlo* mutants as any other non-adapted powdery mildew fungus.

1.5.3.3 *mlo* mutants display pleiotropic phenotypes

Mutations in barley and Arabidopsis *MLO* genes are accompanied by additional developmentally controlled pleiotropic phenotypes. Developmentally controlled spontaneous callose deposition in mesophyll cells and early leaf chlorosis/necrosis were reported in *mlo* mutants (Wolter et al., 1993; Piffanelli et al., 2002; Consonni et al., 2006; Consonni et al., 2010). In Arabidopsis, callose accumulation in *mlo2* mutants is mediated by PMR4/GSL5-activity and chlorosis/necrosis represents an accelerated progression of leaf senescence (Consonni et al., 2006; Consonni et al., 2010). Both phenotypes are fully dependent on SA accumulation, which is not essential for *mlo2*-mediated powdery mildew resistance, demonstrating that the pleiotropic effects can be uncoupled from *mlo*-based resistance (Consonni et al., 2006). Comparative metabolomic profiling revealed altered levels of Trp-derived indolic compounds during vegetative development of *mlo2*/*mlo6*/*mlo12* mutants. However, additional mutations in the biosynthesis of indolic metabolites did not suppress the early leaf senescence phenotype of *mlo2* mutants, indicating that these compounds are not the major cause of *mlo2*-associated leaf senescence (Consonni et al., 2010).

1.5.3.4 The MLO protein structure resembles mammalian G-protein coupled receptors

Results from scanning N-glycosylation mutagenesis and MLO-Lep fusion protein assays demonstrated that barley MLO is an integral plasma membrane-resident protein with seven transmembrane (7TM) helices, an extracellularly located N-terminus and a cytoplasmic C-

terminus (Devoto et al., 1999). Thus the domain architecture and subcellular localization of MLO proteins is reminiscent of the G-protein coupled receptor (GPCR) superfamily in metazoans (Temple and Jones, 2007; Oldham and Hamm, 2008). In animals and fungi, GPCRs relay extracellular stimuli into intracellular signaling events by the activation of heterotrimeric G-proteins (see 1.6). To date knowledge about potential plant GPCRs is still sparse and although no significant sequence similarity between mammalian GPCRs and MLO proteins exists, these plant-specific 7TM domain proteins remain obvious receptor candidates for G-protein binding and signaling. Moreover, a common strategy of several human parasites is the corruption of host GPCRs for successful infection. Prominent examples include the malaria parasite *Plasmodium vivax*, the human immunodeficiency virus type 1 (HIV-1) and the pathogenic bacterium *Streptococcus pneumoniae* (Pease and Murphy, 1998). Together, these facts raise the question whether MLO proteins may play a similar role during plant colonization by powdery mildew fungi.

A potential involvement of heterotrimeric G-proteins in MLO function was tested in a combined pharmacological and genetic study in barley (Kim et al., 2002b). However, transient overexpression of constitutive active and dominant negative mutant variants of the single copy HvGα subunit in single barley leaf epidermal cells did not affect powdery mildew infection in susceptible wild-type or resistant *Hvmlo* mutant barley plants. Similarly, application of G-protein modulating chemicals had no effect on powdery mildew susceptibility, therefore rather precluding a role of heterotrimeric G-protein signaling in MLO-dependent powdery mildew pathogenesis.

1.6 Arabidopsis heterotrimeric G-protein signaling

Like MLO proteins, GPCRs possess 7TM domains with an extracellular N- and intracellular C-terminus. GPCRs activate heterotrimeric G-protein signaling, one of the most evolutionarily conserved signaling pathways in metazoans, by relying extracellular signals through their intracellularly associated G-proteins (Fig. 1.2) (Oldham and Hamm, 2008). The canonical G-protein consists of three distinct subunits, Gα, Gβ and Gγ, which form a heterotrimeric complex in the inactive state. Ligand binding to a GPCR induces the conversion of an inactive G-protein into its active conformation via exchange of GDP by GTP bound to Gα. As a result, Gα–GTP separates from the Gβγ dimer and both, Gα–GTP and the Gβγ dimer, can activate downstream effectors ultimately leading to a cellular response. The intrinsic hydrolytic GTPase activity of Gα recovers the GDP-bound state, which promotes

reassociation of the complex into its inactive form. RGS (Regulator of G-protein Signaling) proteins accelerate the GTPase activity of Gα.

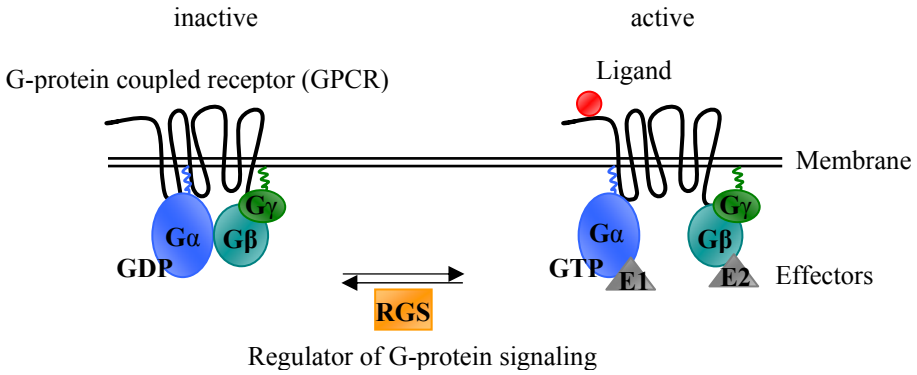

Figure 1.2. Classical model for heterotrimeric G-protein signaling. Ligand binding by the corresponding GPCR activates the heterotrimeric G-protein complex causing Gα to exchange GDP for GTP. Subsequently, Gα-GTP separates from the Gβγ dimer. Both, Gα and Gβγ can then activate downstream effectors (E). The intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP and returns the G-protein complex into its inactive form. Regulator of G-protein Signaling (RGS) proteins accelerate the Gα GTPase activity.

Signal transduction via G-proteins is a conserved mechanism found in all eukaryotes. Mammals have 23 different Gα, 5 Gβ and 12 Gγ subunits mediating signaling in processes such as vision, olfaction, taste and in neuroendocrine pathways (Temple and Jones, 2007; Oldham and Hamm, 2008). Therefore, it is not surprising that heterotrimeric G-protein cascades are targets for approximately 30 % of all approved pharmaceuticals (Temple and Jones, 2007; Oldham and Hamm, 2008; Williams and Hill, 2009). In sharp contrast the set of known heterotrimeric signaling components in plants is much simpler. The fully sequenced reference plant *A. thaliana* has a single canonical Gα subunit (GPA1), one Gβ subunit (AGB1), two Gγ subunits (AGG1 and AGG2) and a single RGS protein (RGS1) (Chen et al., 2003; Temple and Jones, 2007). Interestingly, kinetic studies revealed that the Arabidopsis Gα subunit has one of the slowest GTPase activity and the fastest GDP release properties, which is the limiting step in all previously described non-plant Gα proteins (Johnston et al., 2007). These findings indicate that unlike in mammals the Arabidopsis Gα protein is in the activated GTP-bound state by default. Despite its simplicity, the Arabidopsis heterotrimeric G-protein complex has been implicated in a broad diversity of biological processes, including seed germination, cell division, hormone sensitivity, sugar sensing, pathogen defense and abiotic stress responses (Assmann, 2002; Jones and Assmann, 2004; Perfus-Barbeoch et al., 2004; Chen, 2008). However, elucidation of a complete cascade of G-protein signaling in plants is lacking. To date, neither the downstream signaling components nor the upstream GPCRs have been unequivocally identified. Intriguingly, currently no bona fide plant GPCR has been described and only few candidate proteins exist, whereas approximately 1000

GPCRs have been estimated for mammals. Among these putative Arabidopsis GPCRs GCR1 (G-Coupled Receptor 1) shares the highest sequence similarity (~25 %) with known GPCRs and physically interacts with the Arabidopsis Gα subunit, GPA1. However, a ligand for GCR1 has not yet been identified (Josefsson and Rask, 1997; Plakidou-Dymock et al., 1998; Pandey and Assmann, 2004). The Arabidopsis RGS1 protein is also a well-characterized candidate plant GPCR. This protein has an unusual domain structure composed of an N-terminal 7TM domain and a C-terminal RGS box (Chen et al., 2003). Therefore, unlike typical GPCRs, which activate Gα proteins, RGS1 may function as a receptor that accelerates Gα GTPase activity resulting in inactivation of Gα (Chen et al., 2003). Genetic analysis revealed D-glucose as a putative ligand for RGS1 (Grigston et al., 2008). Recent studies reported about novel GPCR-type G-proteins termed GTG1 and GTG2 that interact with the Arabidopsis Gα subunit (Pandey et al., 2009). Interestingly, GTGs have a predicted nine TM domain topology with a classic GTP-binding as well as a GTP-hydrolysis domain. With a total of 15 members the plant specific MLO proteins constitute one of the largest family of 7TM domain proteins in Arabidopsis (Devoto et al., 1999). However, studies performed in barley rather preclude a role for MLO proteins as GPCRs in the context of powdery mildew pathogenesis (Kim et al., 2002b).

In addition to the canonical heterotrimeric G-protein and small G-proteins, plants have also some unconventional GTP-binding proteins including the above mentioned GTGs as well as XLGs (extra-large GTP-binding proteins) and DRGs (developmentally regulated GTP-binding proteins) (Lee and Assmann, 1999; Li and Trueb, 2000; Assmann, 2002; Ding et al., 2008; Pandey et al., 2009). XLGs contain a Gα-like domain at the C-terminus, which is essential for GTP-binding and GTP-hydrolysis (Ding et al., 2008). Additionally XLGs have an N-terminus with a predicted nuclear localization signal (NLS) that is plant-specific and not found in any non-plant species. DRGs have a conserved GTPase domain motif found in conventional G-proteins but not any other sequence similarities with conventional G-proteins (Li and Trueb, 2000). While plant XLGs are involved in the regulation of root development, responses to sugars, hormones, abiotic stress and pathogens the function of DRGs in plants remains unknown (Ding et al., 2008; Pandey et al., 2008; Zhu et al., 2009).

1.6.1 Arabidopsis heterotrimeric G-protein signaling and plant immunity

A number of early pharmacological analysis originally suggested a role for the heterotrimeric G-protein in plant defense (Legendre et al., 1992; Vera-Estrella et al., 1994; Beffa et al., 1995; Gelli et al., 1997; Mahady et al., 1998; Rajasekhar et al., 1999; Han and Yuan, 2004).

Meanwhile genetic studies with Arabidopsis G-protein mutants have provided conclusive evidence for a function of heterotrimeric G-protein signaling in plant immunity. Arabidopsis mutants lacking the Gβ (AGB1) or the Gγ1 (AGG1) subunit displayed increased susceptibility to the necrotrophic fungi *Plectosphaerella cucumerina*, *Alternaria brassicicola*, *Fusarium oxysporum* and *Botrytis cinerea*, whereas Gα-deficient plants exhibited slightly enhanced resistance to these pathogens (Llorente et al., 2005; Trusov et al., 2006; Trusov et al., 2007). Infection phenotypes of double mutants lacking both Gα and Gβ subunits or Gγ1 and Gγ2 were indistinguishable from Gβ- or Gγ1-single mutants, respectively, suggesting that Gβγ1- and not Gβγ2-mediated signaling plays the leading role in Arabidopsis defense against necrotrophic fungi, which was shown to act independently of SA-, JA- and ET-mediated defense signaling (Trusov et al., 2006; Trusov et al., 2007; Trusov et al., 2008). The increased resistance of Gα mutants was proposed to result from an increase in free Gβγ dimer leading to enhanced Gβγ-mediated defense responses. In contrast, pathogenesis of biotrophic or hemibiotrophic pathogens, including the oomycete *H.arabidopsidis* and the bacterium *P. syringae*, was not affected in G-protein mutants (Llorente et al., 2005; Trusov et al., 2006). However, other data provided evidence for a role of heterotrimeric G-protein signaling in bacterial MAMP responses. For instance, inhibition of stomatal opening by flg22 as part of MTI implicates the Gα subunit, as Gα mutants were impaired in flg22-mediated stomatal closure (Zhang et al., 2008). Moreover, the Gβ subunit was reported to integrate MAMP perception into downstream ROS production, as Gβ-deficient mutants exhibited reduced ROS formation upon treatment with flg22 and elf18 (Ishikawa, 2009). Recent studies reported a function of the unconventional GTP-binding protein XLG2 in defense against *P. syringae* bacteria as well as the non-adapted powdery mildew fungus *E. pisi* (Zhu et al., 2009; Humphry et al., 2010).

1.7 Thesis aims

To date knowledge about potential plant GPCRs is sparse and although no significant sequence similarity between mammalian GPCRs and MLO proteins exist, these plant-unique proteins are considered receptor candidates for G-protein signaling. Previous analysis did not provide any evidence for a requirement of heterotrimeric G-protein signaling for MLO-dependent powdery mildew pathogenesis in barley (Kim et al., 2002b). However, these earlier studies have to be regarded with caution because of the following arguments: 1. The genetic approach used in that analysis was based on transient overexpression and dsRNAi-mediated gene silencing in single barley leaf epidermal cells. 2. Solely a putative function of the barley

Gα subunit was tested. 3. Residue substitutions of the barley Gα subunit supposedly leading to constitutive active or dominant negative protein variants were deduced from the animal or yeast system, questioning a similar function in plants. 4. Pharmacological analysis was performed using chemicals known to manipulate G-protein signaling in the animal system, again questioning an equal effect in plants. In contrast, identification of *mlo2*-mediated powdery mildew resistance in Arabidopsis (Consonni et al., 2006) combined with availability of the Arabidopsis genome sequence and stable sequence-indexed T-DNA insertion mutants allows a more reliable analysis. Consequently, a genetic approach using stable Arabidopsis G-protein signaling mutants was chosen in this study to unequivocally test a potential molecular link between MLO proteins and heterotrimeric G- protein signaling (Section 2.1).

Emerging evidence points to a role of heterotrimeric G-protein signaling in the integration of MAMP perception into downstream responses. Therefore, a putative role of the Arabidopsis heterotrimeric G-protein in MAMP-triggered responses and immunity was investigated in this study (Section 2.3).

The current knowledge about the molecular mechanisms underlying *mlo*-based powdery mildew resistance is sparse. Absence of MLO may result in a constitutive and/or faster activation of defense responses and/or may prohibit defense suppression by powdery mildew fungi. In order to differentiate between these possibilities and to learn more about the molecular basis of *mlo*-mediated resistance a comprehensive comparative analysis of defense marker activation in Arabidopsis wild-type and *mlo2* mutant plants was performed. This analysis was conducted at the transcriptional, hormone, protein and metabolite level (Section 2.2).

2. Results

2.1 A putative function of MLO2 in heterotrimeric G-protein signaling during powdery mildew pathogenesis in Arabidopsis

2.1.1 The Gβ-deficient mutant exhibits increased susceptibility to powdery mildew fungi

To investigate the role of MLO proteins as putative GPCRs, stable T-DNA insertion lines disrupting either the *Arabidopsis thaliana* (here referred to as Arabidopsis) *MLO2* gene or individual members of the heterotrimeric G-protein complex (Gα/GPA1, Gβ/AGB1, Gγ1/AGG1, Gγ2/AGG2 or RGS1) were assessed for their powdery mildew infection phenotype. In addition, a set of respective *mlo2* and G-protein double mutants was generated and likewise inspected following powdery mildew challenge.

Host cell penetration by powdery mildew fungi is essential for successful infection and thus a critical step during pathogenesis. Quantitative analysis of host cell entry by the adapted powdery mildew fungus *Golovinomyces orontii* (*G. orontii*) was performed at 48 hours post inoculation (hpi) and penetration success was calculated as the percentage of germinated fungal spores that developed secondary hyphae. Like wild-type plants, single mutants defective in G-protein signaling were fully susceptible to *G. orontii* (Fig. 2.1A). Slightly enhanced fungal entry rates were observed on Gβ-deficient mutants, *agb1*-2 (T-DNA insertion line) and *agb1*-9 (EMS-mutant) (Fig. 2.1A). This effect was even more pronounced on the respective *mlo2*-6 double mutants, *agb1*-2/*mlo2*-6 and *agb1*-9/*mlo2*-6 (Fig. 2.1A). All other double mutants behaved like resistant *mlo2*-6 plants (Fig. 2.1A).

In the compatible interaction with *G. orontii* high pathogenicity of the host-adapted fungus may mask a contribution of the G-protein complex to powdery mildew defense. Therefore, entry rates of the non-adapted powdery mildew fungus *Erysiphe pisi* (*E. pisi*) on the G-protein mutants were determined at 7 dpi. Again, the *agb1*-2 and *agb1*-9 single mutants exhibited an increased penetration rate of the pea powdery mildew fungus, which was up to 10 % higher compared to wild-type plants (Fig. 2.1B). Similarly, *agb1*-2/*mlo2*-6 and *agb1*-9/*mlo2*-6 double mutants but also *agg1*-1/*mlo2*-6 allowed enhanced plant cell invasion of *E. pisi* (Fig. 2.1B). Taken together, these data suggest a role of the Gβ subunit in preventing plant cell penetration by powdery mildew fungi, termed pre-invasion resistance. Moreover, this function is independent of the presence or absence of the MLO2 protein.

Resistance of Arabidopsis *mlo2* mutants against the compatible powdery mildew fungus *G. orontii* is incomplete, as 40 – 50 % of the spores successfully invade the plant epidermal cells (Consonni et al., 2006). However, fungal development is ultimately terminated by post-

invasion defense mechanisms largely inhibiting fungal conidiation. In order to analyze a putative contribution of the heterotrimeric G-protein to post-invasion defense, quantitative analysis of fungal conidiation was performed. Therefore, the number of conidiophores per single fungal colony was determined at 7 dpi. Remarkably, conidiophore formation of the fungus was at least doubled on all G-protein single mutants compared to wild-type plants, indicating a role of all heterotrimeric G-protein components in post-invasion defense against the adapted powdery mildew fungus *G. orontii* (Fig. 2.1C).

Furthermore, *mlo2*-6 mutants with an additional mutation in either the *agb1*, *agg1* or *agg2* gene allowed increased conidiophore formation of the fungus compared with *mlo2*-6 single mutant plants (Fig. 2.1C).

In summary the presented results point to a function of the Gβ subunit in pre-invasion defense against the adapted and non-adapted powdery mildew fungi *G. orontii* and *E. pisi*, respectively, which is independent of the MLO2 protein. Interestingly, all heterotrimeric G-protein components seem to be involved in post-invasion defense mechanisms against *G. orontii*.

2.1.2 The Gγ1-deficient mutant exhibits reduced *mlo2*-mediated callose deposition

When grown under pathogen-free conditions, barley and Arabidopsis *mlo* mutant plants display pleiotropic phenotypes such as developmentally controlled spontaneous callose accumulation (Wolter et al., 1993; Peterhänsel et al., 1997; Piffanelli et al., 2002; Consonni et al., 2006). In order to test if heterotrimeric G-protein signaling is involved in this process, callose was visualized by aniline blue staining in non-infected G-protein single mutants and respective *mlo2*-6 double mutant plants. G-protein single mutants exhibited no spontaneous callose deposition, whereas the corresponding *mlo2*-6 double mutants behaved like *mlo2*-6 control plants and accumulated callose (Fig. 2.2A). Notably, the *agg1*-1/*mlo2*-6 double mutant showed markedly reduced callose accumulation, indicating a requirement of the Gγ1 subunit for *mlo2*-dependent callose deposition.

To investigate if the Gγ1 subunit may also be involved in callose formation in other processes, callose accumulation after leaf wounding and powdery mildew inoculation was determined. As shown in Figure 2.2B callose was formed at wound sites of Col-0 wild-type, *agg1*-1, *mlo2*-6 and *agg1*-1/*mlo2*-6 leaves. However, *agg1*-1/*mlo2*-6 double mutant plants did not display a callose ring structure around the wound site (see white arrowheads). Callose accumulation after infection with the powdery mildew fungus *E. pisi* was not affected in any of the tested genotypes (Fig. 2.2C).

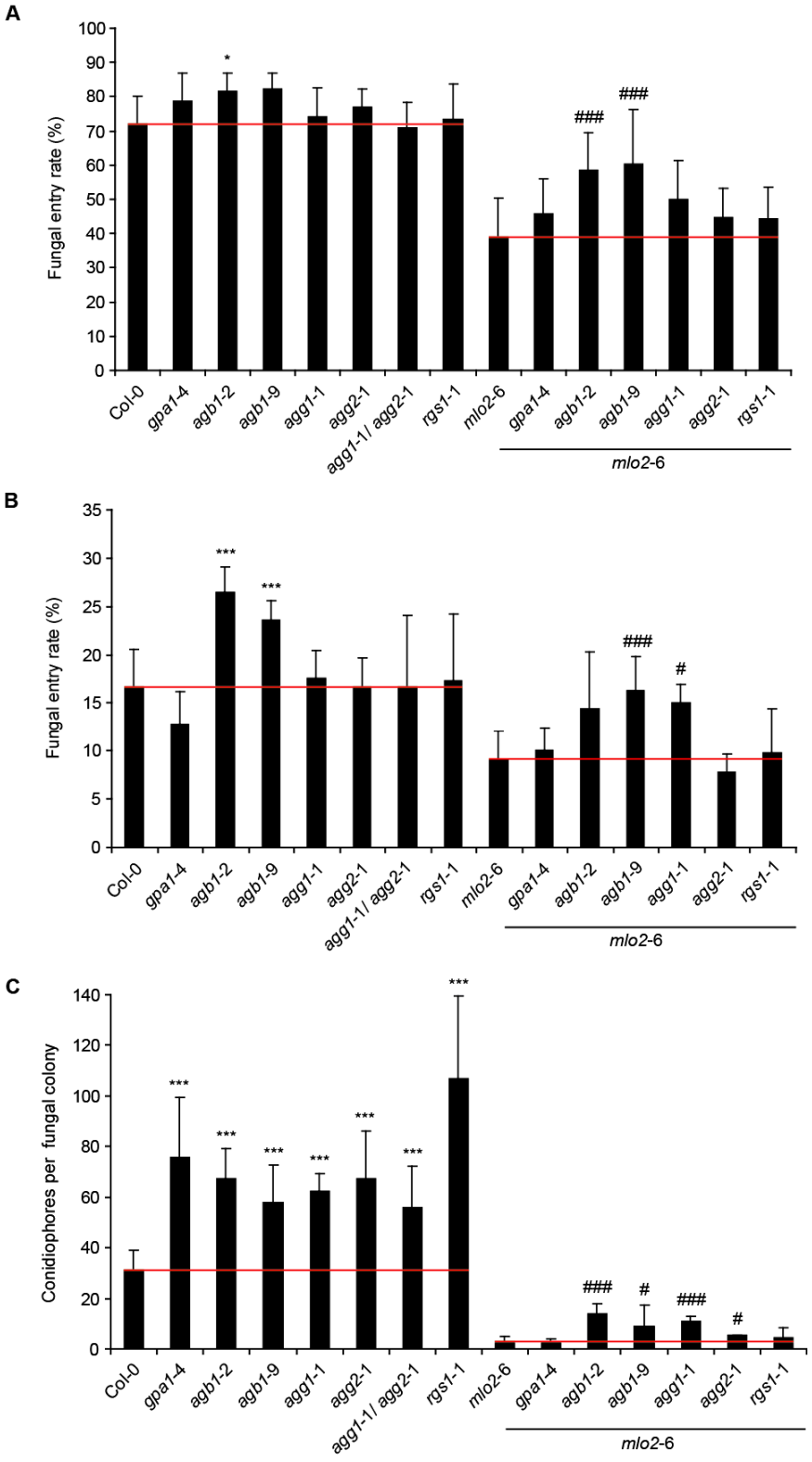

Figure 2.1. Quantitative analysis of powdery mildew infections of G-protein signaling single mutants and corresponding *mlo2* double mutants. Powdery mildew inoculations were performed with conidiospores of the respective fungus on rosette leaves of 4-week-old plants. **(A)** *G. orontii* host cell entry scored at 48 hpi. **(B)** *E. pisi* plant cell entry determined at 7 dpi. **(C)** *G. orontii* conidiophore formation per single fungal colony assessed at 7 dpi. Results represent mean ± SD (n=6 for (A) and (C), n=12 for (B)) of at least three independent experiments. Asterisks indicate a significant difference from Col-0 wild-type (*** P < 0.01, * P < 0.05, Student's t-test) and number signs indicate a significant difference from *mlo2*-6 mutant (### P < 0.01, # P < 0.05, Student's t-test). n: represents the number of leaves tested per genotype in one experiment.

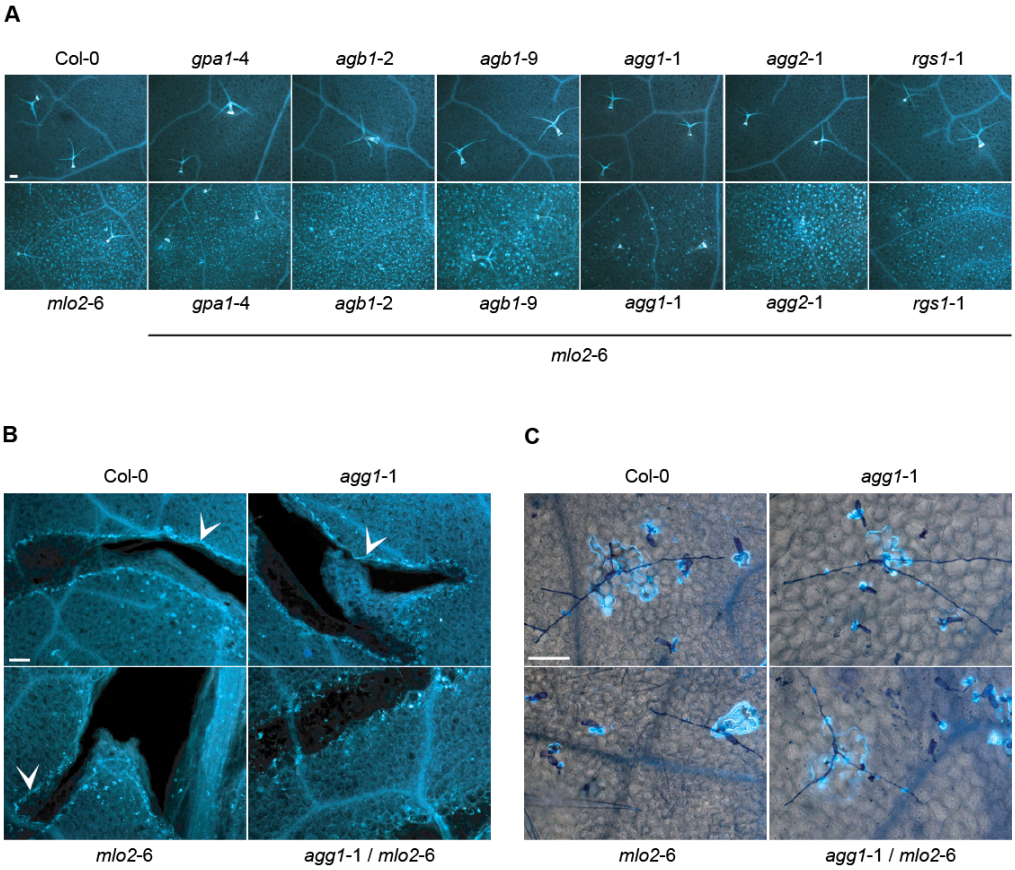

Figure 2.2. Callose accumulation in rosette leaves of G-protein signaling single mutants and corresponding *mlo2* double mutants. Callose was stained with aniline blue. **(A)** Representative micrographs demonstrating spontaneous callose deposition in leaves of 6-week-old plants grown in pathogen-free conditions. The experiment was repeated at least twice (n=6) with similar results. Bar = 100 µm. **(B)** Leaves from 4-weeks-old plants injured with forceps showing callose accumulation at wound sites. White arrowheads point to callose ring structure around the wound site. The experiment was performed once. Bar = 100 µm. **(C)** Four-week-old leaves at 7 dpi with the non-adapted powdery mildew fungus *E. pisi* exhibit callose deposition at sites of fungal interaction. The experiment was performed once (n=6). Bar = 100µm. n: represents the number of leaves tested per genotype in one experiment.

2.2 The molecular basis of *mlo*-mediated powdery mildew resistance in Arabidopsis

2.2.1 A transcriptomic approach to elucidate the molecular basis of *mlo* resistance

To elucidate the molecular mechanisms underlying the powdery mildew resistance of the *mlo2 mlo6 mlo12* (here referred to as *mlo2*/*6*/*12*) mutant, a global gene expression analysis using the Affymetrix ATH1 GeneChip comparing *mlo2*/*6*/*12* mutants with Col-0 wild-type plants upon inoculation with the host powdery mildew fungus *G. orontii* was performed.

Whole rosette leaves from Col-0 wild-type control plants and the *mlo2*/*6*/*12* mutant were harvested at 0 (prior to inoculation), 8 and 12 hpi in three independent replicates. To be able to compare the transcriptional reprogramming events of both genotypes, early time points before host cell entry were chosen for analysis in order to ensure an equivalent developmental stage of the pathogen on both genotypes.

The transcript accumulation data was analyzed to identify candidate genes that exhibited differential transcript levels in the *mlo2*/*6*/*12* mutant compared with Col-0 wild-type plants in response to *G. orontii* challenge. A selection of genes with a significant ($P \leq 0.05$) and at least two-fold change in transcript abundance was used to categorize the genes into two main groups. Group I comprises genes that showed higher transcript levels upon challenge with *G. orontii* in the *mlo2*/*6*/*12* mutant compared to the wild-type. Group II represents genes that exhibited lower transcript accumulation in the mutant compared to Col-0 wild-type plants. Full lists of the genes in group I and II are provided in the Supplementary Tables 6.1 and 6.2, respectively. Venn diagrams in Figure 2.3A illustrate the number of differentially expressed genes in both groups.

Within group I, a set of 116 and 66 genes exhibited elevated transcript levels in the *mlo2*/*6*/*12* mutant compared to the wild-type at 8 hpi and 12 hpi, respectively (Fig. 2.3A). Thirty seven genes overlapped between both time points and 78 genes had specifically higher transcript levels at 8 hpi, whereas only 28 genes showed enhanced transcript accumulation specific to the later time point after inoculation, at 12 hpi. These results indicate a more rapid and/or stronger transcriptional activation in response to powdery mildew challenge in the *mlo2*/*6*/*12* mutant at early stages after *G. orontii* inoculation. Notably, only one gene, *QQS* (*Qua-Quine Starch*), exhibited higher mRNA levels in non-treated mutant plants (at 0 hpi), suggesting that no major constitutive gene expression occurs in the *mlo2*/*6*/*12* mutant.

Within group II, only 21 genes exhibited a statistically significant decrease in transcript levels in the *mlo2*/*6*/*12* mutant compared to the wild-type upon treatment with *G. orontii* (Fig. 2.3A). Twenty of these genes were specifically reduced at 8 hpi. With respect to the number of genes with altered transcript accumulation, transcriptional activation rather than inhibition appears to dominate the response of *mlo2*/*6*/*12* mutant plants in response to *G. orontii* inoculation.

2.2.1.1 Transcript levels of defense-related genes and genes encoding components for the biosynthesis of tryptophan-derived secondary metabolites increase in the *mlo2*/*6*/*12* mutant upon *G. orontii* inoculation

Detailed analysis of group I genes, which showed elevated transcript levels in the *mlo2*/*6*/*12* mutant upon *G. orontii* inoculation, revealed a high prevalence of genes related to defense. Examples are the plant defensin genes *PDF1.2a* and *PDF1.2b*, genes encoding the pathogenesis-related proteins PR1, PR2 and PR4 as well as a chitinase (Supplementary Table 6.1).

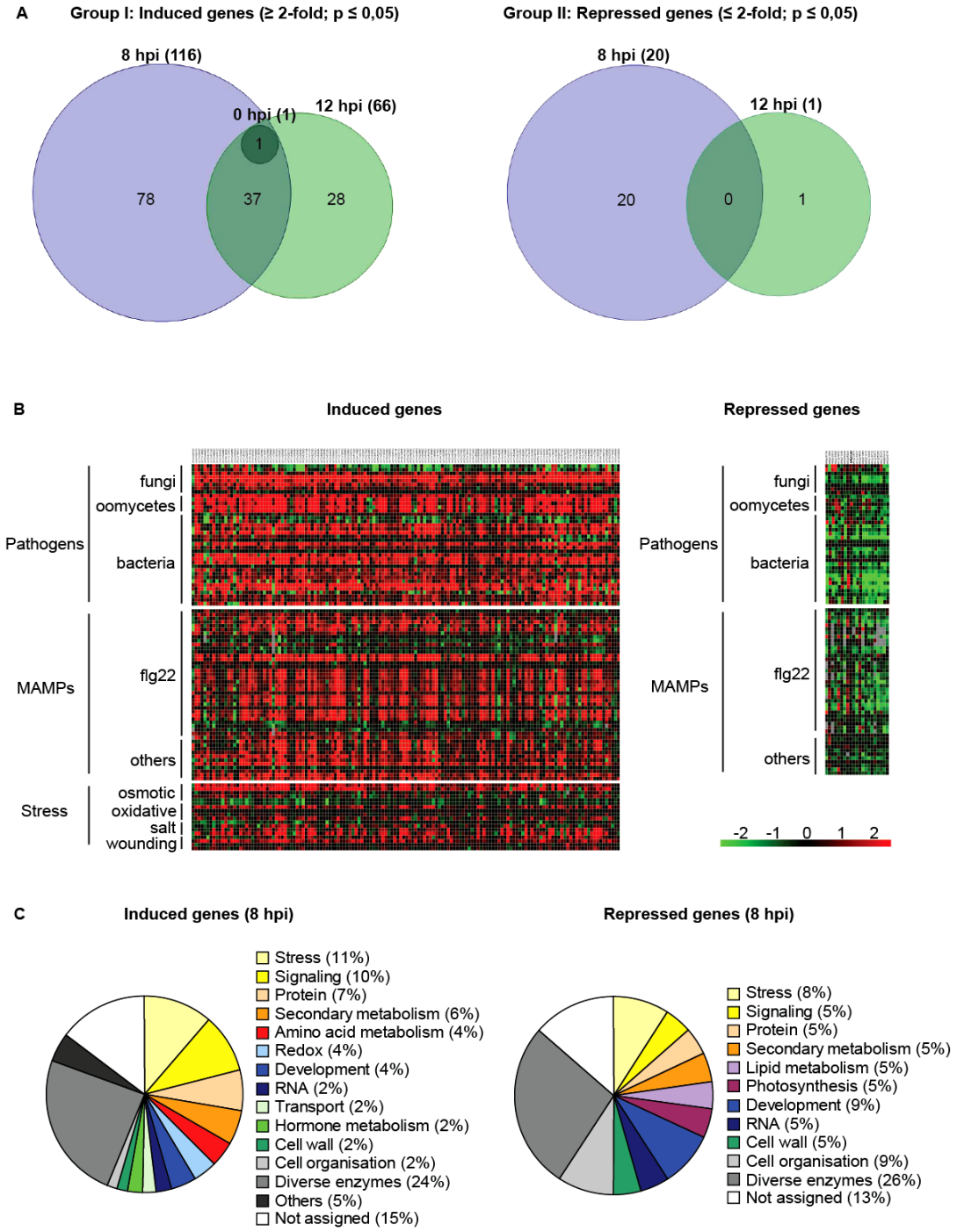

Figure 2.3. Analysis of differentially regulated genes in the *mlo2/6/12* mutant compared to the Col-0 wild-type in response to the adapted powdery mildew fungus *G. orontii* at 8 and 12 hpi. Whole rosette leaves of 4-week-old Col-0 and *mlo2/6/12* plants were inoculated with *G. orontii* spores and sampled at 0 (prior to inoculation), 8 and 12 hpi for comparative transcriptome analysis using the Affymetrix ATH1 GeneChip. All samples were analyzed in triplicates. **(A)** Venn diagram displaying genes significantly ($P \leq 0.05$) induced (group I; left) or repressed (group II; right) by ≥ or ≤ 2-fold in the *mlo2/6/12* mutant at the indicated time points after *G. orontii* inoculation, respectively. **(B)** *In silico* analysis of induced (group I, left) and repressed (group II, right) genes in the *mlo2/6/12* mutant upon *G. orontii* inoculation using the tool Genevestigator V3 on the basis of displayed stimuli. Relative gene expression is indicated in red or green for representing up- or down-regulated gene expression, respectively (Hruz et al., 2008). **(C)** Functional classification of induced (group I; left) and repressed (group II, right) genes in the *mlo2/6/12* mutant in response to *G. orontii* inoculation at 8 dpi according to MapMan (Thimm et al., 2004).

Furthermore genes coding for the pathogen-responsive AP2/ERF (APETALA2/Ethylene Response Factor)-domain transcription factors ORA59, ERF1 and ERF2 as well as a member of the WRKY transcription factor family were present among the genes in group I.

The web-based tool Genevestigator is a large microarray gene expression database that allows the identification of expression patterns of individual genes throughout selected environmental conditions, growth stages or organs in Arabidopsis (Hruz et al., 2008). *In silico* analysis using Genevestigator revealed that genes present in group I were also predominantly induced in response to various pathogens, including fungi other than powdery mildew, oomycetes and bacteria (Fig. 2.3B). Furthermore, MAMPs as well as abiotic stresses enhanced transcript levels of these genes. In agreement with this observation, MapMan analysis, which categorizes selected genes according to their biological function, revealed that most genes present in group I predominantly assign to processes related to stress (Fig. 2.3C, Supplementary Table 6.3) (Thimm et al., 2004). Moreover, genes related to signaling processes were also highly abundant among group I genes, suggesting an enhanced signaling activity upon *G. orontii* inoculation in the *mlo2/6/12* mutant. Together these data indicate a hyperactivation of genes related to defense and stress in the *mlo2/6/12* mutant in response to *G. orontii* challenge.

MapMan analysis of genes in group II, which exhibited decreased transcript levels in the *mlo2/6/12* mutant upon *G. orontii* inoculation, showed that most of these genes associate with processes related to stress and signaling (Fig. 2.3C, Supplementary Table 6.4). Comparative analysis using the expression profile database Genevestigator revealed that these genes are generally repressed by pathogens as well as after treatment with MAMPs (Fig. 2.3B).

Further analysis of the microarray data revealed that transcripts of several genes encoding tryptophan (Trp) biosynthetic and metabolic enzymes hyperaccumulated in the *mlo2/6/12* mutant upon inoculation with the powdery mildew fungus *G. orontii* (Table 2.1). Examples are genes encoding the tryptophan biosynthesis enzymes TSA1 (Trytophan Synthase Alpha subunit) as well as IPGS (Indole-3-Glycerol Phosphate Synthase). Moreover, genes coding for several cytochrome P450 monooxygenases which catalyze steps during the indole glucosinolate and camalexin biosynthesis were found to be induced by *G. orontii* in *mlo2/6/12* mutant plants. The transcription factor MYB51, a key regulator of the genes encoding indole glucosinolate biosynthesis enzymes was also present among group I genes (Gigolashvili et al., 2007). Together these findings emphasize an important role for Trp-derived indolic secondary metabolites in *mlo*-mediated powdery mildew defense, which is in agreement with recently

published data from Consonni et al. (2010), who demonstrated a requirement of these metabolites for resistance in the *mlo2* mutant.

Table 2.1. Genes related to Trp biosynthesis and metabolism hyperaccumulate in the *mlo2/6/12* mutant in response to inoculation with the powdery mildew fungus *G. orontii*. Genes were identified by comparative transcriptome analysis and manually selected from Supplementary Table 6.1. Some of the following genes do not fit the selection criteria of a significant ($P \leq 0.05$) and at least a 2-fold change in transcript abundance but were included for comparison. Trp: tryptophan, IG: indole glucosinolate.

AGI code	Gene annotation and putative function	Process	Fold change *mlo2/6/12* vs Col-0 *G. orontii* (8 hpi)	Fold change *mlo2/6/12* vs Col-0 *G. orontii* (12 hpi)	P-value 8 hpi	P-value 12 hpi
AT5G05730	ASA1 (Anthranilate Synthase Alpha 1)	Trp synthesis	1,6	1,9	2,7E-02	3,9E-03
AT2G04400	IGPS (Indole-3-Glycerol Phosphate Synthase)	Trp synthesis	2,3	2,0	1,0E-04	5,1E-04
AT5G17990	PAT1 (Phosphoribosylanthranilate Transferase 1)	Trp synthesis	1,5	1,6	2,4E-02	1,2E-02
AT3G54640	TSA1 (Tryptophan Synthase Alpha subunit 1)	Trp synthesis	2,7	2,5	5,1E-06	9,7E-06
AT4G39950	CYP79B2 (Cytochrome P450)	IG & camalexin synthesis	1,5	1,7	1,8E-01	8,5E-02
AT5G57220	CYP81F2 (Cytochrome P450)	IG synthesis	2,3	1,5	1,1E-02	1,7E-01
AT4G31500	CYP83B1 (Cytochrome P450)	IG synthesis	2,1	1,6	6,7E-03	6,7E-02
AT1G18570	MYB51 (MYB domain transcription factor 51)	IG synthesis	2,5	2,0	4,1E-03	2,2E-02
AT1G74100	SOT16 (Sulfotransferase 16)	IG synthesis	2,0	1,8	8,1E-03	1,9E-02
AT1G24100	UGT74B1 (UDP-glucosyltransferase)	IG synthesis	1,3	1,4	3,2E-01	1,8E-01
AT2G30770	CYP71A13 (Cytochrome P450)	Camalexin synthesis	6,4	4,0	1,2E-04	1,4E-03
AT3G26830	PAD3 (Phytoalexin Deficient 3)	Camalexin synthesis	6,1	2,3	1,6E-04	3,1E-02

2.2.1.2 Transcript levels of JA/ET-responsive genes increase in the *mlo2/6/12* triple mutant upon *G. orontii* inoculation

Analysis of the transcriptomic data revealed an overrepresentation of jasmonate (JA)- and ethylene (ET)-responsive genes in the *mlo2/6/12* triple mutant after *G. orontii* inoculation, including the members of the AP2/ERF (APETALA2/Ethylene Response Factor)-domain transcription factor family, *ORA59*, *ERF1*, *ERF2* as well as the major JA/ET marker genes encoding the plant defensins PDF1.2a and PDF1.2b and other (Table 2.2). These results suggest an increased JA/ET signaling activity in *mlo*-mutant plants upon *G. orontii* challenge. This hypothesis was tested in the following experiments with the focus on *PDF1.2a* and *PDF1.2b* (here referred to as *PDF1.2*) gene expression. Some of these experiments were performed with the kind help of Katia Becker (master's student).

First, elevated *PDF1.2* transcript levels in the *mlo2/6/12* mutant after *G. orontii* challenge were confirmed by qantitative real-time PCR analysis (Fig. 2.4A). In contrast to previously published results (Zimmerli et al., 2004), *PDF1.2* gene expression was also induced in Col-0 wild-type plants, although always to a lower degree compared to the *mlo2/6/12* mutant (Fig. 2.4A).

JA/ET signaling pathways are activated in response to wounding (León et al., 2001; Gfeller et al., 2010).

Table 2.2. Transcripts of JA/ET-responsive genes are elevated in *mlo2/6/12* mutant plants after inoculation with the powdery mildew fungus *G. orontii*. Genes were identified by comparative transcriptome analysis, manually selected (Supplementary Table 6.1) and compared with published expression data from Col-0 wild-type plants treated with JA or JA and ET. [a]Induced genes in Col-0 wild-type plants after treatment with JA or JA and ET (Pré et al., 2008). [b]These genes were characterized using Genevestigator V3 (Hruz et al., 2008).

AGI code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 *G. orontii* (8 hpi)	Fold change *mlo2/6/12* vs Col-0 *G. orontii* (12 hpi)	Col-0[a] JA	Col-0[a] JA + ET
AT5G44420	PDF1.2a (Plant Defensin 1.2a)	5,0	6,3	yes	yes
AT5G61160	AACT1 (Anthocyanin-5-Aromatic Acyltransferase 1)	5,0	6,2	yes	yes
AT3G49620	DIN11 (Dark Inducible 11)	4,7	7,3	yes	yes
AT3G04720	PR4 (Pathogenesis-Related Protein 4)	4,3	2,8	-	yes
AT4G16260	Glycosyl hydrolase family 17 protein	4,0	5,8	-	yes
AT2G26020	PDF1.2b (Plant Defensin 1.2b)	3,9	7,1	yes	yes
AT2G26560	PLP2 (Phospholipase A 2A)	3,5		yes	yes
AT1G67810	Fe-S metabolism associated domain-containing protein	3,5	2,7	-	yes
AT3G16530	Lectin like protein	3,2	2,2	yes	yes
AT3G23550	MATE efflux family protein	3,0		yes	yes
AT1G26380	FAD-binding domain-containing protein	2,9	2,1	yes	yes
AT5G25250	Unknown protein	2,8	2,2	-	yes
AT1G17745	PGDH (3-Phosphoglycerate Dehydrogenase)	2,7	2,5	yes	yes
AT3G28930	AIG2, avrRpt2-induced gene	2,6		yes	yes
AT2G38860	Protease I (pfpI)-like protein YLS5	2,5	2,0	-	yes
AT1G06160	ORA59 (AP2/ERF domain transcription factor)	2,4	2,8	-	yes
AT1G02920	GST7 (Glutathione-S-Transferase 7)	2,4		yes	yes
AT2G04400	IGPS (Indole-3-Glycerol Phosphate Synthase)	2,3	-	-	yes
AT5G47220	ERF2 (AP2/ERF domain transcription factor)	2,2	-	yes[b]	yes[b]
AT5G10520	RBK1 (ROP binding protein kinases 1)	-	3,8	-	yes
AT1G30135	JAZ8 (Jasmonate-Zim-domain protein 8)	-	2,7	yes[b]	-[b]
AT1G07260	UGT71C3 (UDP-glycosyltransferase 71C3)	-	2,6	-	yes
AT4G08770	Peroxidase, putative	-	2,6	yes	yes
AT4G24350	Phosphorylase family protein	-	2,3	yes	yes
AT1G51760	JR3 (Jasmonic Acid Responsive 3)	-	2,3	yes[b]	-[b]
AT4G17500	ERF1 (AP2/ERF domain transcription factor 1)	-	2,2	yes[b]	yes[b]
AT4G24340	Phosphorylase family protein	-	2,2	yes	yes
AT5G54960	PDC2 (Pyruvate Decarboxylase 2)	-	2,2	-	yes
AT1G17380	JAZ5 (Jasmonate-Zim-domain protein 5)	-	2,2	yes[b]	-[b]
AT4G34200	EDA9 (Embryo Sac Development Arrest 9)	-	2,0	yes	yes

To exclude that increased *PDF1.2* transcript levels are a consequence of leaf wounding during the inoculation procedure (streaking infected leaf over leaf to be tested), leaves were alternatively inoculated by brushing spores form infected leaves on sample leaves. This non-contact inoculation method also led to increased *PDF1.2* mRNA levels in the *mlo2/6/12* mutant compared to wild-type plants, excluding an impackt of the inoculation method on *PDF1.2* gene expression (data not shown).

Beyond the inoculation procedure, wound responses may also be triggered during attempted fungal penetration when a combination of high turgor pressure and hydrolytic enzyme activity is applied on the plant cell. To directly test the impact of wounding on *PDF1.2* gene expression in the *mlo2/6/12* triple mutant, plant leaves were injured with forceps and sampled at 1, 12 and 24 hours after wounding. *PDF1.2* transcript abundance increased at 24 hours in injured leaves of both genotypes but was slightly higher in the *mlo2/6/12* mutant compared to Col-0 wild-type plants (Fig. 2.4B). However, wound- induced *PDF1.2* gene expression was much lower in both genotypes than in plants challenged with *G. orontii* (compare to Fig. 2.4A).

According to published data, transcript levels of *PDF1.2* genes were induced in response to the non-adapted powdery mildew fungus *Bgh* (Zimmerli et al., 2004). To test the impact of a less-adapted powdery mildew fungus on *PDF1.2* expression in *mlo2/6/12* mutant plants, *PDF1.2* transcript abundance was determined after inoculation with the barley powdery mildew fungus *Bgh*. Again, *PDF1.2* gene expression was higher in the *mlo2/6/12* mutant compared to the wild-type but increased later at 24 hpi and always less than in plants inoculated with the adapted fungus *G. orontii* (Fig. 2.4C; compare to Fig. 2.4A). Similar results were observed with the pea powdery mildew fungus *E. pisi* (data not shown).

To assess if accumulation of *PDF1.2* transcripts in the *mlo2/6/12* mutant is a response specific to powdery mildew fungi or also occurs upon challenge with other pathogens, *PDF1.2* gene expression was anlyzed after inoculation with the nectrotrophic fungus *Botrytis cinerea* and the hemibiotrophic fungus *Colletotrichum higginsianum*. However, due to high variability in the results, conclusions cannot yet be drawn from these expriments (data not shown).

In order to test if *PDF1.2* gene expression oscillates during the day, samples of non-treated plants were taken at the same time points during the day as after powdery mildew treatment. *PDF1.2* expression was low during the first 4 hours and increased at 8 hours, a time point where *PDF1.2* transcript levels also elevated in response to powdery mildew treatment (Fig. 2.4D). Thereafter *PDF1.2* expression dropped again. Notably, increase in *PDF1.2* transcript abundance at 8 hours was higher in the *mlo2/6/12* mutant than in Col-0 wild-type plants. However, overall *PDF1.2* gene expression in non-treated plants was much lower than in plants challenged with *G. orontii* (compare to Fig. 2.4A).

In addition to *PDF1.2,* also the genes *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) and *LOX2* (*LIPOXYGENASE 2*) are highly expressed in response to JA signaling activity (Bell and Mullet, 1993; Benedetti et al., 1995). Therefore, the effect of *G. orontii* inoculation on the expression of these additional two JA marker genes was analyzed in *mlo2/6/12* mutant plants. As shown in Figure 2.4E mRNA abundance of both *VSP2* and *LOX2* transiently increased in wild-type and *mlo2/6/12* mutant plants upon challenge with *G. orontii*. However, the induction of these two JA marker genes was much weaker than that of *PDF1.2a* and *PDF1.2b* (compare to Fig. 2.4A). Moreover, transcript levels of *VSP2* and *LOX2* were not remarkably higher in the *mlo2/6/12* mutant compared to the wild-type upon *G. orontii* inoculation and followed a different pattern compared to *PDF1.2*. Taken together, these findings suggest that at least in the context of powdery mildew challenge *PDF1.2* expression is regulated independently from the expression of *VSP2* and *LOX2*.

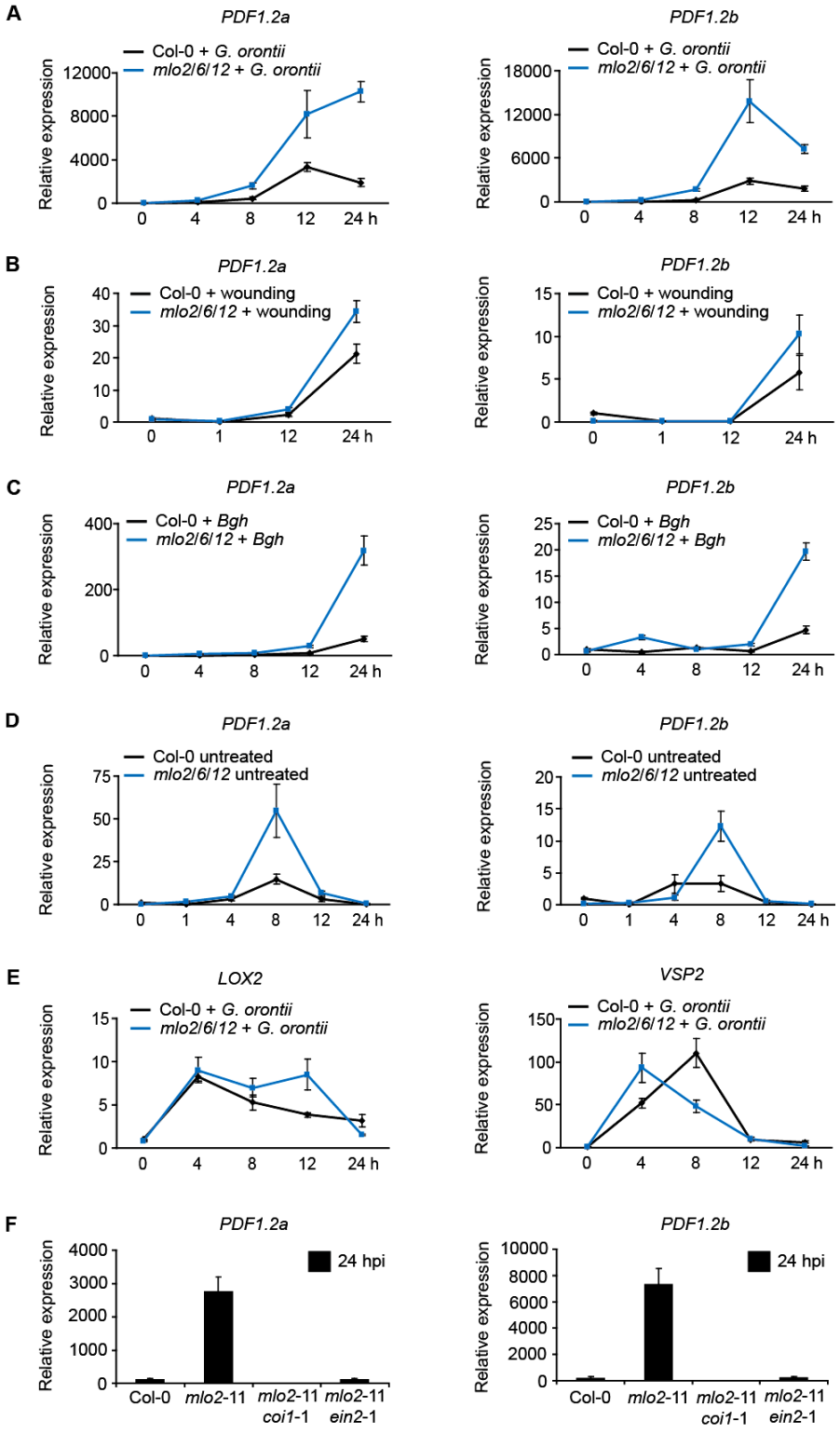

Figure 2.4. Expression levels of JA/ET marker genes in *mlo* mutant plants in response to various stimuli determined by quantitative RT-PCR. Expression of the *PDF1.2a* (left) and *PDF1.2b* (right) gene in *mlo2*/*6*/*12* mutant plants **(A)** upon *G. orontii* inoculation, **(B)** after wounding with forceps, **(C)** upon *Bgh* inoculation, **(D)** under non-treated conditions. **(E)** Transcript levels of *LOX2* (left) and *VSP2* (right) in *mlo2*/*6*/*12* mutant plants after *G. orontii* inoculation. **(F)** Expression of *PDF1.2a* (left) and *PDF1.2b* (right) in Col-0 wild-type, *mlo2*-11, *mlo2*-11/*coi1*-1 and *mlo2*-11/*ein2*-1 plants after *G. orontii* inoculation. Quantitative RT-PCR analysis was performed with rosette leaves from 4-week-old plants at the indicated time points after treatment. Gene expression was normalized to the transcript levels of *At4g26420* encoding a methyltransferase and is presented relative to the transcript abundance of Col-0 wild-type at 0 hpi. A representative data set with mean ± SD of three technical replicates per genotype and time point is shown. The experiments were repeated twice (A and C)

or once (B, D and E) gaining similar results. The experiment in F was performed once. Measurements in A, C and E were carried out by Katia Becker in the context of her master thesis.

To determine if levels of endogenous jasmonates were altered in the *mlo2*/*6*/*12* mutant upon powdery mildew challenge, abundance of free jasmonic acid (JA), its bioactive amino acid-derivative JA-Ile (JA-Isoleucine) as well as the JA precursors, OPDA (12-oxo-phytodienoic acid) and dn-OPDA (10-oxo-dinor-phytodienoic acid) were measured in *G. orontii* and *Bgh* inoculated leaves of *mlo2*/*6*/*12* mutant and Col-0 wild-type plants at 4, 8, 12 and 24 hpi. These experiments were kindly performed in collaboration with Prof. Dr. Ivo Feußner (Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Göttingen, Germany). As shown in Figure 2.5 JA and JA-Ile concentrations were very low in both genotypes before challenge and upon powdery mildew inoculation, and no significant differences in these compounds between Col-0 wild-type and *mlo2*/*6*/*12* mutant plants were observed. Notably, in a second biological replicate JA and JA-Ile were undetectable in the samples (Supplementary Figure 6.2). Interestingly, the JA precursors OPDA and dn-OPDA were specifically induced in response to the non-adapted powdery mildew fungus *Bgh* in both genotypes (Fig. 2.5). However, this observation was not validated in a second biological replicate (Supplementary Figure 6.2). Generally, the variability between the experiments hampers a reliable conclusion regarding the role of OPDA in plant powdery mildew interactions and requires further investigation. In summary the data indicate that JA biosynthesis is not differentially altered in *mlo2*/*6*/*12* mutant and Col-0 wild-type plants in response to powdery mildew fungi.

The genes *COI1* (*CORONATINE INSENSITIVE 1*) and *EIN2* (*ETHYLENE INSENSITIVE 2*) encode for major regulators of JA- and ET-responsive genes, respectively (Xie et al., 1998; Alonso et al., 1999). To elucidate the importance of JA- and ET signaling in *PDF1.2* gene expression in *mlo* mutant plants after powdery mildew infection, analysis of double mutants defective in the *MLO2* and *COI1* or *EIN2* gene, respectively, were performed. Similar to the *mlo2*/*6*/*12* triple mutant *mlo2*-11 single mutant plants also exhibited increased *PDF1.2* expression in response to *G. orontii* challenge (Fig. 2.4F). Noteworthy, *PDF1.2* transcript levels in powdery mildew-treated Col-0 wild-type plants were lower than in previous experiments (compare to Fig. 2.4A). Double mutants defective in *MLO2* and *COI1* failed to trigger *PDF1.2* gene expression upon *G. orontii* inoculation, indicating that *G. orontii*-induced *PDF1.2* transcription in the *mlo* mutant is dependent on COI1. Mutants deficient in *MLO2* and *EIN2* exhibited strongly decreased *PDF1.2* transcript levels in response to *G. orontii*, which were similar to Col-0 wild-type plants. These results suggest that also EIN2 is required for *PDF1.2* expression in *G. orontii* inoculated *mlo* mutant plants.

Interestingly, neither the presence of COI1 nor EIN2 could compensate for *PDF1.2* expression in the absence of the other, indicating a requirement of both signaling pathways in this context.

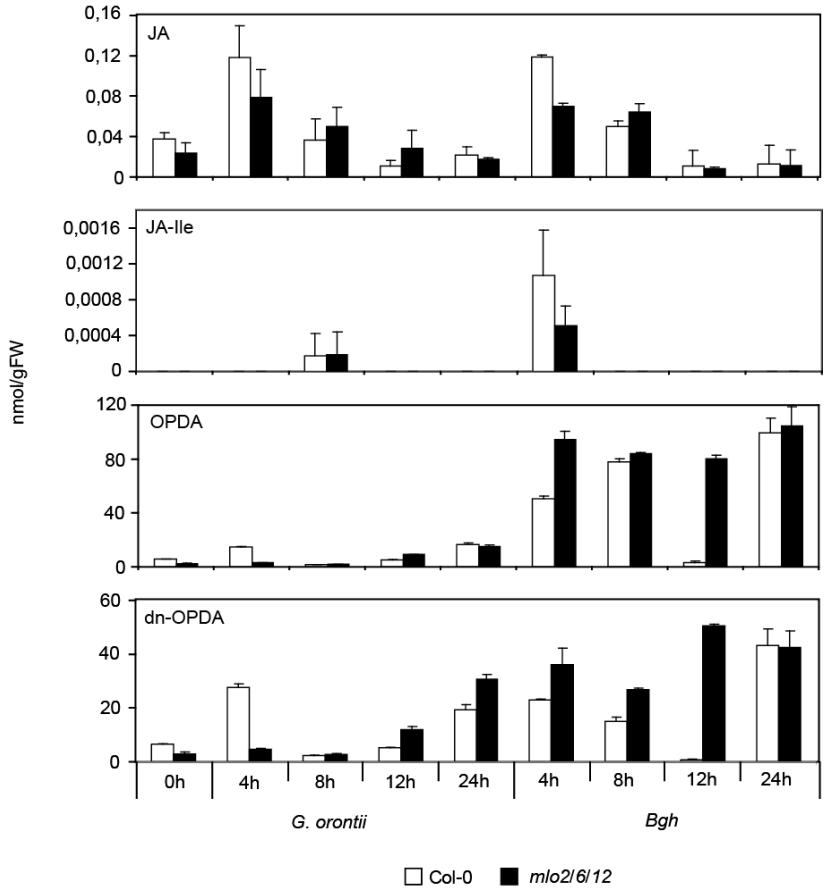

Figure 2.5. Analysis of jasmonic acid (JA), its conjugate, JA-Ile, and the JA precursors OPDA and dn-OPDA in Col-0 wild-type and *mlo2/6/12* mutant plants after inoculation with powdery mildew fungi. Rosette leaves from 4-week-old plants were inoculated with spores form the adapted and non-adapted powdery mildew fungus *G. orontii* and *Bgh*, respectively, and harvested for jasmonate analysis at the indicated time points. Results represent mean ± SD of two measurements of the same experiment. The experiment was repeated once with different observations resulting in no JA and JA-Ile detection and with OPDA and dn-OPDA levels similar after inoculation with *G. orontii* and *Bgh* (compare Suplemmentary Figure 6.2). Measurements were performed in cooperation with Prof. Dr. Ivo Feußner (Albrecht-von-Haller-Institute für Pflanzen-wissenschaftern, Göttingen, Germany).

2.2.1.3 Summary of transcriptome analysis of the *mlo2/6/12* mutant after challenge with the powdery mildew fungus *G. orontii*

Analysis of the transcriptomic data revealed an overrepresentation of genes associated with defense and stress responses that showed higher mRNA abundance in the *mlo2/6/12* mutant upon *G. orontii* inoculation compared to Col-0 wild-type plants. Amongst those, transcripts of several genes encoding tryptophan biosynthetic and metabolic enzymes were found, emphasizing an important role for Trp-derived indolic secondary metabolites in *mlo*-mediated powdery mildew defense. Furthermore, increased transcript abundance of JA/ET-responsive

genes such as *PDF1.2a* and *PDF1.2b* in *G. orontii*-inoculated *mlo2*/*6*/*12* mutant plants was observed and was shown to be dependent on COI1 and EIN2. However, JA biosynthesis and accumulation appears to be not affected in *mlo2*/*6*/*12* mutant plants after powdery mildew inoculation.

2.2.2 Metabolomic analysis of tryptophan-derived indolic compounds in the *mlo2*/*6*/*12* mutant in response to powdery mildew inoculation

Recent studies revealed the requirement of a PEN2-dependent glucosinolate metabolism pathway for Arabidopsis non-host resistance against powdery mildew fungi (Bednarek et al., 2009). More recently, it was found that the same Trp-derived indolic secondary metabolites are essential for *mlo2*-mediated powdery mildew resistance (Consonni et al., 2010). In this study comparative transcriptional profiling revealed a hyperactivation of genes encoding enzymes involved in the biosynthesis of these Trp-derived metabolites in the *mlo2*/*6*/*12* triple mutant in response to the powdery mildew fungus *G. orontii* (Table 2.1). These results suggest an enhanced activity of metabolic processes leading to the formation of indolic metabolites in *G. orontii*-inoculated *mlo2*/*6*/*12* plants compared to the wild-type. To examine if the transcriptional hyperactivation in fact correlates with enhanced accumulation of indolic metabolites in *mlo2*/*6*/*12* mutant plants upon challenge with powdery mildew fungi, a comparative metabolite profile was conducted in cooperation with Paweł Bednarek (MPI for Plant Breeding Research, Cologne, Germany). Therefore, 4-week-old plants of the Col-0 wild-type, *pen2*-1, *mlo2*/*6*/*12* triple and *pen2*-1/*mlo2*/*6*/*12* quadruple mutant genotypes were inoculated with the adapted or non-adapted powdery mildew fungus *G. orontii* and *Bgh*, respectively. Whole leaf samples of the indicated genotypes were harvested at 0 (prior to inoculation), 8, 16 and 24 hpi and concentrations were measured of the indol-3-ylmethylglucosinolate (I3G), its PEN2-mediated hydrolysis products, indol-3-ylmethylamine (I3A) and raphanusamic acid (RA), as well as its 4-methoxylated conjugate 4-methoxyindol-3-ylmethylglucosinolate (4MI3G), which is converted to yet unknown antifungal products by the atypical PEN2 myrosinase (Bednarek et al., 2009).

Constant levels of the indole glucosinolate, I3G, were detected in all tested genotypes, throughout all time points after inoculation and independent of the pathogen (Fig. 2.6). This indicates that I3G accumulation is not affected in any of the genotypes in response to powdery mildew challenge. The atypical PEN2 myrosinase hydrolyzes I3G to the compounds I3A and RA. In accordance with this, I3A and RA only accumulated in Col-0 wild-type and *mlo2*/*6*/*12* triple mutant plants (Fig. 2.6). Mutants defective in the *PEN2* gene failed to accumulate these

compounds, confirming the requirement of a functional PEN2 myrosinase for I3A and RA production. Interestingly, concentrations of I3A and RA in wild-type and *mlo2*/*6*/*12* plants treated with the barley powdery mildew fungus *Bgh* were remarkably higher compared to plants inoculated with the adapted fungus *G. orontii*. This either indicates an overall low hydrolysis of I3G into I3A and RA by PEN2 or a rapid depletion of these compounds in *G.orontii*-treated Col-0 wild-type and *mlo2*/*6*/*12* mutant plants. The indole glucosinolate 4MI3G, which is activated by PEN2 for antifungal defense, did not accumulate in Col-0 and *mlo2*/*6*/*12* triple mutant plants after powdery mildew inoculation independent of the pathogen. Mutants lacking a functional PEN2 myrosinase were enriched in 4MI3G after treatment with the non-adapted fungus *Bgh*, confirming that 4MI3G is a PEN2 substrate. This *Bgh*-induced 4MI3G accumulation was solely dependent on the *pen2* mutation and independent of the presence or absence of functional MLO proteins. Interestingly, 4MI3G accumulation in response to *G. orontii* was not observed in *pen2*-1 single mutants, suggesting that the adapted powdery mildew fungus either fails to induce 4MI3G production or interferes with its accumulation. Quadruple mutants defective in *PEN2* and the three *MLO* genes exhibited 4MI3G accumulation after *G. orontii* challenge, indicating that the adapted powdery mildew fungus is able to induce 4MI3G formation. Therefore, the host fungus *G. orontii* may either be able to metabolize 4MI3G or to suppress its biosynthesis, a process which seems to be entirely dependent on the presence of all three MLO proteins. Nonetheless, transcriptional hyperactivation of genes encoding for biosynthetic enzymes of Trp-derived metabolites does not correlate with an enhanced accumulation of the tested indolic metabolites in the *mlo2*/*6*/*12* mutant in response to powdery mildew pathogens.

2.2.3 PEN1 and PEN2 protein accumulation in *mlo* mutants in response to powdery mildew inoculation

The results presented in 2.2.2 indicate that powdery mildew-triggered amounts of the measured indolic metabolites are not differentially altered in *mlo2*/*6*/*12* mutant plants compared to the wild-type. However, it is possible that PEN2 protein levels are elevated in *mlo* mutant plants upon powdery mildew challenge, enabling an enhanced 4MI3G turnover into the yet unknown antifungal products. Therefore, PEN2 protein levels were determined in Col-0 and *mlo2*-6 single as well as *mlo2*/*6*/*12* triple mutant plants at 0 (prior to infection), 12, 24, 36, 48 and 72 hpi with the adapted and non-adapted powdery mildew fungi *G. orotnii* and *Bgh*, respectively.

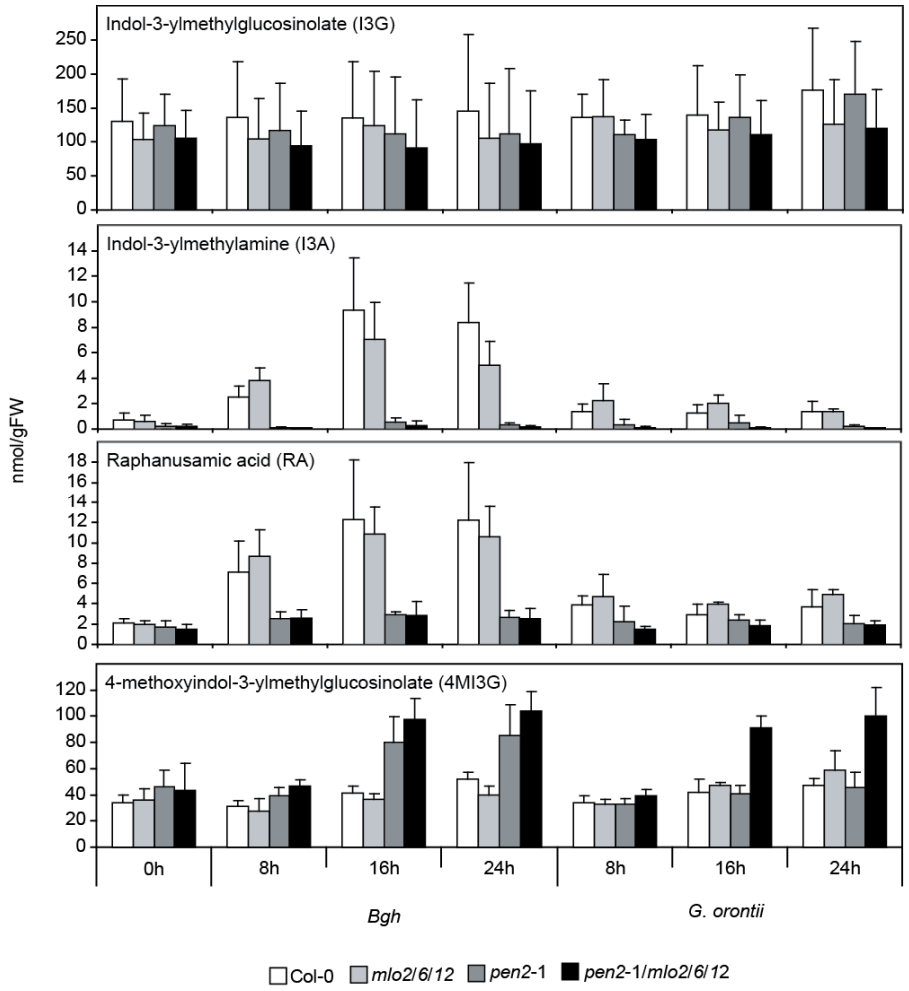

Figure 2.6. Accumulation of selected Trp-derived metabolites in response to inoculation with powdery mildew fungi. Rosette leaves of 4-week-old Col-0 wild-type, *mlo2*/*6*/*12*, *pen2*-1 and *pen2*-1/*mlo2*/*6*/*12*/ plants were inoculated with spores from the adapted and non-adapted powdery mildew fungus *G. orontii* and *Bgh*, respectively. Samples for the metabolite profile were harvested at the indicated time points after inoculation. Results are represented as mean ± SD (n=3; n: represents the number of plants tested per genotype in one experiment) of three independent experiments. Measurements were performed in cooperation with Paweł Bednarek (MPI for Plant Breeding Research, Cologne, Germany). n: represents number of plants tested per genotype in one experiment.

As shown in Figure 2.7A constitutively high PEN2 protein abundance was detected before pathogen challenge (0 hpi), which sustained throughout all time points independent of the genotype and pathogen. This finding indicates that PEN2 protein levels are not differentially elevated in *mlo* mutant plants upon powdery mildew challenge.

Published data demonstrated the requirement for a PEN1-dependent vesicle-mediated secretory pathway in *mlo*-based powdery mildew resistance (Consonni et al., 2006). In order to test if PEN1 protein accumulation was altered in *mlo*-resistant mutants, PEN1 protein levels were determined in *mlo2*-6 single and *mlo2*/*6*/*12* triple mutants in response to the powdery mildew fungi *G. orontii* and *Bgh*. Similarly to PEN2, non-infected plants of all tested genotypes showed high steady-state PEN1 protein levels (Fig 2.7B).

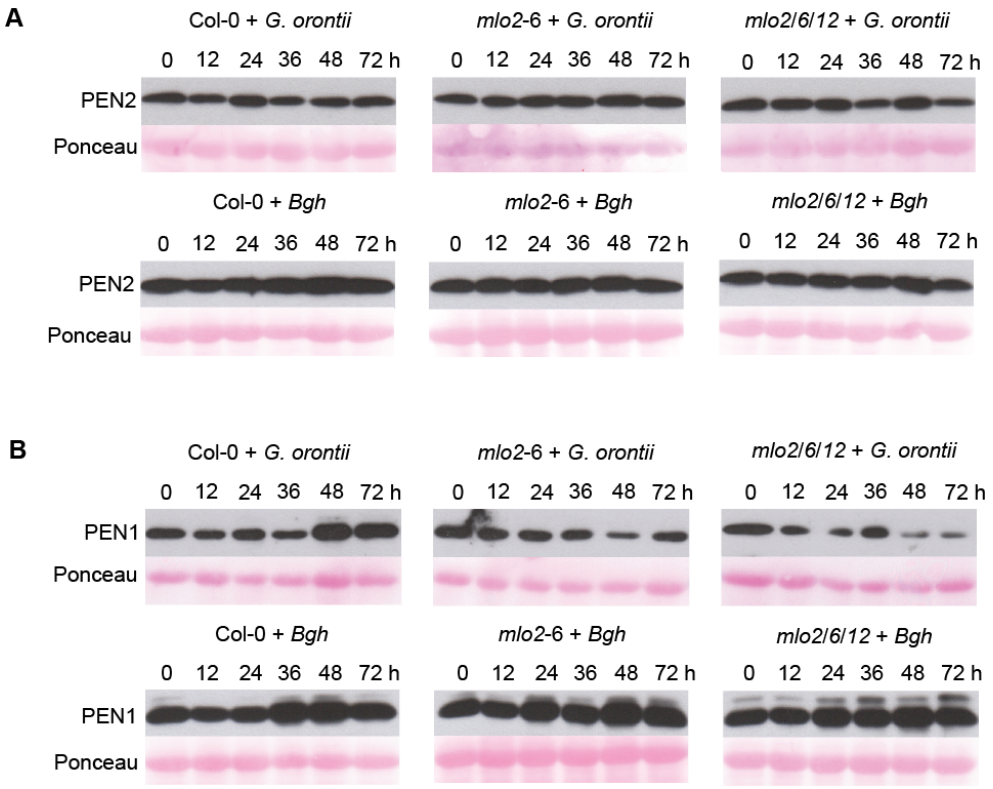

Figure 2.7. PEN2 and PEN1 protein accumulation in *mlo* mutants in response to powdery mildew fungi inoculation. Total protein extracts from 4-week-old plants inoculated with the powdery mildew fungus *G. orontii* or *Bgh* for the indicated time points were separated by SDS-PAGE and probed with either α-PEN2 **(A)** or α-PEN1 **(B)** antiserum. Ponceau staining served as a loading control. The experiment was repeated once with similar results.

Consistent with previously published results, PEN1 protein amount slightly increased in *Bgh* challenged plants at later time points after inoculation, indicating an enhanced contribution of the PEN1-dependent defense pathway (Pajonk et al., 2008). The accumulation of PEN1 occurred irrespective of the presence or absence of functional MLO proteins.

In response to *G. orontii* treatment PEN1 protein abundance was generally less than in *Bgh*-challenged plants. Col-0 wild-type plants exhibited a slight increase in PEN1 protein amount in response to *G. orontii* but it occurred later (at 48 hpi) compared to *Bgh*-inoculated wild-type plants. Strikingly, PEN1 levels decreased in *mlo* mutant plants at 48 hpi, with a stronger decrease in the *mlo2*/*6*/*12* triple mutant, suggesting no major contribution of the PEN1-mediated defense pathway to *mlo*-mediated resistance against *G. orontii* during later time points after fungal inoculation.

2.2.4 MAMP-triggered responses in *mlo* mutants

MAMP perception triggers a broad set of early and late cellular responses leading to an enhanced state of immunity that limits invasion and propagation of potential microbial intruders (Boller and Felix, 2009). To investigate if MAMP-mediated signaling is altered in

mlo mutant plants, MAMP-triggered responses such as the formation of reactive oxygen species (ROS), also termed oxidative burst, activation of mitogen-activated protein kinase (MAPK) cascades and transcriptional reprogramming were analyzed. However, measurement of some of these responses after treatment with powdery mildew fungi is technically not trivial. Therefore, elicitors such as the fungal chitin and flg22, the active epitope of bacterial flagellin, were used as elicitors of immune responses.

As shown in Figure 2.8A flg22-induced ROS formation was not altered in *mlo2*-6 single and *mlo2/6/12* triple mutants compared to the wild-type. In contrast, ROS production in response to chitin was abolished in *mlo2/6/12* triple mutants, whereas *mlo2*-6 single mutant plants showed wild-type like oxidative burst after chitin treatment. These results indicate a putative role for MLO6 and/or MLO12 in chitin-triggered ROS formation, which is independent of MLO2.

Activity of MAPKs was strongest at 15 minutes after treatment with flg22 or chitin and decreased at 30 minutes after MAMP-application in both genotypes (Fig. 2.8B). Notably, in *mlo2/6/12* mutant plants chitin-induced MAPK activity was lower at 30 minutes after treatment.

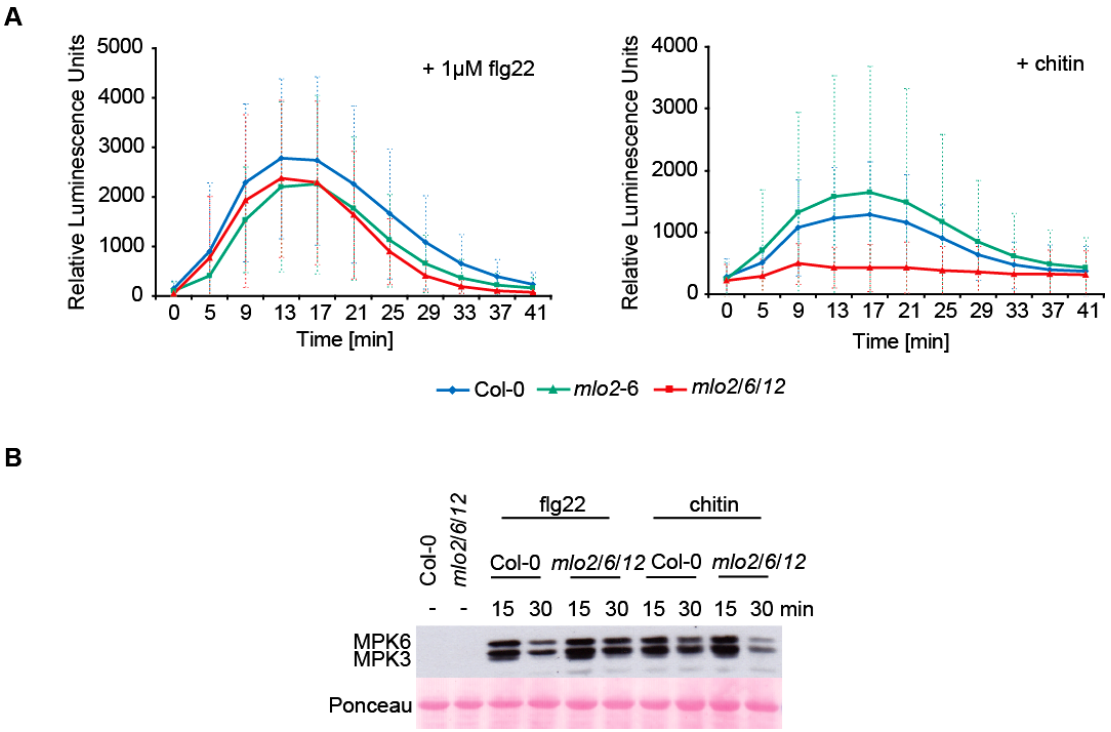

Figure 2.8. MAMP-induced oxidative burst and MAPK activation in the *mlo2/6/12* mutant. Leaf discs from 4-week-old plants were treated with 1 µM flg22 or crab shell chitin (see 4.1.7). **(A)** ROS formation was measured in a chemiluminescence assay for the indicated time points after MAMP application. Results are represented as mean ± SD (n=10-12; n: represents the number of leaf discs tested per genotype in one experiment) of at least three independent experiments. **(B)** MAPK activity was determined by immunoblot analysis at the indicated time points after MAMP treatment. Ponceau staining served as a loading control. The experiment was repeated twice with similar results.

Downstream of MAPK activation transcriptional reprogramming events are induced in order to generate defense ouputs. Induction of *WRKY22* and *WRKY29* gene expression, encoding members of the WRKY transcription factor family, was widely reported in response to different MAMPs (Asai et al., 2002; Navarro et al., 2004; Libault et al., 2007; Zhang et al., 2007). Therefore, expression of these genes was analyzed in Col-0 wild-type and *mlo2*/*6*/*12* triple mutant seedlings during the time course of flg22 and chitin treatment. Consistent with previous data, both *WRKY* genes were rapidly induced within 1 h of MAMP treatment (Fig. 2.9A, B) (Lu et al., 2009). However, no significantly different *WRKY* gene expression between the Col-0 wild-type and *mlo2*/*6*/*12* mutant was observed in response to both MAMPs. *PDF1.2b* gene expression was also tested in response to MAMP treatment and highest *PDF1.2b* transcript levels were observed at 8 h in both genotypes independent of the elicitor (Fig. 2.9C). Interestingly, no different *PDF1.2b* transcript abundance was detected between the *mlo2*/*6*/*12* mutant and wild-type as it was observed after *G. orotii* inoculation (compare to Fig. 2.4A).

Interrogation of the Arabidopsis gene co-expression database ATTED-II (Obayashi et al., 2009) revealed that the genes *PROPEP2* and *PROPEP3* were co-expressed with *MLO6* and *MLO12*, respectively. Both *PROPEP* genes encode endogenous defense peptides and showed high levels of expression in response to pathogen attack as well as to microbe-derived elicitors including flg22 (Huffaker et al., 2006). Therefore, *PROPEP2* and *PROPEP3* gene expression was analyzed in the *mlo2*/*6*/*12* mutant upon flg22 and chitin treatment. Both *PROPEP* genes were rapidly induced within 1 h after MAMP-application (Fig. 2.9D, E). Interestingly, *PROPEP2* and *PROPEP3* transcript levels were higher in the *mlo2*/*6*/*12* mutant than in the wild-type upon treatment with either MAMP.

Recent studies revealed the involvement of a signaling network between MAPKs and calcium-dependent protein kinases (CDPKs) in activation of MAMP-triggered transcriptional reprogramming (Boudsocq et al., 2010). In this context the authors proposed the involvement of four different regulatory programs in the activation of early flg22-responsive genes. Genes such as *FRK1* (*FLG22-INDUCED RECEPTOR KINASE1*) were specifically activated by MAPKs, whereas *PHI1* (*PHOSPHATE-INDUCED1*) seemed to be CDPK-specific. Other flg22-induced genes were either activated equally by both, the MAPK- and CDPK signaling pathway, for example *NHL10* (*NDR1*/*HIN1-LIKE 10*), or were induced to much higher levels by MAPKs.

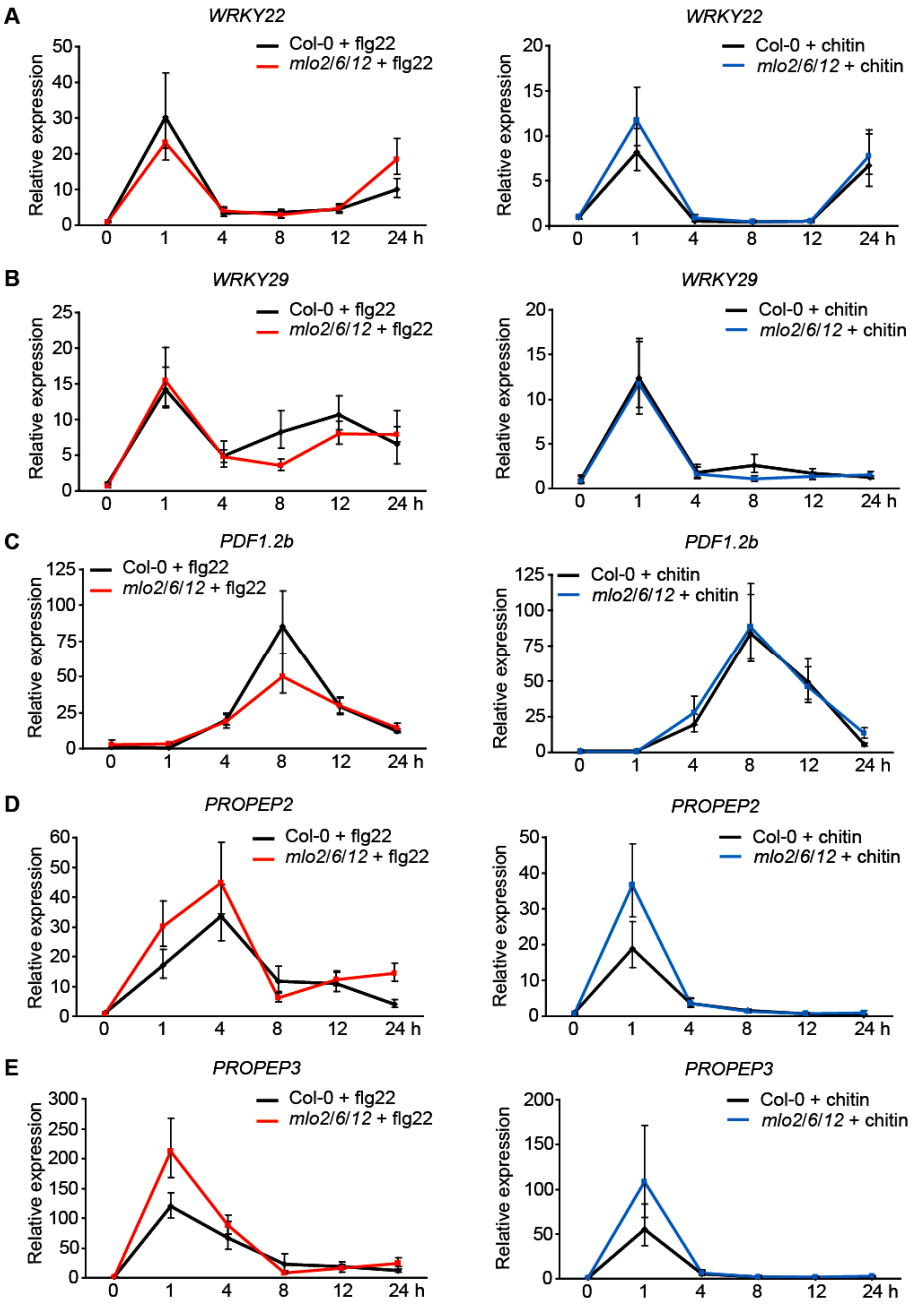

Figure 2.9. Expression levels of selected MAMP-induced genes in the *mlo2/6/12* mutant after treatment with flg22 or chitin. Two-week-old seedlings were treated with 1 µM flg22 (left) and crab shell chitin (see 4.1.7) (right) and sampled at the indicated time points after MAMP application. Transcript levels were determined by quantitative RT-PCR. **(A)** *WRKY22*, **(B)** *WRKY29*, **(C)** *PDF1.2b*, **(D)** *PROPEP2* and **(E)** *PROPEP3* gene expression was normalized to the transcript levels of *At4g26420* encoding a methyltransferase and is presented relative to the transcript abundance of Col-0 wild-type at 0 hpi. A representative data set with mean ± SD of three technical replicates per genotype and time point is shown. The experiments were repeated twice gaining similar results.

Interestingly, Boudsocq and colleagues (2010) observed that flg22-induced expression of *PROPEP2* and *PROPEP3* was CDPK-dependent, raising the question if elevated *PROPEP* transcript levels in the *mlo2/6/12* mutant are a consequence of an enhanced CDPK signaling pathway. In accordance with this hypothesis transcript abundance of the early CDPK-specific gene *PHI1* was elevated in the *mlo2/6/12* mutant in response to chitin, whereas expression of

the MAPK-specific gene *FRK1* was not altered (Fig. 2.10A, B). Expression levels of *NHL10*, a gene equally induced by MAPK and CDPK signaling cascades, was also stronger in the *mlo2/6/12* mutant in response to chitin treatment compared to the wild-type (Fig. 2.10C).

In summary the results presented in this section revealed a defect in the chitin-induced generation of ROS in the *mlo2/6/12* triple but not in the *mlo2*-6 single mutant. However, MAPK activation in response to MAMPs was not effected in the mutant. Consistently, MAMP-triggered activation of MAPK-specific genes was not altered in the *mlo2/6/12* genotype. Interestingly, genes specifically regulated by CDPKs were stronger expressed in the mutant in response to MAMPs, indicating an enhanced activity of the CDPK signaling pathway in *mlo2/6/12* plants upon MAMP treatment.

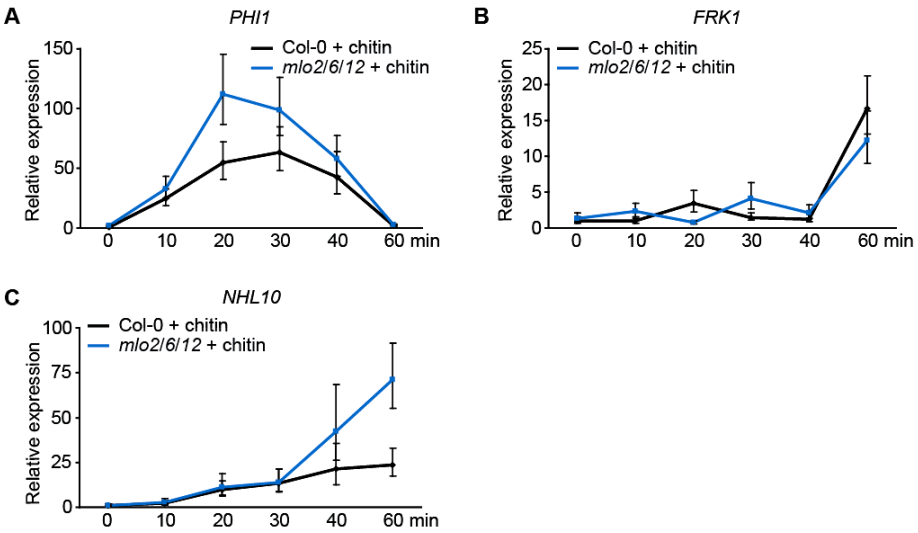

Figure 2.10. Expression levels of selected MAMP-induced genes in the *mlo2/6/12* mutant after treatment with chitin. Two-week-old seedlings were treated with crab shell chitin (see 4.1.7) and sampled at the indicated time points after MAMP application. Transcript levels were determined by quantitative RT-PCR. **(A)** *PHI1*, **(B)** *FRK1* and **(C)** *NHL10* gene expression was normalized to the transcript levels of *At4g26420* encoding a methyltransferase and is presented relative to the transcript abundance of Col-0 wild-type at 0 hpi. A representative data set with mean ± SD of three technical replicates per genotype and time point is shown. The experiments were repeated once gaining similar results.

2.3 The role of heterotrimeric G-protein signaling in MTI

2.3.1 The Gβ-deficient mutant exhibits altered MAMP-triggered responses

Recent publications demonstrated a function for the Arabidopsis Gβ subunit, AGB1, in MAMP-induced oxidative burst control (Ishikawa, 2009). These results were confirmed in this study using two independent *agb1* mutants, the T-DNA insertion line, *agb1*-2, and the EMS mutant, *agb1*-9, which showed reduced ROS production upon flg22 treatment (Fig.

2.11A). In contrast, MAPK activation upon flg22 and elf18 treatment was not disturbed in *agb1* mutant plants as has been shown by Ishikawa (2009).

Consistent with this, MAPK-specific *FRK1* gene activation in response to flg22 was not altered in *agb1* mutant seedlings (Fig. 2.12A) (Boudsocq et al., 2010). Furthermore, induction of *WRKY22*, another MAPK-activated flg22-responsive gene was also not affected in the mutants (Fig. 2.12B) (Asai et al., 2002). However, expression levels of *NHL10*, a gene which is synergistically induced by MAPK and CDPK signaling cascades, was notably lower in *agb1*-2 and *agb1*-9 mutants, assuming that the CDPK-dependent singnaling cascade may be impaired in Gβ-deficient mutants (Fig. 2.12C). In agreement with this hyothesis transcript levels of the CDPK-activated genes *PROPEP2* and *PROPEP3* were also reduced in *agb1* mutant plants after flg22 treatment (Fig. 2.12D, E).

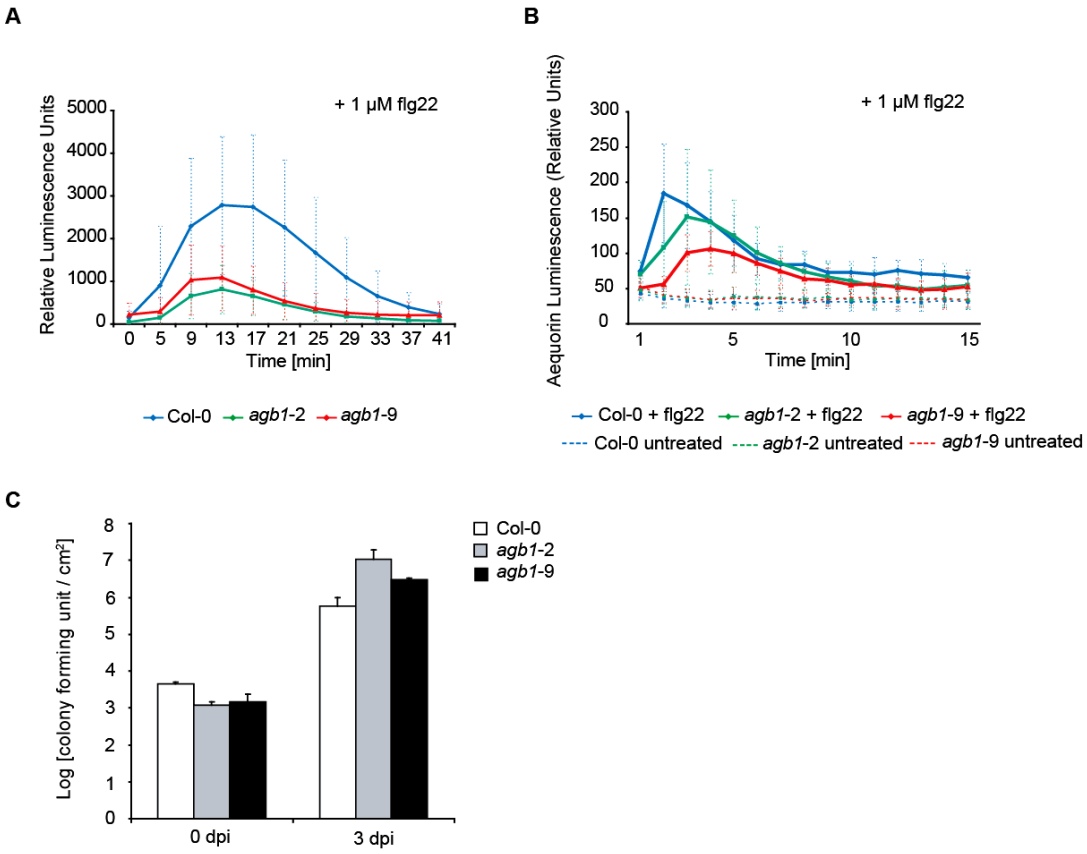

Figure 2.11. Flg22-induced ROS and Ca^{2+} spiking as well as growth of *Pst* DC3000 bacteria in *agb1* mutants. (A) Leaf discs from 4-week-old plants were treated with 1 µM flg22 and ROS formation was measured in a chemiluminescence assay for the indicated time points after flg22 application. Results are presented as mean ± SD (n=10-12; n: represents the number of leaf discs tested per genotype in one experiment) of at least three independent experiments. **(B)** Mesophyll protoplasts expressing the Ca^{2+} sensor aequorin were treated with 1 µM flg22 and changes in Ca^{2+} concentration were measured in a luminescence assay for the indicated time points. Results are presented as mean ± SD (n=5; n: represents the number of wells containing protoplasts tested per genotype) of one experiment. **(C)** Four-week-old plants were sprayed with *Pst* DC3000 bacteria at OD_{600}=0.2 and bacterial titres were determined 3 h (0 dpi) after inoculation and at 3 dpi. A representative data set with mean ± SD (n=9; n: represents the number of leaves tested per genotype in one experiment) of one experiment is shown. The experiment was repeated once with similar results.

However, inconsistent with this assumption, expression of *PHI1*, which has been shown to be specificaly induced by the CDPK signaling pathway, was not affected in the *agb1* mutant (Fig 2.12E) (Boudsocq et al., 2010).

In order to elucidate if Ca^{2+} signatures are modified in the *agb1* mutant in response to MAMP treatment, Ca^{2+} spiking was measured in *agb1* mesophyll protoplasts expressing the Ca^{2+} sensor aequorin. Preliminary data suggest a reduction in the cytosolic Ca^{2+} concentration in *agb1* mutant plants after flg22 treatment (Fig. 2.11B). Notably, Ca^{2+} spiking in the Col-0 wild-type occurred quicker and was higher than in the *agb1* mutant protoplasts.

Finally, the contribution of the Gβ subunit to bacterial defense was investigated. Notably, published studies contradict a role of the Gβ subunit in bacterial growth restriction (Trusov et al., 2006). However, these conclusions were based on bacteria infiltration assays, an inoculation method that may circumvent the first steps of natural infection process, as has also been observed for the *fls2* mutant (Zipfel et al., 2004). Naturally, bacteria enter host plant leaves through wounds or natural openings such as stomata. Thus, *Pst* DC3000 bacteria were sprayed on plant leaves in order to mimic the natural infection conditions. As shown in Fig. 2.11C *agb1* mutant plants showed a slight increase in bacterial growth which was more pronounced on the T-DNA insertion line *agb1*-2 than on the EMS mutant, *agb1*-9. Although the differences in bacterial growth were not statistically significant, a minor contribution of the Gβ subunit to defense against bacteria can be assumed.

2.3.2 The role of Gγ1 and Gγ2 subunits in MTI

The Arabidopsis genome encodes two canonical Gγ subunits, Gγ1 and Gγ2 (AGG1 and AGG2, respectively) but only one Gβ subunit, AGB1, indicating the presence of two Gβγ dimers, Gβγ1 and Gβγ2, in Arabidopsis (Temple and Jones, 2007). In the following section the putative function of the two Gγ subunits in MAMP-triggered signaling and immunity was assessed. Given that Gγ1 and Gγ2 may act redundantly, the double mutant *agg1*-1/*agg2*-1 (here referred to as *agg1*/*agg2*) was added to the analysis.

In agreement with redundant functions of both Gγ subunits, the *agg1*/*agg2* double mutant but not the single mutants were impaired in ROS formation after flg22 and elf18 treatment (Fig. 2.13A). MAPK activation upon treatment with the different MAMPs flg22, elf18 and chitin was not affected in the single mutants (Fig. 2.13B). Intriguingly, flg22-triggered MAPK activity was completely abolished in the *agg1*/*agg2* double mutant, whereas it was wild-type like in response to elf18 and chitin, suggestive of a redundant role of the two Gγ subunits specifically in flg22-induced MAPK signaling.

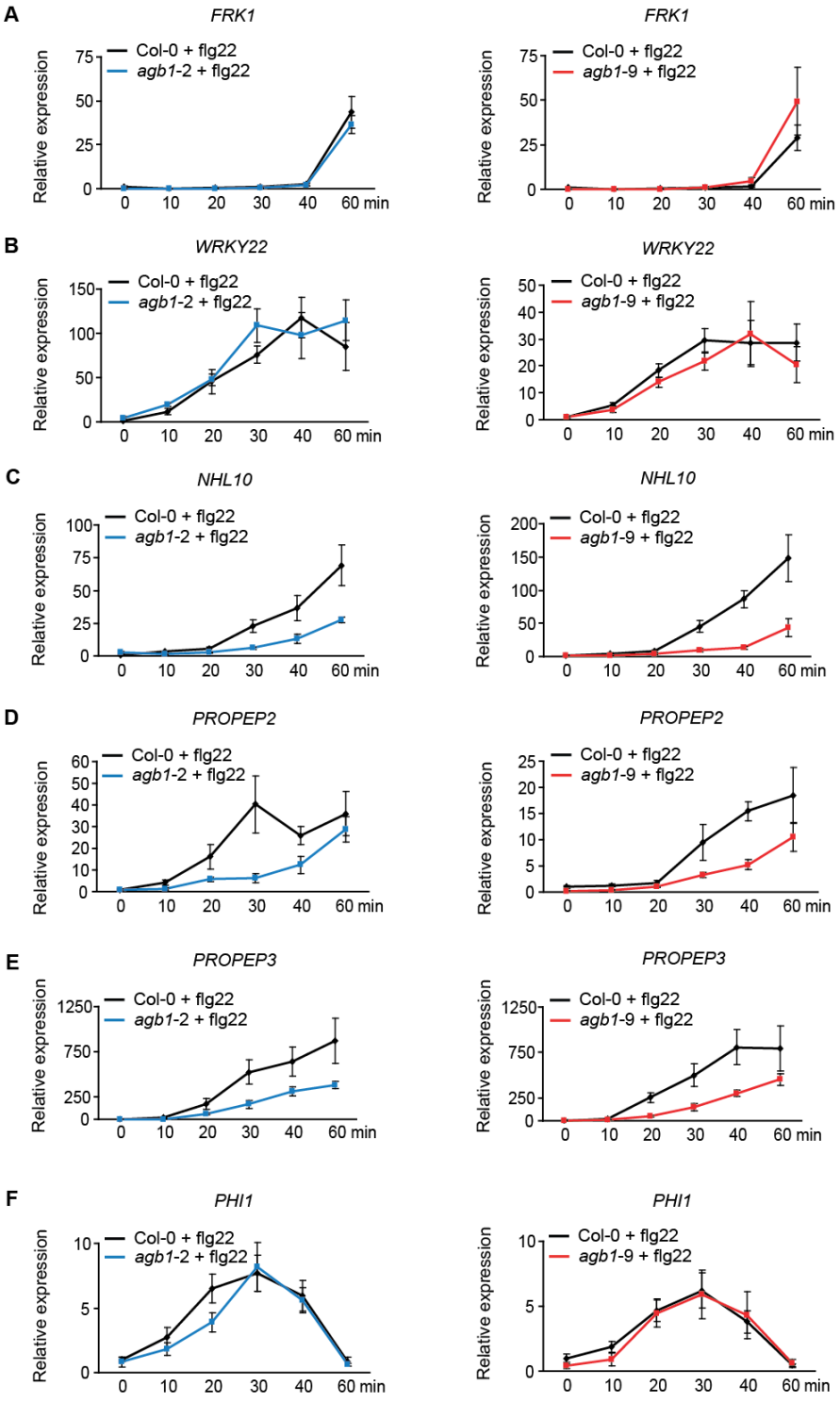

Figure 2.12. Expression levels of selected MAMP-induced genes in the *agb1* mutant after treatment with flg22. Two-week-old *agb1*-2 (left) and *agb1*-9 (right) seedlings were treated with 1 µM flg22 and sampled at the indicated time points after MAMP application. Transcript levels were determined by quantitative RT-PCR. **(A)** *FRK1*, **(B)** *WRKY22*, **(C)** *NHL10*, **(D)** *PROPEP2*, **(E)** *PROPEP3* and **(F)** *PHI1* gene expression was normalized to the transcript levels of *At4g26420* encoding a methyltransferase and is presented relative to the transcript abundance of Col-0 wild-type at 0 hpi. A representative data set with mean ± SD of three technical replicates per genotype and time point is shown. The experiments were repeated once gaining similar results.

In bacterial spray assays no difference in growth of the wild-type bacterial strain *Pst* DC3000 was observed between the tested genotypes (Fig. 2.13C). However, using the disarmed bacterial strain *Pst* DC3000 *hrcC*, which is defective in effector delivery via the type III secretion system, bacterial growth was slightly enhanced in the *agg1*-1 and *agg2*-1 single mutants and more pronounced in the respective *agg1*/*agg2* double mutant, suggesting a redundant function of the two Gγ subunits in MAMP-triggered immunity.

Importantly, the *agg1*-1 single mutation originates from the Arabidopsis accession Ws-0 and was subsequently introgressed into the Col-0 ecotype by eight backcrosses (Trusov et al., 2007). This designated *agg1*-1 Col-0 mutant was used to generate the *agg1*/*agg2* double mutant. Of particular importance is that the Ws-0 accession carries a natural mutation in the gene encoding the flagellin receptor, FLS2, and is therefore insensitive to flg22 treatment (Gómez-Gómez and Boller, 2000). Hence, it was possible that the *agg1*/*agg2* plants lack a functional FLS2 receptor, although the *agg1*-1 single mutant, which was used to generated the double mutant plants, was still responsive to flg22 (Fig. 2.13A, B). Sequencing of the *FLS2* gene in the *agg1*/*agg2* double mutant revealed a deletion at 3014 bp that leads to an early stop codon and therefore a non-functional FLS2 receptor as it is known for the natural *fls2* mutation in the Arabidopsis Ws-0 accession (Fig. 2.14A) (Silke Robatzek, personal communication). The *agg1*-1 single mutant carried a non-mutated *FLS2* version (Fig. 2.14A), which is consistent with its wild-type-like responses to flg22. Interestingly, the elf18-mediated oxidative burst was also strongly impaired in the *agg1*/*agg2* double mutant, indicating an influence of either the Ws-0-associated *fls2* mutation or yet another mutation in the Ws-0 genetic background on elf18-triggered MAMP signaling (Fig. 2.13A).

However, Gγ1 and Gγ2 may still act redundantly in MAMP-triggered signaling and immunity. In agreement with this is the weak increase in susceptibility to bacterial challenge of both single mutants, *agg1*-1 and *agg2*-1 (Fig. 2.13C). To test this possibility a transient *FLS2* complementation approach in mesophyll protoplast was performed. Protoplasts from the Col-0, *fls2* and *agg1*/*agg2* genotypes were transfected with a functional YFP-tagged *FLS2* gene and treated with flg22 to monitor MAPK activity in response to the elicitor. Non-transfected protoplasts served as a control. As shown in Figure 2.14B (left) solely non-transfected Col-0 protoplasts exhibited flg22-induced MAPK activity, whereas non-transfected *fls2* and *agg1*/*agg2* mutant protoplast, which lacked a functional *FLS2* gene, were insensitive to flg22-triggered MAPK activation. Transfection of *FLS2*-*YFP* into protoplasts of the *fls2* mutant restored flg22 sensitivity and MAPK activity, indicating that *FLS2* complementation in protoplasts is functional (Fig. 2.14B).

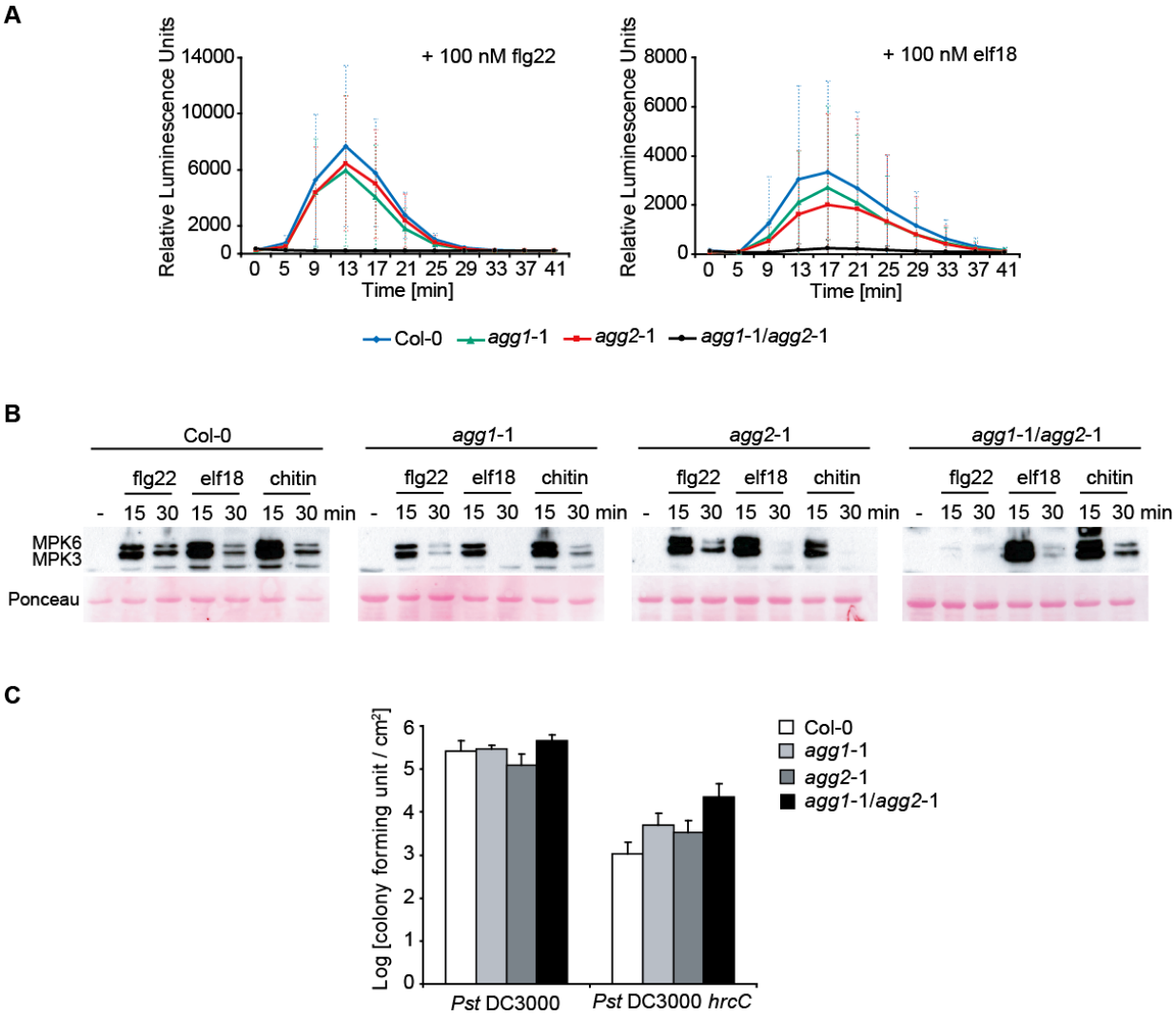

Figure 2.13. MAMP-induced oxidative burst and MAPK activation as well as growth of *Pst* DC3000 wild-type and *hrcC* bacteria in *agg1*, *agg2* single and double mutants. (A) Leaf discs from 4-week-old plants of the indicated genotypes were treated with 100 nM flg22 and 100 nM elf18. ROS formation was measured in a chemiluminescence assay for the indicated time points after MAMP application. A representative data set with mean ± SD (n=10-12; n: represents the number of leaf discs tested per genotype in one experiment) of one experiment is shown. The experiment was repeated once with the same MAMP concentration as well as with 1 µM flg22 or elf18 in at least three independent replicates (data not shown) gaining similar results. **(B)** MAPK activity was induced in leaf discs of 4-week-old plants by application of 1 µM flg22 or 1 µM elf18 or crab shell chitin (see 4.1.7) for the indicated time points and determined by immunoblot analysis. Ponceau staining served as a loading control. The experiment was repeated once with similar results. **(C)** Four-week-old plants were sprayed with *Pst* DC3000 and *hrcC* bacteria at OD_{600}=0.2 and bacterial titres were determined at 3 dpi. A representative data set with mean ± SD (n=9; n: represents the number of leaves tested per genotype in one experiment) of one experiment is shown. The experiment was repeated once with similar results.

FLS2 transfected *agg1*/*agg2* protoplast also successfully responded to flg22 with activation of the MAPK signaling cascade, indicating that flg22-induced MAPK activity is not affected in the *agg1*/*agg2* double mutant. However, Gβ-deficient plants are also not impaired in flg22-mediated MAPK activation but show defects in other flg22 responses including oxidative burst, Ca^{2+} spiking and defense gene activation (see Fig. 2.11, 2.12). Therefore the generation

of a true *agg1*/*agg2* double mutant is indispensable to assess the contribution of the Gγ subunits to MTI.

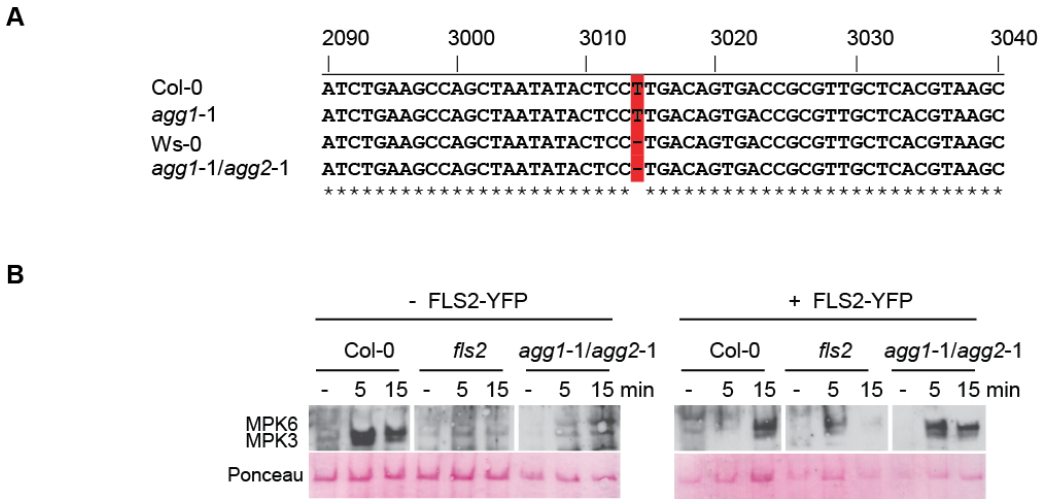

Figure 2.14. The *agg1*/*agg2* double mutant carries a mutation in the flagellin receptor FLS2. (A) Partial sequence of the *FLS2* gene of the indicated genotypes. Similar to the Arabidopsis ecotype Ws-0, the *agg1*/*agg2* double mutant carries a deletion at 3014 bp (red bar) in the *FLS2* gene leading to flg22 insensitivity. The *agg1* single mutant has a functional Col-0 wild-type-like *FLS2* gene. **(B)** Flg22-induced MAPK activation in mesophyll protoplasts of Col-0, *agg1*/*agg2* and *fls2* genotypes transiently expressing a functional *FLS2* gene tagged with YFP (right). The protoplasts were treated with 1 µM flg22 for the indicated time points and sampled for detection of MAPK activity by immunoblot analysis. Non-transfected protoplasts were treated in the same way and served as control (left). Ponceau staining served as a loading control. The experiment was performed once.

3. Discussion

3.1 The role of heterotrimeric G-protein signaling in Arabidopsis defense

3.1.1 MLO2 function in powdery mildew pathogenesis is independent of heterotrimeric G-protein signaling

To date knowledge about potential plant G-protein coupled receptors (GPCRs) is sparse and currently no bona fide plant GPCR has been identified. The plant-specific MLO proteins constitute one of the largest heptahelical protein families in Arabidopsis. The topology and subcelullar localization of MLO proteins is reminiscent of the GPCR superfamily in metazoans and although no significant sequence similarity between mammalian GPCRs and MLO proteins exist, these plant-unique proteins are considered receptor candidates for G-protein signaling. A previous combined pharmacological and genetic study in barley did not provide any evidence for a function of heterotrimeric G-protein signaling in MLO-mediated powdery mildew pathogenesis (Kim et al., 2002b). However, these studies have to be regarded critically as they were based on transient overexpression and dsRNAi-mediated gene silencing in single leaf epidermal cells of barley Gα variants that were deduced from the animal or yeast system. A similar function of these mutant variants in plants seems likely but has not been proven. Moreover, pharmaceutical agents used in that study were known to modulate G-protein signaling in the animal system, however, their specificity to manipulate the plant G-protein has been questioned and criticized (Fujisawa et al., 2001; Miles et al., 2004). Consequently, a genetic approach using stable *Arabidopsis thaliana* (here referred to as Arabidopsis) G-protein signaling mutants was chosen in this study to unequivocally test a potential molecular link between MLO proteins and heterotrimeric G-protein signaling. Therefore, T-DNA insertion lines disrupting the *MLO2* gene or individual members of the heterotrimeric G-protein complex (Gα/GPA1, Gβ/AGB1, Gγ1/AGG1, Gγ2/AGG2 or RGS1) as well as the corresponding *mlo2*-6 double mutants were assessed for their powdery mildew infection phenotype with either the adapted or non-adapted fungus *G. orontii* or *E. pisi*, respectively. Results obtained in this study demonstrated that absence of the Gβ subunit, AGB1, either alone or in the *mlo2*-6 mutant background, enhanced penetration rates of both the adapted and non-adapted powdery mildew fungus (Fig. 2.1A, B). Similarly, irrespective of the presence or absence of the MLO2 protein, lack of Gβ or one of the Gγ subunits, AGG1 or AGG2, increased post-invasive conidiophore formation of the host powdery mildew fungus *G. orontii* (Fig. 2.1C). Taken together, the contribution of Gβ, Gγ1 and Gγ2 to powdery mildew defense is independent of the presence or absence of the MLO2 protein. Thus these

findings support previous results form Kim et al. (2002b), indicating that powdery mildew susceptibility conferred by the presence of MLO does not implicate heterotrimeric G-protein signaling and therefore precluding a role of MLO2 as a GPCR in this context. Furthermore, an interaction between MLO2 and either of the heterotrimeric G-protein components in the split-ubiquitin system have not been observed (Consonni C. unpublished, Ralph Panstruga personal communication). However, although results obtained in this study and analysis performed by Kim and co-workers (2002b) strongly suggest that MLO-dependent powdery mildew pathogenesis functions independently of heterotrimeric G-proteins, this remains a possibility for other yet unknown MLO-dependent processes. Calmodulin (CaM) binding is a common feature of MLO proteins and numerous examples for CaM binding to members of mammalian GPCR families were reported, including the μ-opioid, metabotropis glutamate, serotonin 5-HT(1A) and the D2-dopamine GPCRs (Minakami et al., 1997; Wang et al., 1999; Bofill-Cardona et al., 2000; Belcheva et al., 2001; Kim et al., 2002a; Turner et al., 2004; Bhat et al., 2005; Liu et al., 2007). Alternatively, MLO proteins may transmit extracellular signals through mechanisms that function independently of G-protein coupling (signaling 'at zero G') as has been proven for GPCRs in *Dictyostelium discoideum* and mammalian cells (Brzostowski and Kimmel, 2001). Consistent with this hypothesis, the putative Arabidopsis GPCR, GCR1, which shares extended sequence similarity (~ 20 %) with the *D. discoideum* CAR1 receptor, has been shown to act in both, G-protein-dependent and -independent pathways (Chen et al., 2004; Pandey et al., 2006). On the other hand kinetic studies demonstrated that the sole Arabidopsis Gα subunit is in the activated GTP-bound state by default, suggesting that the heterotrimeric G-protein may act without any canonical GPCRs in Arabidopsis (signaling 'at zero GPCR') (Johnston et al., 2007).

Alternatively, it is conceivable that the function of MLO proteins is entirely unrelated to ligand binding but may involve transduction of signals perceived by other receptors such as receptor-like kinases (RLKs) or receptor-like proteins (RLPs). The Arabidopsis genome contains more than 600 RLKs that represent nearly 2.5 % of the annotated protein-coding genes (Shiu and Bleecker, 2001). Interestingly, applying the split-ubiquitin system to unravel the Arabidopsis membrane interactome within the 'Arabidopsis membrane interactome project' suggests a preferential interaction of the MLO2 protein with different RLKs. The corresponding data can be accessed via the web-based tool Associomics (http://www.associomics.org/index.php), but have to be regarded carefully and need further confirmation. However, recent studies demonstrated that the MLO7/NORTIA protein and FERONIA (FER), a RLK, both control pollen tube reception (Kessler et al., 2010). Moreover,

fer and *mlo7* as well as *mlo2* mutants share several phenotypic similarities, suggesting that FER and various MLO proteins function together to control pollen tube reception as well as powdery mildew pathogenesis and maybe other yet unknown processes. However, an interaction between MLO proteins and FER has not been observed. It, nevertheless, remains also possible that the function of MLO proteins is entirely unrelated to signal transduction.

3.1.2 The heterotrimeric G-protein signaling facilitates defense against powdery mildew fungi

Results obtained in this study demonstrated that absence of the Gβ subunit, AGB1, either alone or in the *mlo2*-6 mutant background, increased penetration rates of both, the adapted and non-adapted powdery mildew fungus *G. orontii* and *E. pisi*, respectively (Fig. 2.1A, B). This finding strongly suggests a role of the Gβ subunit in pre-invasion defense against adapted and non-adapted powdery mildew fungi, which is independent of the presence or absence of MLO2. This is in agreement with the previously published function of the Gβ subunit in defense against necrotrophic fungi including *Plectosphaerella cucumerina*, *Alternaria brassicicola*, *Fusarium oxysporum* and *Botrytis cinerea* (Llorente et al., 2005; Trusov et al., 2006; Trusov et al., 2007). In contrast, previous findings did not support a role of Gβ in Arabidopsis defense against pathogens with a biotrophic life style such as *P. syringae* bacteria or the oomycete *P. parasitica* (Llorente et al., 2005; Trusov et al., 2006). Therefore, data acquired in this study for the first time provide evidence for a function of heterotrimeric G-protein signaling in Arabidopsis defense against biotrophic pathogens, the powdery mildew fungi.

According to previous reports defense against necrotrophic fungi is selectively mediated by Gβγ1 and not Gβγ2 signaling (Trusov et al., 2007). In this study, none of the *agg1*-1 single or respective *mlo2*-6 double mutants showed altered powdery mildew penetration rates, except for the *agg1*-1/*mlo2*-6 double mutant, which displayed a statistically significant increase of *E. pisi* entry success (Fig. 2.1A, B). Furthermore, Gγ2-deficient single and respective *mlo2*-6 double mutants exhibited unaltered powdery mildew penetration rates (Fig. 2.1A, B). However, a functional redundancy between Gγ1 and Gγ2 in the context of powdery mildew defense can be likely excluded, as the respective double mutant *agg1*-1/*agg2*-1 exhibited wild-type like penetration levels of both powdery mildew fungi (Fig. 2.1A, B). Together, these findings do not support a selective function of Gβγ1 or Gβγ2 signaling in defense against powdery mildew fungi and thus contrasts the findings for necrotrophic pathogens (Trusov et al., 2007).

The Gβ and Gγ subunits generally act together as a dimer and therefore mutations in one of the proteins should result in the same phenotype. Paradoxically, while Gβ-deficient mutants displayed enhanced powdery mildew penetration success, mutants lacking the Gγ1 or Gγ2 subunit showed entry rates similar to control plants. This finding indicates a Gγ-independent function of the Arabidopsis Gβ subunit in powdery mildew pre-invasion defense comparable to the mammalian Gβ5 subunit, which interacts with novel binding partners other than Gγ (Snow et al., 1998; Snow et al., 1999; Dupré et al., 2009).

In contrast, single or the corresponding *mlo2*-6 double mutants defective in the Gβ, Gγ1 or Gγ2 subunit allowed increased conidiophore formation of the adapted powdery mildew fungus *G. orontii*, suggesting an involvement of both, Gβγ1 and Gβγ2 dimers, in post-invasion defense (Fig. 2.1C). Taken together, results obtained in this study provide evidence for Gγ-independent as well as -dependent functions of the plant Gβ subunit in pre-and post-invasion defense to powdery mildew fungi, respectively.

Remarkably, conidiophore formation of the adapted powdery mildew fungus *G. orontii* was at least twice as high on all G-protein single mutants compared to wild-type plants, indicating a role of all heterotrimeric G-protein components in post-invasion defense (Fig. 2.1C). This phenotype was surprising, because it implicates that all components of the plant heterotrimeric G-protein act in common cellular processes that affect defense against *G. orontii* following successful host cell invasion. Given that plant heterotrimeric G-protein signaling modulates various biological processes it is conceivable that single mutations in the G-protein complex affect different cellular functions that positively contribute to fungal fitness, therefore ultimately leading to enhanced condidiophore formation of the fungus (Assmann, 2002; Jones and Assmann, 2004; Perfus-Barbeoch et al., 2004; Chen, 2008). Moreover, preliminary data suggest that not only *G. orontii* condiophore formation is increased on all G-protein single mutants but also fungal colony size, indicating a generally better performance of the fungus on these mutants (data not shown).

Of particular importance is that the *agg1*-1 mutation originates from the Arabidopsis Ws-0 accession and was subsequently introgressed into the Col-0 ecotype (Trusov et al., 2007). Therefore, an influence of the Ws-0 genetic background on powdery mildew defense cannot be excluded. In general all *agg1*-1 associated phenotypes have to be regarded with caution.

3.1.3 The Gγ1 subunit is involved in *mlo2*-dependent spontaneous callose accumulation

When grown under axenic conditions *mlo2* mutants display pleiotropic phenotypes such as spontaneous callose accumulation during later developmental stages (Consonni et al., 2006;

Consonni et al., 2010). Recent studies demonstrated that the PMR4/GSL5 callose synthase is accountable for the developmentally controlled biosynthesis of spontaneous callose deposits in *mlo2* plants (Consonni et al., 2010). Analysis of the G-protein single and corresponding *mlo2*-6 double mutants in this study revealed a putative involvement of the Gγ1 subunit in spontaneous callose accumulation in *mlo2* mutant plants. Notably, *agg1*-1/*mlo2*-6 plants showed reduced callose deposition, indicating that Gγ1 contributes to, but is not absolutely required for PMR4-dependent callose accumulation in *mlo2* plants (Fig. 2.2A).

Spontaneous callose formation in *mlo2* mutants depends on functional SA-signaling, as double mutants defective in MLO2 and components of the SA pathway such as *eds5*, *npr1*, *pad4* and *sid2* or when expressing the bacterial salicylate hydroxylase gene, *NahG*, were suppressed in developmental callose deposition (Consonni et al., 2006). Moreover, *mlo* mutant plants displayed elevated levels of free and conjugated SA during the development from six weeks onwards (Consonni et al., 2006). It is therefore tempting to speculate that Gγ1-mediated signaling positively contributes to the SA pathway. Consequently, a mutation in the Gγ1 encoding gene, *AGG1*, may result in a compromised SA pathway and hence account for a reduced spontaneous callose formation in the *mlo2* genetic background. Thus, determination of SA levels in *agg1*-1/*mlo2*-6 plants would be of interest.

PMR4-generated callose is also deposited at wound sites and in pathogen-triggered papillae (Jacobs et al., 2003; Nishimura et al., 2003). Callose staining after leaf wounding of *agg1*-1/*mlo2*-6 mutant plants showed callose speckles around the wound site, but the wound-induced callose ring structure was absent, suggesting that also PMR4-mediated wound callose deposition may be reduced in this double mutant (Fig. 2.2B) (Jacobs et al., 2003). In contrast, pathogen-triggered callose accumulation by the non-adapted powdery mildew fungus *E. pisi* seemed not to be altered in the *agg1*-1/*mlo2*-6 mutant, suggesting that PMR4-mediated pathogen-induced callose production was not affected in this mutant (Fig. 2.2C). However, these results are based on one experiment. Therefore more data are required to investigate a putative function of the Gγ1 subunit in wound- and pathogen-induced callose formation.

The Gβ and Gγ subunits generally act together as a dimer and therefore depletion of one of these proteins should result in the same phenotype. Interestingly, while *agg1*-1/*mlo2*-6 double mutants displayed reduced developmentally controlled callose deposition, *agb1*-2/*mlo2*-6 double mutants lacking the Gβ subunit and MLO2 exhibited callose formation indistinguishable from *mlo2*-6 control plants (Fig. 2.2A). Since a Gβ-independent Gγ-mediated signaling has not been reported yet, it is likely that a yet unknown polymorphism in the Ws-0 genetic background in the *agg1*-1 mutant may account for the reduced spontaneous

callose deposition in the *agg1*-1/*mlo2*-6 mutant. As already mentioned above, all phenotypes associated with the *agg1*-1 mutant have to be regarded critically.

To date only one study provided some evidence for a putative function of the heterotrimeric G-protein complex in pathogen-induced callose deposition. Llorente and colleagues (2005) observed specifically impaired callose accumulation in Gβ-deficient mutants, *agb1*-1, upon inoculation with the necrotrophic fungus *P. cucumerina*, whereas pathogen-induced callose formation upon *P. parasitica* challenge as well as after wounding was not altered in *agb1*-1 mutant plants. Preliminary data obtained in this study showed that pathogen-triggered callose formation upon inoculation with the adapted powdery mildew fungus *G. orontii* was not affected in *agb1*-2 mutants, therefore precluding a role of Gβ in powdery mildew-induced callose accumulation (data not shown).

3.1.4 The Gβ subunit mediates flg22-induced defense responses

Perception of MAMPs by the corresponding PRRs is associated with the activation of diverse physiological responses that are thought to contribute to robust immunity. However, mechanisms by which MAMP-induced responses are achieved are largely unknown. Emerging evidence points to a role of heterotrimeric G-protein signaling in the integration of MAMP-perception into downstream responses. A recent publication revealed a function for the Arabidopsis Gβ subunit in MAMP-induced ROS formation (also oxidative burst) positioning Gβ-mediated signaling upstream of the NADPH oxidase activity (Ishikawa, 2009).

In Arabidopsis the plasma membrane-resident NADPH oxidase homologs RbohD and RbohF (respiratory burst oxidase homologs D and F) are the mediators of apoplastic ROS production in defense and other stresses (Torres et al., 2002; Kwak et al., 2003; Galletti et al., 2008). Whereas RbohD is essential for flg22-induced oxidative burst, RbohF plays a minor role in this context (Zhang et al., 2007).

The results obtained by Ishikawa (2009) were confirmed in this study with two independent *agb1* mutants (*agb1*-2 and *agb1*-9). Both mutants showed reduced ROS production after treatment with flg22 (Fig. 2.11A). Residual ROS spiking in *agb1* mutants suggests the involvement of other components in flg22-triggered oxidative burst.

Different studies already implicated plant heterotrimeric G-protein signaling in Arabidopsis ROS formation during ozone (O_3) stress and stomatal closure as well as in response to an elicitor from the rice blast fungus in rice (Suharsono et al., 2002; Joo et al., 2005; Lieberherr et al., 2005; Li et al., 2009). While the Gα subunit accounts for ROS production in the two

latter cases, Gα and Gβ act synergistically and separately in activating ROS during O_3-stress. The early ROS peak of the bimodal O_3-induced oxidative burst is mediated by both subunits, whereas the late peak is activated by Gα only (Joo et al., 2005).

Although flg22-induced oxidative burst was reduced in Gβ-deficient mutants, MAMP-triggered activation of MAPK cascades was not affected, indicating that Gβ-signaling is not required for MAPK activity (Ishikawa, 2009). Together these results suggest that either both events occur independently of each other or that MAPKs act upstream of Gβ-mediated ROS formation. There have been many disputations about the relationship between ROS and MAPK cascades in plant defense and while some results favour the action of MAPKs upstream of ROS formation (Zhang et al., 2007) other data contradict this hypothesis (Lu et al., 2009; Boudsocq et al., 2010).

Besides MAPK cascades calcium-dependent protein kinases (CDPKs) play a central role in plant immune responses. Recently, Arabidopsis CDPK4, 5, 6 and 11 were reported to mediate MAMP-triggered responses including transcriptional reprogramming and oxidative burst (Boudsocq et al., 2010). Moreover double and triple *cdpk* mutant combinations were compromised in flg22-induced bacterial disease resistance. Results obtained by Boudsocq and colleagues (2010) point to a network of four different regulatory pathways involved in the activation of early flg22-induced genes. Genes such as *FRK1* were specifically activated by MAPKs, whereas induction of *PHI1* was CDPK-specific. Other genes including *NHL10* were synergistically activated by MAPK- and CDPK-signaling.

Consistent with unaltered flg22-induced MAPK activation in Gβ-deficient mutants, induction of *FRK1* and *WRKY22*, another MAPK-activated flg22-responsive gene, was not affected in *agb1* mutants upon flg22 treatment (Fig. 2.12A, B). In contrast expression levels of *NHL10* as well as *PROPEP2* and *PROPEP3*, which have been shown to be specifically activated by CDPK5 and CDPK11 in response to flg22, were reduced in Gβ-deficient mutants (Fig. 2.12C-E) (Boudsocq et al., 2010). Hence it is tempting to speculate that flg22-mediated CDPK-signaling cascade is impaired in *agb1* mutants. To prove this hypothesis, in-gel kinase assays to detect flg22-induced CDPK activity in *agb1* mutants would be of interest. Inconsistent with this assumption, *PHI1* gene activation was not altered upon flg22 treatment in Gβ-deficient mutants (Fig. 2.12F). However, it is conceivable that mutations in the Gβ subunit only affect the activity of a subset and not all CDPKs, thereby allowing normal flg22-induced *PHI1* gene expression in mutant plants. Moreover, a CDPK-independent induction of *PHI1* gene expression in *agb1* mutants cannot be excluded.

Activation of CDPKs occurs through binding of Ca^{2+} ions, and cytosolic Ca^{2+} elevation is an early event in response to MAMP-recognition (Harper et al., 2004; Ma and Berkowitz, 2007). Assuming that reduced flg22-mediated CDPK-signaling in Gβ-deficient mutants might be caused by impaired Ca^{2+} influxes, cytosolic Ca^{2+} signatures upon flg22 treatment were determined in *agb1* mutant protoplasts expressing the Ca^{2+} sensor aequorin. Preliminary data suggest a reduction in cytosolic Ca^{2+} levels in *agb1* mutant protoplasts in response to flg22, leading to a delayed and lower Ca^{2+} spiking in comparison to the wild-type (Fig. 2.11B). Although these results derive from a single experiment and therefore must be judged carefully, it is tempting to speculate that the Gβ subunit may regulate Ca^{2+} influxes in response to flg22. This hypothesis is further supported by studies which showed a function of the plant heterotrimeric G-protein in activation of Ca^{2+} influxes in response to extracellular nucleotides or ABA during stomatal closure as well as during pollen tube germination (Wu et al., 2007; Tanaka et al., 2010; Zhao et al., 2010). While ABA-induced Ca^{2+} influx in guard cells during stomatal closure as well as Ca^{2+} influx during pollen development require the Gα subunit, Ca^{2+} influx induced by extracellular nucleotides is inhibited by the Gβ subunit, as *agb1* mutants exhibited increased cytosolic Ca^{2+} concentrations upon treatment with extracellular nucleotides. This seems to contrast the observations made in this study. However, it is conceivable that Ca^{2+} influxes mediated by flg22 and extracellular nucleotides are coordinated by distinct types of Ca^{2+} channels requiring different regulatory modes of the Gβ subunit. Other studies also implicate heterotrimeric G-protein signaling in ABA- and flg22-induced inhibition of K^+ influxes during stomatal opening (Wang et al., 2001; Fan et al., 2008; Zhang et al., 2008).

Plant cyclic nucleotide gated channels (CNGCs) are supposed to represent non-selective cation channels that are activated by cAMP or cGMP binding and that contribute to cytosolic Ca^{2+} rise upon pathogen perception (Ali et al., 2007; Ma and Berkowitz, 2007; Qi et al., 2010). Mammalian heterotrimerc G-protein signaling regulates adenylate cyclase, the cAMP producing enzyme (Piñeyro, 2009). Interestingly, plants lack a canonical adenylate cyclase, however, there is evidence for a cAMP system in plants (Lomovatskaya et al., 2008). Given that Ca^{2+} influxes in response to flg22 may be disturbed in *agb1* mutants, determination of cyclic nucleotide concentrations in Gβ-deficient plants would be of interest to investigate a putative link between Gβ and CNGC-mediated Ca^{2+} influxes. However, other candidate channels for facilitating cytosolic Ca^{2+} spiking in plant defense such as glutamate receptors cannot be excluded (Ma and Berkowitz, 2007).

Finally, the contribution of the Gβ subunit to antibacterial defense was investigated. Notably, published studies did not provide any evidence for a role of the Gβ subunit in bacterial growth restriction (Trusov et al., 2006). However, these conclusions were based on bacteria infiltration assays, an inoculation method that may bypass the first steps of the natural infection process, as has also been observed for the *fls2* mutant (Zipfel et al., 2004). Naturally, bacteria enter host plant leaves through wounds or natural openings such as stomata. Thus in this study, *P. syringae* bacteria were sprayed on plant leaves to mimic the natural infection conditions, resulting in slightly increased bacterial growth on *agb1* mutant plants (Fig. 2.11C). Although it is largely unknown to which extent MAMP-triggered responses add to plant immunity, it is conceivable that reduced but not abolished flg22-induced defense responses and the functional MAPK signaling in *agb1* mutants account for the subtle bacterial infection phenotype of *agb1* plants. Moreover, it is also possible that subsequent sustained defense responses, which have been shown to be important for robust immunity against bacteria, may not be affected in *agb1* mutants (Lu et al., 2009). Hence, analysis of late induced genes such as *PR1* in Gβ-deficient mutants upon flg22 treatment would be of interest. Taken together, although differences in bacterial proliferation were not statistically significant, a minor contribution of the Gβ subunit to defense against bacteria cannot be excluded.

Analysis of a putative redundant function of the Gγ subunits, Gγ1 and Gγ2, in MTI was hampered by the discovery of an *fls2* mutation in the *agg1*/*agg2* double mutant, explaining the flg22 insensitivity as well as enhanced susceptibility to bacteria of this mutant (Fig. 2.13A-C, 2.14A). Transient complementation of the wild-type *FLS2* gene in *agg1*/*agg2* protoplasts restored flg22-triggered MAPK activation comparable to wild-type (Fig. 2.14B). However, this does not exclude a redundant role of Gγ1 and Gγ2 in flg22-induced defense responses as Gβ-deficient mutants also displayed wild-type like MAPK activation but showed defects in other flg22 responses (Ishikawa 2009 and this study). Bacterial susceptibility of *agg1*-1 and *agg2*-1 single mutants was weakly but not statistically significantly increased, indicating a putative redundant function of Gγ1 and Gγ2 in antibacterial defense. Interestingly, enhanced bacterial susceptibility was only observed with the disarmed bacterial strain *Pst* DC3000 *hrcC*, which is defective in effector delivery into the plant cell, supporting a role of the Gβγ dimer in MTI. However, the generation of a genuine *agg1*/*agg2* double mutant is indispensable to investigate the contribution of the Gγ subunit to MTI.

Interestingly, elf18-mediated oxidative burst was strongly reduced in the *agg1*/*agg2* double mutants (Fig. 2.13A). Similar results were obtained with the Ws-0 ecotype, indicating an

influence of either the Ws-0-associated *fls2* mutation or yet another mutation in the Ws-0 genetic background on elf18-triggered MAMP signaling (Kunze et al., 2004).

3.1.6 A model for the function of heterotrimeric G-protein signaling in MTI and powdery mildew defense

MAMP perception in plants is accompanied by a diverse set of defense responses that are thought to contribute to robust immunity. However, mechanisms by which MAMP-induced responses are achieved remain largely elusive. Published reports and data obtained in this study point to an involvement of heterotrimeric G-protein signaling in the integration of MAMP-perception into downstream responses. In the following, these results are summarized and assembled into a speculative model for the function of the heterotrimeric G-protein in flg22-induced signaling (Fig. 3.1A). Results acquired in this study and by Ishikawa (2009) support a role for the Gβ subunit in flg22-induced ROS production therefore placing Gβ activity upstream of RbohD oxidase function (Fig. 2.11A). Activation of Rboh oxidases involves Ca^{2+} signaling as has been shown in tomato and tobacco and is indicated by the Ca^{2+} binding EF-hand motifs in the N-terminus of Rboh proteins (Keller et al., 1998; Sagi and Fluhr, 2001). Moreover, CDPKs were recently shown to modulate the oxidative burst by directly phosphorylating an Rboh oxidase in potato (Kobayashi et al., 2007). Preliminary data obtained in this study indicate a function of Gβ in flg22-triggered Ca^{2+} influxes (Fig. 2.11B). Reduced Ca^{2+} levels in *agb1* mutant plants may therefore account for decreased oxidative burst as well as impaired CDPK signaling, which is supported by decreased *PROPEP2*, *PROPEP3* and *NHL10* gene activation after flg22 treatment (Fig. 2.12C-E). Unaltered *PHI1* gene expression in *agb1* mutants rather contradicts a reduced CDPK signaling cascade (Fig. 2.12F). However, the existence of CDPK-independent *PHI1* activation cannot be excluded. Together, these findings support a putative role for Gβ in Ca^{2+} channel activation upon flg22 perception. In contrast, MAPK activation is independent of Gβ, which is consistent with unaltered gene induction of *FRK1* and *WRKY22* in *agb1* mutant plants after flg22 treatment (Fig. 2.12A, B) (Ishikawa, 2009). Interestingly, Gβ functions are not restricted to flg22 singnaling, as elf18-induced ROS production was also reduced in the *agb1* mutant (Ishikawa, 2009). It is therefore conceivable that Gβ has a general function in integrating the perception of various MAMPs into downstream signaling.

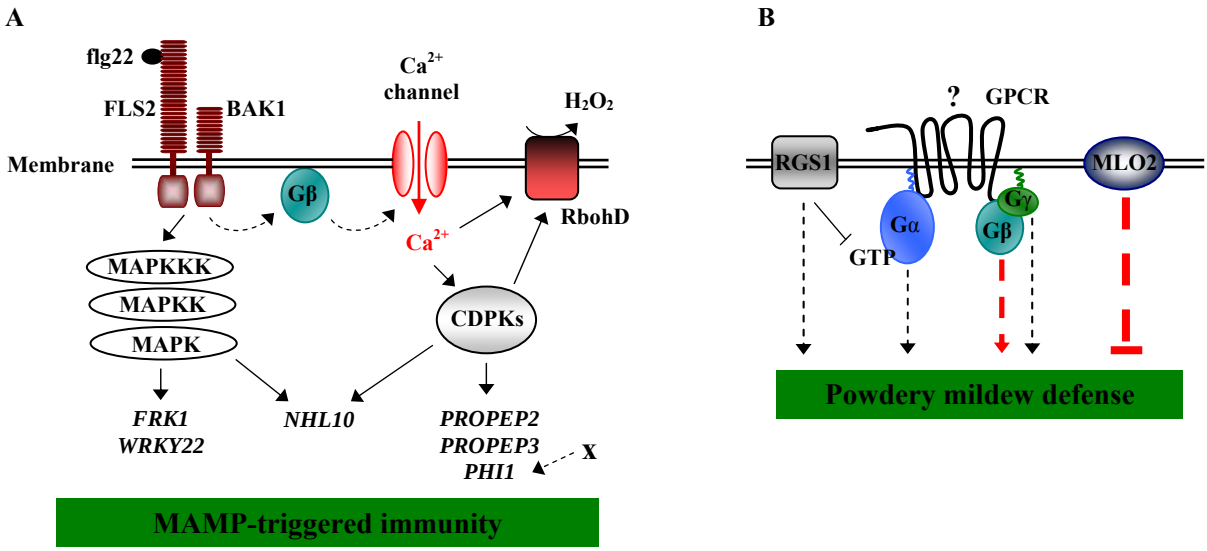

Figure 3.1. A hypothetical model for the function of heterotrimeric G-protein signaling in MTI and powdery mildew defense. (A) Perception of flg22 by the FLS2 receptor stimulates Ca^{2+} influxes, which are partially modulated by the Gβ subunit. Cytoplasmic Ca^{2+} activates CDPK cascades as well as the NADPH oxidase RbohD resulting in an oxidative burst. CDPK signaling mediates transcriptional reprogramming and oxidative burst through phosphorylation of RbohD. In parallel, flg22-perception induces MAPK signaling cascades resulting in transcriptional activation. For simplicity, only the Gβ subunit of the heterotrimeric G-protein complex is depicted. For details see text. **(B)** The contribution of heterotrimeric G-protein signaling to powdery mildew defense is independent of MLO2 function(s). Red lines indicate the involvement in pre-invasion and dashed lines in post-invasion powdery mildew defense. The Gβ subunit plays the predominant role and contributes to both pre- and post-invasion resistance, either alone or as Gβγ dimer. All heterotrimeric G-protein components partly confer post-invasion resistance. Thickness of the lines indicates the degree of the contribution to powdery mildew defense. For simplicity, transmembrane domains of RGS1 and MLO2 are not depicted.

Moreover, besides the involvement of heterotrimerc G-protein signaling in defense against necrotrophic fungal pathogens in Arabidopsis, data acquired in this study also support a role of the heterotrimeric G-protein in defense against virulent and non-virulent biotrophic powdery mildew fungi (Fig. 3.1B, 2.1A-C) (Llorente et al., 2005; Trusov et al., 2006; Trusov et al., 2007). In this context signaling mediated by the Gβ subunit appears to play the predominant role in Arabidopsis powdery mildew defense, as Gβ-deficient mutants were impaired in pre-invasion defense against the adapted and non-adapted *G. orontii* and *E. pisi*, respectively, as well as in post-invasion defense against the adapted fungus. Mutants lacking Gγ1 and Gγ2 exhibited normal powdery mildew penetration rates, indicating a Gγ-independent function of Gβ in pre-invasion defense against powdery mildew fungi (Fig. 2.1A, B). In contrast, post-invasion defense against *G. orontii* seems to involve the action of both G-protein dimers, Gβγ1 and Gβγ2 (Fig. 2.1C). Interestingly, all heterotrimeric G-protein components contribute to powdery mildew post-invasion defense (Fig. 2.1C). However, a direct or indirect function in this context remains elusive. In any way, the contribution of the heterotrimeric G-protein to powdery mildew defense is independent of the presence or

absence of the MLO2 protein. In other words, MLO2 function in powdery mildew pathogenesis does not implicate heterotrimeric G-protein signaling, therefore precluding a role of MLO2 as a GPCR in this context.

3.2 MLO: a negative regulator of powdery mildew defense

3.2.1 MLO proteins negatively regulate the transcriptional activation of defense-related genes in response to *G. orontii* inoculation

Successful powdery mildew pathogenesis in the dicotyledonous plant species Arabidopsis as well as the monocot barley requires the presence of specific isoforms of the plant-unique MLO proteins (Jørgensen, 1992; Consonni et al., 2006). Currently the knowledge about the molecular mechanisms underlying *mlo*-based powdery mildew resistance is sparse. However, the following scenarios are conceivable to explain *mlo*-mediated immunity. Absence of MLO may result in (1) a constitutive and/or (2) faster activation of defense responses and/or (3) may eliminate defense suppression by powdery mildew fungi. In order to differentiate between these possibilities and to learn more about the molecular basis of *mlo*-mediated resistance, a comprehensive comparative analysis of defense marker activation in Arabidopsis wild-type and *mlo2* mutant plants was conducted in the presented study.

In this context a whole leaf-based transcriptome analysis comparing *mlo2*/*6*/*12* triple mutant with Col-0 wild-type plants upon inoculation with the adapted powdery mildew fungus *G. orontii* was conducted at 0, 8 and 12 hpi. Time points before host cell entry were chosen for analysis in order to be able to compare transcriptional reprogramming events of both genotypes. Analysis of the expression data revealed a predominance of genes with elevated transcript levels in the *mlo2*/*6*/*12* triple mutant compared to the wild-type at 8 and 12 hours after *G. orontii* inoculation (Fig. 2.3A). In contrast, the number of genes with decreased transcript accumulation was low, indicating that transcriptional activation and not inhibition dominates the response of *mlo2*/*6*/*12* plants to powdery mildew inoculation. The majority of genes with enhanced expression levels was already present at 8 hpi and detailed analysis revealed a high prevalence of genes known to be associated with defense and stress, which was further supported by *in silico* analysis using MapMan and Genevestigator (Supplementary Table 6.1, 6.3, Fig. 2.3A-C). Similarly, barley *mlo* mutants also displayed elevated transcript accumulation of pathogenesis-related genes compared to the wild-type in response to the barley powdery mildew fungus *Bgh* (Peterhänsel et al., 1997; Gjetting et al., 2004; Zierold et al., 2005). Together, these findings point to a faster defense gene activation in Arabidopsis and barley *mlo* mutant plants at early stages after inoculation with the

compatible powdery mildew fungus. Recently published microarray data showed that a similar set of defense-related genes was also activated in Col-0 wild-type plants after *G. orontii* inoculation, indicating that these genes are generally activated in response to *G. orontii* challenge (Chandran et al., 2009; Chandran et al., 2010). Importantly, transcriptome analysis performed by Chandran and colleagues (2009, 2010) focused on later time points after *G. orontii* inoculation (from 0 – 7 dpi) when the fungus already successfully invaded and colonized the host plant. In contrast, activation of defense genes in the *mlo2/6/12* mutant was already present before host cell penetration. Taken together, these findings suggest that the presence of MLO proteins may negatively regulate defense gene activation in response to powdery mildew challenge, corroborating the model of MLO as a negative regulator of plant defense (Büschges et al., 1997; Panstruga, 2005; Consonni et al., 2010). If this holds true, then MLO-mediated defense gene suppression in wild-type plants may account for the delayed formation of respective antimicrobial metabolites, thus leading to compatibility of *G. orontii*. In contrast, the absence of MLO proteins, resulting in a faster activation of defense genes upon *G. orontii* challenge, may contribute to early termination of pathogenesis and thus confer resistance against the fungus. However, it is conceivable and therefore important to note that negative regulation of defense responses by loss of MLO proteins may either be a direct or an indirect consequence of *mlo* mutations. The first scenario implicates a direct involvement of MLO proteins in defense suppression. In the latter case, yet unknown changes that are associated with *mlo* mutations, which may implicate alterations in cell wall composition, the apoplastic environment or other, may primarily cause early termination of fungal pathogenesis thereby preventing host cell invasion and effective defense suppression by the fungus. In this case, hyperactivation of defense genes in the *mlo2/6/12* mutant upon *G. orontii* challenge would be an indirect consequence. However, data obtained in this study rather contradict this scenario, as early defense gene activation upon MAMP treatment also occurred faster in *mlo2/6/12* triple mutant than in wild-type plants, suggesting that MLO proteins negatively regulate this MAMP response (see 3.2.6, Fig. 2.9, 2.10).

Rapid transcriptional activation of defense genes in the *mlo2/6/12* mutant may not only be induced by powdery mildew fungi, but occur generally upon any pathogen challenge. Therefore, transcriptional analysis of *mlo2/6/12* mutant plants upon inoculation with pathogens other than powdery mildew is required to investigate this possibility. However, absence of barley MLO enhanced transcript accumulation of pathogenesis-related genes in response to the rice blast fungus *Magnaporthe oryzae* (Jarosch et al., 2003). Furthermore, as shown in this study defense gene activation was also enhanced in Arabidopsis *mlo2/6/12*

mutant plants upon MAMP treatment (Fig. 2.9, 2.10). Taken together, these findings disagree with a powdery mildew-specific activation of defense genes in barley and Arabidopsis *mlo* mutants, further corroborating a general function of MLO proteins as negative regulators of defense.

A constitutive gene expression was not observed in *mlo2*/*6*/*12* mutant plants under the tested conditions, but constitutively enhanced transcript levels in epidermal cells may be diluted in whole-leaf extracts. Moreover, it cannot be ruled out that constitutive activation of other, transcription-independent defense responses may account for *mlo*-based immunity.

In whole leaf-based transcriptomic analysis of *G. orontii* - Arabidopsis interaction, genes specifically activated by the fungus at infection sites are masked and diluted by infection-independent gene expression in distal cell tissues. To avoid the dilution effect, Chandran and colleagues (2010) employed the laser microdissection (LMD) technology to isolate single *G. orontii*-infected epidermal cells, thereby increasing the sensitivity to detect responses that act specifically at the site of infection. Using the LMD approach, 1089 differentially expressed genes were exclusively identified in the site-specific analysis, allowing the identification of novel host processes and components previously hidden in whole-leaf global expression analysis. Although transcriptome analysis of powdery mildew-infected epidermal cells of barley *mlo* mutants was performed previously, it would be of importance to apply the LMD technology to isolate epidermal cells from *G. orontii*-challenged Arabidopsis *mlo2*/*6*/*12* mutant plants in order to get more detailed insights into the molecular basis of *mlo*-mediated transcriptional reprogramming in response to powdery mildew inoculation (Zierold et al., 2005).

3.2.2 Non-host resistance and *mlo*-mediated immunity share similar transcriptional reprogramming events in response to powdery mildew inoculation

Comparative transcriptome analysis performed in this study revealed that transcript levels of genes related to plant defense increased more rapidly in *mlo2*/*6*/*12* mutant plants upon inoculation with the adapted powdery mildew fungus *G. orontii* (Supplementary Table 6.1). Among these genes the plant defensins genes *PDF1.2a* and *PDF1.2b* as well as genes encoding components relevant for the Trp-derived indole glucosinolate pathway were present (Table 2.1, 2.2). Indolic glucosinolates were previously described in the context of powdery mildew non-host resistance, but have been recently shown to be required for *mlo*-mediated immunity (Bednarek et al., 2009; Consonni et al., 2010). Interestingly, genes of the *PDF1* family including *PDF1.2a* and *PDF1.2b* as well as particular indole glucosinolate pathway-

relevant genes were also shown to be uniquely induced in wild-type plants following inoculation with the non-adapted powdery mildew fungus *Bgh* (Zimmerli et al., 2004). This suggests that the adapted powdery mildew fungus may elicit the same transcriptional responses in the *mlo2*/*6*/*12* mutant as *Bgh* in Arabidopsis wild-type plants, indicating that non-host resistance to *Bgh* and *mlo*-mediated immunity to *G. orontii* share similar transcriptional reprogramming events in response to the respective pathogen. Consistently, when comparing histological and phytopathological characteristics as well as genetically defined requirements of powdery mildew non-host and *mlo*-based resistance in Arabidopsis and barley, it becomes evident that both types of resistance share analogous features (see 1.5.3.2). It has been therefore hypothesized that non-host resistance and *mlo*-based immunity are mechanistically related or possibly identical (Humphry et al., 2006). Taken together, results obtained by Zimmerli and colleagues (2004) as well as in this study suggest similar transcriptional responses in wild-type and *mlo2*/*6*/*12* mutant plants upon challenge with *Bgh* and *G. orontii*, respectively, therefore substantiating a mechanistic link between powdery mildew non-host resistance and *mlo*-mediated immunity.

3.2.3 MLO proteins negatively regulate the accumulation of the defense-relevant indolic glucosinolate, 4MI3G, in response to *G. orontii* inoculation

It has recently been demonstrated that Trp-derived indolic metabolites that are hydrolyzed by the atypical myrosinase PEN2 are required for Arabidopsis non-host resistance (Bednarek et al., 2009). Loss of *PEN2* enhanced the penetration rate of the non-adapted powdery mildew fungi *Bgh* and *E. pisi*. Similarly, double mutants defective in *CYP79B2* and *CYP79B3*, the two core enzymes leading to most known Trp-derived metabolites, also showed enhanced susceptibility to the non-adapted fungi, pointing to the importance of indole metabolites in powdery mildew non-host resistance. Specifically, the 4-methoxyindol-3-ylmethylglucosinolate (4MI3G) is supposed to be the biologically relevant PEN2 substrate leading to the formation of yet unknown toxic metabolites that are important for the restriction of fungal growth (Supplementary Figure 6.1) (Bednarek et al., 2009). More recently, it was found that the same Trp-derived indolic secondary metabolites are likely also essential for *mlo*-mediated powdery mildew resistance (Consonni et al., 2010). The absence of *PEN2* or both *CYP79B2* and *CYP79B3* in the *mlo2* mutant background restored susceptibility of the adapted powdery mildew fungus *G. orontii* to wild-type-like levels, indicating that MLO2 negatively regulates this defense pathway (Consonni et al., 2006; Consonni et al., 2010). In this study, comparative transcriptional profiling revealed a hyperactivation of genes

encoding enzymes involved in the biosynthesis of Trp-derived metabolites in the *mlo2*/*6*/*12* triple mutant in response to the powdery mildew fungus *G. orontii*, corroborating the importance of these metabolites in Arabidopsis *mlo2*-based powdery mildew immunity (Table 2.1). Interestingly, a similar set of genes was also found to hyperaccumulate in the *mlo2*/*6*/*12* mutant under pathogen-free conditions during vegetative development in 7-week-old plants (Consonni et al., 2010). It is therefore tempting to speculate that the increased spontaneous activation of defense responses including the biosynthesis of indolic secondary metabolites in *mlo2*/*6*/*12* mutant plants may account for the pleiotropic effects in this mutant (Consonni et al., 2006; Consonni et al., 2010).

To study if the transcriptional hyperactivation in powdery mildew-inoculated *mlo2*/*6*/*12* mutant correlates with enhanced accumulation of indolic metabolites, comparative profiling of the Trp-derived compounds I3G, I3A, RA and 4MI3G was performed. Interestingly, no differnetial accumulation of these metabolites was observed between Col-0 wild-type and *mlo2*/*6*/*12* mutant plants after *G. orontii* or *Bgh* inoculation (Fig. 2.6). However, it cannot be excluded that higher accumulation of indolic metabolites in the *mlo2*/*6*/*12* mutant may be compensated by a concomitant increase in glucosinolate turnover, therefore resulting in unaltered compound steady-state levels between the mutant and wild-type. Moreover, a differential indolic metabolite accumulation might be diluted in whole-leaf extracts. Importantly, it is currently not possible to properly estimate the turnover of the indole glucosinolate pathway, because the indolic end products remain elusive (Bednarek et al., 2009).

Consistent with previous findings, accumulation of the indole glucosinolate 4MI3G, which is activated by PEN2 for antifungal defense, was absent in *pen2*-1 mutants challenged with *Bgh*, confirming that 4MI3G is a PEN2 substrate (Fig. 2.6) (Bednarek et al., 2009). A similar phenotype was observed in the *pen2*-1/*mlo2*/*6*/*12* quadruple mutant. Interestingly, absence of *PEN2* did not cause 4MI3G accumulation in response to the adapted powdery mildew fungus *G. orontii*, suggesting that the host fungus may fail to elicit 4MI3G formation. Inconsistent with this hypothesis, the quadruple mutant defective in *PEN2*, *MLO2*, *MLO6* and *MLO12* displayed 4MI3G accumulation upon *G. orontii* inoculation, indicating that the presence of MLO proteins negatively regulates 4MI3G accumulation in response to *G. orontii* challenge. This is in agreement with the hypothesis that the adapted powdery mildew fungus might specifically corrupt MLO proteins to modulate plant defense for successful pathogenesis (Panstruga, 2005). Consequently, absence of MLO-mediated defense suppression by the fungus may enable the formation of 4MI3G and its subsequent activation by PEN2 for

antifugal defense and thus leading to resistance of *mlo2/6/12* mutant plants against *G. orontii*. Alternatively, suppression of 4MI3G accumulation in the *mlo2/6/12* mutant may be an indirect consequence of the mutations in *MLO* genes and primarily an effect of abolished fungal host cell invasion, indicating that post-invasive fungal infection structures might be required for successful suppression of 4MI3G accumulation (see 3.2.1).

3.2.4 The adapted powdery mildew fungus, *G. orontii*, induces PEN1 protein accumulation dependent on the MLO proteins

The PEN1-dependent vesicle secretory pathway is required for Arabidopsis non-host resistance to powdery mildew fungi (Lipka et al., 2005). Absence of the PEN1 syntaxin greatly enhanced the entry rates of the barley and pea powdery mildew fungi, *Bgh* and *E. pisi*, respectively. It has been proposed that the cargo delivered by PEN1-dependent vesicles may comprise antimicrobials and cell wall components in order to defeat the pathogen and reinforce the cell wall at attempted penetration sites (Kwon et al., 2008). PEN1 as well as its barley homolog, ROR2, are also essential for *mlo*-mediated powdery mildew resistance in Arabidopsis and barley, respectively (Freialdenhoven et al., 1996; Collins et al., 2003; Consonni et al., 2006). Loss of wild-type ROR2 or PEN1 in *Hvmlo* or *Atmlo2* genotypes, respectively, restored susceptibility to the corresponding adapted powdery mildew fungus, suggesting that syntaxin activity is required for effective *mlo* resistance. Moreover, PEN1, ROR2 as well as the MLO protein focally accumulate at sites of attempted fungal cell-wall penetration (Assaad et al., 2004; Bhat et al., 2005; Meyer et al., 2009).

Consistent with previously published data, results obtained in this study demonstrated an induced PEN1 protein accumulation in response to the non-adapted powdery mildew fungus *Bgh*, likely reflecting an enhanced requirement for PEN1-mediated defense against this pathogen (Fig. 2.7B) (Pajonk et al., 2008). These observations corroborate the importance of PEN1-dependent activity in Arabidopsis non-host resistance. PEN1 accumulation in response to *Bgh* occurred irrespective of the presence or absence of functional MLO proteins (Fig.2.7B).

PEN1 protein levels also increased in Col-0 wild-type plants in response to the adapted powdery mildew fungus *G. orontii*, although delayed and to a lesser degree compared to *Bgh*-inoculated wild-type plants (Fig. 2.7B). Consistently, transcriptomic analysis of single *G. orontii*-infected wild-type epidermal cells revealed that genes associated with the PEN1-secretory defense pathway such as *PEN1* itself, *SNAP33*, *VAMP721* and *VAMP722* displayed elevated transcript levels at 5 dpi, indicating a transcriptional activation of the PEN1 defense

pathway upon *G. orontii* challenge (Chandran et al., 2010). Interestingly, PEN1 protein levels decreased in *G. orontii* inoculated *mlo* mutant plants with a stronger decrease in the *mlo2*/*6*/*12* triple compared to the *mlo2*-6 single mutant, suggesting no major contribution of the PEN1-mediated defense pathway to *mlo*-mediated resistance against *G. orontii*. This is consistent with the powdery mildew infection phenotype of Arabidopsis *mlo2*/*pen1* double mutant plants. While the Arabidopsis powdery mildew fungus *G. cichoracearum* was able to propagate on *mlo2*/*pen1* mutant plants, these plants were still resistant to the adapted fungus *G. orontii*, suggesting that *G. orontii* might be insensitive to PEN1-mediated defense (Consonni et al., 2006).

As demonstrated in this study, *G. orontii*-induced PEN1 protein accumulation appears to be dependent on the presence of functional MLO proteins, indicating that the adapted powdery mildew fungus *G. orontii* may trigger PEN1 protein accumulation through the MLO proteins. With respect of the different *G. orontii* and *G. cichoracearum* infection phenotypes on *mlo2*/*pen1* mutant plants and the results obtained in this study, it is tempting to speculate that the adapted fungus *G. orontii*, but not *G. cichoracearum*, may have evolved means to subvert the PEN1-mediated secretory pathway for its own advantage. Such advantage may implicate the delivery of beneficial cargo for successful fungal propagation. An example for successful abuse of host trafficking vesicles by pathogens is the human bacterium *Salmonella enterica*. The pathogen replicates within host cells in a specialized membrane-bound compartment, the *Salmonella*-containing vacuole (SCV) and uses effectors to exploit host vesicle trafficking for biogenesis and maintenance of the SCV (Knodler and Steele-Mortimer, 2005; Bujny et al., 2008). However, if *G. orontii* in fact is able to manipulate the PEN1-dependent exocytosis apparatus for the secretion of beneficial cargo, than the contribution of such mechanisms to the establishment of successful *G. orontii* pathogenesis is rather minor or redundant, as *pen1* mutants are still susceptible to *G. orontii* and not resistant (Lipka et al., 2005). In future, it would be of interest to analyse PEN1 protein accumulation in *mlo* mutant plants in response to *G. cichoracearum* challenge.

3.2.5 Plant defensins are dispensable for *mlo*-mediated powdery mildew resistance

Global gene expression analysis performed in this study revealed an overrepresentation of JA- and ET-responsive genes among the genes that showed elevated expression levels in the *mlo2*/*6*/*12* triple mutant after *G. orontii* inoculation (Table 2.2, Supplementary Table 6.1). In the microarray analysis, the plant defensin genes *PDF1.2a* and *PDF1.2b*, which represent major marker genes for JA and ET signaling pathways, displayed 5 – 7-fold enhanced

transcript abundance in *mlo2*/*6*/*12* mutant plants after *G. orontii* challenge compared to the wild-type. These results suggest an increased JA/ET signaling activity in *mlo2*/*6*/*12* mutant plants after *G. orontii* inoculation. As previously published, enhanced *PDF1.2* transcript abundance was also observed in wild-type plants upon challenge with the non-adapted powdery mildew *Bgh* but not in response to the host fungus *G. cichoracearum* (Zimmerli et al., 2004). It was thus concluded that the host pathogen must either suppress or fail to elicit the JA/ET signaling pathways. Differential transcript levels of *PDF1.2* genes in *G. orontii*-challenged *mlo2*/*6*/*12* mutant plants disagree with the latter hypothesis and rather favour the first assumption, pointing to a negative regulation of JA and/or ET signaling by MLO proteins in response to *G. orontii*. Consequently, an increased activity of these phytohormones in powdery mildew-challenged *mlo2*/*6*/*12* mutant plants may contribute to resistance. Inconsistent with this hypothesis, jasmonate levels did not differ between *mlo2*/*6*/*12* and wild-type plants in response to *G. orontii* inoculation (Fig. 2.5). However, a possible dilution effect in whole leaf-based analysis or altered ET accumulation and signaling in *G. orontii*-challenged *mlo2*/*6*/*12* mutant plants cannot be excluded. Nonetheless, *mlo2* double mutants affecting the JA or ET pathway still displayed a resistant powdery mildew infection phenotype, which was comparable to *mlo2* single mutants (Consonni et al., 2006). Taken together these findings rather preclude a role for JA/ET signaling in *mlo*-based powdery mildew immunity, although a compensatory effect of both pathways cannot be ruled out. Therefore, a triple mutant defective in *MLO2* and both signaling pathways is required to conclusively investigate this possibility. As demonstrated in this and previous studies, the major regulators of JA and ET signaling, COI1 and EIN2, are required for *PDF1.2* gene expression in *G. orontii* treated *mlo* plants, but they are dispensable for *mlo*-mediated powdery mildew resistance, indicating that the plant defensins PDF1.2 do not contribute to *mlo*-based immunity (Fig. 2.4F) (Consonni et al., 2006). Nevertheless, it remains elusive how JA/ET-responsive genes are activated in *mlo2*/*6*/*12* mutant plants upon *G. orontii* inoculation. Notably, *PDF1.2* gene expression seems to underlie circadian oscillations resulting in elevated transcript accumulation at the same time point when *PDF1.2* gene expression was induced in *mlo2*/*6*/*12* mutant plants by *G. orontii* (Fig. 2.4D). However, this contribution is rather minor, as non-treated *mlo2*/*6*/*12* plants displayed a much lower *PDF1.2* gene expression than *G. orontii*-challenged plants. Currently it cannot be excluded that the fungus induces a wound response during its penetration attempts, resulting in enhanced *PDF1.2* transcript abundance.

3.2.6 MLO proteins negatively regulate CDPK-mediated gene activation in response to MAMPs

MAMP perception triggers a broad set of cellular responses that are supposed to enhance the state of plant immunity to limit invasion and propagation of potential microbial intruders. To date knowledge about a potential function of MLO proteins in MAMP-induced signaling pathways is lacking. Consequently, analysis of MAMP-triggered responses in *mlo* mutant plants was undertaken in this study. Interestingly, the oxidative burst in response to chitin, but not flg22 treatment, was abolished in *mlo2*/*6*/*12* triple mutant, but not in *mlo2* single mutant plants (Fig. 2.8A). This result implicates a role of MLO6 and/or MLO12 in ROS formation that is specific to chitin and independent of MLO2. To clarify if one or both MLO proteins mediate chitin-induced ROS formation, analyses of the single or respective *mlo6*/*12* double mutant are required. Consistent with unaltered MAPK induction upon treatment with flg22 or chitin, activation of MAPK-induced genes including *FRK1*, *WRKY22* and *WRKY29* was not changed in *mlo2*/*6*/*12* mutant plants, indicating that MAMP-triggered MAPK signaling is not affected in the mutant (Fig. 2.8B, 2.9A, B, 2.10B). In contrast, CDPK-activated genes such as *PHI1*, *PROPEP2*, *PROPEP3* and *NHL10* displayed elevated transcript levels in the *mlo2*/*6*/*12* mutant in response to flg22 and chitin (Fig. 2.9D, E, 2.10A, C). These findings suggest a negative regulation of MAMP-induced CDPK signaling by the MLO proteins. Consistent with this notion, transient expression of a putative constitutive active variant of the barley *Hv*CDPK3 partially compromised *mlo*-mediated *Bgh* resistance in barley, corroborating a negative regulation of CDPK signaling by MLO proteins (Freymark et al., 2007). A similar break in *mlo* resistance to *Bgh* was also observed upon expression of the junction domain of the paralog *Hv*CDPK4, which supposed to exert a dominant negative effect on multiple CDPKs, pointing to antagonistic roles of individual CDPKs (Freymark et al., 2007). However a potential link between MLO function and CDPK signaling requires further investigation and in-gel kinase assays to detect MAMP-induced CDPK activity in *mlo* mutant plants as well as *cdpk* mutant analysis would be of interest in this context.

3.2.7 A model for MLO-mediated control of powdery mildew defense

Mutations affecting the PEN1- or PEN2- (PEN2, PEN3, CYP79B2, CYP79B3) defense pathway restore powdery mildew susceptibility in the *mlo2* mutant, indicating that presence of MLO2 negatively regulates these defense pathways (Consonni et al., 2006; Consonni et al., 2010). Consistently, results obtained in this study demonstrated that dependent on the presence of MLO proteins the host fungus, *G. orontii*, is able to suppress the accumulation of

the defense-relevant PEN2 substrate, 4MI3G, thereby inhibiting the PEN2-dependent glucosinolate defense pathway (Fig. 2.6). However, it remains elusive if this suppression acts through the MLO proteins or requires the formation of post-invasive fungal infection structures.

In addition, data presented in this study point to a negative regulation of transcriptional defense gene activation by MLO proteins in response to powdery mildew challenge (Supplementary Table 6.1). Unexpectedly, defense gene activation upon MAMP treatment appears also to be suppressed by MLO proteins, suggesting an impact of MLO functions on MAMP-triggered signaling, possibly through the regulation of CDPK-cascades (Fig.2.9, 2.10). Consistent with previous findings, results from this study preclude a role of JA signaling in *mlo*-mediated resistance (Fig. 2.4, 2.5) (Consonni et al., 2006). In any way, findings from this work rather disagree with a constitutive activation of defense responses in *mlo* mutants. Taken together, powdery mildew resistance in *mlo* mutants appears to be the consequence of multiple more rapidly activated defense response pathways (Fig. 3.2).

The presence of MLO is absolutely required for powdery mildew fungi to successfully infect the host plant. It was therefore proposed that these pathogens possibly abuse MLO function(s) to suppress host defense (Panstruga, 2005). Alternatively, MLO proteins were suggested to function as a negative regulator (modulator) of plant defense (Panstruga and Schulze-Lefert, 2003). Data obtained in this study rather support the second hypothesis. Early defense gene activation upon MAMP treatment, which did not implicate the influence of the fungus, occurred faster in the Arabidopsis *mlo2*/*6*/*12* triple mutant than in the wild-type, suggesting that MLO proteins dampen early MAMP signaling. However, it can not be ruled out that powdery mildew fungi potentiate the negative regulatory role of MLO proteins, e.g. by manipulation of MLO activity via secreted effector proteins.

It has been previously hypothesized that powdery mildew non-host resistance and *mlo*-mediated immunity share common defense execution machinery (Humphry et al., 2006). The results obtained in this study corroborate this hypothesis, pointing to similar transcriptional reprogramming events in wild-type and *mlo* mutant plants in response to the non-adapted and adapted powdery mildew fungus, respectively.

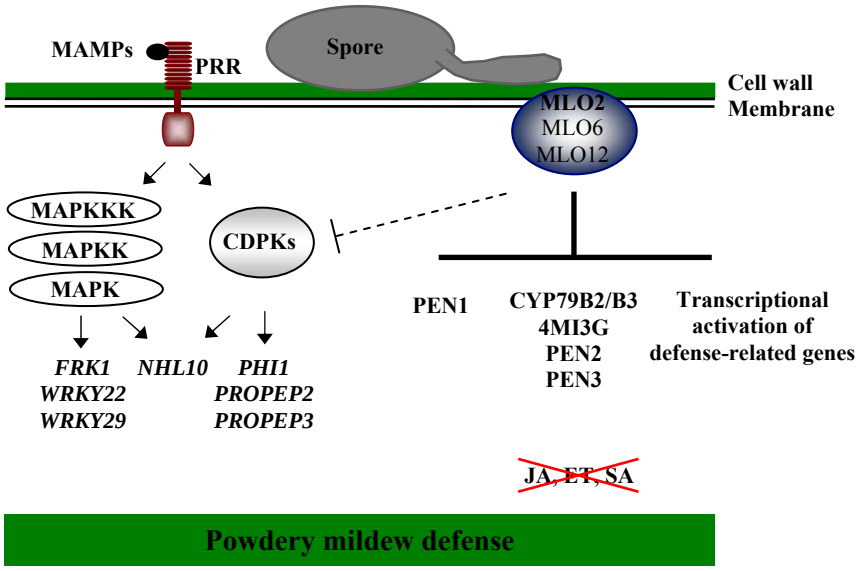

Figure 3.2 A hypothetical model for MLO-mediated control of Arabidopsis defense. MAMP recognition mediates MAPK and CDPK signaling resulting in transcriptional activation. MLO proteins negatively regulate defense, including the PEN1-dependent secretory pathway, biosynthesis of Trp-derived secondary metabolites, transcriptional activation of defense-related genes and MAMP-induced CDPK signaling. Powdery mildew fungi may potentiate the negative regulatory role of MLO proteins. Components marked by the red cross are not required for *mlo*-mediated resistance. JA: jasmonates, ET: ethylene, SA: salicylic acid, PRR: pathogen recognition receptor, MAMP: microbe-associated microbial pattern.

3.3 Perspectives

Results presented in this study point to a role of the Gβ subunit in the regulation of flg22-induced CDPK signaling cascades. In order to prove this assumption in-gel kinase assays to detect potentially altered flg22-induced CDPK activity would be of interest. Preliminary data indicate that Gβ functions may be involved in the modulation of Ca^{2+} influxes in response to flg22. However, these data have to be confirmed in additional experiments. Nonetheless, it would be of interest to determine cyclic nucleotide concentrations in flg22-treated *agb1* mutant plants to investigate a putative link between Gβ and CNGC-mediated Ca^{2+} influxes. Moreover, the generation of a genuine *agg1*/*agg2* double mutant is indispensable to analyze a putative redundant function between the Gγ1 and Gγ2 subunits in MAMP-triggered responses.

Data obtained in this study indicate an impact of MLO function(s) in MAMP signaling. Interestingly, MLO6 and/or MLO12, but not the MLO2 protein, seem to regulate ROS production that is specific to chitin. Mutant analysis using the single or respective *mlo6*/*12* double mutant is required to entangle the role of these MLO proteins to chitin-mediated oxidative burst.

4. Material and Methods

4.1 Material

4.1.1 Plant materials

Arabidopsis thaliana (here referred to as Arabidopsis) wild-type and mutant lines used in this study are listed in Table 4.1 and 4.2, respectively. G-protein *mlo2* double mutants were generated by crossing the *mlo2*-6 mutant with the corresponding G-protein single mutants listed below and homozygous plants were selected by PCR genotyping from F_2 progeny.

Table 4.1. Wild-type Arabidopsis accessions used in this study

Accession	Abbreviation	Original source
Columbia	Col-0	J. Dangl[a]
Wassilewskaija	Ws-0	K. Feldmann[b]

[a]University of North Carolina, Chapel Hill, NC, USA
[b]University of Arizona, Tuscon, AZ, USA

Table 4.2. Mutant Arabidopsis lines used in this study

Mutant allele	Accession	Mutagen	Reference/Source
agb1-2	Col-0	T-DNA	Ullah et al., 2003
agb1-9	Col-0	EMS	A. M. Jones[a]
agg1-1	Col-0/(Ws-0)[b]	T-DNA	Trusov et al., 2007
agg2-1	Col-0	T-DNA	Trusov et al., 2007
agg1-1/*agg2*-1	Col-0/(Ws-0)[b]	T-DNA	Trusov et al., 2007
fls2	Col-0	T-DNA	Zipfel et al., 2004
gpa1-4	Col-0	T-DNA	Jones et al., 2003
mlo2-6	Col-0	T-DNA	Consonni et al., 2006
mlo2-11	Col-0	EMS	Consonni et al., 2006
mlo2-11/*coi1*-1	Col-0	EMS	C. Consonni[c]
mlo2-11/*ein2*-1	Col-0	EMS	C. Consonni[c]
mlo2-5/*mlo6*-2/*mlo12*-1	Col-0	T-DNA/T-DNA/transposon	Consonni et al., 2006
pen2-1	Col-3, *gl1*	EMS	Lipka et al., 2005
pen2-1/*mlo2*-5/6-2/*12*-1	Col-0/Col-3, *gl1*	EMS/ T-DNA/T-DNA/transposon	R. Panstruga[c]
rgs1-1	Col-0	T-DNA	Chen et al., 2003

[a]University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; unpublished
[b]Ws-0 *agg1*-1 allele introgressed into Col-0 genetic background, 8th backcrossed generation
[c]Max-Planck Institute for Plant Breeding Research, Cologne, Germany; unpublished
EMS: ethyl methanesulfonate; T-DNA: transfer DNA

4.1.2 Pathogens

4.1.2.1 *Pseudomonas syringae* pv. *tomato* (*Pst*)

Pseudomonas syringae pv. *tomato* (*Pst*) wild-type strain DC3000 (R. Innes, Indiana University, Bloomington, Indian, USA) and *Pst* DC3000 *hrcC* defective in the type III secretion system (K. Shirasu, The Sainsbury Laboratory, John Innes Centre, Norwich, UK) were used throughout this study.

4.1.2.2 Powdery mildew fungi

Anonymous powdery mildew isolates of *Golovinomyces orontii* (*G. orontii*) and *Erysiphe pisi* (*E. pisi*) kept at the Max-Planck Institute for Plant Breeding Research (Cologne, Germany) as well as the *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolate K1 were used in this study (Büschges et al., 1997; Lipka et al., 2005).

4.1.3 Vectors

Vectors used in this study are listed in Table 4.3.

Table 4.3. Vectors used in this study

Vector	Description
pAM-PAT-35S-mCherry-AEQ-NES	Binary Gateway® vector expressing mCherry-tagged aequorin carrying a NES motif obtained from Dr. M. Kwaaitaal (Max-Planck Institute for Plant Breeding Research, Cologne, Germany).
pEX-35S-FLS2-YFP	Binary Gateway® vector expressing YFP-tagged FLS2 obtained by Dr. N. Frey dit Frei (Max-Planck Institute for Plant Breeding Research, Cologne, Germany).

4.1.4 Oligonucleotides

Oligonucleotides used in this study are listed in Tables 4.4 and 4.5 and were purchased from Invitrogen (Karlsruhe, Germany).

Table 4.4. Oligonucleotides used in this study for qRT-PCR analysis (F: forward, R: reverse)

Gene	AGI code	Sequence 5'→3'	Reference
At4g26420	At4g26420	F: GAGCTGAAGTGGCTTCCATGAC R: GGTCCGACATACCCATGATCC	Czechowski et al., 2005
FRK1	At2g19190	F: CGGTCAGATTTCAACAGTTGTC R: AATAGCAGGTTGGCCTGTAATC	Boudsocq et al., 2010
LOX2	At2G45140	F: TGAATTGCAAGCTGTTGCTC R: GCAGAAGCTACAAGACCACC	unpublished
NHL10	At2g35980	F: TTCCTGTCCGTAACCCAAAC R: CCCTCGTAGTAGGCATGAGC	Boudsocq et al., 2010
PDF1.2a	At5g44420	F: TAAGTTTGCTTCCATCATCACCC R: GTGCTGGGAAGACATAGTTGCAT	Zimmerli et al., 2004
PDF1.2b	At2g26020	F: ACGCTGCTCTTGTTCTCTTTGCA R: AAGTACCACTTGGCTTCTCGCAC	Zimmerli et al., 2004
PHI1	At1g35140	F: TTGGTTTAGACGGGATGGTG R: ACTCCAGTACAAGCCGATCC	Boudsocq et al., 2010
PROPEP2	At5g64890	F: AGAAAAGCCTAGTTCAGGTCGTC R: CTCCTTATAAACTTGTATTGCCGC	unpublished
PROPEP3	At5g64905	F: GTTCCGGTCTCGAAAGTTCATC R: TGAACTCTAATTGTGTTTGCCTCC	unpublished
VSP2	At5g24770	F: ACCGTTGGAAGTTGTGGAAG R: CCAAATCAGCCCATTGATCT	unpublished
WRKY22	At4g01250	F: CATCCGATCAACAGACGAGTAAAT R: AAATTCGTCGGCTGAAGTCAC	Lu et al., 2009
WRKY29	At4g23550	F: TCCTATGATCCCATCCGCTG R: CGCTTGGTGCGTACTCGTT	Lu et al., 2009

Table 4.5. Oligonucleotides used in this study for sequencing (F: forward, R: reverse)

Gene	AGI code	Sequence 5'→3'	Reference
FLS2	At5g46330	F: TCTTAGGGTTTGCGTGGGAAAGCG R: AATCGATTTCTCCACCAATTGGCGC	unpublished

4.1.5 Enzymes

Restriction enzymes were purchased from New England Biolabs (Frankfurt/Main, Germany) and Fermentas (St. Leon-Rot, Germany). Restriction digestions were performed following the manufacturer's recommendations, using the provided 10 x reaction buffer. Standard and quantitative RT-PCR reactions were performed using home-made *Taq* DNA polymerase.

4.1.6 Chemicals and antibiotics

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Serva (Heidelberg, Germany) unless otherwise stated. Rifampicin (Rif) antibiotic stock solution (1000 x) was prepared in DMSO, sterile filtrated and used in the concentration of 100 µg/ml.

4.1.7 Elicitors

Peptides were synthetized by EZBiolab Inc. (Carmel, USA) with the following sequences: flg22 – QRLSTGSRINSAKDDAAGLQIA and elf18 – AcSKEKFERTKPHVNVGTIG. Crab shell chitin (Sigma-Aldrich, Deisenhofen, Germany) was prepared in water at a concentration of 10 mg/ml, ground with a pestle, heated at 56 °C for 15 – 30 min and centrifuged at 13,000 rpm for 15 min. The supernatant was used to trigger MAMP responses.

4.1.8 Antibodies

Primary and secondary antibodies used in this study for immunoblot analysis (4.2.6.3) are listed in Table 4.6.

Table 4.6. Primary and secondary antibodies used in this study

Antibody	Source	Conjugate	Dilution	Reference
α-PEN1	rabbit, polyclonal	-	1:5000	Zhang et al., 2007
α-PEN2	rabbit, polyclonal	-	1:5000	Lipka et al., 2005
α-phospho p44/p42 MAPK	rabbit, polyclonal	-	1:1000	Cell Signaling,USA
α-rabbit IgG	goat, monoclonal	horseradish peroxidase	1:5000	Santa Cruz, USA

4.1.9 Media, buffers and solutions

Media were sterilised by autoclaving at 121 °C for 20 min. For the addition of antibiotics and other heat labile compounds the solution or media were cooled. Heat labile compounds were sterilised using filter sterilisation units prior to addition.

Pseudomonas syringae media

NYG broth

Bactopeptone	5 g/l
Yeast extract	3 g/l
Glycerol	20 ml/l
pH 7.0	

For NYG agar plates 1.5 % (w/v) bacto agar (Becton, Franklin Lakes, USA) was added to the above broth.

Arabidopsis thaliana media

½ MS (Murashige and Skoog) medium

MS powder including vitamins	2.2 g/l
Sucrose	8 g/l
pH 5.8	

For MS agar plates 0.8 % (w/v) plant agar (Duchefa, Haarlem, Netherlands) was added to the above medium. MS powder including nitch vitamins (MSN) was purchased from Duchefa (Haarlem, Netherlands).

Buffers and solutions used in this study are described below each method. If not otherwise stated, buffers were prepared in dH2O and aqueous solutions were sterilized by autoclaving at 121 °C for 20 min.

4.2 Methods

4.2.1 Maintenance and cultivation of Arabidopsis plants and pathogens

Arabidopsis seeds were germinated by sowing directly onto moist compost (Stender, Schermbeck, Germany) or on jiffy pellets (Jiffy Products International AS, Stange, Norway) (Sections 2.2.1.1, 2.2.1.2, 2.2.2). Before sowing the jiffy pellets were moistened in water supplied with 1 ml/l Wuxal (Stender, Schermbeck, Germany) fertilizer. Seeds were stratified for three days at 4 °C in darkness and subsequently transferred to a controlled environment growth chamber and maintained under short day conditions (10 h light/14 h darkness, 23 °C

during light period, 20 °C during darkness and 60 % humidity). For pathogen treatment plants were transferred to growth chambers designated for the respective pathogen (see below). For MAMP-triggered gene expression assays (Sections 2.2.4, 2.3.1) seedlings were grown on ½ MS agar plates for 5 days and subsequently on ½ MS liquid medium under controlled conditions (10 h light/14 h darkness, 21 °C during light period, 21 °C during darkness and 70 % humidity).

The barley powdery mildew fungus *Bgh* isolate K1 was maintained on seven-day-old barley seedlings I10, a near-isogenic line of Ingrid, and plants were kept at 14 h light/10 h darkness, 21 °C during light period and during darkness and 70 % humidity. *Bgh* inoculated Arabidopsis plants were transferred into a climate chamber suitable for Arabidopsis cultivation (10 h light/14 h darkness, 23 °C during light period, 21 °C during darkness and 60 % humidity).

The pea powdery mildew fungus *E. pisi* was propagated on 3-week-old pea plants cultivar Linga and plants were kept at 12 h light/12 h darkness, 22 °C during light period and during darkness and 70 % humidity.

The Arabidopsis powdery mildew fungus *G. orotnii* was maintained on Arabidopsis *pad4*/*sag101* double mutants cultivated at 10 h light/14 h darkness, 22 °C during light period and during darkness and 70 % humidity.

Pseudomonas syringae DC3000 strains (4.1.2.1) were streaked onto selective NYG agar plates containing rifampicin (100 µg/ml) and were incubated at 28 °C for 48 h. Plants sprayed with *Pst* bacterial strains were kept at 10 h light/14 h darkness, 23 °C during light period, 22 °C during darkness and 65 % humidity.

4.2.2 Arabidopsis seed surface sterilization

Seeds were placed in 2 ml tubes and washed with 70 % ethanol for 30 sec followed by a second washing step with 100 % ethanol. The ethanol was removed and seeds were dried in a sterile hood.

4.2.3 Generation of Arabidopsis protoplasts

Arabidopsis leaf mesophyll protoplasts were prepared from 4 - 5-week-old plants according to Asai et al., 2002, with some modifications. Fifteen leaves were cut in strips and placed in the enzyme solution (0.4 M Mannitol; 20 mM KCl; 20 mM MES pH 5.7) containing 1 % cellulase R-10 (Merck, Darmstadt, Germany), 0.2 % macerozyme R-10 (Serva, Heidelberg, Germany) and 0.1 % BSA (Sigma-Aldrich, Deisenhofen, Germany). The solution was

vacuum-infiltrated for 15 - 30 min and incubated for 3 h in the dark. The protoplasts were released by shaking for 1 min and W5 solution (154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl; 2 mM MES pH 5.7) was added. The protoplast solution was filtered through a nylon mesh and centrifuged at 100 g for 2 min. The protoplast pellet was resuspended in W5 solution, centrifuged again and dissolved in MMg solution (0.4 M Mannitol; 15 mM $MgCl_2$; 4 mM MES pH 5.7). Protoplasts were diluted in MMg solution to a concentration of $2 * 10^5$/ml.

4.2.4 Pathogen infection assays

4.2.4.1 *Pseudomonas syringae* pv. *tomato* infections

Four-week-old Arabidopsis plants were watered and kept under a humidified lid before treatment. *P. syringae* spray inoculation was performed with a bacterial suspension (OD_{600}=0.2) in 10 mM $MgCl_2$ supplemented with 0.04 % Silwet L-77. Infected plants were kept in a covered container and samples were harvested at 1 h (0 dpi) and 3 days (3 dpi) post-inoculation. A total of 9 surface-sterilized leaf discs (5 mm diameter) excised from 9 plants per genotype were separated into 3 pools, and subjected to the quantification of leaf bacteria. Bacterial titers were determined by shaking the leaf discs in 10 mM $MgCl_2$ supplemented with 0.01 % Silwet L-77 at 28 °C for 1 h (Tornero and Dangl, 2001). The resulting bacterial suspensions were serially diluted 1:10 and an aliquot of 20 µl per dilution was grown on selective NYG agar medium with rifampicin (100 µg/ml) at 28 °C for 48 h before colony numbers were determined. Bacterial infections per genotype were performed in two to three independent experiments.

4.2.4.2 Powdery mildew infections

Powdery mildew inoculations were carried out as described before (Consonni et al., 2006). In brief, 4-week-old Arabidopsis plants were inoculated with *G. orontii* or *E. pisi* spores and quantitative analysis of fungal entry rates were performed at 48 hpi or at 7 dpi, respectively. Quantification of *G. orontii* conidiophore formation was assessed at 7 dpi. For visualization of epiphytic fungal structures, detached leaves were cleared with ethanol/acidic acid (3/1 (v/v)) and subsequently stained with 0.6 % Coomassie Brillant Blue in ethanol. For quantification of fungal entry rate, the proportion of germinated fungal sporelings that developed secondary hyphae was assessed on at least 6 leaves (two leaves per plant) per genotype (minimum of 40 germinated sporelings/leaf evaluated). Fungal penetration success on each genotype was quantified in at least three independent experiments. The quantification of conidiophore

formation was performed by counting conidiophores on a single fungal colony. A minimum of 24 leaves (two leaves per plant) per genotype were counted in at least three independent experiments.

4.2.5 Molecular biological methods

4.2.5.1 Genomic DNA extraction

Genomic DNA was isolated as previously described (Edwards et al., 1991). In brief, Arabidopsis leaf tissue was ground in Edwards buffer (200 mM Tris-HCl ph 7.5; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS) and centrifuged at 13,000 rpm for 5 min. The DNA in the supernatant was precipitated with isopropanol and centrifuged. The DNA pellet was washed with 70 % ethanol, dried and resuspended in sterile water.

4.2.5.2 RNA extraction and cDNA synthesis

Total RNA from 2-week-old seedlings (Fig.2.9, 2.10, 2.12) was isolated using the RNeasy Plant Kit (QIAGEN, Hilden, Germany) according to the manual provided. RNA from mature leaves of 4-week-old plants (Fig. 2.3, 2.4) was extracted with 1 ml Trizol (Invitrogen, Karlsruhe, Germany) per 100 mg leaf material according to the manufacturer's instructions. In this case, further purification of the isolated RNA was performed using mini columns from the RNeasy Plant Kit as suggested by the manufacturers (QIAGEN, Hilden, Germany). cDNA was synthesized using 5 µg total RNA, oligo(dT) primers and the SuperScript II reverse transcriptase according to the manual provided (Invitrogen, Karlsruhe, Germany).

4.2.5.3 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using home-made *Taq* DNA polymerase and were carried out in a Peltier Thermal Cycler PTC-225 (GMI Inc., Ramsey, USA). A typical PCR reaction mix and thermal profile is shown below using the 10 x PCR buffer containing 750 mM Tris-HCl pH 8.8; 200 mM $(NH_4)_2SO_4$; 0.1 % (v/v) Tween 20; 25 mM $MgCl_2$.

Reaction mix	
genomic DNA	2 µl
PCR buffer (10 x)	2,5 µl
dNTPs (10 mM)	0.5 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq polymerase	0.5 µl
H_2O	ad 25 µl

PCR programme			
Initial denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	(35 x)
Annealing	55 °C	30 sec	
Extension	72 °C	1.5 min	
Final extension	72 °C	3 min	
	16 °C	5 min	

4.2.5.4 Quantitative RT-PCR

Quantification of transcripts of the indicated genes was performed on the IQ5 real-time PCR Thermocycler (Bio-Rad, Hercules, USA) using the primers listed in Table 4.4. A typical PCR reaction mix and thermal profile is shown below. The expression of the genes of interest, normalized to *At4g26420* encoding a methyltransferase and relative to the transcript abundance in Col-0 wild-type control samples at 0 hpi was calculated according to the comparative cycle threshold (ΔΔCt) method (Libault et al., 2007). The reference gene *At4g26420* was previously described to be stably expressed upon biotic stresses (Czechowski et al., 2005). Three technical replicates per sample were included. Experiments were performed once (Fig. 2.4F), in two (Fig. 2.4B, D, E and 2.10A-C and 2.12A-F) or in three independent (Fig. 2.4A, C and 2.9A-E) biological replicates.

Reaction mix	
cDNA (1:10)	1 µl
PCR buffer (10 x) see 2.2.53	2.5 µl
dNTPs (10 mM)	0.5 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
SYBR® Green (1:3000)	1.25 µl
Glycerol (50 %)	4 µl
DMSO (100 %)	0.75 µl
Taq polymerase	0.5 µl
H_2O	ad 25 µl

PCR programme			
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	20 sec	(40 x)
Annealing	59 °C	30 sec	
Extension	72 °C	25 sec	
	95 °C	1 min	
	55 °C	1 min	
Melting curve	55 – 95 ° C	10 sec; à 0.5 °C; 81 x	

4.2.5.5 Microarray experiment and data analysis

Powdery mildew inoculated mature rosette leaves from 4-week-old plants (three per genotype and time point) were harvested at 0 (prior to inoculation), 8 and 12 hpi and frozen in liquid nitrogen. Total RNA was extracted according to 4.2.5.2. Copy RNA (cRNA) was prepared following the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx). Labeled cRNA transcripts were purified using the sample cleanup module (Affymetrix). Fragmentation of cRNA transcripts, hybridization, and scanning of the high-density oligonucleotide microarrays (Arabidopsis ATH1 genome array; Affymetrix) were performed according to the manufacturer's GeneChip Expression Analysis Technical Manual. Three replicates per time point and genotype were performed. The quality of the data was evaluated at probe level by examining the arrays for spatial effects, distribution of absent and presents calls, and the intensity of spike-in controls. The robust multiarray average procedure (Irizarry et al., 2003) was used to correct for background effects and chip effects and to summarize the probe values

into probe set values, resulting in 22,811 normalized expression values per array. R/Bioconductor (Gentleman et al., 2004) was used to preprocess the raw microarray data. The ANOVA statistical test was applied in combination with the false discovery rate test method to correct for the P-values (Benjamini and Hochberg, 1995). To identify candidate genes with potentially altered transcript accumulation in the *mlo2 mlo6 mlo12* triple mutant after powdery mildew inoculation genes were selected that showed a significant ($P \leq 0.05$) and at least 2-fold higher transcript accumulation at 0, 8 and 12 hpi in the mutant (M) than in the wild-type (WT) resulting in the group I list (group I: M 0 hpi $\geq$ 2 x WT 0 hpi: 1 gene; M 8 hpi $\geq$ 2 x WT 8 hpi: 116 genes; M 12 hpi $\geq$ 2 x WT 12 hpi: 66 genes). Furthermore genes that showed a significant ($P \leq 0.05$) at least 2-fold lower transcript accumulation at 0, 8 and 12 hpi in the mutant than in the wild-type were selected and resulted in the group II gene list (group II: M 0 hpi $\leq$ 2 x WT 0 hpi: 0 genes; M 8 hpi $\leq$ 2 x WT 8 hpi: 20 genes; M 12 hpi $\leq$ 2 x WT 12 hpi: 1 gene). Analysis of overrepresented gene processes was carried out by using the online tool MapMan (www.mapman.gabipd.org/web/guest/mapman) (Thimm et al., 2004). Genevestigator V3 (https://www.genevestigator.com/gv/index.jsp) was used for meta-analysis of gene expression (Hruz et al., 2008).

4.2.5.6 DNA sequencing and analysis

DNA sequences were determined by the "Automatische DNA Isolierung und Sequenzierung" (ADIS) service unit at the Max-Planck Institute for Plant Breeding Research (Cologne, Germany) on Abi Prism 377 and 3700 sequencers(Applied Biosystems, Weiterstadt, Germany) using Big Dye-terminator chemistry. Sequence data were analysed using EditSeq™ version 5.00 (DNAStar, Madison, USA).

4.2.5.7 Agarose gel electrophoresis of DNA

DNA fragments were separated by agarose gel electrophoresis in gels consisting of 1 - 3 % (w/v) agarose (Bio-Budget Technologies, Krefeld, Germany) supplied with ethidium bromide solution (1:40000) in TAE buffer (400 mM Tris; 10 mM EDTA; 200 mM acetic acid; pH 8.5).

4.2.5.8 PEG transfection of Arabidopsis protoplasts

Leaf mesophyll protoplasts ($2 * 10^5$/ml) were transfected with plasmid DNA (1 µg/ml) and PEG solution (40 % (v/v) PEG4000 (Fluka); 0.2 M Mannitol; 0.1 M $Ca(NO_3)_2$) and were

incubated for 15 min. The protoplast solution was washed with W5 solution (see 4.2.3), centrifuged at 150 g for 2 min and resuspended in W5 solution. Protoplasts used for analysis of flg22-induced MAPK activation were treated with WI solution (0.5 M Mannitol; 20 mM KCl; 2 mM $CaCl_2$; 4 mM MES pH 5.7) and instead of W5 as protoplasts for Ca^{2+} measurements.

4.2.6 Biochemical methods

4.2.6.1 Total protein extraction

Total protein extracts were prepared from leaves of 4 - 5-week-old plants. Plant material was frozen in liquid nitrogen and homogenized with stainless steel beads (1,5 mm; Mühlheimer, Bärnau, Germany) in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, USA). Lysis buffer (20 mM HEPES pH 7.5; 13 % Sucrose; 1 mM EDTA; 1 mM DTT; 0.01 % Trition, 1x complete protease inhibitor cocktail (Roche, Mannheim, Germany)) was added (120 µl/100 mg FW) and samples were centrifuged at 13,000 rpm, 4 °C for 10 min. The supernatant was collected and the protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. Subsequently, 2x loading buffer (125 mM Tris-HCl pH 6.8; 5 % SDS; 25 % Glycerol (v/v); 0,025 % Bromphenol blue (w/v); 0.2 M DTT) was added, boiled for 10 min and samples were loaded onto SDS-polyacrylamide gels.

4.2.6.2 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out using the Mini-PROREAN® 3 system (Bio-Rad, Hercules, USA) and discontinuous SDS-polyacrylamide gels according to standard procedures (Laemmli, 1970). A 10 % separating gel (Table 4.7) was poured between two glass plates and overlaid with ethanol. After polymerization the ethanol was removed and a stacking gel was poured onto the separating gel (Table 4.7).

Table 4.7 Formulation for separating and stacking gel

Separating gel	**10 %**	**Stacking gel**	
30 % Acrylamide/Bis solution, 29:1 (Roth)	5 ml	30 % Acrylamide/Bis solution, 29:1 (Roth)	6.8 ml
1 M Tris-HCl pH 8.8	5.7 ml	1 M Tris-HCl pH 6.8	1.26 ml
10 % SDS	150 µl	10 % SDS	100 µl
10 % APS	150 µl	10 % APS	100 µl
TEMED	6 µl	TEMED	15 µl
dH_2O	4 ml	dH_2O	6.8 ml

SDS-polyacrylamide gel was placed into the electrophoresis tank and submerged in 1x SDS-running buffer (25 mM Tris; 250 mM Glycine; 0.1 % SDS). A pre-stained molecular weight marker (Precision plus protein standard dual colour, Bio-Rad, Hercules, USA) and denatured protein samples were loaded onto the gel and run at 100 V (stacking gel) and 130 V (separating gel).

4.2.6.3 Immunoblot analysis

Proteins that were separated on SDS-polyacrylamide gels were transferred to a Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) in a Mini Trans-Blot® Cell (Bio-Rad, Hercules, USA) according to the manufacturer's instruction. The transfer was carried out in 1 x transfer buffer (15 mM $NaPO_4$; 0.05 % SDS; 20 % Ethanol) at 70 V for 2 h. Subsequently, membranes were stained with Ponceau S (0.5 % Ponceau S (w/v) in 5 % (v/v) acedic acid) for 10 min, washed 3 x 5 min with PBS-T (10 mM Na_2HPO_4; 2 mM NaH_2PO_4; 137 mM NaCl; 1:1000 Tween20; pH 7.5) and blocked for 1 h in PBS-T containing 5 % (w/v) non-fat dry milk (AppliedChem, Germany). The blocking solution was removed and membranes were washed 3 x 5 min with BBS-T. Incubation with primary antibodies was carried out overnight by slowly shaking at 4 °C in PBS-T. The primary antibody solution was removed and membranes were washed 3 x 5 min with PBS-T at room temperature. Bound primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies diluted in PBS-T for 1 h at room temperature. For antibody details see 4.18. The antibody solution was removed and membranes were washed 3 x 5 min with PBS-T. Chemiluminescence detection using the SuperSignal® West Pico Chemimuminescent kit or a 8:1 mixture of the SuperSignal® West PicoChemimuminescent- and SuperSignal® West Femto Maximum Sensitivity-kits (Pierce, Rockford, USA) was carried out according to the manufacturer's instructions. Luminescence was detected by exposing the membrane to photographic film.

4.2.7 Analysis of indolic metabolites and oxylipins

4.2.7.1 Extraction of indolic metabolites and HPLC analysis

Extraction of secondary metabolites was performed in collaboration with Dr. Paweł Bednarek (Max-Planck Institute for Plant Breeding Research, Cologne, Germany). Powdery mildew-inoculated plants were collected at 8, 16 and 24 hpi and frozen in liquid nitrogen. Non-inoculated control plants were harvested prior to inoculation. Extraction and HPLC analysis

of secondary metabolites was performed as previously described (Bednarek et al., 2009). After addition of DMSO (50 µl/20 mg FW), the tissue was homogenized with zirconium beads (1 mm; Roth, Karlsruhe, Germany) in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, USA) and centrifuged for 15 min at 20,000 g. The supernatant was collected and subjected to HPLC on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, USA) equipped with diode array detector and fluorescence detector. Samples were analyzed on an Atlantis T3 C18 column (150 mm x 2.1 mm, 3 µm; Waters) with 0.1 % trifluoroacetic acid as solvent A and 98 % acetonitrile/ 0.1 % trifluoroacetic acid as solvent B at a flow rate of 0.25 ml/min at 22°C (gradient of solvent A: 100 % at 0 min, 100 % at 2 min, 90 % at 9 min, 72 % at 30 min, 50 % at 33 min, 20 % at 40 min, 100 % at 41 min). The concentrations of the metabolites of interest were quantified based on the comparison of their peak areas with those obtained during HPLC analyses of known amounts of the respective compounds purified from plant tissue (I3G, 4MI3G), synthetic (I3A) or commercial (RA) standards. Each sample was tested in three independent experiments.

4.2.7.2 Extraction of oxylipins and HPLC-MS/MS analysis

Extraction of jasmonic acid (JA), JA-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA) and 10-oxo-dinor-phytodienoic acid (dinor-OPDA, dn-OPDA) was performed in collaboration with Prof. Dr. Ivo Feußner (Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Göttingen, Germany) as previously described with some modifications (Luo et al., 2009). Powdery mildew-inoculated leaves were collected at 4, 8, 12 and 24 hpi and plant material (200 mg) was extracted with 0.75 ml of methanol containing 10 ng D_6-JA, 30 ng D_5-OPDA (kindly provided by Otto Miersch, Halle/Saale, Germany) each as internal standard. After vortexing, 2.5 ml of methyl-*tert*-butyl ether (MTBE) were added and the extract was shaken for 1 h at room temperature. For phase separation, 0.625 ml water was added, incubated for 10 min at room temperature and centrifuged at 450 *g* for 15 min. The upper phase was collected and the lower phase was re-extracted with 0.7 ml methanol and 1.3 ml MTBE as described above. The combined upper phases were dried under streaming nitrogen and resuspended in 100 µl of acetonitrile/water/acetic acid (20:80:0.1, v/v/v). The analysis of constituents was performed using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, Germany) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (MDS Sciex, Ontario, Canada). Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, USA). Reversed-phase HPLC separation was

performed on an EC 50/2 Nucleodure C18 gravity 1.8 µm column (50 x 2.1 mm, 1.8 µm particle size; Macherey and Nagel, Düren, Germany). The binary gradient system consisted of solvent A, acetonitrile/water/acetic acid (20:80:0.1, v/v/v) and solvent B, acetonitrile/acetic acid (100:0.1, v/v) with the following gradient program: 10 % solvent B for 2 min, followed by a linear increase of solvent B up to 90 % within 6 min and an isocratic run at 90 % solvent B for 2 min. The flow rate was 0.3 ml/min. For stable nanoESI, 130 µl/min of 2-propanol/acetonitrile/water/formic acid (70:20:10:0.1, v/v/v/v) delivered by a 2150 HPLC pump (LKB, Bromma, Sweden) were added just after the column via a mixing tee valve. By using another post column splitter 740 nl/min of the eluent were directed to the nanoESI chip. Ionization voltage was set to -1.7 kV. Phytohormones were ionized in a negative mode and determined in multiple reaction monitoring mode. Mass transitions were as follows: 215/59 [declustering potential (DP) -45 V, entrance potential (EP) -9.5 V, collision energy (CE) -22 V] for D_6- JA, 209/59 (DP -45 V, EP -9.5 V, CE -22 V) for JA, 296/170 (DP -70 V, EP -8.5 V, CE -28 V) for D_5-oPDA, 291/165 (DP -70 V, EP -8.5 V, CE -28 V) for OPDA, 263/59 (DP -70 V, EP -8.5 V, CE -28 V) for dinor-OPDA and 322/130 (DP -80 V, EP -4 V, CE -30 V) for JA-Ile. The mass analyzers were adjusted to a resolution of 0.7 amu full width at half-height. The ion source temperature was 40 °C, and the curtain gas was set at 10 (given in arbitrary units). Quantification was carried out using a calibration curve of intensity (*m/z*) ratios of [unlabeled]/[deuterium-labeled] *vs.* molar amounts of unlabeled (0.3 – 1000 pmol).

4.2.8 Analysis of MAMP responses

4.2.8.1 ROS measurement

ROS measurements were performed as described previously (Felix et al., 1999). Briefly, leaf discs (5 mm diameter) excised from 4-week-old plants were incubated over night in water before they were transferred into microtiter plates (CulturePlate-96, Perkin Elmer, Waltham, USA) containing 50 µl water. ROS production was triggered by the addition of 1 µM MAMP (unless otherwise stated; 4.1.7), applied in a reaction mixture containing 50 µl water, 20 µM luminol (Fluka, Deisenhofen, Germany) and 1 µg horseradish peroxidase (Sigma-Aldrich, Deisenhofen, Germany). Luminescence was measured in a Centro LB 960 microplate luminometer (Berthold Technologies, Wildbach, Germany).

4.2.8.2 MAPK measurement

Leaf discs (5 mm diameter) excised from 4-week-old plants were incubated over night in water before MAMP treatment. Three leaf discs per genotype or 100 µl mesophyll protoplasts (2 * 10^5/ml) were treated with 1 µM flg22 or chitin (4.1.7) for the indicated time points. Total proteins were extracted using a lysis buffer containing 50 mM Tris at pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM NaF, 25 mM beta-glycerophosphate, 2 mM sodium orthovanadate, 10 % (w/v) glycerol, 0.1 mM Tween 20, 0.5 mM DTT, 1 mM PMSF, and 1x complete protease inhibitor cocktail (Roche, Mannheim, Germany) as previously described (Saijo et al., 2008) and separated by SDS-PAGE (4.2.6.2). MAPK activation was detected by immunoblot analysis (4.2.6.3) using anti-phospho p44/p42 MAPK antibody (4.1.8).

4.2.8.3 Calcium measurement

Coelenterazine (10 µM in methanol) was added to leaf mesophyll protoplasts (2 * 10^5/ml) expressing the mCherry-tagged calcium sensor aequorin and incubated in darkness for at least two hours. Protoplasts (100 µl/well) were transferred into black microtiter plates (CulturePlate-96, Perkin Elmer, Waltham, USA) and mCherry fluorescence was quantified using the Synergy™ 4 Hybrid Microplate Reader (BioTek, Winooski, USA). Calcium spiking was triggered with 1 µM flg22 and chemiluminescence was monitored using the LAS-4000 detection system (FujiFilm, Tokio, Japan).

4.2.9 Analysis of callose deposition

Leaves from 6-week-old plants grown under axenic conditions were detached, cleared with ethanol/acidic acid (3/1 (v/v)) and subsequently stained for 24 h with 0.01 % aniline blue in 150 mM KH_2PO_4 (pH 5.8). Callose deposits were visualized by epifluorescence microscopy using a UV filter set.

5. Literature

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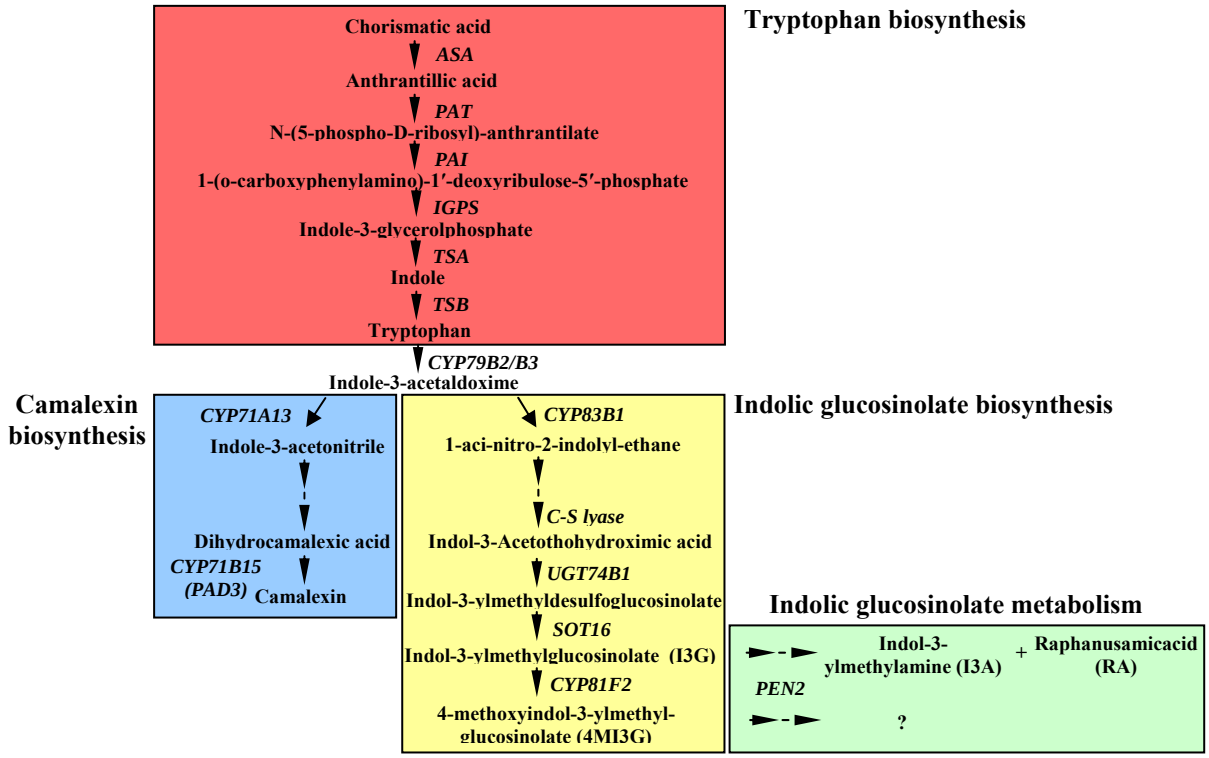
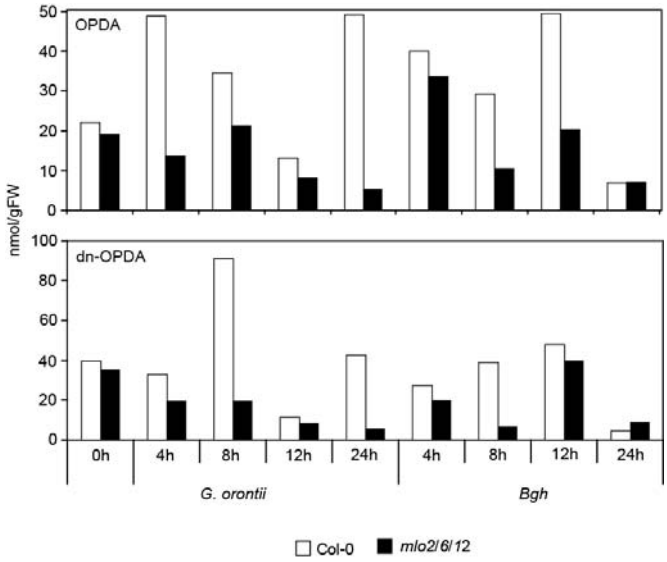
6. Supplementary Material



Supplementary Figure 6.1. Scheme of the tryptophan, camalexin and indolic glucosinolate biosynthesis as well as the indolic glucosinolate metabolism. Tryptophan is synthesized from chorismatic acid (red) and subsequently hydrolyzed to indole-3-acetaldoxime by the cytochrome P450 monooxygenases CYP79B2 and CYP79B3. Indole-3-acetaldoxime is either converted to camalexin (blue) or indolic glucosinolates (yellow). PEN2 is an atypical myrosinase that hydrolyzes 4MI3G for antifungal defense (green).



Supplementary Figure 6.2. Analysis of the JA precursors OPDA and dn-OPDA in Col-0 wild-type and *mlo2/6/12* mutant plants after inoculation with powdery mildew fungi. Independent biological replicate of the experiment shown in Figure 5. Rosette leaves from 4-week-old plants were inoculated with spores form the adapted and non-adapted powdery mildew fungus *G. orontii* and *Bgh*, respectively, and harvested for jasmonate analysis at the indicated time points

Supplementary Table 6.1. List of group I genes with significantly (P ≤ 0.05) at least two-fold increased transcript levels in the *mlo2 mlo6 mlo12* mutant at 0, 8 and 12 hpi upon challenge with *G. orontii*. Genes written in bold are associated with defense according to analysis with Genevestigator V3, ATTED-II and Tair (Hruz et al. 2008; Obayashi et al. 2009).

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 0 hpi	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 0 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT3G30720	256940_at	QQS (Qua-Quine Starch)	8,7	7,9	8,6	8,7E-07	1,5E-06	9,1E-07
AT2G30770	**267567_at**	**CYP71A13 (Cytochrome P450)**	**-**	**6,4**	**4,0**	**-**	**1,2E-04**	**1,4E-03**
AT3G26830	**258277_at**	**PAD3 (Phytoalexin Deficient 3)**	**-**	**6,1**	**2,3**	**-**	**1,6E-04**	**3,1E-02**
AT1G14870	262832_s_at	Unknown protein	-	6,0	-	-	1,2E-03	-
AT5G44420	**249052_at**	**PDF1.2a (Plant Defensin 1.2a)**	**-**	**5,0**	**6,3**	**-**	**1,6E-05**	**4,4E-06**
AT5G61160	247573_at	AACT1 (Anthocyanin-5-Aromatic Acyltransferase 1)	-	5,0	6,2	-	9,8E-04	3,5E-04
AT3G57240	**251673_at**	**Beta-1,3-Glucanase 3**	**-**	**4,7**	**-**	**-**	**4,4E-04**	**-**
AT3G49620	252265_at	DIN11 (Dark Inducible 11)	-	4,7	7,3	-	8,0E-03	1,5E-03
AT3G04720	**258791_at**	**PR4 (Pathogenesis-Related Protein 4)**	**-**	**4,3**	**2,8**	**-**	**5,4E-05**	**9,8E-04**
AT2G02930	266746_s_at	GST16 (Glutathione-S-Transferase 16)	-	4,1	2,4	-	1,2E-04	5,5E-03
AT2G29460	266267_at	GST22 (Glutathione-S-Transferase 22)	-	4,1	3,7	-	4,9E-03	7,6E-03
AT4G16260	245393_at	Glycosyl hydrolase family 17 protein	-	4,0	5,8	-	6,9E-05	6,3E-06
AT1G13470	259385_at	Unknown protein	-	3,9	-	-	2,5E-03	-
AT2G24850	263539_at	TAT3 (Tyrosine Aminotransferase 3)	-	3,9	2,8	-	1,9E-04	1,6E-03
AT2G26020	**257365_x_at**	**PDF1.2b (Plant Defensin 1.2b)**	**-**	**3,9**	**7,1**	**-**	**1,1E-03**	**4,1E-05**
AT2G14610	**266385_at**	**PR1 (Pathogenesis-Related Protein 1)**	**-**	**3,6**	**2,0**	**-**	**8,5E-04**	**3,3E-02**
AT2G26560	**245038_at**	**PLP2 (Phospholipase A 2A)**	**-**	**3,5**	**-**	**-**	**5,4E-03**	**-**
AT1G67810	245193_at	Fe-S metabolism associated domain-containing protein	-	3,5	2,7	-	8,4E-03	2,8E-02
AT2G43570	**260568_at**	**Chitinase, putative**	**-**	**3,5**	**2,8**	**-**	**4,6E-05**	**2,5E-04**
AT4G18250	254660_at	Receptor serine/threonine kinase, putative	-	3,4	2,2	-	1,1E-03	1,6E-02
?	248083_at	?	-	3,4	-	-	5,2E-03	-
AT5G38900	249481_at	DSBA oxidoreductase family protein	-	3,3	2,6	-	2,7E-03	1,1E-02
AT3G16530	**257206_at**	**Lectin like protein**	**-**	**3,2**	**2,2**	**-**	**4,3E-04**	**6,6E-03**
AT3G50480	**252170_at**	**HR4, Homolog of RPW8**	**-**	**3,2**	**-**	**-**	**8,7E-03**	**-**
AT5G18470	249983_at	Lectin family protein	-	3,1	-	-	2,1E-03	-
AT3G23550	258100_at	MATE efflux family protein	-	3,0	-	-	2,9E-02	-
AT2G35980	**263948_at**	**YLS9 (Yellow-Leaf-Specific 9) / NDR1-like**	**-**	**3,0**	**-**	**-**	**2,1E-03**	**-**
AT1G76960	264958_at	Unknown protein	-	3,0	4,0	-	3,9E-03	7,2E-04
AT1G57630	**246405_at**	**Disease Resistance Protein (TIR class), putative**	**-**	**3,0**	**2,3**	**-**	**2,0E-04**	**1,7E-03**
AT1G78410	260804_at	VQ motif-containing protein	-	3,0	-	-	3,1E-03	-
AT1G26380	261021_at	FAD-binding domain-containing protein	-	2,9	2,1	-	2,9E-03	2,2E-02
AT5G52760	248322_at	Heavy-metal-associated domain-containing protein	-	2,9	-	-	2,3E-02	-
AT3G26210	257623_at	CYP71B23 (Cytochrome P450)	-	2,9	-	-	3,5E-03	-

Supplementary Table 6.1. continued

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 0 hpi	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 0 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT1G04980	261167_at	PDIL2-2 (Protein Disulfide Isomerase-like protein)	-	2,8	-	-	2,7E-04	-
AT2G26440	245052_at	Pectinesterase	-	2,8	-	-	5,8E-04	-
AT5G25250	246927_s_at	Unknown protein	-	2,8	2,2	-	1,9E-03	1,0E-02
AT3G09940	**258941_at**	**MDAR3 (Monodehydroascorbate Reductase)**	**-**	**2,8**	**2,1**	**-**	**7,0E-04**	**8,0E-03**
AT3G01830	258947_at	Calmodulin-like protein, putative	-	2,8	-	-	2,4E-03	-
AT1G17745	259403_at	PGDH (3-Phosphoglycerate Dehydrogenase)	-	2,7	2,5	-	7,7E-05	1,9E-04
AT1G21120	261449_at	O-Methyltransferase, putative	-	2,7	-	-	1,6E-03	-
AT5G52750	248327_at	Heavy-metal-associated domain-containing protein	-	2,7	-	-	6,7E-03	-
AT1G26420	261005_at	FAD-binding domain-containing protein	-	2,7	-	-	2,0E-03	-
AT3G54640	**251847_at**	**TSA1 (Tryptophan Synthase Alpha Chain 1)**	**-**	**2,7**	**2,5**	**-**	**5,1E-06**	**9,7E-06**
AT3G60540	251422_at	Sec61beta family protein	-	2,6	-	-	9,4E-04	-
AT4G38540	252993_at	Monooxygenase, putative	-	2,6	2,4	-	4,7E-03	8,4E-03
AT3G57260	**251625_at**	**Beta-1,3-Glucanase 2**	**-**	**2,6**	**3,4**	**-**	**9,7E-03**	**1,9E-03**
AT3G28930	**258002_at**	**AIG2, avrRpt2-induced gene**	**-**	**2,6**	**-**	**-**	**7,7E-05**	**-**
AT3G09410	259033_at	Pectinacetylesterase family protein	-	2,6	2,6	-	3,9E-03	4,0E-03
AT3G22600	256933_at	Lipid transfer protein family protein	-	2,6	-	-	6,9E-04	-
AT1G21110	261450_s_at	O-Methyltransferase, putative	-	2,5	-	-	6,5E-03	-
AT1G18570	**255753_at**	**MYB51 (MYB domain transcription factor 51)**	**-**	**2,5**	**2,0**	**-**	**4,1E-03**	**2,2E-02**
AT2G38860	266167_at	Protease I (pfpI)-like protein YLS5	-	2,5	2,0	-	8,4E-04	4,8E-03
AT5G20960	246133_at	AAO1 (Aldehyde Oxidase 1)	-	2,5	-	-	2,6E-04	-
AT3G26820	258259_s_at	Esterase/Lipase/Thioesterase family protein	-	2,5	-	-	1,6E-03	-
AT3G51330	252098_at	Aspartyl protease family protein	-	2,4	-	-	2,0E-03	-
AT4G11890	254869_at	Serine/threonine kinase	-	2,4	-	-	1,3E-03	-
AT1G06160	**260783_at**	**ORA59 (AP2/ERF domain transcription factor)**	**-**	**2,4**	**2,8**	**-**	**1,9E-02**	**8,1E-03**
AT1G02920	**262119_s_at**	**GST7 (Glutathione-S-Transferase 7)**	**-**	**2,4**	**-**	**-**	**6,1E-03**	**-**
AT3G21520	257540_at	Unknown protein	-	2,4	-	-	7,1E-04	-
AT4G03450	255406_at	Ankyrin repeat family protein	-	2,4	-	-	8,3E-04	-
AT3G54960	251840_at	PDIL1-3 (Protein Disulfide Isomerase-like protein)	-	2,4	-	-	3,5E-03	-
AT5G64120	**247327_at**	**Peroxidase**	**-**	**2,4**	**-**	**-**	**1,5E-02**	**-**
AT5G52810	248330_at	Ornithine cyclodeaminase/mu-crystallin family protein	-	2,4	-	-	4,1E-04	-
AT1G24807	247864_s_at	Anthranilate Synthase Beta Subunit, putative	-	2,4	2,0	-	5,0E-04	2,2E-03
AT1G19020	259479_at	Unknown protein	-	2,3	-	-	2,4E-02	-
AT4G33050	**253414_at**	**EDA39 (Embryo Sac Development Arrest 39)**	**-**	**2,3**	**-**	**-**	**2,1E-02**	**-**
AT1G30900	265161_at	VSR (Vacuolar Sorting Receptor), putative	-	2,3	-	-	5,7E-03	-

Supplementary Table 6.1. continued

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 0 hpi	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 0 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT3G20510	257088_at	Unknown protein	-	2,3	-	-	4,8E-05	-
AT1G45145	**260943_at**	**TRX5 (Thioredoxin 5)**	**-**	**2,3**	**-**	**-**	**1,4E-02**	**-**
AT4G20110	254500_at	VSR (Vacuolar Sorting Receptor), putative	-	2,3	-	-	3,4E-05	-
AT4G14365	245329_at	Zinc finger family protein	-	2,3	-	-	3,7E-02	-
AT5G57220	**247949_at**	**CYP81F2 (Cytochrome P450)**	**-**	**2,3**	**-**	**-**	**1,1E-02**	**-**
AT5G27420	246777_at	CNI1 (Carbon/Nitrogen Insensitive zinc finger family protein)	-	2,3	-	-	5,7E-03	-
AT5G48540	248686_at	Secretory protein-like	-	2,3	-	-	2,3E-02	-
AT1G77510	259757_at	PDIL1-2 (Protein Disulfide Isomerase-like protein)	-	2,3	-	-	1,7E-03	-
AT3G24982	**257592_at**	**Disease resistance / LRR family protein, putative**	**-**	**2,3**	**-**	**-**	**4,4E-02**	**-**
AT3G22060	257264_at	Unknown protein	-	2,3	-	-	3,1E-02	-
AT2G04400	263807_at	IGPS (Indole-3-Glycerol Phosphate Synthase)	-	2,3	-	-	1,0E-04	-
AT3G54420	251895_at	EP3 chitinase	-	2,3	-	-	1,4E-03	-
AT3G54150	251884_at	Embryo-abundant protein, putative	-	2,3	-	-	1,8E-02	-
AT2G18690	266017_at	Unknown protein	-	2,3	-	-	4,7E-03	-
AT1G66690	256376_s_at	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	-	2,3	2,3	-	7,9E-03	7,0E-03
AT1G61560	**265008_at**	**MLO6 (Mildew Resistance Locus O 6)**	**-**	**2,2**	**2,4**	**-**	**6,1E-03**	**3,8E-03**
AT4G12490	**254832_at**	**LTP (Lipid transfer protein)**	**-**	**2,2**	**-**	**-**	**3,1E-02**	**-**
AT1G02850	262118_at	BGLU11 (Beta-Glucosidase 11)	-	2,2	2,4	-	2,8E-02	1,5E-02
AT5G40760	249372_at	G6PD6 (Glucose-6-Posphate Dehydrogenase)	-	2,2	-	-	8,9E-05	-
AT5G47220	**248794_at**	**ERF2 (AP2/ERF domain transcription factor)**	**-**	**2,2**	**-**	**-**	**7,8E-03**	**-**
AT3G47480	252417_at	Calcium-binding EF hand family protein	-	2,2	-	-	5,0E-03	-
AT2G47130	266761_at	SDR (Short-chain Dehydrogenase/Reductase family protein)	-	2,2	-	-	2,0E-03	-
AT1G33030	261216_at	O-methyltransferase family 2 protein	-	2,1	2,4	-	1,1E-03	4,1E-04
AT3G11010	**256431_s_at**	**RLP34 (Receptor-like protein 34)**	**-**	**2,1**	**-**	**-**	**4,0E-02**	**-**
AT2G32160	265698_at	Unknown protein	-	2,1	-	-	5,1E-03	-
AT3G13910	258201_at	Unknown protein	-	2,1	-	-	1,3E-02	-
AT2G29120	266782_at	GLR2.7 (Glutamate Receptor 2.7)	-	2,1	-	-	4,8E-03	-
AT4G31500	**253534_at**	**CYP83B1 (Cytochrome P450)**	**-**	**2,1**	**-**	**-**	**6,7E-03**	**-**
AT1G65820	262932_at	Glutathione-S-Transferase, putative	-	2,1	-	-	3,4E-04	-
AT5G04340	245711_at	Zinc finger transcription factor, putative	-	2,1	2,6	-	3,3E-02	8,8E-03
AT5G02780	250983_at	GSTL1 (Glutathione-S-Transferase Lambda 1)	-	2,1	2,1	-	1,2E-03	9,1E-04
AT5G25930	246858_at	Receptor serine/threonine kinase	-	2,1	-	-	1,2E-02	-
AT4G25900	254040_at	Aldose 1-Epimerase family protein	-	2,1	-	-	1,4E-03	-
AT3G28940	**258000_at**	**AIG (Avirulence induced protein), putative**	**-**	**2,1**	**-**	**-**	**4,7E-03**	**-**

Supplementary Table 6.1. continued

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 0 hpi	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 0 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT4G18430	254673_at	RABA1E (Rab GTPase homolog A1E)	-	2,1	-	-	3,5E-03	-
AT1G13750	256100_at	Calcineurin-like phosphoesterase family protein	-	2,0	-	-	4,7E-05	-
AT5G46050	**248932_at**	**PTR3 (Peptide Transporter Protein)**	**-**	**2,0**	**-**	**-**	**4,4E-02**	**-**
AT4G22690	254331_s_at	CYP706A1 (Cytochrome P450)	-	2,0	-	-	3,4E-03	-
AT1G13990	262607_at	Unknown protein	-	2,0	-	-	3,3E-03	-
AT2G02000	265221_s_at	GAD3 (Glutamate Decarboxylase 3)	-	2,0	-	-	2,9E-02	-
AT3G04210	**258537_at**	**Disease resistance protein (TIR-NBS class), putative**	**-**	**2,0**	**-**	**-**	**2,0E-02**	**-**
AT1G63840	260327_at	Zinc finger family protein	-	2,0	-	-	2,8E-02	-
AT2G41410	266371_at	Calmodulin, putative	-	2,0	-	-	5,6E-03	-
AT2G32680	**267546_at**	**RLP23 (Receptor-like protein 23)**	**-**	**2,0**	**-**	**-**	**2,3E-02**	**-**
AT5G60950	247604_at	COBL5 (Cobra-like protein 5 precursor)	-	2,0	-	-	3,3E-04	-
AT1G27730	261648_at	STZ (Salt Tolerant zinc finger)	-	2,0	-	-	1,5E-02	-
AT1G74100	**260387_at**	**SOT16 (Sulfotransferase 16)**	**-**	**2,0**	**-**	**-**	**8,1E-03**	**-**
AT5G54490	248164_at	PBP1 (Pinoid-binding protein 1)	-	2,0	-	-	2,0E-02	-
AT5G40780	249346_at	LHT1 (Lysine Histidine Transporter 1)	-	2,0	-	-	1,2E-02	-
AT5G10520	250443_at	RBK1 (ROP binding protein kinases 1)	-	-	3,8	-	-	2,7E-04
AT1G10070	264524_at	BCAT 2 (Branched-Chain Amino Acid Transaminase 2)	-	-	3,6	-	-	1,0E-02
AT2G39030	266142_at	GCN5-related N-acetyltransferase (GNAT) family protein	-	-	3,1	-	-	2,2E-03
AT1G16420	262731_at	MC8 (Metacaspase 8)	-	-	2,9	-	-	7,0E-04
AT1G30135	256159_at	JAZ8 (Jasmonate-Zim-domain protein 8)	-	-	2,7	-	-	1,1E-03
AT1G15520	**261763_at**	**PDR12, Pleiotropic Drug Resistance 12**	**-**	**-**	**2,7**	**-**	**-**	**1,9E-04**
AT1G07260	256053_at	UGT71C3 (UDP-glycosyltransferase 71C3)	-	-	2,6	-	-	2,9E-06
AT4G08770	255110_at	Peroxidase, putative	-	-	2,6	-	-	6,7E-05
AT5G67080	247026_at	MAPKKK19 (Mitogen-Activated Protein Kinase Kinase Kinase 19)	-	-	2,5	-	-	9,0E-05
AT4G19370	254571_at	Unknown protein	-	-	2,4	-	-	5,2E-04
AT4G24350	254150_at	Phosphorylase family protein	-	-	2,3	-	-	2,0E-06
AT1G51760	256178_s_at	JR3 (Jasmonic Acid Responsive 3)	-	-	2,3	-	-	3,3E-02
AT3G26440	256883_at	Unknown protein	-	-	2,3	-	-	6,4E-05
AT4G17500	**245252_at**	**ERF1 (AP2/ERF domain transcription factor 1)**	**-**	**-**	**2,2**	**-**	**-**	**3,4E-03**
AT4G24340	254163_s_at	Phosphorylase family protein	-	-	2,2	-	-	1,8E-04
AT4G21850	254387_at	Methionine Sulfoxide Reductase domain-containing protein	-	-	2,2	-	-	6,0E-03
AT3G28580	256989_at	AAA-type ATPase family protein	-	-	2,2	-	-	1,7E-05
AT5G54960	248138_at	PDC2 (Pyruvate Decarboxylase 2)	-	-	2,2	-	-	2,2E-02
AT3G63380	251176_at	Calcium-transporting ATPase, putative	-	-	2,2	-	-	8,3E-03

Supplementary Table 6.1. continued

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 0 hpi	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 0 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT1G17380	**261033_at**	**JAZ5 (Jasmonate-Zim-domain protein 5)**	**-**	**-**	**2,2**	**-**	**-**	**2,8E-02**
AT1G80840	**261892_at**	**WRKY40 (Pathogen-induced transcription factor)**	**-**	**-**	**2,1**	**-**	**-**	**2,1E-02**
AT4G11280	**254926_at**	**ACS6 (1-Aminocyclopropane-1-Carboxylate Synthase 6)**	**-**	**-**	**2,1**	**-**	**-**	**6,7E-04**
AT3G50770	252136_at	CML41 (Calmodulin-like 41)	-	-	2,1	-	-	1,3E-02
AT5G40990	249333_at	GLIP1 (GDSL Lipase 1)	-	-	2,1	-	-	6,3E-04
AT4G23700	254215_at	CHX17 (Cation/H+ Exchanger 17)	-	-	2,1	-	-	3,4E-03
AT2G15080	**265917_at**	**RLP19 (Receptor-like protein 19)**	**-**	**-**	**2,0**	**-**	**-**	**3,7E-02**
AT2G36380	263904_at	PDR6 (Pleiotropic Drug Resistance 6)	-	-	2,0	-	-	8,6E-05
AT4G34200	253274_at	EDA9 (Embryo Sac Development Arrest 9)	-	-	2,0	-	-	1,7E-02

Supplementary Table 6.2. List of group II genes with significantly ($P \leq 0.05$) at least two-fold decreased transcript levels in the *mlo2 mlo6 mlo12* mutant at 8 and 12 hpi upon challenge with *G. orontii*.

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT4G28780	253736_at	GDSL-like Lipase	0,40	-	1,06E-02	-
AT4G31000	253571_at	Calmodulin-binding protein	0,42	-	8,77E-03	-
AT5G20630	246004_at	GER3 (Germin-like protein 3)	0,43	-	2,26E-02	-
AT2G15090	265918_at	Fatty acid elongase, putative	0,43	-	5,38E-03	-
AT1G54050	263150_at	Heat shock protein, class III (HSP17.4-CIII)	0,43	-	4,21E-02	-
AT4G31240	253519_at	Electron carrier; similar to DC1 domain-containing protein	0,44	-	2,26E-04	-
AT3G50240	252215_at	Kinesin-related protein	0,44	-	1,29E-04	-
AT2G45920	266910_at	U-box domain-containing protein	0,45	-	3,92E-05	-
ATCG01060	244932_at	PsaC subunit of photosystem I	0,45	-	4,85E-02	-
AT1G72970	262376_at	EDA17 (Embryo Sac Developent Arrest 17)	0,45	-	1,30E-02	-
AT1G18620	255774_at	Unknown protein	0,46	-	4,65E-02	-
AT3G04630	258796_at	Gene family with a KLEEK domain	0,46	-	8,40E-05	-
AT4G01460	255579_at	bHLH (basic helix-loop-helix) family protein	0,46	-	6,77E-05	-
AT5G01015	251142_at	Unknown protein	0,46	-	1,63E-03	-
AT2G10940	265400_at	Protease inhibitor/ seed storage/ lipid transfer protein family protein	0,49	-	2,79E-03	-
AT1G19715	261133_at	Mannose-binding lectin superfamily protein	0,49	-	4,09E-03	-

Supplementary Table 6.2. continued

AGI code	Probe code	Gene annotation and putative function	Fold change *mlo2/6/12* vs Col-0 8 hpi	Fold change *mlo2/6/12* vs Col-0 12 hpi	P-value *mlo2/6/12* vs Col-0 8 hpi	P-value *mlo2/6/12* vs Col-0 12 hpi
AT1G78970	264100_at	Lupeol synthase	0,49	-	8,73E-04	-
AT1G55260	259660_at	Protease inhibitor/ seed storage/ lipid transfer protein family protein	0,49	-	1,69E-02	-
AT3G16370	259375_at	GDSL-like Lipase	0,49	-	1,63E-02	-
AT4G03210	255433_at	XTH9 (Xyloglucan Endotransglucosylase/ Hydrolases 9)	0,49	-	1,38E-02	-
AT3G44735	252624_at	Phytosulfokine 3 precursor	-	0,50	-	3,78E-02

Supplementary Table 6.3. Functional MapMan classification (Thimm et al. 2004) of group I genes (≥ 2-fold; P ≤ 0.05) in the *mlo2 mlo6 mlo12* mutant upon challenge with *G. orontii*.

Bin	Bin name	*mlo2/6/12* 8 hpi	*mlo2/6/12* 12 hpi
		Count	Count
2	**major CHO metabolism**	**1**	**1**
3	**minor CHO metabolism**	**1**	-
5	**fermentation**	-	**1**
7	**OPP**	**1**	-
10	**cell wall**	**2**	**1**
10.8.1	cell wall.pectin*esterases.PME	1	-
10.8.2	cell wall.pectin*esterases.acetyl esterase	1	1
13	**amino acid metabolism**	**5**	**5**
13.1.1.1.1	amino acid metabolism.synthesis.central amino acid metabolism.GABA.Glutamate decarboxylase	1	-
13.1.4.1.4	amino acid metabolism.synthesis.branched chain group.common.branched-chain amino acid aminotransferase	-	1
13.1.5.1.1	amino acid metabolism.synthesis.serine-glycine-cysteine group.serine.phosphoglycerate dehydrogenase	1	2
13.1.6.5.1	amino acid metabolism.synthesis.aromatic aa.tryptophan.anthranilate synthase	1	1
13.1.6.5.4	amino acid metabolism.synthesis.aromatic aa.tryptophan.indole-3-glycerol phosphate synthase	1	-
13.1.6.5.5	amino acid metabolism.synthesis.aromatic aa.tryptophan.tryptophan synthase	1	1
15	**metal handling**	**2**	**1**
16	**secondary metabolism**	**7**	**3**
16.5.1.1.3.3	secondary metabolism.sulfur-containing.glucosinolates.synthesis.indole.synthesis.indole-3-methyl-desulfoglucosinolate sulfotransferase	1	-
16.5.1.1.3.4	secondary metabolism.sulfur-containing.glucosinolates.synthesis.indole.synthesis.cytochrome P450 monooxygenase	1	-
16.5.1.1.4.1	secondary metabolism.sulfur-containing.glucosinolates.synthesis.shared.CYP83B1 phenylacetaldoxime monooxygenase	1	-
16.5.1.2.3	secondary metabolism.sulfur-containing.glucosinolates.regulation.indole	1	1
16.8.1	secondary metabolism.flavonoids.anthocyanins	1	1

Supplementary Table 6.3. continued

Bin	Bin name	*mlo2/6/12* 8 hpi	*mlo2/6/12* 12 hpi
		Count	Count
16.10	secondary metabolism.simple phenols	1	1
16.99	secondary metabolism.unspecified	1	-
17	**hormone metabolism**	**3**	**5**
17.2.1	hormone metabolism.auxin.synthesis-degradation	-	1
17.5.1.1	hormone metabolism.ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate synthase	-	1
17.5.2	hormone metabolism.ethylene.signal transduction	2	2
17.8.1	hormone metabolism.salicylic acid.synthesis-degradation	1	1
18	**Co-factor and vitamine metabolism**	**1**	**2**
20	**stress**	**14**	**9**
20.1	stress.biotic	14	8
20.1.3	stress.biotic.signalling	2	1
20.1.3.1	stress.biotic.signalling.MLO-like	1	1
20.1.7	stress.biotic.PR-proteins	6	4
20.1.7.12	stress.biotic.PR-proteins.plant defensins	2	2
20.2	stress.abiotic	-	1
21	**redox**	**5**	**1**
21.1	redox.thioredoxin	4	-
21.2.1	redox.ascorbate and glutathione.ascorbate	1	1
26	**misc**	**30**	**15**
26.1	misc.misc2	1	-
26.2	misc.UDP glucosyl and glucoronyl transferases	-	1
26.3	misc.gluco-, galacto- and mannosidases	1	1
26.4.1	misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	3	2
26.5	misc.acyl transferases	1	-
26.6	misc.O-methyl transferases	2	-
26.7	misc.oxidases - copper, flavone etc.	1	1
26.9	misc.glutathione S transferases	5	3
26.10	misc.cytochrome P450	6	2
26.12	misc.peroxidases	1	1
26.13	misc.acid and other phosphatases	1	-
26.16	misc.myrosinases-lectin-jacalin	2	1
26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2	-
26.22	misc.short chain dehydrogenase/reductase (SDR)	1	-
26.24	misc.GCN5-related N-acetyltransferase	-	1
26.25	misc.sulfotransferase	1	-
26.28	misc.GDSL-motif lipase	-	1

Supplementary Table 6.3. continued

Bin	Bin name	*mlo2/6/12* 8 hpi	*mlo2/6/12* 12 hpi
		Count	Count
27	**RNA**	**3**	**3**
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	2	1
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	1	1
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	-	1
29	**protein**	**8**	**5**
29.3.4.3	protein.targeting.secretory pathway.vacuole	2	-
29.3.4.99	protein.targeting.secretory pathway.unspecified	1	-
29.4	protein.postranslational modification	2	3
29.4.1.56	protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VI	-	1
29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	2	-
29.5.4	protein.degradation.aspartate protease	1	-
29.5.9	protein.degradation.AAA type	-	1
30	**signalling**	**12**	**3**
30.1.1	signalling.in sugar and nutrient physiology	1	-
30.2.11	signalling.receptor kinases.leucine rich repeat XI	1	-
30.2.15	signalling.receptor kinases.thaumatin like	1	1
30.2.17	signalling.receptor kinases.DUF 26	1	-
30.2.99	signalling.receptor kinases.misc	2	-
30.3	signalling.calcium	5	2
30.5	signalling.G-proteins	1	-
31	**cell**	**2**	**-**
33	**development**	**5**	**2**
34	**transport**	**3**	**3**
34.3	transport.amino acids	1	-
34.12	transport.metal	-	1
34.13	transport.peptides and oligopeptides	1	-
34.16	transport.ABC transporters and multidrug resistance systems	-	2
34.99	transport.misc	1	-
35	**not assigned**	**18**	**9**

Supplementary Table 6.4. Functional MapMan classification (Thimm et al. 2004) of group II genes (≤ 2-fold; P ≤ 0.05) in the *mlo2 mlo6 mlo12* mutant upon challenge with *G. orontii*.

Bin	Bin name	*mlo2/6/12* 8 hpi
		Count
1	**PS**	**1**
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	**1**
10	**cell wall**	1
10.7	cell wall.modification	1
11	**lipid metabolism**	**1**
11.1.11	lipid metabolism.FA synthesis and FA elongation.fatty acid elongase	1
16	**secondary metabolism**	**1**
16.1.5	secondary metabolism.isoprenoids.terpenoids	1
20	**stress**	**2**
20.2.1	stress.abiotic.heat	1
20.2.99	stress.abiotic.unspecified	1
26	**misc**	**6**
26.8	misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	1
26.16	misc.myrosinases-lectin-jacalin	1
26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2
26.28	misc.GDSL-motif lipase	2
27	**RNA**	**1**
27.3	RNA.regulation of transcription	1
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	1
29	**protein**	**1**
29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	1
30	**signalling**	**1**
30.3	signalling.calcium	1
31	**cell**	**2**
31.1	cell.organisation	2
33	**development**	**2**
33.99	development.unspecified	2
35	**not assigned**	**3**

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Erklärung

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Köln, 28. März 2011 _____

Justine Lorek